EVALUATION OF PORCINE ACELLULARIZED DERMAL MATRIX AS A BIOMATERIAL: IN VITRO FIBROPLASIA

By

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A thesis submitted in conformity with the requirements for the degree of
Master of Science
Department of Surgery, Institute of Medical Science
University of Toronto

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Master of Science 2001

Alexis Devon Armour

Department of Surgery, Institute of Medical Science, University of Toronto

Abstract

Acellularized pig dermis (pig ACM) is a biomaterial under study for use as a xenograft dermal substitute, and for modeling dermal matrix biology in vitro. The specific objectives of the present project were threefold. (1) To characterize the structure and acellularity of pig ACM, in comparison with pig dermis and human ACM. (2) To study the effects of pig ACM on fibroplasia in vitro in comparison with other in vitro models of fibroplasia. (3) To evaluate the effects of hyaluronan and heparin on fibroplasia in vitro, after incorporation into pig ACM. Pig ACM was found to be inferior to human ACM as a scaffold for human fibroblasts. Fibroblasts proliferated more rapidly on pig ACM and differentiated into myofibroblasts to a greater degree. Hyaluronan-enriched pig ACM was associated with decreased myofibroblast differentiation, whereas a greater percentage of fibroblasts differentiated to myofibroblasts on heparin-enriched ACM. Hyaluronan, unlike heparin, may therefore be considered a useful ACM dermal substitute component. Pig ACM was found to be a promising material for in vitro modeling of myofibroblast-mediated contraction of dermis. Structural modification of pig ACM would likely improve its clinical suitability as a xenograft dermal substitute.
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# List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>1ºHFb</td>
<td>primary or passaged human dermal fibroblasts</td>
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<tr>
<td>AC</td>
<td>acetyl group</td>
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<tr>
<td>ACM</td>
<td>acellular dermal matrix</td>
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<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
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<tr>
<td>CEA</td>
<td>cultured epidermal autograft</td>
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<tr>
<td>cpm</td>
<td>counts per minute</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle Medium</td>
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<tr>
<td>DMSO</td>
<td>dimethylsulfoxide</td>
</tr>
<tr>
<td>DNase</td>
<td>deoxyribonuclease</td>
</tr>
<tr>
<td>EBM-2</td>
<td>endothelial cell basal medium</td>
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<tr>
<td>ECM</td>
<td>extracellular matrix</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylene diamine tetra-acetate</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
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<tr>
<td>FD</td>
<td>freeze-dried</td>
</tr>
<tr>
<td>FITC</td>
<td>fluorescein</td>
</tr>
<tr>
<td>FTSG</td>
<td>full thickness skin graft</td>
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<tr>
<td>GAG</td>
<td>glycosaminoglycan</td>
</tr>
<tr>
<td>Gly</td>
<td>glycine</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>hematoxylin and eosin</td>
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<tr>
<td>HA</td>
<td>hyaluronan</td>
</tr>
<tr>
<td>HA-ACM</td>
<td>hyaluronan-enriched acellular dermal matrix</td>
</tr>
<tr>
<td>HACM</td>
<td>human acellular dermal matrix</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hank’s balanced salt solution</td>
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<tr>
<td>HCl</td>
<td>hydrochloric acid</td>
</tr>
<tr>
<td>HP</td>
<td>heparin</td>
</tr>
<tr>
<td>HP-ACM</td>
<td>heparin-enriched acellular dermal matrix</td>
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<tr>
<td>MCDB-131</td>
<td>Molecular Cellular Developmental Biology medium #131</td>
</tr>
<tr>
<td>MEC</td>
<td>microvascular endothelial cells</td>
</tr>
<tr>
<td>MTT</td>
<td>3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide</td>
</tr>
<tr>
<td>MW avg</td>
<td>average molecular weight</td>
</tr>
<tr>
<td>n</td>
<td>total sample size</td>
</tr>
<tr>
<td>NaOH</td>
<td>sodium hydroxide</td>
</tr>
<tr>
<td>O.C.T.</td>
<td>trade name, not an abbreviation</td>
</tr>
<tr>
<td>o.d.</td>
<td>outer diameter</td>
</tr>
<tr>
<td>P#</td>
<td>passage number</td>
</tr>
<tr>
<td>PACM</td>
<td>pig acellular dermal matrix</td>
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<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
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<tr>
<td>PG</td>
<td>proteoglycan</td>
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<tr>
<td>PMSF</td>
<td>phenyl-methyl-sulfonyl-fluoride</td>
</tr>
<tr>
<td>r</td>
<td>number of repeats per experiment</td>
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<tr>
<td>RHPS</td>
<td>Recombined Human-Pig Skin</td>
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<tr>
<td>RTF</td>
<td>rat tail fibroblasts</td>
</tr>
<tr>
<td>s</td>
<td>sample size per group, per repeat</td>
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<tr>
<td>SEM</td>
<td>scanning electron microscopy</td>
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<td>SMA</td>
<td>alpha-smooth muscle actin</td>
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<td>STSG</td>
<td>split-thickness skin graft</td>
</tr>
<tr>
<td>TCA</td>
<td>trichloroacetic acid</td>
</tr>
<tr>
<td>TEM</td>
<td>transmission electron microscopy</td>
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<tr>
<td>TGFβ</td>
<td>transforming growth factor-beta</td>
</tr>
<tr>
<td>Section</td>
<td>Page</td>
</tr>
<tr>
<td>-------------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>Abstract</td>
<td>ii</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>iii</td>
</tr>
<tr>
<td>List of Abbreviations</td>
<td>iv</td>
</tr>
<tr>
<td>List of Tables</td>
<td>x</td>
</tr>
<tr>
<td>List of Figures</td>
<td>xi</td>
</tr>
<tr>
<td><strong>Chapter One: INTRODUCTION</strong></td>
<td>1</td>
</tr>
<tr>
<td>1.1 CLINICAL PROBLEM</td>
<td>1</td>
</tr>
<tr>
<td>1.1.1 Burns</td>
<td>1</td>
</tr>
<tr>
<td>1.1.2 Chronic Wounds</td>
<td>2</td>
</tr>
<tr>
<td>1.2 DERMAL SUBSTITUTE ENGINEERING</td>
<td>2</td>
</tr>
<tr>
<td>1.3 HYPOTHESES AND OBJECTIVES</td>
<td>4</td>
</tr>
<tr>
<td><strong>Chapter Two: BACKGROUND</strong></td>
<td>6</td>
</tr>
<tr>
<td>2.1 ANATOMY OF SKIN</td>
<td>6</td>
</tr>
<tr>
<td>2.1.1 Dermal extracellular matrix</td>
<td>6</td>
</tr>
<tr>
<td>2.1.1.1 Collagen</td>
<td>7</td>
</tr>
<tr>
<td>2.1.1.2 Hyaluronan</td>
<td>8</td>
</tr>
<tr>
<td>2.1.1.3 Heparan sulfates</td>
<td>9</td>
</tr>
<tr>
<td>2.1.2 Comparison of pig and human skin</td>
<td>10</td>
</tr>
<tr>
<td>2.2 CELL-MATRIX INTERACTIONS IN WOUND HEALING</td>
<td>11</td>
</tr>
<tr>
<td>2.2.1 Stages of wound healing</td>
<td>11</td>
</tr>
<tr>
<td>2.2.2 Inflammation</td>
<td>12</td>
</tr>
<tr>
<td>2.2.3 Angiogenesis</td>
<td>12</td>
</tr>
<tr>
<td>2.2.4 Epithelialization</td>
<td>13</td>
</tr>
<tr>
<td>2.2.5 Fibroplasia</td>
<td>13</td>
</tr>
<tr>
<td>2.2.6 Wound contraction</td>
<td>16</td>
</tr>
<tr>
<td>2.2.7 Summary</td>
<td>18</td>
</tr>
<tr>
<td>2.3 HOST RESPONSE TO BIOMATERIALS</td>
<td>19</td>
</tr>
<tr>
<td>2.3.1 Foreign body response</td>
<td>19</td>
</tr>
<tr>
<td>2.3.2 Biomaterial integration into the wound</td>
<td>19</td>
</tr>
<tr>
<td>2.4 EXTRACELLULAR MATRIX-DERIVED BIOMATERIALS</td>
<td>20</td>
</tr>
<tr>
<td>2.4.1 Collagen</td>
<td>21</td>
</tr>
<tr>
<td>2.4.1.1 Immunogenicity</td>
<td>21</td>
</tr>
<tr>
<td>2.4.1.2 Structure modification</td>
<td>23</td>
</tr>
<tr>
<td>2.4.2 Hyaluronan</td>
<td>23</td>
</tr>
<tr>
<td>2.5 DERMAL SUBSTITUTE DESIGN</td>
<td>24</td>
</tr>
<tr>
<td>2.5.1 Human acellular dermal matrix</td>
<td>25</td>
</tr>
<tr>
<td>2.5.2 Pig acellular dermal matrix</td>
<td>26</td>
</tr>
<tr>
<td>2.5.2.1 Pig dermis as a wound dressing</td>
<td>26</td>
</tr>
<tr>
<td>2.5.2.2 Immunogenicity and vascularization</td>
<td>27</td>
</tr>
<tr>
<td>2.5.2.3 Development of current acellularization protocol</td>
<td>28</td>
</tr>
<tr>
<td>2.5.2.4 Acellular pig dermal matrix as a dermal substitute</td>
<td>29</td>
</tr>
<tr>
<td>2.5.2.5 Cell seeding of acellular pig dermis</td>
<td>31</td>
</tr>
</tbody>
</table>
3.3.4 Collagen synthesis ................................................. 61
3.3.5 Cell infiltration ..................................................... 62
3.3.6 Myofibroblast differentiation .................................... 62
3.3.7 Statistics ............................................................. 63

Chapter Four: RESULTS AND DISCUSSION .......................... 64
4.1 PIG ACELLULAR MATRIX CHARACTERIZATION ................. 64
  4.1.1 Acellularity ....................................................... 64
    4.1.1.1 Pig ACM .................................................... 64
    4.1.1.2 Human ACM .............................................. 68
  4.1.2 Acellular matrix structure ...................................... 70
    4.1.2.1 Pig dermal ACM versus pig dermis .................... 70
    4.1.2.2 Pig ACM versus human ACM .......................... 74
  4.1.3 Summary .......................................................... 79

  4.2 FIBROPLASIA IN A PIG ACELLULAR DERMAL MATRIX ......... 81
    4.2.1 Cell morphology .............................................. 81
    4.2.2 Cell proliferation ............................................ 84
      4.2.2.1 MTT assay ............................................ 84
      4.2.2.2 CyQuant assay ....................................... 86
    4.2.3 Collagen synthesis ........................................... 89
    4.2.4 Cell infiltration .............................................. 91
    4.2.5 Comparison of pig ACM to human ACM as a scaffold ...... 93
      4.2.5.1 Time in culture ..................................... 97
      4.2.5.2 Level of dermis ..................................... 97
      4.2.5.3 Freeze-drying ....................................... 99
    4.2.6 Myofibroblast differentiation ................................ 100
    4.2.7 Contraction .................................................. 103
    4.2.8 Summary ...................................................... 109

  4.3 FIBROPLASIA IN GLYCOSAMINOGLYCAN-ENRICHED PIG ACELLULAR DERMAL MATRIX .... 111
    4.3.1 Glycosaminoglycan incorporation ................................ 111
    4.3.2 Glycosaminoglycan quantification ................................ 113
    4.3.3 Cell proliferation ............................................ 118
    4.3.4 Collagen synthesis ........................................... 119
    4.3.5 Cell infiltration .............................................. 120
    4.3.6 Myofibroblast differentiation ................................ 124
    4.3.7 Summary ...................................................... 125

Chapter Five: CONCLUSIONS AND RECOMMENDATIONS ............ 127
5.1 CONCLUSIONS ........................................................ 127
  5.1.1 Acellular pig dermal matrix as a model of cell-matrix interactions ... 127
  5.1.2 Acellular pig dermal matrix as a dermal substitute .......................... 128
  5.1.3 Hyaluronan and heparin as dermal substitute components .................. 130

      5.2 RECOMMENDATIONS ............................................. 131
        5.2.1 Dermal substitute development ................................ 131
        5.2.2 In vitro modeling ........................................ 132

      5.3 SUMMARY ........................................................ 133

REFERENCES .................................................................. 135

APPENDIX
Culture of human dermal microvascular endothelial cells on pig ACM

A-1
List of Tables

Table 1. Hypotheses and objectives.................................................................5
Table 2. Design requirements of wound dressings and skin substitutes in the treatment of full-thickness wounds.........................................................25
Table 3. Areas of cell-matrix interactions in cutaneous wound healing studied in 2D......33
Table 4. Acellularized pig and human dermis batches used in experiments.................41
Table 5. Acellularization technique for pig and human dermis............................41
Table 6. ACM sterilization technique ..................................................................41
Table 7. Antibodies used for immunohistochemical staining of the ACM..................42
Table 8. Donor information for primary human dermal fibroblast harvesting ..........47
Table 9. Summary of cell proliferation experiments for CyQuant assay...............50
Table 10. Cell infiltration experiments................................................................54
Table 11. Cell infiltration experiments continued ..............................................54
Table 12. Levels of harvested dermis, defined by depth from surface ...................55
Table 13. Summary of contraction experiments..................................................57
Table 14. Effect of diffusion on the quantity of HA and HP in ACM over time.........61
Table 15. Effect of HA or HP on fibroblast proliferation in PACM.........................61
Table 16. Structural properties of pig and human dermal ACM PACM....................78
Table 17. Comparison of human fibroblast infiltration in fresh pig ACM, freeze-dried pig ACM and freeze-dried human ACM...........................................93
Table 18. Distance of cells from monolayer surface, in representative fields of vimentin-stained sections of cell-seeded ACM samples at 3 weeks.........................94
Table 19. Effect of culture time on primary human fibroblast infiltration into pig ACM...97
Table 20. Effect of dermal level on fibroblast infiltration of pig ACM .....................98
Table 21. Effect of freeze-drying on fibroblast infiltration of pig ACM....................99
Table 22. Expression of α-smooth muscle actin by fibroblasts cultured on pig ACM after different times in culture .........................................................101
Table 23. Percentage of fibroblasts on each ACM type expressing α-smooth muscle actin (SMA) at 2 weeks.................................................................102
Table 24. Effect of ACM thickness on fibroblast-mediated contraction..................107
Table 25. Evaluation of GAG incorporation methods...........................................115
Table 26. Effect of HA and HP on distance of fibroblast migration from the cell-seeded surface into the pig ACM.....................................................124
Table 27. Effect of HA and HP on fibroblast smooth muscle actin expression at 2 weeks. 124
Table 28. Comparison of pig ACM with existing in vitro models of fibroplasia.........130
**List of Figures**

**Figure 1.** Structural levels of collagen ................................................................. 7
**Figure 2.** Chemical structure of hyaluronan ............................................................... 9
**Figure 3.** Chemical structure of heparin ................................................................. 9
**Figure 4.** Temporal sequence of wound healing in adult humans .............................. 12
**Figure 5.** Histological processing of an ACM sample ................................................ 53
**Figure 6.** Diagram of ACM and HA in centrifuge filter tube for HA incorporation .... 58
**Figure 7.** Pig dermal ACM, H&E, 10x ..................................................................... 65
**Figure 8.** Pig ACM, stained for pan keratin, 10x ...................................................... 65
**Figure 9.** Pig dermal ACM and dermis stained for vimentin, 10x ......................... 67
**Figure 10.** Pig ACM and dermis staining with smooth muscle actin, 10x .............. 67
**Figure 11.** Pig ACM stained for smooth muscle actin, showing non-specific background staining, 20x .............................................................. 68
**Figure 12.** Human fibroblast-seeded pig ACM, smooth muscle actin stain, with hematoxylin counter-stain, 20x and 40x .............................................................. 68
**Figure 13.** Human dermal ACM acellularity with different stains .......................... 69
**Figure 14.** Pig ACM, Movats stain, 10x .................................................................. 70
**Figure 15.** A comparison of pig ACM and pig dermis by scanning electron microscopy ... 71
**Figure 16.** Pig ACM by transmission electron microscopy (16,000x) ..................... 72
**Figure 17.** Pig ACM structure under different conditions, by histology ................... 73
**Figure 18.** Pig ACM stained with alcian blue ......................................................... 74
**Figure 19.** Gross appearance of pig and human dermal ACM ................................. 74
**Figure 20.** Histological comparison of pig and human dermal ACM collage matrix structure ........................................................................................................... 75
**Figure 21.** Pig and human ACM, scanning electron micrographs of surface structure of tangentially cut surface, 500x ................................................................. 77
**Figure 22.** Pig and human ACM, freeze-fracture scanning electron micrographs of ACM in cross-section, 100x ................................................................. 77
**Figure 23.** Confocal microscopy of passaged human dermal fibroblasts, 10x ........... 82
**Figure 24.** Confocal microscopy of passaged human dermal fibroblasts, 63x .......... 83
**Figure 25.** Pig ACM without cells, after four hour incubation with MTT and extraction with isopropanol .............................................................. 85
**Figure 26.** Calibration curves with the MTT assay of passaged human dermal fibroblasts in the presence and absence of ACM ......................................................... 85
**Figure 27.** Passaged human dermal fibroblast viability on different substrates over time, by the MTT assay ................................................................. 86
**Figure 28.** Calibration curves with the CyQuant assay of human dermal fibroblasts in the presence and absence of digested ACM ......................................................... 87
**Figure 29.** Fibroblast proliferation on different substrates as determined by the CyQuant assay ................................................................. 88
**Figure 30.** Protein synthesis by human fibroblasts seeded on different substrates .... 89
**Figure 31.** Percentage of radiolabeled-proline incorporated into newly synthesized collagen by human fibroblasts cultured on different substrates ............................. 90
Chapter One: INTRODUCTION

1.1 Clinical Problem
In many clinical situations encountered in plastic surgery, it is necessary to replace lost skin. Clinically, the epidermis can be easily replaced. Thin split-thickness skin grafts are commonly transplanted to deliver intact epidermis to the wound. Alternatively, in the absence of available donor skin, the patient's keratinocytes can be expanded in culture to form a multilayered, cultured epidermal autograft (CEA). However, the epidermis represents only a small percentage of normal skin. The dermis is responsible for the skin's strength and durability, as well as for scar formation in cutaneous wounds. Despite the functional importance of the dermis, dermal replacement presents an ongoing challenge in the treatment of full thickness wounds (i.e. burns and chronic wounds).

1.1.1 Burns
In North America, approximately 107,000 people are victims of severe burn injury every year. The cost of their treatment is estimated to be twice that of other hospitalized patients (Dimick, Potts et al., 1986). The variables most commonly related to length of care for burn injury are: size of the burn, patient age, inhalation injury, infection and the length of time the wounds are left open (Bowser, Caldwell et al., 1983). In addition, a prolongation of the time before wound debridement and healing can foster complications such as pneumonia and multiple organ failure (Demling and Lalonde, 1990), (Stratta, Warden et al., 1986), as well as increases the risk of hypertrophic scar formation (Deitch, Wheelahan et al., 1983). A particular challenge in the area of burns lies therefore in the inadequate availability of autograft donor sites. It has become increasingly necessary to seek a solution in the use of temporary or permanent skin substitutes.
1.1.2 Chronic Wounds

For chronic wounds, which may take months or even years to close, the impact on patients' quality of life during this time is significant. Furthermore, systemic complications such as septicemia, anemia and failure to thrive, as well as the enormous costs of nursing care, antibiotics, and specialized care, are additional frustrations in the management of chronic skin wounds. In the case of wounds such as these, the healing process itself is impaired for a variety of underlying reasons. As a result, skin grafting often fails to achieve the desired level or quality of wound closure. A growing area of research is concerned with designing skin substitutes specifically to enhance the healing process in chronic wounds.

Further to the acute problems associated with full thickness wounds, keloids, hypertrophic scars and contractures in inadequately treated wounds can lead to significant functional and cosmetic morbidity in the long term. Effective dermal replacement can play a major role in improving the final scarring outcome.

1.2 Dermal Substitute Engineering

Dermal replacement allows the skin's cosmetic and protective functions to be restored. Specifically, dermal substitutes may be designed to (1) replace lost tissue, (2) accelerate healing and (3) reduce scarring, thereby decreasing the personal and financial costs of full thickness wounds. The field of dermal substitute engineering encompasses both materials science and cell biology. The term tissue engineering refers to the development and application of materials which maintain, restore or improve tissue function (Langer and Vacanti, 1993). These biomaterials may be naturally occurring or synthetic materials. Naturally occurring materials have a number of advantages in skin engineering applications, as we have learned from the success of cadaveric allograft dermal transplants. Unfortunately the supply of allograft (human source) materials can be limited. An attractive alternative lies in the use of xenograft (non-human source) biological materials, given their greater availability and frequent homology with human tissues.
For each of these approaches, it is important to appreciate the influence of the materials themselves on cell behaviour in wound healing. Specific healing processes occur in the presence of a foreign material or device in the body. The ideal dermal substitute biomaterial would not only minimize a foreign body reaction, but also modulate other wound healing events. Candidates for biologically active, natural materials are largely found in the extracellular matrix. Many matrix components have been shown to beneficially influence wound healing in specific clinical situations (Roy, DeBlois et al., 1993), (Harris, di Francesco et al., 1999), (Dawson, Goberdhan et al., 1996).

A thorough understanding of the manner in which the ultrastructure of the extracellular matrix modulates function, and its applications in wound healing, is fundamental to tissue engineering. It is also necessary to understand the effect of different matrix components on the various and precise responses in the wound matrix environment. After the initial inflammatory phase, wound fibroblasts are the dermal cells predominantly responsible for the regeneration and remodeling of skin. The specific effects on fibroblasts, of acellular pig dermal matrix and the glycosaminoglycans hyaluronan and heparin, are of the focus of this thesis.

Acellularized pig dermal matrix is evaluated as a biomaterial for two possible applications. First, it is investigated as a scaffold for modeling dermal fibroplasia in vivo. By isolating particular interactions during wound healing, in vitro experiments attempt to explain the mechanism of events observed in vivo. Acellular pig dermal matrix offers a useful opportunity to investigate how fibroblasts are influenced by dermal collagen in its original architecture. This material would be expected to be more representative of human dermis than the current models of fibroplasia, particularly in terms of contraction. As well, this novel material is used here to study the effects of matrix glycosaminoglycans on fibroplasia in a dermal matrix. It is hoped that, in this way, specific matrix molecules could be evaluated as potential dermal substitute components. Hyaluronan may potentially facilitate cell infiltration into an acellular dermal matrix, while heparin has pro-angiogenic effects.
Second, the \textit{in vitro} methods in this study are intended to complement \textit{in vivo} research on acellular pig dermal matrix as a xenograft dermal substitute. Acellularized pig dermis is currently not used as a dermal substitute in humans. There are a number of possible limitations with its use, which have not been fully elucidated. The acellular xenograft dermis may elicit a significant immune response, causing delayed take and chronic inflammation. Alternatively, the structure of xenograft dermis may not provide the appropriate microenvironment to support host cell infiltration. Another option is that both these factors are at play in the limited clinical effectiveness of acellular xenograft dermal substitutes.

This study focuses on the scaffold properties of and fibroblast responses to acellular pig dermal matrix. By using simplified \textit{in vitro} conditions, these fibroblast-matrix interactions can be studied in isolation from any inflammatory or immune-mediated effects. The results of this study may therefore help sort out the relative contribution of the material’s structure to its effectiveness as a dermal replacement. Immunogenicity of the material will be examined in a separate study, either under \textit{in vitro} conditions or in an animal model. To evaluate its potential both as a dermal substitute and as an \textit{in vitro} model, acellular pig dermis will be compared to human dermis in terms of its matrix structure and fibroblast-matrix interactions.

\subsection*{1.3 Hypotheses and Objectives}

The overall objective of this project is to evaluate the acellular pig dermis produced in our laboratory, as a scaffold for human fibroblasts \textit{in vitro}. Where the results of this project may be relevant to the development of a xenograft dermal substitute, it is hoped they will, as well, lay the groundwork for an \textit{in vitro} model of dermal cell-matrix interactions, including contraction (Table 1).
<table>
<thead>
<tr>
<th>Hypothesis</th>
<th>Structure</th>
<th>Objectives</th>
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<tbody>
<tr>
<td>Pig and human dermis may be acellularized without significant modification of the collagen matrix structure</td>
<td>• To acellularize pig and human dermis</td>
<td>• To evaluate acellularity and matrix structure</td>
</tr>
<tr>
<td>Acellular pig dermis is equivalent to acellular human dermis as a three-dimensional scaffold for human fibroblast culture</td>
<td>• To compare acellular pig dermis to acellular human dermis and bovine collagen gel as a scaffold for human fibroblasts.</td>
<td>• To characterize aspects of \textit{in vitro} fibroplasia in acellular pig and human dermal matrices: - Fibroblast morphology - Proliferation - Collagen synthesis - Infiltration of matrix - Myofibroblast differentiation</td>
</tr>
<tr>
<td>The glycosaminoglycans hyaluronan and heparin influence fibroplasia in a dermal matrix, as demonstrated using acellular pig dermis as a model</td>
<td>• To develop methods for incorporating and quantifying hyaluronan and heparin into the pig dermal matrix</td>
<td>• To evaluate the effects of hyaluronan and heparin in the matrix on fibroblast proliferation, infiltration, differentiation and collagen synthesis</td>
</tr>
<tr>
<td>Acellular pig dermis may be used as an \textit{in vitro} model for studying the mechanisms of fibroblast-mediated contraction of the dermis</td>
<td>• To determine \textit{in vitro} conditions that will allow fibroblast-mediated contraction of the ACM to occur</td>
<td>• To compare acellular pig dermis with bovine collagen gel as a model of contraction</td>
</tr>
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Table 1. Hypotheses and objectives of this project.
Chapter Two: BACKGROUND

2.1 Anatomy of Skin
The skin is a highly specialized bilaminate structure which serves as an organ of protection to the body. It controls the invasion of microorganisms, regulates fluid loss or infiltration, monitors temperature, protects against injury from radiation and electricity and provides immunologic surveillance (Wilkins, Chung et al., 1996). Human skin is generally 1.2 mm thick but varies from 0.5 to 6 mm. The outer highly cellular epidermal layer measures 0.06 to 0.8 mm in thickness and is in contact with the dermis through irregular interpapillary ridges and grooves. The dermis is 20 to 30 times thicker than the epidermis (Wilkins, Chung et al., 1996). While the dermis also contains the nervous, vascular, lymphatic and epidermal appendage structures, the functions of the dermis are mainly attributable to its extracellular matrix.

2.1.1 Dermal extracellular matrix
The dermal extracellular matrix contains fibrous and nonfibrous matrix molecules. The fibrous collagen and elastin proteins impart bulk, density and tensile properties to skin and also allow for pliancy and elasticity. The papillary and reticular dermis comprise the two main dermal zones. The papillary dermis is only slightly thicker than the epidermis. It is composed primarily of type III collagen. The collagen in the reticular dermis, of which the majority of the dermis is composed, is primarily type I. In addition, the blood vessels, pilosebaceous units, eccrine and apocrine glands of the dermis are encircled by a thin meshwork of type III collagen fibres similar to those present in the papillary dermis (Junqueira, Montes et al., 1983).

The elastic fibres of the dermis are much finer than its collagen fibres. The function of elastin fibres is to restore the collagen network to its relaxed condition after deformation (Elder, Elenitsas et al., 1997). The elastic network is in part responsible for natural skin tension. The nonfibrous matrix molecules form the ground substance, which influences the osmotic properties of the skin, promotes cellular migration in a more fluid milieu,
and serves as a continuous medium for the other matrix elements (Mac Neil, 1994). The ground substance includes glycosaminoglycans, proteoglycans and certain glycoproteins.

### 2.1.1.1 Collagen

Type I collagen is the predominant protein within adult pig and human dermal extracellular matrices. Collagen's structure is comprised of a hierarchy (primary, secondary, tertiary and quaternary) of levels (Figure 1). The primary structure denotes the amino acid sequence along each of three polypeptide chains, as well as the location of interchain cross-links in relation to this sequence. Mature collagen features more cross-linking than newly synthesized collagen (Yannas, 1996). The secondary structure refers to the local configuration of a polypeptide chain. This configuration is dictated by the stereochemistry requirements and hydrogen-bonding potential of the amino acids. The structure of type I collagen is characterized by the frequent repetition of the amino acid triplet Gly-X-Y, where X and Y are frequently proline or hydroxyproline, respectively. This sequence enables the polypeptide chain to form a tightly coiled left-handed helix, or α chain.

<table>
<thead>
<tr>
<th>Primary: Amino acid sequence</th>
<th>Secondary: Configuration of polypeptide chain</th>
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<tr>
<td>...-Gly-Pro-Hyp-Gly-Ala-...</td>
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<table>
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<tr>
<th>Tertiary: Triple helix</th>
<th>Quaternary: Formation of microfibrils and collagen fibre</th>
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![Figure 1. Structural levels of collagen.](image)

The tertiary structure refers to the global configuration of the polypeptide chains in space. For type I collagen, three helical α chains pack together to form a triple helix. The absence of side chains in the abundant glycine residues in type I collagen permits
the close alignment of polypeptide chains in a triple helix arrangement. The peptide bonds linking adjacent amino acids are oriented towards the interior of the helix, rendering the triple-helix highly resistant to general protease attack. The quaternary structure denotes the repeating supermolecular unit structure, comprising several molecules packed in a specific lattice, i.e. a microfibril. Adjacent molecules in the microfibril are approximately parallel to the fibre axis; they all point in the same direction along the fibre and are staggered regularly. This gives rise to the well-known D-period of about 64 nm, which is visible by transmission electron microscopy (Linsenmayer, 1991).

Collagen synthesis is a complex, multi-step progression through each of these four levels. The collagen molecules acquire their primary, secondary and tertiary structures intracellularly, and are secreted as triple-helical, procollagen molecules. Extracellular processing involves proteolytic cleavage of terminal propeptides as well as self-assembly into microfibrils and fibres. Collagen fibres become further stabilized by interfibre cross-linking of lysine residues by the enzyme lysyl oxidase (Linsenmayer, 1991). Type I collagen is characterized by its stability as a molecule; this stability is enhanced over time by the ongoing formation of intermolecular cross-links in mature collagen.

2.1.1.2 Hyaluronan

Hyaluronan (HA) is a linear polysaccharide of up to 25,000 disaccharide units composed of D-glucuronic acid and N-acetyl-D-glucosamine (Figure 2). Its high molecular mass and numerous mutually repelling anionic carboxyl groups on the glucuronic acid residues make HA a rigid and hydrated molecule. Accordingly, HA occupies a volume in solution approximately 1000 times greater than in its dry state (Voet and Voet, 1990). Besides providing structural support within the extracellular matrix, HA maintains water homeostasis of the tissues. Even at low concentrations, the individual HA chains entangle and form a continuous network in the solution, causing a pronounced viscoelasticity (Laurent, Fraser et al., 1986). HA is found in variable amounts in all tissues and fluids of adult animals and is especially abundant in soft connective tissues (Alberts, Bray et al., 1994). HA occurs bound to plasma membranes, aggregated with other macromolecules, or as a free polysaccharide (Laurent, Fraser et al., 1986).
Figure 2. Chemical structure of hyaluronan. Abbreviations: Ac = acetyl group.

2.1.1.3 Heparan sulfates
The heparan sulfates, composed of heparin and heparan sulfate, are both sulfated glycosaminoglycans composed of glucuronic acid or iduronic acid and N-acetylglucosamine with an α1,4 linkage (Wight, Heinegard et al., 1991). The degree of sulfation differs between heparin and heparan sulfate; less than 50% of the N-acetyl groups in heparan sulfate are sulfated, while more than 70% are sulfated in heparin (Figure 3) (Wight, Heinegard et al., 1991). Heparin is the major intracellular heparan sulfate, found within mast cells and basophils. Commercial heparin, clinically used as an anticoagulant, is a degradative product of mast cell heparin.

Figure 3. Chemical structure of heparin, indicating the target side groups for sulfation.

Heparin is released from mast cells and basophils in early wounds and has a number of important effects on cells during wound healing (Section 2.2). Heparin and other sulfated glycosaminoglycans (GAGs) anchor soluble proteins in the matrix by their highly anionic sulfate groups. These soluble proteins may be growth factors, proteinases or proteinase inhibitors. It is not clear whether GAGs act as reservoirs to keep these growth factors in the vicinity of the cells, help prevent their proteolytic breakdown or actually present the growth factors to the cells in a more efficient manner. However, the association between small soluble growth factors and these extracellular matrix molecules is effective in modulating cell behaviour (Roy, DeBlois et al., 1993).
2.1.2 Comparison of Pig and Human Skin

The pig is widely used in research as a representative model of human wound healing (Sullivan, Eaglstein et al., 2001). Given the many morphological similarities between pig and human skin, acellularized pig skin may be a suitable, less expensive alternative to human dermis for dermal replacement. There are many similarities between the morphological and functional characteristics of pig and human skin. These features include relatively sparse hair, a thick epidermis with distinct rete pegs and corresponding dermal papillae, as well as similarities in the structure of the collagenous tissue framework and the adipose chambers of the subcutis (Meyer, Schwarz et al., 1978). Pig dermal collagen is similar to human dermal collagen biochemically, accounting for its use in a number of wound healing products (Heinrich, Lange et al., 1971). There are significant parallels in the composition of the lipid film of the skin surface, and both similarities in epidermal tissue turnover time and the character of keratinous proteins (Meyer, Schwarz et al., 1978). Like human dermis, pig dermis has a well-defined papillary component, consisting of delicate collagen fibres intermingled with connective tissue cells. This layer is continuous with the broad reticular dermis, which shows a well-developed interlacing of connective tissue fibres.

There are also several differences to note between pig and human dermis. In contrast to man, the elastic fibre content of pig dermis is relatively low but is still higher than in other species (Meyer and Neurand, 1987). As well, pig hair is coarser than human hair (Hinrichsen, Birk-Sorensen et al., 1998). The subcutis of the pig is similar to that of the human, with some differences. In the most superficial layer, the stratum adiposum subcutis, elastic and collagen fibers show pocket formation in which adipose tissue is deposited abundantly, much like in humans. The deeper stratum fibrosum subcutis of the pig is represented by a connective tissue sheet which, in contrast to man, contains the panniculus carnosus (Rose, Vistnes et al., 1978). The pattern of vascularization in the pig displays subepidermal, lower and mid-dermal networks. It differs from man in that the sub-epidermal network is less dense in the pig. However the vascularization of the lower region of the hair follicle including the hair papilla, parallels that in humans. Pig skin contains no eccrine glands, and unlike man, apocrine glands are distributed through the skin surface (Meyer, Schwarz et al., 1978).
Given the success of acellular human dermis as a dermal substitute (Section 2.5.1), the similarities between pig and human dermis suggest that acellular pig dermis may too have potential as a dermal substitute. It is hypothesized in this thesis that acellular pig dermis is equivalent to acellular human dermis as a scaffold for human fibroblasts in vitro.

2.2 Cell-Matrix Interactions in Wound Healing

For all stages of wound healing, cellular responses are to a large extent dictated by their interaction with the surrounding extracellular matrix. Cells are found to bind to extracellular matrices as a means of anchoring themselves to the scaffold and to derive traction for migration (Martin, 1997). Ongoing research into the structure and composition of the extracellular matrix now strongly indicates that a major role of the matrix is to regulate cell behaviour, rather than to passively support cells. Matrices also impart signals for growth and differentiation. Extracellular matrix components involved in scar regulation and neovascularization, in particular, are desirable as dermal substitute materials. This section will highlight some of the ways in which the cell-matrix interactions of collagen, hyaluronan and proteoglycans can influence specific wound healing events.

2.2.1 Stages of wound healing

The healing process can be broken down into early, intermediate, late and terminal phases (Figure 4). Specific biologic processes characterize each phase. The primary activities involved in the early phase of healing are hemostasis and inflammation. Mesenchymal cell proliferation and migration, epithelialization and angiogenesis are the primary events of the intermediate phase. In the late phase (after one week), the synthesis of collagen and other matrix proteins begin. As well, wound contraction can be observed from this point on. The terminal phase of wound healing is characterized by wound remodeling to form a scar. The role of the fibroblast in wound healing will be summarized here, as well as the influence of the extracellular matrix on cellular responses in wound healing.
Figure 4. Temporal sequence of wound healing in adult humans.

2.2.2 Inflammation
After hemostasis has been accomplished, inflammatory cells such as neutrophils migrate out of leaky blood vessels and into the wound. This chemotaxis is achieved through cell-cell signaling via cytokines, as well as by cell-matrix interactions with the provisional fibrin matrix (Tuan, Song et al., 1996). As monocytes migrate from the capillaries into the extravascular space, they transform into macrophages by a process mediated by serum factors and fibronectin (Wilkins, Chung et al., 1996). Macrophages are a primary source of cytokines that stimulate fibroblast proliferation, collagen production and other healing processes. Structural molecules of the early extracellular matrix (such as fibrin, fibronectin and hyaluronan) provide a scaffold to guide cell migration. Hyaluronan in particular facilitates cell mobility, and proteoglycans act as a reservoir for cytokines. Furthermore, hyaluronan has been shown to affect the activity of neutrophils by modulating adherence and chemotactic responsiveness (Forrester and Wilkinson, 1981), (Forrester and Lackie, 1981).

2.2.3 Angiogenesis
Angiogenesis reconstructs the vasculature where it has been damaged by wounding. Endothelial cell migration and tube formation are facilitated by changes in the
extracellular matrix. The synthesis and degradation of type I and type IV collagen surrounding the capillary sprouts are tightly regulated to provide support and guidance to the newly formed capillaries (Clark, 1996). Cytokines directly and indirectly stimulate endothelial cell migration and proliferation. Many angiogenic cytokines have been identified, and include basic fibroblast growth factor (bFGF), of which heparin is a co-factor, as well as vascular endothelial growth factor and transforming growth factor-β (Wilkins, Chung et al., 1996). The pro-angiogenic effects of heparin are believed to be attributable to its delivery of bFGF and acidic FGF to the cells (Mueller, Thomas et al., 1989), (Folkman and Sking, 1992), but this has not been conclusively shown (Sasisekharan, Moses et al., 1994). Heparin has been investigated in cardiovascular applications for its pro-angiogenic properties, which have been shown to be molecular weight-dependent (Bombardini and Picano, 1997). The degradative products of hyaluronican, specifically fragments 4 to 25 disaccharides in length, also have angiogenic properties (Cockerill, Gamble et al., 1995).

2.2.4 Epithelialization
The sequence of events which gives rise to epithelialization include cellular detachment, migration, proliferation and differentiation. Marginal basal cells elongate, detach from the underlying basement membrane and migrate into the wound. Without collagenolytic activity, keratinocytes do not migrate within a dermal collagen matrix (Pilcher, Sudbeck et al., 1998). It is hypothesized that keratinocytes use collagenase-1 to cleave collagen to gelatin, thereby providing a substrate which is more conducive to migration. It is believed that altered cell-matrix interactions in wound healing regulate the spatially precise pattern of collagenase production in migrating keratinocytes (Clark, 1996). Evidence shows that keratinocytes lose contact with the basement membrane and instead bind to the provisional matrix and the type I collagen-rich dermal matrix. This transfer is a critical determinant of increased collagenase production, and initiation of migration (Pilcher, Sudbeck et al., 1998).

2.2.5 Fibroplasia
Fibroplasia denotes fibroblast cellular activities during tissue repair. These include migration, proliferation, differentiation, organization and degradation of the transient
Matrices, and synthesis of the new matrices. In this study, each of these aspects of fibroplasia is demonstrated in isolation in vitro. The in vivo relevance of each aspect of fibroplasia, and the role of cell-matrix interactions in its regulation, will be reviewed here and in Sections 2.2.6 and 2.3.2.

In response to injury, resident dermal fibroblasts near the edge of the wound first proliferate. They then migrate into the fibrin matrix of the wound clot in a tightly regulated manner, where early matrix changes facilitate fibroblast migration and differentiation. Once the initial fibrin matrix is cleared by proteolysis, invading fibroblasts deposit a second provisional matrix rich in fibronectin and hyaluronan (Martin, 1997). In open cutaneous wounds, hyaluronan content in the granulation tissue increases until day 5, falls until day 10, then remains fairly constant. HA appears to promote cell movement, as supported by the concomitant occurrence of HA and cell migration during both tissue repair and organ generation. During regeneration and morphogenesis, HA is present during cell movement and mitosis, and disappears at the onset of differentiation (Ueno, Chakrabarti et al., 1992).

There are at least three possible ways in which hyaluronan influences cell motility. First, HA may facilitate adhesion-disadhesion between the cell membrane and the matrix substratum during cell movement (Clark, 1996). Second, since hyaluronan becomes extremely hydrated, the expanded interstitial space at sites of deposition might allow more cell recruitment and proliferation in these areas (Docherty, Forrester et al., 1989). Finally, specific cell surface receptors exist for hyaluronan, and cell movement into hyaluronan-rich areas is likely to be mediated, in part, by such transmembrane proteins (Turley, Bowman et al., 1985). In addition to evidence that hyaluronan may facilitate cell movement, several lines of investigation suggest that hyaluronan may instigate cell division. Hyaluronan receptors are preferentially expressed on proliferating epithelial cells, suggesting that proliferating cells may be specifically interactive with HA (Clark, 1996).

Approximately a week after wounding, the wound clot will have been fully invaded and all but replaced by activated fibroblasts. These cells are stimulated by TGF-β and other
growth factors to synthesize and remodel a new collagen-rich matrix (Martin, 1997). The rate of collagen synthesis increases rapidly and continues at an accelerated rate for 2 to 4 weeks. Collagen makes up more than 50% of the protein found in scar tissue. After four weeks, collagen synthesis rates decline, eventually balancing the rate of collagen destruction by collagenase (Wilkins, Chung et al., 1996).

The dynamic between extracellular matrix and fibroblasts is especially evident during granulation tissue development. It has been shown that the provisional matrix promotes an early granulation tissue fibroblast phenotype. In contrast, collagen matrix promotes a relatively quiescent dermal fibroblast phenotype. Extracellular matrix fibrils strongly influence the direction of fibroblast migration, since the cells tend to align and migrate along discontinuities in substrata to which they are attached (Clark, 1996). Type I collagen appears to cause directed migration of cells through this process (Postlethwaite, Seyer et al., 1978). Thus chemotactic, adhesion and contact guidance signals may all influence fibroblast migration into the provisional matrix.

The effects of extracellular matrix collagen on fibroplasia are mediated in part through activation of the integrin collagen receptors α1β1 and α2β1. Collagen matrices reduce fibroblast proliferation and collagen synthesis, but induce procollagenase and α2β1 integrin expression (Mac Neil, 1994). It is likely that the collagen-rich extracellular matrix—which accumulates as granulation tissue matures—at the same time reduces the ability of wound fibroblasts to produce further collagenous matrix, and promotes the ability of these cells to remodel the collagen-rich matrix already present (Grinnell and Lamke, 1984). While the specificity of the cell-matrix interactions seems to come from the integrin binding, other adhesive matrix molecules also contain sites that can interact with the glycosaminoglycan component of proteoglycans. The binding of cell surface proteoglycans to such sites is likely to also play a regulatory role in cell adhesion (Clark, 1996).

In open cutaneous wounds the level of galactosaminoglycans, i.e. chondroitin-4-sulfate and dermatan sulfate, begins to increase at approximately one week post-wounding
Collagen and the sulfated galactosaminoglycans replace hyaluronan, forming a matrix of collagen and proteoglycans. These substances provide the tissue with more tensile strength and resilience, but accommodate cell movement and proliferation less well (Clark, 1996). Chondroitin-4-sulfate occurs at high levels in granulation tissue, but not in mature scar. Both collagen synthesis and chondroitin-4-sulfate levels are elevated in hypertrophic scars (Bertheim, 1994). The presence of chondroitin-4-sulfate may therefore facilitate collagen deposition during the matrix formation and remodeling phases of wound healing.

Fibroblast proliferation, migration and collagen synthesis are key steps in successful dermal wound healing. In chronic wounds, these aspects of fibroplasia are often impaired by underlying systemic illness (Lawrence, 1996). Treatments to accelerate dermal healing of chronic wounds may therefore be designed to promote fibroblast proliferation, migration and collagen synthesis. Such an effect would be desirable, in the face of impaired fibroproliferative responses. However, in acute wounds with normal reparative mechanisms (such as a full thickness burn in a healthy person), fibroblast proliferation, migration and collagen synthesis are responsible for scar formation. An effective dermal substitute for acute burns therefore, signals to wound fibroblasts, to down-regulate the fibroproliferative response and thereby achieve optimal scarring.

2.2.6 Wound contraction
Wound contraction is part of normal wound healing, and is referred to as healing by secondary intention. This process, like collagen synthesis, begins approximately 4 to 5 days after wounding. Wound contraction represents the centripetal movement of the wound edge towards the center of the wound. Maximal wound contraction continues for 12 to 15 days, though it will continue for longer periods if the wound remains open. The rate of contraction depends on tissue laxity with great variability among tissues. Wound contraction varies depending on the type of wound (open or incisional), as well as the shape of the wound (Wilkins, Chung et al., 1996). In humans, wound contraction can result in a spectrum of results ranging from wound closure with minimal scar to loss of joint motion and deformation due to contracture.
Since radiation and cytolytic drugs delay contraction, it can be assumed that cellular activity is required in this process. It is now known that contraction is a cell-directed process that requires cell division, not collagen synthesis. The necessity for the functional integrity of the fibroblast cytoskeleton has also been demonstrated (Bell, Ivarsson et al., 1978). Collagen deposition may be involved in fixing the tissues in their final state, however.

Disagreement exists as to the mechanism by which wounds contract. Large numbers of myofibroblasts are found in wounds during wound contraction, and many observers feel these specialized fibroblasts mediate the process. After the infiltration of fibroblasts into the provisional matrix, a proportion of wound fibroblasts differentiate into myofibroblasts. These cells express α-smooth muscle actin and resemble smooth muscle cells in their capacity to generate strong contractile forces (Martin, 1997). Human myofibroblasts grow more slowly than fibroblasts, possess stress fibers and express α-SMA in 20-80% of cells. In contrast, α-SMA is only detected in 10% of human dermal fibroblasts (Moulin, Castillou et al., 1996). All fibroblasts and myofibroblasts contain vimentin, an intermediate filament protein found in the cytoplasm.

This conversion is triggered by growth factors such as TGF-β-1, PDGF and mechanical cues related to the forces resisting contraction (Martin, 1997). Dynamic linkages between the actin bundles within the myofibroblasts and the extracellular matrix are required for wound contraction (Desmouliere and Gabbiani, 1996). Myofibroblasts appear in wounds on the third day post-wounding and persist in large numbers during wound contraction. They are primarily found at the periphery of the wound, giving rise to the theory that they pull the wound edges together in a picture frame fashion (Wilkins, Chung et al., 1996).

The role of the extracellular matrix in regulating wound contraction is the focus of considerable research. Collagen, glycoproteins and proteoglycans are all involved in fibroblast differentiation during wound healing. Most research on the effects of collagen on wound contraction has been done in vivo. Of particular interest is the effect of dermal
collagen thickness (of skin grafts) on fibroblast differentiation. Full thickness skin grafts clearly inhibit myofibroblast differentiation to a greater degree than split thickness skin grafts (Rudolph, 1979). The reasons for this are not fully elucidated. Research in this area is limited by the lack of a life-like in vitro model for studying the relationship between dermal collagen and fibroblast-mediated contraction (Section 2.6).

Hyaluronan has been of particular interest to researchers of scarless fetal wound healing. Hyaluronan is found in high levels in fetal extracellular matrices where wounds heal by marked cell migration and tissue regeneration, and not by contraction and fibrosis (Bertolami, Berg et al., 1992). Experimentally however, the causal link between hyaluronan and wound contraction is controversial. Heparin is able to induce α-smooth muscle actin mRNA and protein expression in some cultured fibroblasts (Desmouliere, Rubbia-Brandt et al., 1992). It is suggested that heparin facilitates the presentation of differentiation or maturation factors present in serum to cell receptors. The role of heparin in wound contraction is also limited by the lack of a reliable in vitro model of dermal contraction.

Increased skin tension contributes to increased scarring (Burgess, Morin et al., 1990). Fibroblast-seeded-collagen gels demonstrate how fibroblasts respond to mechanical stress. As long as the collagen material is under stress, cell proliferation and biosynthetic activity persists. Once mechanical stress is relieved, usually by a combination of wound contraction and biosynthetic activity, cells switch to a non-proliferative phenotype and begin to regress even in the continued presence of growth factors (Grinnell, 1994). Mechanical resistance is also believed to regulate myofibroblast differentiation (Welch, Odland et al., 1990).

**2.2.7 Summary**

The composition and structure of the extracellular matrix regulates many cellular aspects of wound healing. Each phase of wound healing is characterized by temporal changes in matrix composition. The introduction of extracellular matrix-derived biomaterials into the wound may provide an opportunity to modulate cell-matrix interactions in wound healing. Intact dermal collagen is associated with down-regulation of fibroblast-
mediated contraction. Hyaluronan facilitates cell migration, and may play a role in scarless wound healing. Hyaluronan's degradation products, as well as heparin, are pro-angiogenic. The significance of these matrix molecules as components of an acellular dermal matrix, in terms of their effects on fibroplasia, is the focus of this thesis.

2.3 Host Response to Biomaterials
Biomaterials for dermal substitutes may be designed to replace lost tissue or to further alter the host’s healing response. To effectively replace the dermis, the material must become vascularized and infiltrated by the host’s dermal cells, in order to become successfully integrated into the wound. The design of biomaterials must take into account wound healing mechanisms involved in biomaterial integration into the body.

2.3.1 Foreign body response
The occurrence of a foreign body reaction is considered a typical wound healing response to implanted biomaterials (Anderson, 1996). This is typified by the presence of foreign body giant cells and the known components of granulation tissue. Generally, the foreign body reaction results in fibrosis at the surface, which isolates the implant from the host tissue (Yannas, 1996). With biocompatible materials, the foreign body reaction in the implant site may be controlled by the properties of the biomaterial. The degree of fibroproliferative response in most cases parallels the extent of inflammatory response of a material. Materials which elicit a marked inflammatory reaction become quickly walled off with a thick fibrous capsule. For dermal substitute materials, where the goal is to achieve minimal scarring, it is critically important that fibrous capsule formation is prevented. Materials for this application must therefore be specifically designed to modulate fibroblast responses such as fibroblast proliferation and collagen synthesis, and to provoke a minimal inflammatory reaction.

2.3.2 Biomaterial Integration into the Wound
Repair of implanted biomaterials can involve two distinct processes: replacement of the implant by fibrous capsule connective tissue, or regeneration, i.e. the replacement of injured tissue by parenchymal cells of the same type. These processes are generally controlled by (1) the proliferative capacity of the cells in the recipient tissue, and (2) the extent of injury as it relates to the destruction or persistence of the original tissue
The condition of the underlying framework or supporting stroma of the parenchymal cells following an injury plays an important role in the restoration of normal tissue structure. Retention of the framework may lead to restitution of the normal tissue structure, while its destruction most commonly leads to fibrosis (Yannas, 1996).

Biomaterials may be designed as scaffolds for host cell infiltration, thus replacing the supporting framework of the injured tissue. Biomaterials intended for integration must be designed to modulate cellular responses. This is necessary to prevent fibrous capsule formation and its sequestration as an avascular implant in the wound. The material must possess the necessary features for cell infiltration to occur: adequate pore size for cell migration and nutrient diffusion, pro-angiogenic properties, and suitable adhesiveness for migrating cells. A key step in the integration of foreign materials into the wound is the ingrowth of host capillaries. As the material becomes vascularized, the migrating endothelial cells signal to surrounding fibroblasts and macrophages to begin migration into the material (Yannas, 1997). Ideally, the material’s degradation rate is matched to the biosynthetic rates of the infiltrating host cells, thus remodeling a new extracellular matrix. Upon integration of the ideal biomaterial, the typical fibrotic response of wound healing would be down-regulated, so as to instead favour a regenerative response.

2.4 Extracellular Matrix-Derived Biomaterials

Most of the naturally occurring materials in use as biomaterials today are constituents of the extracellular matrix (Griffith, Osbourne et al., 1999). Natural allograft and xenograft materials offer the advantage of being similar or identical to those found within the human body, enabling their recognition and degradation in the biological environment. The problems of toxicity and chronic inflammation, which are frequently provoked by synthetic polymers, may therefore be avoided (Yannas, 1996). The promising compatibility offered by natural materials allows the possibility of designing biomaterials which function biologically at the molecular, rather than at the macroscopic, level.
An intriguing characteristic of these materials lies in their high degradation level by naturally occurring enzymes. It is therefore possible to control the degradation rate of the implanted material, by specifically modifying its structure with the enzyme specificity in mind (Mauch, Adelmann-Grill et al., 1989). A disadvantage, however, in the use of biomaterials presents itself in a sometimes significant level of immunogenicity, which of course derives precisely from their similarity to biological materials. The immunological reaction of the host to the implant is directed against selected antigenic determinants in the protein molecule, with eventual degradation or encapsulation of the implant (Anderson, 1996). It must be noted that, in some cases, the immune reactivity of proteins can be virtually eliminated by chemical or physical modification of the antigenic determinants. The immunogenicity of polysaccharides is typically far lower than that of proteins. As well, collagens are generally weak immunogens relative to the majority of proteins (Yannas, 1996).

2.4.1 Collagen
Collagen from both animal and human sources is widely used as a biomaterial. Type I collagen is the most abundant and important type for use as a biomaterial (Bailey, 2000). Collagen-based materials can be separated into two categories: (1) biomaterials retaining the original tissue structure (used as sutures, cardiac valves, ligamentary prostheses), and (2) biomaterials prepared from purified collagen, with or without additives, and prepared in different forms depending on their applications. These in turn are used as sponges, tubes, spheres, membranes etc. Examples of applications of collagen-based biomaterials include: injectable collagen for cosmetic skin defects, vitreous replacement and coating of bioprostheses, collagen sponge for wound dressings, hemostatic agents, sponge scaffolds for tissue regeneration of peripheral nerves, dermis collagen microspheres for drug delivery and cell culture, collagen membrane as dialysis membranes and corneal shields, and collagen powder for bone filling and repair (Chevallay and Herbage, 2000).

2.4.1.1 Immunogenicity
The immunogenicity of collagen has been extensively studied. The clinical significance of collagen's immunogenicity has been shown to be very low, and is often considered to be negligible. The apparent reason for the low antigenicity of type I collagen stems from
the small species difference among type I collagens, as in cow and human (Yannas, 1996). Such similarity is, in turn, borne out by the inability of the triple helical configuration to incorporate the substantial amino acid substitutions characteristic of other proteins (Linsenmayer, 1991).

In order to modify the immunogenicity of collagen, it is useful to consider the location of its antigenic determinants, i.e. the specific chemical groups which are recognized as foreign by the immune system. In general, the procollagen form is more antigenic. However, when native collagen molecules are used as immunogens, antibodies can still be elicited. When procollagen is used as an immunogen, most of the antibodies are directed against epitopes within the propeptide extensions at the terminal ends. These antigenic determinants include both sequential (i.e. dependant on amino acid sequence) and conformational (i.e. dependant on tertiary or quaternary structure) determinants. The conformational determinants of collagen depend on the presence of the intact triple helix and, consequently, are abolished when collagen is denatured into gelatin. The process of gelatinization briefly exposes the sequential determinant of collagen before it is cleared away by enzymatic digestion (Clark, 1996). Controlling the stability of the triple helix during processing of collagen, therefore, would prevent the display of the sequential determinants.

Sequential determinants exist in the nonhelical end (telopeptide region) of the collagen molecule. This region has been associated with most of the immunogenicity of collagen-based implants (Bailey, 2000). Several enzymatic treatments have been devised to cleave the telopeptide region without destroying the triple helix. Treating collagen with cross-linking agents also permits a reduction in antigenicity (Weadock, Olsen et al., 1984). Intermolecular cross-linking of collagen by physical treatment or chemical agents modifies other properties of biomaterials, as well as their antigenicity. For example, cross-linking can reduce solubility, water absorption and biodegradability of the collagen biomaterial. Its mechanical properties are also enhanced (Chevallay and Herbage, 2000).
2.4.1.2 Structure Modification

Cross-linking and structural changes to the collagen molecule can be achieved by exposure to ultraviolet or gamma rays, as well as by thermal dehydration. Chemical cross-links may also be formed by chemical treatment with dialdehydes such as glutaraldehyde, or with carbodiimide (Chevallay and Herbage, 2000). Changes in pH can be used to selectively abolish the quaternary structure of collagen while maintaining the tertiary structure. The lattice of aggregated triple helices may be lost reversibly when the pH of a suspension of collagen fibres in acid is lowered below 4.25. During this transformation, the triple helical structure remains unchanged. The triple helix may be converted to random coil gelatin by heating above the helix-coil temperature, or by exceeding a critical concentration of anions in a solution of collagen molecules (Yannas, 1996). Collagen may also be processed through solubilization and freeze-drying to achieve a porous sponge-like structure, with mean pore diameters ranging from 1 to 800 um. Collagen gels used in this study are prepared from a solution of acid-denatured bovine dermal collagen. The suspension of non-aggregated triple-helical molecules is neutralized with base and heated to 37°C, to allow the molecules to quickly re-aggregate into microfibrils. This form of collagen differs from intact dermal collagen in the extent of intermolecular cross-linking, as well as in the fibril size (Chevallay and Herbage, 2000).

2.4.2 Hyaluronan

The structure of hyaluronan (HA) is conserved across species, and therefore is not antigenic (Chen and Abatangelo, 1999). The fact that HA elicits no immune response makes it a desirable biomaterial. The high molecular weight and hydrophilicity of HA form the basis for most of its uses as a biomaterial. Almost all applications make use of the exceptionally high viscosity and facility to form gels, characteristic of large polysaccharides. HA is used in ophthalmologic applications such as in cataract surgery and retinal reattachment. It is also used as an injectable synovial fluid additive in the treatment of osteoarthritis. Benzyl-esterified HA has been used to create Laserskin®, a biocompatible delivery system for keratinocytes to improve cultured epidermal graft take rates. The keratinocyte-seeded laser microperforated HA membranes are coupled to fibroblast-seeded meshes in order to obtain a dermal-epidermal composite structure
The main drawbacks associated with the use of HA are its short residence time and the limited range of its mechanical properties (Laurent, Fraser et al., 1986).

### 2.5 Dermal Substitute Design

The skin serves a variety of functions, the most important being a barrier to the environment. To be effective, a potential skin replacement must achieve functional wound coverage. Although a satisfactory degree of wound coverage is attained through the use of temporary wound dressings, permanent skin replacement has been shown to improve survival rates and functional outcomes for patients with large burns (Cooper, Andree et al., 1993). Permanent skin replacements, unlike temporary replacements, should also control scar formation. A major advance lies in the use of dermal replacements along with epidermal grafts. This approach can control scar formation, limit wound contraction and maximize epidermal maturation to form a functional stratum corneum (Berthod and Rouabhia, 1997). Table 2 compares the features of a skin substitute with those of a wound dressing.

<table>
<thead>
<tr>
<th>Required Features</th>
<th>Wound Dressing</th>
<th>Skin Substitute</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Physical</strong></td>
<td>Bacterial barrier</td>
<td>Bacterial barrier</td>
</tr>
<tr>
<td></td>
<td>Appropriate water flux</td>
<td>Appropriate water flux</td>
</tr>
<tr>
<td></td>
<td>Adherence</td>
<td>Proper suturing characteristics</td>
</tr>
<tr>
<td><strong>Biological</strong></td>
<td>Hemostatic</td>
<td>Adherence to wound bed</td>
</tr>
<tr>
<td></td>
<td>Sterilizability</td>
<td>Appropriate mechanical properties</td>
</tr>
<tr>
<td></td>
<td>Absorbancy</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Nonantigenic and nontoxic</td>
<td>Nonantigenic and nontoxic</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Minimal inflammatory or foreign body response</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sterile</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Controlled biological degradation</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Facilitate angiogenesis, host cell infiltration</td>
</tr>
<tr>
<td><strong>Desirable Features</strong></td>
<td>Comfort</td>
<td>Improve cosmetic result</td>
</tr>
<tr>
<td></td>
<td>Conformability</td>
<td>(minimize scarring and contraction)</td>
</tr>
<tr>
<td></td>
<td>Drug delivery</td>
<td>Comfort</td>
</tr>
<tr>
<td></td>
<td>Durability</td>
<td>Inexpensive</td>
</tr>
<tr>
<td></td>
<td>Ease of application and removal</td>
<td>Available</td>
</tr>
</tbody>
</table>
Table 2. Design requirements of wound dressings and skin substitutes in the treatment of full-thickness wounds.

<table>
<thead>
<tr>
<th>Appropriate mechanical properties</th>
<th>Ease of handling and application</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gaseous exchange</td>
<td>Allow growth of skin appendages</td>
</tr>
</tbody>
</table>

2.5.1 Human Acellular Dermal Matrix

Acellular dermal matrix is used as a dermal substitute in the treatment of full thickness burns, in association with a thin split thickness autograft. AlloDerm® processed allograft dermis (LifeCell Corporation, The Woodlands, Texas) was developed as a permanent dermal transplant for full-thickness burn wounds and has been in use since 1992 (Wainwright, 1995). Human cadaver skin, obtained from skin banks, is processed to remove the epidermal, fibroblast and endothelial cells which are targets for the immune response. This process yields an acellular, immunologically inert, dermal collagen matrix, while retaining elastin, proteoglycans, and the basement membrane complex. The availability of this dermal tissue allows for successful use of ultrathin autografts while maximizing the amount of dermis delivered to the wound site.

Because full-thickness skin grafts (FTSGs) inhibit wound contraction better than split-thickness grafts (STSGs), the presence of dermis is believed to be an important factor in controlling wound contraction and limiting scar formation. If thinner STSGs are used, the lack of an adequate dermal layer leads to wound contracture and hypertrophic scarring. However, neither FTSGs nor thick STSGs can generally be used to treat large full thickness wounds because of donor site considerations. The risk of donor site infection, hypertrophic scarring, blistering and hyper- or hypo-pigmentation is increased with harvesting deeper grafts (Boyce, Goretsky et al., 1995). Typically, meshed, thin STSG are used, to maximize the surface area of the wound covered by the graft. This technique, however, does little to reduce scarring and wound contraction because there is no dermis beneath the mesh interstices. Conversely, areas of the hands and feet grafted with AlloDerm exhibit excellent elasticity and good pigmentation with minimal scarring or wound contracture (Lattari, Jones et al., 1997). An initial multicenter study
documents that results obtained with acellular dermis and ultrathin STSG grafts were at least as equal to those of standard thickness STSGs (Wainwright, 1995).

The transmission of infection remains a concern in the use of allograft materials. As a result, all allograft donor tissue is well screened. In addition, the acellularization process safeguards against the transmission of viral disease. Because replication of human pathogenic viruses occurs only intracellularly, the removal of cells from the extracellular matrix eliminates sites for viral propagation. In addition, the process includes treatment of the allograft tissue with antiviral agents (Herndon, 1997).

2.5.2 Pig Acellular Dermal Matrix

Tissues of animal origin have been used for thousands of years as dressings for extensive burns. Porcine skin is the most common source of xenograft skin because of its similarity to human skin. Of the non-primate species, porcine dermis is probably the most structurally and immunologically similar to human dermis (Srivastava, Jennings et al., 1999). There exists the potential for cross-species transmission of pathogens, especially viruses and prions, when xenografts are used. Xenograft dermis can be treated with ionizing radiation, freeze-dried or soaked in glycerin for sterilization (Berthod and Rouabhia, 1997).

2.5.2.1 Pig dermis as a wound dressing

Pig skin can be used to dress second degree burns, especially after early excision (Gao, Hao et al., 1992). In addition, pig skin provides a suitable overlay to cover widely meshed (1:8 to 1:12) autografts. Two studies have analysed the cost-effectiveness of pig skin as a temporary dressing (<4 days between changes) for partial thickness and excised full thickness wounds (Basile, 1982), (Wood and Hale, 1972). These studies concluded that rehydrated freeze-dried pig skin adhered firmly to the wound surface with diminution of fluid, electrolyte, protein and heat loss. With frequent dressing changes to assist in wound debridement and to lower the degree of immune rejection, the authors also described a reduction in pain and bacterial growth. Pig dermis was not however found to be advantageous over petrolatum gauze, in the treatment of donor sites (Breach, Davies et al., 1979). Biological skin dressings are also of value in cleaning leg ulcers, decubitus ulcers and many types of infected wounds (Artz, Rittenbury et al., 1972).
2.5.2.2 Immunogenicity and vascularization of pig dermis

For most types of xenograft transplants, immediate hyperacute graft rejection becomes increasingly likely as the phylogenetic distance between the donor and recipient grows. Interestingly, hyperacute rejection does not seem to be a problem with pig skin grafts, but such grafts are susceptible to both cell-mediated and late antibody-mediated rejection after revascularization (Heslop and Shaw, 1986). This rejection is hypothesized to be due to the presence of xenogeneic endothelial cells, other cell types, or extracellular matrix materials present in the graft (Wang, Chen et al., 1997). There is some debate as to the immunogenicity of acellularized porcine dermis. Research in this area has focused on either fresh pig skin or nonviable dermis without the cells removed. It has not been clearly demonstrated which aspect of the graft elicits antibody production, and whether vascularization is necessary for this sensitization to occur. Acellularized pig dermal substitute, however, must support the ingrowth of host capillaries to remain viable and achieve wound closure.

There is experimental evidence on both sides, indicating that fresh xenografts do and do not acquire a blood supply. One study demonstrated, through the use of microangiograms, vascularization of fresh full-thickness pig skin in rabbits (Toranto, Salyer et al., 1974). Preserved split-thickness pig skin was used to treat partial thickness wounds in humans. The pig skin did not become viable, although vessels were seen in the graft at 7 days. Using microangiographic techniques, the vessels could not be distinguished from those accompanying the invasion of the grafts by granulation tissue (Toranto, Salyer et al., 1974). Another approach to accurately estimate blood flow through the graft was to quantify the degree of radioactivity four days after injecting hosts with radiolabelled red cells. Here, it was found that autogenic, allogenic and mammalian xenogenic skin grafts contained approximately the same quantity of blood when examined four days after grafting in the rat (Heslop and Shaw, 1986). These results are promising for the use of acellular pig dermis as a dermal substitute, where a material must be able to support host blood vessel ingrowth.
For biological materials to be suitable for implantation in an allogenic or xenogenic host, the material's immunogenicity and potential for infection must be modified. Acellularization techniques have been designed to conserve the tissue architecture of the extracellular matrix while removing the more antigenic cellular and soluble protein components. Most acellularization protocols are also successful in sterilizing the tissue for implantation. There are many variations in acellularization techniques, depending on the desired features of the resulting acellular matrix.

The acellularization protocol used in this project was originally developed by Courtman to produce acellular vascular allografts (Courtman, Pereira et al., 1994). The tissue is first immersed in a hypotonic solution, which causes cells to lyse as they absorb water through osmosis. The next solution is isotonic, but contains a mild detergent which further dissolves the phospholipid-based cellular membranes. These two solutions contain protease inhibitors, to prevent enzymatic degradation of the collagen matrix by the released cellular material. The only enzymatic step involves a combination of DNAse and RNAse to destroy the genetic material of the cells. The next rinsing step uses a strong anionic detergent (sodium dodecyl sulfate, or SDS) to remove residual soluble proteinaceous and phospholipid material. Adipose tissue is not removed in this step. Finally, the acellular material is soaked in 70% ethanol to remove the residual SDS, and rehydrated in PBS prior to sterilization. The process causes cell disruption with a leaching of cell contents, cellular antigens and lipid dissolution, yet inhibits collagen and elastin matrix proteolysis (Wilson, Yeger et al., 1990).

This acellularization protocol was designed for tissues where preserved mechanical properties and the ability of the resulting material to support host cell infiltration are critically important. The acellularization process was applied to the production of acellular xenograft pericardium and heart valves. Mechanical testing confirmed that the acellular pericardium possessed mechanical characteristics almost identical to those of fresh pericardium (Courtman, Pereira et al., 1994). Implantation of acellularized allograft carotid arteries resulted in poor cellular ingrowth into the acellular material after one month. It was suggested that this may be due to the retention of SDS in the
vessel extraction process (Wilson, Courtman et al., 1995). The duration of soaking of the acellularized tissue in ethanol was therefore extended to achieve complete removal of SDS, in order to allow cellular ingrowth (Robinson-Seurig, 1999).

### 2.5.2.4 Acellular pig dermal matrix as a dermal substitute

In recent years, many attempts have been made to produce a nonimmunogenic, xenograft dermal substitute which is capable of supporting thin split-thickness skin grafts (STSG) or cultured epithelial autografts. Dermal substitutes composed of denatured xenogenic collagen gels have been studied extensively in humans. Gels composed of bovine type I collagen have been found to have some efficacy in the treatment of full-thickness burn injuries. Few adverse reactions directly related to the implanted materials have been reported following those grafts. Anti-bovine collagen and serum antibody titers do not increase in recipients of these grafts, and no evidence of immediate or delayed immune reactivity to xenogenic collagen has been observed (Boyce, Goretsky et al., 1995). However, the implanted gels degrade too rapidly to prevent contraction and fibrosis (Compton, Butler et al., 1998).

The efficacy of undenatured xenograft collagen for the treatment of skin wounds has also been examined. Xenograft porcine and human skins, both with the epidermis removed, have been found to have modest value in the treatment of full-thickness skin wounds in conjunction with immediate STSG autografting. When trypsinized porcine skin or cryopreserved dispase-treated human skin was implanted into full-thickness wounds on rats, acute inflammation, some loss of the overlying autograft, and variable amounts of wound contraction were observed (Wang, Chen et al., 1997), (Fang, Robb et al., 1990). Lyophilized rehydrated porcine and bovine dermis have given poor results in human studies (Breach, Davies et al., 1979). Trypsinized acellular porcine skin was frankly rejected when it was implanted into human cutaneous wounds. Trypsinization effectively removes the cellular matter from the dermis, but alters the structure of dermal collagen by cleaving collagen telopeptides. Dispase treatment of skin does not remove all cell debris from the dermis (Srivastava, Jennings et al., 1999). Dermal matrices produced by these methods may therefore exhibit antigenicity which would account for their unsatisfactory biomaterial performance.
A recent study examined “acellular” porcine dermis, covered with thin autografts, implanted in full thickness wounds in rats (Srivastava, Jennings et al., 1999). The acellularity of the xenograft material was not verified histologically before implantation, however. Wounds treated with xenografts contracted to 46% of the initial 1.5 x 1.5 cm square wound size, while allografts treated with the same acellularization process maintained 74% of the initial surface area. Some graft survival was noted in the presence of xenograft dermal matrix (52% of graft area survived), but was less than that of allografts (82%). Histologically, the xenografts showed significant inflammatory response, occasional foreign body giant cells, swelling and disorganization of collagen bundles at 10, 14, 20 and 30 days post implantation. Some inflammatory cell infiltration was also noted in the allograft-treated wounds. It is not known whether similar results would be found if porcine dermis, acellularized in the same manner, were to be implanted in humans. Histologically however, studies of this type have revealed a clearly intense local immune response, with significant disruption of the dermal collagen meshwork. It is suggested that this response was induced by cellular material present in the xenogenic dermal matrix.

Using the same animal model with a larger wound size (5 x 9 cm), another study compared the contraction and epithelialization rates of wounds treated with three types of skin replacements (Wang, Chen et al., 1997). The groups compared in this study were meshed acellular allograft dermis with autograft epidermis, meshed acellular pig xenograft dermis with autograft and finally, split thickness autograft alone. In that study, the acellularity of the allo- and xenografts was confirmed histologically prior to implantation. Wounds in all three groups had completely epithelialized by three weeks. Post-operative contraction was followed for 6 weeks. Allograft dermis-treated wounds contracted to 79 ± 1% of the original wound size; xenograft treated-wounds, to 76 ± 6%; and split-thickness autografted wounds, to 58 ± 4% by 6 weeks. The histological responses to the grafts were monitored up to 4 months post-grafting. At 3 weeks, xenograft-treated wounds demonstrated acute inflammation between the porcine dermal elements, and the collagen fibres were swollen and irregularly arranged. Mast cell infiltration with dense granules was also identified. These findings were in contrast to
the normal appearance of granulation tissue seen in the allograft- and autograft-treated wounds. Interestingly, follow-up studies at 4 months showed that the inflammatory process and mast cells faded away completely in all groups. H&E stains showed normal scarring in all three groups. It was therefore concluded that both allogemis and xenodermis were equivalent, long term, at enhancing the healing of widely expanded meshed skin grafts.

2.5.2.5 Cell seeding of acellular pig dermis
Matouskova et al describe a method of preparing recombined human/pig skin (RHPS) (Matouskova, Vogtova et al., 1993). Human keratinocytes are cultured on the epidermal side of the dried cell-free pig dermis, using lethally irradiated 3T3 cells as feeders, according to the method of Rheinwald and Green (Rheinwald and Green, 1975). After reaching confluency, the matrix was turned over and fibroblasts seeded on the dermal side. Fibroblasts were seeded at a density of 2 x 10^4 cells/cm^2 for 1-3 days. The fibroblasts grew as a monolayer, and did not migrate into the internal structure of the dermis.

In order to evaluate the suitability of animal dermal matrix preparations for human use, Oliver tried to determine whether they could support the growth of human cells in vitro. Adult human skin fibroblasts were cultured on sheets of rat and pig acellular dermal matrices. The results showed that the cells grew and survived for at least seven weeks, and that the majority of cells formed a near-confluent layer on the surface with "some" (Oliver, Barker et al., 1982) cell infiltration into the collagen matrix. No histological data was provided in this study. Clearly, many questions remain unanswered in the development of acellularized pig dermis as a dermal substitute in humans.

2.6 Fibroplasia In Vitro
This topic is relevant to both biomaterial development and wound healing research. The many variables, which must be taken into account in the design of biomaterials, allow for a large number of possible outcomes. Several design features may therefore be more conveniently optimized through the use of in vitro cell culture methods. For example, as discussed in the previous section, the effect of acellularization on subsequent cell
infiltration can be studied in vitro. Ideally, the in vitro conditions reproduce in vivo wound healing events as closely as possible. In this way, any information obtained from the experimental model would be reliable in predicting the actual result were the biomaterial placed in the body.

For all aspects of biomaterial design and wound healing research, it is desirable to experimentally model each wound healing event in isolation. Such models allow for direct interpretation of cause and effect relationships, provided all the experimental variables are accounted for. This type of in vitro model is often used to determine the underlying mechanism of an effect seen in an in vivo model. For understanding the effect of specific extracellular matrix molecules on specific cells, in vitro methods allow for a simplified system where most of the variables are known and controlled. The information gained from these simplified in vitro models could in turn be applied to the development of more life-like in vitro models. Research in this area has contributed a great deal towards the development of sophisticated in vitro tissue equivalents (Berthod and Auger, 1997), (Griffith, Osbourne et al., 1999).

Ongoing development and refinement of experimental methodologies allow for a growing understanding of cell-matrix interactions. The bulk of our current knowledge of cell-matrix interactions in wound healing has been derived from various experimental models. Each of these models has demonstrated particular strengths and limitations. In this section, experimental approaches to study cell-matrix interactions in cutaneous wound healing will be categorized and briefly discussed. The emphasis will be on fibroblast migration, proliferation, collagen synthesis, contraction and differentiation.

2.6.1 Two dimensional models
The most commonly used technique to study specific aspects of cell biology, including matrix biology, is two-dimensional in vitro cell culture. Coating a tissue culture dish with a specific matrix molecule, and subsequently culturing cells in a monolayer on the coated dish, allows for the focused investigation of many aspects of cell biology. Some aspects of cutaneous wound healing that have been studied in 2D are listed in Table 3.
- Fibroblast and keratinocyte attachment, migration, intracellular protein expression and proliferation
- Cell proliferation "post-injury"
- Inflammatory cell activation
- Endothelial cell migration, proliferation and lumen formation
- Intracellular signalling after matrix binding
- Matrix degradation
- Cytotoxicity of modified matrix molecules
- Matrix-specific cell surface receptors
- Cell morphology
- Cell growth rate or protein synthesis

Table 3. Areas of cell-matrix interactions in cutaneous wound healing studied with two-dimensional cell culture techniques.

Existing two-dimensional designs have attempted to explain the role of fibroblasts in wound healing. Soluble matrix molecules can be dissolved in the cell culture medium, bathing fibroblasts grown as a monolayer. This design allows for the study of specific receptor-mediated interactions between matrix molecules and cells. However, the structural and physical roles of matrix molecules in cell-matrix interactions cannot be addressed in this way. Fibroblasts display an intimate awareness of the structure and composition of their surrounding environment (Coulomb, Dubertet et al., 1984). In fact, studies suggest that the growth cycle and phenotype of fibroblasts in three-dimensional scaffolds in vitro more closely approximates that of cells grown in vivo (Greco, Iocono et al., 1998), (Doane and Birk, 1991).

2.6.2 Three-dimensional models
As a result of in vivo research into matrix biology, the importance of structural relationships among the matrix components has become recognized. The limitations of two-dimensional cell culture, as well as the cost and ethical issues involved with in vivo research, have spurred the development of three-dimensional in vitro models for cell biology research. The goal of 3D models is to provide a simplified environment in which cells behave as they would in the body. When cultured in monolayer, fibroblasts display high proliferation rates and high collagen synthesis, while their mitotic behaviour and collagen production are inhibited if placed in a collagen gel. Fibroblast
behaviour may differ in different models. It is hypothesized that a mature dermal matrix would present a more life-like scaffold for studying fibroplasia, and by extension, the process of contraction.

A number of in vitro models have been developed to study the mechanisms of fibroblast infiltration into a matrix and the resulting contraction. In vitro contraction models are usually based on various purified extracellular matrix components such as collagen, fibrin, glycosaminoglycans or acellularized dermal matrices from allogenic or xenogenic sources. However, a weakness exists with these models in their applicability to in vivo wound contraction. Wound contraction is a steady, active process that transpires over a relatively long period of time. An in vitro model where rapid lattice contraction occurs does not correctly replicate the slow steady process of in vivo wound contraction underway during wound healing. The collagen gel, collagen sponge, fibrin gel and acellular dermis as in vitro models of fibroplasia will be reviewed in this section.

2.6.2.1 Collagen gels
To study wound contraction in vitro, several models using fibroblasts cultured in collagen matrices have been developed. These fibroblast-laden collagen gels can be either anchored to the culture substratum, or left free-floating on culture media. The anchored model has been shown to more closely resemble granulation tissue, whereas the floating model more closely resembles the normal resting dermis (Frey, Chamson et al., 1995). The collagen gel system has been extensively studied and is the most popular dermal equivalent used since it is easy to produce. Production methods take advantage of the spontaneous ability of collagen monomers to rearrange into fibrils at neutral pH, and of the contractile properties of fibroblasts upon these collagen fibrils. The ability of fibroblasts to contract type I collagen gels is also reliant on the β1 subfamily of the integrins, particularly α2β1 (Klein and Nagy, 1981).

It has been shown that collagen gels made with a higher collagen concentration or seeded with more fibroblasts contract faster. The mechanism of fibroblast contraction of collagen gel has been demonstrated to occur by a gathering of the collagen fibres by the fibroblasts as they migrate along the gel surface, resulting in the exclusion of water from
between the fibrils (Bell, Ivarsson et al., 1978). By testing various substances for their ability to inhibit or stimulate fibroblast-mediated contraction of collagen gel, the mechanism of contraction may be investigated. For example, cytochalasin B is known to inhibit translocation and contractility of non-muscle cells. In the presence of this compound, the fibroblasts do not contract collagen gel, indicating that their translocation and contractility are required for this process (Bell, Ivarsson et al., 1978).

Collagen gel has been widely used by many investigators to compare the contractile properties of different cells. Steinberg et al found that established and transformed fibroblasts are less able to contract collagen gels than normal pre-crisis cells (Steinberg, Smith et al., 1980). Nedelec et al compared the ability of normal and hypertrophic scar fibroblasts to induce lattice contraction (Nedelec, Shen et al., 1995). This study showed no difference in contraction between the two fibroblast types, and was further able to demonstrate differences in contraction by the hypertrophic scar fibroblasts after incubation with α-2b-interferon. The fibroblast-populated collagen gel with, and without, epidermal coverage has been used to model wound contraction by making a punch biopsy in the centre (Ehrlich and Rajaratnam, 1990). Repopulation of the wound space and re-epithelialization was then monitored.

Moreover, collagen gels can be used to study synthesis of extracellular matrix molecules by fibroblasts in a life-like environment. Fibroblasts cultured in collagen gels have been shown to synthesize less collagen and fewer glycosaminoglycans than fibroblasts grown as a monolayer (Edward, 1995). The surrounding collagen matrix is believed to induce a feedback inhibition on collagen synthesis, as well as an inhibition of the mitotic activity of fibroblasts within the gel.

Stimulants and inhibitors of contraction may be added to the medium or to the collagen suspension prior to gelling, with a view to studying their effect on the cell-mediated contraction. Among the modulators tested, DNA synthesis inhibitors, corticosteroids, TGF-β, PDGF, interferon gamma, retinoic acid, as well as agents preventing the organization of actin filaments such as cytochalasin B, have been studied in the collagen gel model (Coleman, Tuan et al., 1998).
The use of three-dimensional in vitro dermal models also allows for easier study of cell-cell interactions in dermal healing. Using a collagen gel model, Berthod et al showed that dermal fibroblasts activate keratinocyte outgrowth on collagen gels, as well as promote the differentiation of a multilayered stratified epithelium covered with a compact stratum corneum (Berthod and Rouabhia, 1997). In contrast, in the absence of fibroblasts, the keratinocytes form disorganized epithelial cell layers. Guidry et al demonstrated that the presence of endothelial cells stimulates collagen gel contraction by fibroblasts (Guidry and Hook, 1991).

2.6.2.2 Collagen sponges

Collagen sponges are obtained by the lyophilisation of a collagen solution. The size and structure of the pores in the sponge are related to the growth of ice crystals during the freezing process (Doillon and Silver, 1986). Unlike collagen gels, porous collagen sponges permit seeded cells to proliferate. Doillon et al showed that fibroblasts placed on a sponge of crosslinked type 1 collagen, multiplied and migrated throughout the sponge (Doillon, Wasserman et al., 1988). Berthod et al obtained a sponge of collagen-GAG-chitosan entirely colonised by human fibroblasts after 10 days of culture (Berthod and Auger, 1997). The fibroblasts' rate of growth was less rapid however than in monolayer culture.

During the first weeks of culture in a collagen sponge, fibroblasts exhibit moderate mitotic activity and high protein synthesis approaching the level found in the healing process. After one month their protein production and proliferation profile is markedly reduced, mimicking the in vivo situation. A major advantage of this model is the production of a high amount of newly synthesized extracellular matrix which fills the porous sponge after a month. Collagen synthesized by fibroblasts in a collagen sponge is arranged in fibrils associated in interwoven bundles that mimic the fibrillar organization of the dermis. Integra® consists of a collagen sponge enriched with chondroitin sulfate. Menard et al used the Integra® collagen-GAG sponge as a scaffold to study the contractile behaviour of osteoblasts in a 3D matrix (Menard, Mitchell et al.,
The cells were seen to migrate throughout the sponge, resulting in contraction and bone extracellular matrix formation in the pores.

### 2.6.2.3 Fibrin gels

Tuan et al proposed a fibrin gel model of fibroblast-mediated contraction, cell proliferation, fibrin degradation and collagen synthesis/deposition. Fibroblasts cultured in fibrin gels display a high rate of collagen synthesis and mitotic activity. This occurs although the fibroblasts are closely surrounded by fibrin fibrils, in contrast with the behaviour of fibroblasts in a collagen gel (Tuan, Song et al., 1996). This fibrin gel model is able to model the behaviour of fibroblasts in an early wound.

### 2.6.2.4 Acellularized human dermis

Care should be taken in assuming clinical application from the ability of fibroblasts to contract collagen gels \textit{in vitro}. Since collagen gels are too simple to mimic the mature extracellular matrix structure of the dermis, recognition of this has led to the development of more complex dermal equivalents. Normal dermis has a particular three-dimensional geography. This overall structure comprises relatively open collagen fibres in the deep reticular dermis, more compact and ordered collagen fibres in the upper papillary dermis, and the complex basement membrane region containing several extracellular matrix proteins in a unique conformation. The development of a skin composite model, consisting of an acellularized human dermal matrix seeded with fibroblasts and keratinocytes, allows for the study of epidermal-mesenchymal interactions in wound healing (Ralston, Layton et al., 1997). This composite model did not however demonstrate any fibroblast-mediated contraction of acellular human dermis \textit{in vitro}.

Ghosh et al studied fibroblast ingrowth within acellular human dermal matrix in an effort to develop a fibroblast-seeded composite skin substitute (Ghosh, Boyce et al., 1997). The matrix was processed by one of several methods. It was soaked in glycerol, or lyophilized followed by ethylene oxide treatment. Both lyophilized and ethylene oxide-treated and glycerol-treated dermis accepted infiltration by fibroblasts. In total, 75% of all samples seeded on the reticular surface were successfully infiltrated by fibroblasts within ten days. No fibroblast ingrowth occurred when the cells were seeded on the
papillary surface of the dermis, unless keratinocytes were co-cultured with the fibroblasts.

In an attempt to study the stimulatory effects of fibroblasts on epithelialization of the dermis by keratinocytes, Krejci et al. seeded human fibroblasts on acellularized, second-cut reticular human dermis (Krejci, Cuono et al., 1991). Fresh acellularized dermis was compared with a lyophilized and rehydrated acellular dermis. The results of the fibroblast infiltration were not reported; only the degree of epithelialization was described.

2.6.3 Summary
The development of three-dimensional in vitro models makes possible the study of contraction and matrix invasion by capillaries, without animal experimentation. Information gained from these models may contribute to knowledge in a variety of fields. Specific mechanisms of dermal wound healing biology, such as epithelialization, contraction, and angiogenesis, may be studied. Potential therapies for excessive scarring, poor healing due to inadequate microvascular blood supply, and tumor vascularization and metastases, are a few long-term objectives of such research. The understanding and development of the cell-seeding techniques used in these models will also prove helpful in the production of composite skin substitutes and other engineered tissues. The availability of novel biocompatible materials for tissue engineering scaffolds has allowed for considerable progress in the development of sophisticated in vitro models of skin as well as other tissues of interest in the field of bioengineered materials.
Chapter Three: MATERIALS AND METHODS

3.1 Acellular Dermal Matrix Production and Characterization

3.1.1 Dermal Acellular Matrix (ACM) Production

3.1.1.1 Pig dermis harvesting

All materials were obtained from Sigma-Aldrich Canada (Oakville, Ont.) unless otherwise specified. Porcine dermis was obtained from freshly sacrificed York pigs (Riemens Fur Ranch, Guelph, Ont.) used for surgical demonstration. The use of pigs for these applications was conducted according to protocols approved by the Animal Research Ethics Board of the University of Toronto. The skin was harvested up until 8 hours post-mortem. Prior to harvesting, the pig’s back, neck, flanks and thighs were shaved with an electric razor and cleaned with soap and water. The depilatory Nair (Carter-Horner Inc., Mississauga, Ont.) was then applied to the pig skin, and left on for at least 20 minutes, until the hair was easily wiped off with a cloth. After removing the Nair and loose hair, the skin was washed with betadine solution (Purdue Frederick Inc., Pickering, Ont.). The skin was rinsed with 70% ethanol (Commercial Alcohols Inc., Brampton, Ont.) two or three times to further decontaminate the skin.

An electroderrmatome (Padgett Instruments, Kansas City, MO) was used to harvest split-thickness second-cut dermis. With the dermatome set at an initial thickness of 0.02", the top layer containing epidermis and papillary dermis was tangentially excised and discarded. The remaining dermis was then harvested with a 10 cm guard, at a dermatome thickness set of 0.02". The level of dermis from the surface was recorded. Only tissue harvested 0.02 to 0.10" below the epidermal surface was used for cell culture experiments. Five levels of dermis were obtained within this depth range (Table 12). In order to produce ACM in a range of thicknesses, the ACM was thinned by cutting tangentially with a dermatome blade (Padgett Instruments, Kansas City, MO) while clamped to a cutting board for resistance. Biopunches of 8 or 16 mm in diameter were
used to make discs of ACM which fitted the wells of a 24 well plate. The ACM was then sterilized and stored in sterile PBS at 4°C or in some cases, freeze-dried for storage. Representative ACM samples from each batch were fixed in formalin or glutaraldehyde, for histological and scanning electron microscopic analysis respectively.

3.1.1.2 Human dermis harvesting
Surgical specimens from routine plastic surgery procedures (abdominoplasty and breast reduction) were provided by Dr. R. Levine at St. Joseph's Health Centre, according to a research protocol approved by the University of Toronto's Ethics Review Board. The skin of the surgical specimens was separated from the subcutaneous tissue with a scalpel. The skin was then clamped to a cutting board and tangentially cut with a dermatome blade (Padgett Instruments, Kansas City, MO) into thin strips. The level of dermis was recorded. Four layers of dermis were obtained, including the epidermal layer (Table 12). Samples of intact dermis were fixed for histological analysis. The remaining dermis was acellularized according to the protocol below.

3.1.1.3 Dermal acellularization
The acellularization protocol used in this project was based on one previously described for pig vascular grafts and pericardial tissue (Courtman, Pereira et al., 1994), (Wilson, Yeger et al., 1990), and optimized by Robinson-Seurig for application to pig dermis (Robinson-Seurig, 1999). This multistep detergent-enzymatic extraction process is designed to remove all cells, phospholipid membranes, membrane-associated antigens and soluble matrix components from the extracellular matrix (Wilson, Courtman et al., 1995), (Courtman, Pereira et al., 1995). The protocol used for the acellularization of both pig and human dermis in this study is described in Table 5, with a catalog of harvested pig and human dermis recorded in Table 4. The technique developed to sterilize the ACM is described in Table 6.
Table 4. Acellularized pig and human dermis batches used in experiments.

<table>
<thead>
<tr>
<th>Date harvested (batch)</th>
<th>Age of subject</th>
<th>Date acellularized (batch)</th>
<th>Date used in experiments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pig</td>
<td>18-02-00</td>
<td>7 weeks</td>
<td>04-03-00</td>
</tr>
<tr>
<td></td>
<td>26-07-00</td>
<td>7 weeks</td>
<td>09-08-00</td>
</tr>
<tr>
<td></td>
<td>10-08-00</td>
<td>7 weeks</td>
<td>24-08-00</td>
</tr>
<tr>
<td></td>
<td>2-11-00</td>
<td>3 months</td>
<td>16-11-00</td>
</tr>
<tr>
<td></td>
<td>17-11-00</td>
<td>7 weeks</td>
<td>1-12-00</td>
</tr>
<tr>
<td></td>
<td>30-11-00</td>
<td>3 months</td>
<td>14-12-00</td>
</tr>
<tr>
<td>Human</td>
<td>23-11-00</td>
<td>26 y</td>
<td>7-12-00</td>
</tr>
<tr>
<td></td>
<td>24-11-00</td>
<td>32 y</td>
<td>8-12-00</td>
</tr>
</tbody>
</table>

- Immerse in 10 mM Tris buffer with EDTA, pH 8.0 containing 5% PMSF and antibiotics.
- Rinse with shaking for three days, with fresh solution twice daily.
- Rinse with shaking in 1% Triton-X in 50 mM Tris buffer, pH 8.0 with EDTA and PMSF for four days, with fresh solution twice daily.
- Rinse with Sorensen’s phosphate buffer.
- Cut skin into smaller pieces, and incubate with 6000 U DNAse and 5 mg RNAse for 24 hours in 40 ml Sorensen’s phosphate buffer
- Rinse with shaking in 1% sodium dodecyl sulfate in 50 mM Tris buffer, pH 9.0, for four days, with fresh solution twice daily.
- Rinse with shaking in water, then in 70% ethanol overnight.
- Rehydrate in sterile PBS. Store at 4°C.
- Re-sterilize in 70% ethanol prior to use.

Table 5. Acellularization technique for pig and human dermis.

- Soak in 70% ethanol in sterile container overnight.
- Aspirate using sterile technique.
- Soak in fresh 70% ethanol in a new sterile container, for 30 minutes. Aspirate and replace with fresh 70% ethanol for 30 minutes, twice.
- Rinse in sterile PBS overnight.
- Aspirate and replace with fresh sterile PBS for 30 minutes, twice.
- Transfer to a new sterile container. Store in sterile PBS or media at 4°C until further use.

Table 6. ACM sterilization technique.
3.1.2 Dermal Acellular Matrix Characterization

3.1.2.1 Acellularity
For each batch of acellularized dermis, a sample was fixed in formalin. The samples were cut in cross-section and stained with hematoxylin-eosin, anti-vimentin antibodies and anti-smooth muscle actin (SMA) antibodies. The antibodies were visualized using the avidin-biotin system. Fixed pig and human dermis were used as positive controls. Successful binding of the anti-vimentin and anti-SMA antibodies (both from Dako, Glostrup, Denmark) was demonstrated in tissue sections from both species. A few preliminary samples were also stained for pankeratin (Dako) to ensure complete de-epithelialization of superficial dermal layers. Table 7 summarizes the antibodies used for immunohistochemistry in this study.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vimentin, monoclonal goat anti-human, clone 1A4</td>
<td>Cytoplasmic intermediate filament in mesenchymal cells (including fibroblasts, myofibroblasts)</td>
</tr>
<tr>
<td>α-Smooth muscle actin, monoclonal goat anti-human</td>
<td>Cytoplasmic actin isoform expressed by contractile vascular smooth muscle cells and wound myofibroblasts</td>
</tr>
<tr>
<td>Pankeratin, AE1/AE3, monoclonal rabbit anti-human</td>
<td>Cytoplasmic proteins expressed by keratinocytes</td>
</tr>
</tbody>
</table>

Table 7. Antibodies used for immunohistochemical staining of the ACM.

3.1.2.2 Acellular matrix structure
Freeze-fracture scanning electron microscopy (SEM) and transmission electron microscopy (TEM) were performed on representative samples of fresh pig ACM. Each sample was examined at low (50x and 100x) and high (500x and 1000x) magnification for collagen bundle size and arrangement as well as ACM surface characteristics. SEM was used to compare the matrix structure of pig ACM and human ACM, freeze-dried and fresh ACM, hyaluronan- and heparin-enriched pig and human ACM and finally, cell-seeded pig and human ACM samples. Level 2 samples (see Table 12) of the 17-11-00 pig ACM batch and level 3 samples of the 23-11-00 human ACM batch were cultured...
with primary human fibroblasts for four weeks, and compared in structure by SEM to equivalent, non-seeded samples.

Samples were fixed in 2% glutaraldehyde and dehydrated in a graded series of ethanol concentrations, followed by critical point drying. Some samples were then freeze-fractured with a scalpel before mounting. The freeze-dried samples were affixed to aluminum stubs with double-sided electrical tape. The edges of the samples were then coated with silver paint to improve electrical conductivity. The mounted samples were sputter-coated with platinum. The samples were examined under a scanning electron microscope (model S-2500, Hitachi, Japan) and the images were captured using a passive image capture system (Quartz PCI, Quartz, Vancouver, B. C.).

TEM was done to verify the quaternary collagen structure in the pig ACM, after the acellularization process. TEM samples were fixed in 2% glutaraldehyde for 24 hours, then post-fixed in 2% osmium tetroxide for 2 hours. Dehydration was through a graded series of ethanol followed by propylene oxide. Samples were embedded in Spurr’s resin, sectioned, and counterstained with uranyl acetate and lead citrate.

3.1.2.3 Glycosaminoglycan content of ACM

The relative glycosaminoglycan content of the ACM was assessed qualitatively by alcian blue staining of freeze-fixed sections. Freshly acellularized samples of pig and human ACM were embedded in O.C.T. embedding compound® (Sakura Finetek U.S.A. Inc., Torrance, CA) without fixation and immediately frozen at −70°C to preserve the matrix glycosaminoglycans, which tend to wash out with formalin fixation. Sections were cut and stained with alcian blue at pH 1.0 and 2.5. The lower pH is better suited to detect the sulfated glycosaminoglycans, and the higher pH allows some visualization of hyaluronan (Elder, Elenitsas et al., 1997). Freshly harvested pig and human dermis were processed and stained in the same manner. The relative intensity of alcian blue staining was compared between fresh and acellularized pig dermis.
3.2 Fibroplasia in a Pig Acellular Dermal Matrix

3.2.1 Cell Culture

All cell culture experiments were performed in a tissue culture facility approved by the University of Toronto Safety Committee as a Biohazard Level Two facility. The room was equipped with several laminar flow biological safety cabinets (NSF accredited, H. E. P. A. Filter Services Inc, Toronto). All metal and glass equipment was sterilized with a steam sterilizer (SM 300, Yamato Scientific American Inc., Orangeburg, NY) prior to contact with cell culture materials. A standard cell culture incubator, set at 37°C with 5% CO₂ infused, was used for all experiments (Sanyo Electric Company Ltd, Japan).

3.2.1.1 Human dermal fibroblast culture

Subsequent cell culture experiments used passaged human dermal fibroblasts. These were isolated from fresh adult surgical specimens by explant culture (Nishiyama, Tsunenaga et al., 1989). The subjects’ age and medical history are listed in Table 6. Briefly, the dermis was obtained from discarded surgical specimens (from breast reduction or abdominoplasty) in a sterile field and cut into small pieces (less than 2 mm x 1 mm x 1 mm) with a sharp scalpel. The tissue pieces were transported on ice from the operating room to the tissue culture room, in sterile PBS with 1 mM calcium chloride. The tissue was rinsed with sterile PBS several times. The fragments were then placed in sterile 35 mm petri dishes with a drop of media to hydrate them, but without causing the pieces to float. The fragments were kept attached to the petri dish for up to three weeks with minimal media, until outgrowth of fibroblasts from the fragments were seen. At this time the tissue was carefully removed and the fibroblasts immersed in 1-2 ml of media until confluency was reached. The cells were then trypsinized using 0.25% trypsin/EDTA and plated in T75 flasks (Becton Dickinson, Franklin Lakes, NJ) for expansion. At 80-100% confluency, the cells were harvested and frozen as passage 2 cells, or used in cell seeding experiments. Prior to seeding on the ACM, a sample of the cells were stained with trypan blue and counted with a hemocytometer (Hausmann Scientific, Horsham, PA). Dulbecco’s Modified Eagles Medium (DMEM) with 10% fetal bovine serum, 1000 U penicillin and 100 μg streptomycin was used for all explant and expansion culturing. The fibroblasts were used in all experiments at passages 2 to 4.
### Table 8. Donor information for primary human dermal fibroblast harvesting.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age</th>
<th>Sex</th>
<th>Health problems</th>
<th>Date harvested</th>
<th>Date frozen</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. M.</td>
<td>37</td>
<td>F</td>
<td>none</td>
<td>11-5-00</td>
<td>12-6-00</td>
</tr>
<tr>
<td>B. T.</td>
<td>25</td>
<td>F</td>
<td>none</td>
<td>17-8-00</td>
<td>12-9-00</td>
</tr>
<tr>
<td>T. L.</td>
<td>29</td>
<td>F</td>
<td>none</td>
<td>5-9-00</td>
<td>2-10-00</td>
</tr>
<tr>
<td>M. W.</td>
<td>31</td>
<td>F</td>
<td>none</td>
<td>29-9-00</td>
<td>25-10-00</td>
</tr>
<tr>
<td>P. H.</td>
<td>26</td>
<td>F</td>
<td>none</td>
<td>23-11-00</td>
<td>26-12-00</td>
</tr>
</tbody>
</table>

3.2.1.2 ACM preparation for cell seeding

Pig and human dermal acellular matrix were cut to fit the wells on either 24 or 96 well tissue culture plates (Nalge Nunc International, Denmark) with 8 mm or 16 mm biopunches (Fray Products Corp., Amherst, NY). The thickness of the ACM samples was measured using a micrometer, and recorded. The ACM samples were then sterilized in sterile tissue culture plates or eppendorf tubes by soaking in a series of 70% ethanol rinses as described in Table 4. All human ACM and half of the pig ACM samples were freeze-dried for storage. Prior to cell seeding, the ACM were rehydrated in PBS and transferred to sterile tissue culture plates and soaked in the appropriate media for 48 hours. As a further sterility check, the turbidity and colour of the media in which the ACM were soaked was noted prior to cell seeding.

3.2.1.3 Fibroblast seeding onto ACM

For all experiments, the day of cell seeding was considered Day 0. Prior to trypsinizing the cells, the plate containing the ACM was removed from the incubator. The media in the wells was aspirated and the ACM pressed down against the bottom of the well to remove any air bubbles or excess media. The flasks containing the confluent cells were removed from the incubator and rinsed with warmed PBS. Trypsin/EDTA (5 ml) was added to each T75 flask for five minutes at 37°C. The selected media containing 10% serum was added (5 ml), rinsing the cells off the side of the flask. The resulting 10 ml cell suspension was transferred to two sterile 15 ml tubes and centrifuged for 10 minutes at 500 rpm (50 x g). The supernatant was aspirated and the cell pellet resuspended in the desired volume to yield an appropriate cell concentration for counting (10^5-10^6 cells/ml).
An aliquot of the resuspended cells was diluted with trypan blue, and counted using a hemocytometer. The cells were then diluted to the required concentration to allow for seeding with a desired amount of cells in an appropriate volume. The plate was returned to the incubator, the time noted, and the wells topped up to the required volume of warmed media 24 hours later. For all cell-seeded ACM samples, the media was changed every 2-3 days. Fresh media was added slowly to the side of the well to avoid washing the cells off the ACM surface.

3.2.1.4 Numbering of experiments
The experiments described in this section were numbered according to the conditions of the experiment. If a variable was changed, the experiment was assigned a sequentially different number. For each objective several experiments were done, each under slightly different conditions. Each experiment is therefore numbered independently within the same section. The number of times each experiment was repeated under identical conditions (r), is listed at the top of each column in Tables 9, 10, 11, 13 and 15. The number of experimental samples in each group (s) is listed in the last row of the table, for each repeat. In the statistical analyses of the results, the sample size (n) used in each set of experimental conditions corresponded to the following equation:

Sample size (n) = r x s

3.2.1.5 Experimental controls
To determine how the presence of pig dermal matrix influenced fibroplasia in an experimental setting, a number of controls were included. Human dermal ACM was used in some experiments to determine whether the effects of pig ACM were species-specific. Bovine collagen gel was used as a comparison to determine whether the effect of a collagen matrix on fibroblasts is structure-dependent. To this end, collagen gels were prepared in the wells of the tissue culture plate the day of the experiment. Briefly, 8 ml of chilled acidified bovine dermal collagen (Cohesion Corp., Palo Alto, CA) were mixed with 1 ml of 10x PBS. The pH was adjusted to 7.4 with 1 ml of 0.1M NaOH. Different volumes (400, 500 or 700 μl) of neutralized, isotonic collagen solution were placed in the wells and incubated at 37°C for at least an hour. The resulting gel was adherent to the sides and bottom of the well. As a final control, fibroblasts were also grown directly on a polystyrene culture dish itself.
3.2.2 Cell Morphology

Specific morphological findings have been associated with fibroblast contraction in different matrix environments (Ehrlich and Rajaratnam, 1990). To observe whether the collagen substrate influenced the morphology of the seeded fibroblasts, confocal microscopy of phalloidin-stained samples was performed.

3.2.2.1 Phalloidin staining

The appearance of human fibroblasts on pig ACM was compared to that of fibroblasts cultured on human ACM, collagen gel and coverslips. After 4 to 6 days in culture, the cell-seeded samples were fixed in formalin for 30 minutes. Alexa Fluor 488 phalloidin (Molecular Probes, Oregon) was used, at a 1:40 dilution with 0.1% bovine serum albumin (BSA) in PBS, to stain the F-actin fibres of the fibroblast’s cytoskeleton. The samples were rinsed in PBS. The samples were immersed in 1:1 PBS:glycerol in 24 well plates and wrapped in foil for transport. The coverslips were mounted on a slide and sealed with clear nail polish.

3.2.2.2 Confocal microscopy

The confocal microscope system used was the Zeiss LSM 510, with an argon laser at a wavelength of 488 nm. The morphology of the fibroblasts was examined with a 10x dry objective and a 63x water-immersion objective, with an optical slice thickness of 2 μm. The morphology of the cells on pig ACM, human ACM, collagen gel and polystyrene coverslips were compared. Three samples were viewed for each group, over twelve optical fields of view at 10x magnification, and five fields at 63x magnification.

3.2.3 Cell Proliferation

3.2.3.1 MTT assay

The MTT (3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay was used to determine how many cells were viable after seeding onto ACM, as compared to cells plated in the absence of ACM. First, a calibration curve was generated to compare the accuracy of the assay when used in the presence of ACM. Hs68 fibroblasts were seeded at different densities (0, 5000, 10,000 and 20,000 cells/cm²) in 2 x 24 well plates either in the absence or in the presence of 15 mm diameter ACM samples. Each curve
was performed in quadruplicate. After 24 and 48 hours incubation in DMEM with 10% serum and antibiotics, the MTT assay was performed as outlined below. The average absorbance of the two readings per sample was plotted against the cell concentration. The calibration curves for cell-seeded ACM were compared to that of cells only, and the 24- and 48-hour data were compared.

The MTT assay was used to determine the number of viable cells on pig ACM (PACM) after different lengths of time in culture. Cell numbers on PACM samples were compared to those on collagen gel and on polystyrene tissue plates. PACM samples (16 mm in diameter) were seeded with dermal fibroblasts at a density of 2500 cells/cm², and incubated for 24 hours, 1 week and 2 weeks. A total of 18 samples were seeded, for 6 samples at each time point (Experiment 1).

In preparation for the MTT assay, the media was aspirated from the wells and the cell-seeded ACM rinsed with warmed PBS. After aspirating the PBS, the cell-seeded ACM samples were transferred to a new 24 well plate to avoid counting the cells adherent to the culture dish. Sterile 5 mg/ml MTT (3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide) solution, in serum-free RPMI-1640 medium, was added to each well (300 µl), and the plates returned to the incubator for 4 hours. After addition of MTT to the wells, the ACM samples were flipped cell-side down to fully immerse the cells adherent to the ACM in the MTT solution. At the end of the incubation period, the MTT solution was aspirated from each well and acidified isopropanol (4% 1 N HCl) was added (300 µl/well). The lid was secured with parafilm, and the plate gently shaken for 1 hour on a rotomix. Each sample was mixed by pipetting up and down. A 100 µl aliquot of each sample was transferred to two wells on a 96 well plate. Fresh MTT solution was used as a blank. The plate was then read spectrophotometrically at 570 nm with a 630 nm reference filter. In one repeat of the experiment, after solubilization with isopropanol, the ACM samples and controls were photographed with a digital camera to demonstrate the MTT colour change.
3.2.3.2 CyQuant assay

In order to verify the reliability of the MTT assay as a measure of cell proliferation on the ACM, a second assay for cell proliferation was also used. The basis of the CyQuant kit (Molecular Probes, Oregon) is the use of a proprietary green fluorescent dye, which exhibits strong fluorescence enhancement when bound to cellular nucleic acids. Unlike the MTT assay, serum components (which are difficult to wash out of the ACM) do not appreciably interfere with the CyQuant assay. By measuring the degree of fluorescence after adding the dye to cell-seeded ACM, the assay provides a means of quantifying the relative amount of cells bound to the ACM. While the cells are not viable at the time of the dye binding, the assay provides an indirect measure of cell viability, as only viable cells will remain adherent to the ACM with repeated changes of media.

Human dermal fibroblasts were grown to confluence in a T75 flask under standard culture conditions. The cells were trypsinized, collected by centrifugation and resuspended in 2 ml of phenol red-free media. The cell concentration was determined by counting live cells (as shown by trypan blue staining). The cell suspension was diluted to a known concentration of around $2 \times 10^5$ cells/ml. The diluted cell suspension was transferred to sterile eppendorf tubes in exactly 1 ml aliquots. The tubes were centrifuged for 5 minutes at 200 x g. The supernatant was carefully removed and discarded without disturbing the cell pellet. The cells were placed immediately in a –70°C freezer.

Two calibration curves were generated: one for cells only, and one for the same number of cells in the presence of a digested 8 mm ACM sample. The second calibration curve was done to account for the error in the experimental samples, which contained cells and digested ACM. Since digested ACM collagen adds to the turbidity of the solution, the possibility of falsely elevated fluorescence values had to be considered. Once the ACM had been digested for 48 hours, the plate was centrifuged at 400 x g for 5 minutes, and gently inverted to blot the media off. The digested ACM was stored at –70°C until the cells were ready for diluting. The cell pellet was thawed at room temperature. A 1 ml volume of the prepared CyQuant dye/cell lysis buffer (see below) was added and the cells were resuspended by vortexing. A dilution series was generated in the wells of a
microplate by diluting the cell suspension with more CyQuant buffer. The range for this series comprised 0, 1000, 5000, 10,000, 15,000, 20,000 and 25,000 cells per well. The samples were incubated for 2-5 minutes, with the plate wrapped in foil. Two calibration curves were made in both the presence and absence of digested ACM (four curves in total).

The CyQuant assay was used to determine cell proliferation on the pig ACM (PACM), human ACM (HACM) and polystyrene plates. The assay allows for spectrofluorometric analysis directly in the cell-seeded well. 8 mm ACM samples were used. Table 9 summarized the different experimental conditions under which the samples were prepared for the CyQuant assay.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Experiment 2 (r = 1)</th>
<th>Experiment 3 (r = 3)</th>
<th>Experiment 4 (r = 2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Groups</td>
<td>PACM, HACM, collagen gel, cells only</td>
<td>PACM, collagen gel, cells only</td>
<td>PACM</td>
</tr>
<tr>
<td>Seeding density (cells/cm²)</td>
<td>20,000</td>
<td>20,000</td>
<td>20,000</td>
</tr>
<tr>
<td>Time points</td>
<td>1d, 1, 2, 3, 4 wks</td>
<td>1d, 1, 3 wks</td>
<td>1d, 2d, 3d, 5d, 1, 2, 4 wks</td>
</tr>
<tr>
<td># of samples per group per repeat</td>
<td>s = 3 or 4</td>
<td>s = 3</td>
<td>s = 3</td>
</tr>
</tbody>
</table>

Table 9. Summary of cell proliferation experiments for CyQuant assay. Abbreviations: PACM = pig ACM; HACM = human ACM; r = number of times the experiment was performed; s = number of samples in each group.

For cell proliferation experiments, controls consisted of cells on polystyrene wells without the presence of ACM were seeded on separate plates for each time point. At each time point, the cell-seeded ACM samples were transferred to unused wells on the plate designated for that time point. This transfer prevented cells growing in the same well as the ACM, but not on the ACM, from being included in cell proliferation determinations. The media was aspirated from the wells with cells only, and the wells filled with warmed HBSS. Type H collagenase (200 ul of 5 mg/ml solution) was added to the cell-seeded ACM. The plate was returned to the incubator for 24 hours. Each cell-ACM solution was pipetted up and down to break up the ACM collagen. The plate was returned to the incubator for another 24 hours. By that time, the ACM had been
completely digested, and the released cells could be easily seen by microscopy. The plate was centrifuged at 400 x g for 5 minutes and gently inverted onto paper towels to blot the supernatant. The plate was placed in a -70°C freezer to lyse the cells prior to quantification.

For the purpose of quantification, the plate was first thawed at room temperature. An adequate volume of CyQuant dye/cell lysis buffer was prepared with the following ratios of reagents: 380x distilled water, 20x cell lysis stock, 1x CyQuant dye stock. A 200 ul volume of the prepared buffer was added to each well. Samples of 200 ul of buffer without cells served as controls. All samples were incubated at room temperature for 2-5 minutes. Fluorescence was measured using a spectrofluorometer with filters appropriate for 485 nm excitation and 538 nm emission maxima. The appropriate calibration curve was used to determine cell number values.

3.2.4 Collagen Synthesis
Radiolabeled-proline incorporation is a common method for quantifying collagen or protein synthesis by cells in vitro. In this study, collagen synthesis by fibroblasts seeded on the PACM was compared to the background radioactivity of PACM without cells. This procedure was adapted from that of Freiberger et al (Freiberger, Grove et al., 1980). The samples were seeded with human dermal fibroblasts at a density of 30,000 cells/cm². They were cultured in DMEM with 10% FBS and penicillin/streptomycin for 4 days, with a change of media at day 2. In each repeat, the samples were performed in quadruplicate, as were the cell-seeded samples to be used for cell proliferation measurement. The experiment was repeated three times (Experiment 5). On day 4, the samples were washed with PBS and changed to pre-labeling medium. The pre-labeling medium consisted of the same medium with 50 µg/ml ascorbic acid and 50 µg/ml β-aminopropionitrile fumarate. The samples were incubated in pre-labelling medium for 7 hours, and then labeled with 200 µl of labeling medium for an additional 23 hours. This medium was identical to the pre-labeling medium, except that it contained 2 µCi of 2,3-³H-proline.
At the end of the incubation, the medium and a 200 µl PBS wash were collected into labeled eppendorf tubes. The ACM were then dissolved in 100 µl of 10 mM HCl for 1 hour at 37°C. The acid-dissolved ACM was collected in separate tubes. One of each the medium and the ACM were digested with collagenase in the following manner. The samples were made up to 1 ml with 50 mM tris-HCl, 10 mM calcium acetate, 2.5 mM N-ethylmaleimide and 10 U of type VII collagenase and incubated at 37°C for 3 hours. Half of the samples were made up to 2.5 mM N-ethylmaleimide and 0.5 mg/ml BSA in the meantime. Once the collagenase digestion was complete, 0.5 mg/ml BSA was added to these samples as well. All samples were then made up to 10% TCA, and left overnight at 4°C to precipitate the protein. The next day, the eppendorf tubes were centrifuged at 12,000 x g for 3 minutes and the supernatant discarded. The pellet was resuspended in 5% TCA and centrifuged; this was repeated twice. The pellet was solubilized by soaking in 10 mM HCl overnight. The acidified protein solution was transferred to numbered scintillation vials and 10 ml of scintillation fluid (Ready-Safe, Beckman Instruments Ltd., Fullerton, CA) was added for counting in a scintillation counter.

The fraction of collagenase soluble counts (cpm) was calculated from the formula:

\[
\frac{\text{Collagenase-digested cpm} - \text{blank cpm}}{\text{Total cpm} - \text{blank cpm}}
\]

The percentage of radiolabeled-proline incorporated into newly synthesized collagen (as opposed to all other protein synthesis) could then be determined:

\[
\left[1 - \frac{\text{Collagenase-digested cpm} - \text{Blank cpm}}{\text{Total cpm} - \text{Blank cpm}}\right] \times 100
\]

The relative amount of collagen synthesis per sample in each group was normalized by the cell numbers in each group. The number of cells per sample was determined by the CyQuant assay of designated samples.

3.2.5 Cell infiltration

Infiltration of human fibroblasts into pig dermal ACM was determined by cross-sectional histology of cell-seeded samples. Fibroblasts were seeded on one surface of the ACM, as in the cell proliferation experiments. At each time point, the cell-seeded ACM samples were removed from the plate and fixed in formalin for 24 hours. The
samples were then carefully sectioned with a #10 scalpel blade into three pieces (see Figure 5). Each section was blotted dry, and the cut edge was marked with mercuriochrome. The tissue pieces were put back into formalin until they could be embedded in paraffin on edge. All three pieces were embedded in a single paraffin block. One section was made from each block, mounted on a slide and stained with hematoxylin and eosin. There were three cross-sectional pieces of tissue per sample.

![Diagram](image)

**Figure 5.** Histological processing of an ACM sample.

The slides were reviewed to find sections containing a monolayer of fibroblasts at the top of the tissue section; this ensured that the tissue was in fact cut perpendicular to the cell-seeded surface. All H&E stained sections containing a monolayer were analysed for cell infiltration of the matrix. In some experiments, new slides were made from the sample blocks for vimentin staining. Cell infiltration was assessed in three ways. First, the percentage of sections in each experimental group demonstrating cell infiltration was determined. A section was considered positive for cell infiltration if at least 50 cells were seen, at least 50 µm below the surface. Second, the number of cells per positive section was determined by manual counting. The cells located in the monolayer, at the surface of the matrix, were not included in the count. Automated counts were done on some samples to correlate with manual count results. Third, the distance of each cell from the surface was recorded using digital photography and MCID image analysis software. Again, the cells of the monolayer were not included in the analysis. Distance measurements were recorded for three microscopic fields per section, at 10x magnification.
Several experiments were done to assess pig ACM as a scaffold for human fibroblast infiltration. Many experimental variables were investigated for any possible influence on cell infiltration. Human ACM samples were seeded under the same conditions, to allow a comparative assessment of pig ACM infiltration. Tables 10 and 11 summarize the cell infiltration experiments.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Exp 6 (r = 2)</th>
<th>Exp 7 (r = 2)</th>
<th>Exp 8 (r = 2)</th>
<th>Exp 9 (r = 3)</th>
<th>Exp 10 (r = 2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACM type and batch tested</td>
<td>PACM 18-02-00</td>
<td>PACM 10-08-00</td>
<td>FD-PACM 17-11-00, FD-HACM 23-11-00</td>
<td>FD-PACM 2-11-00, 17-11-00, 30-11-00, FD-HACM 24-11-00</td>
<td>FD-PACM 2-11-00, 30-11-00</td>
</tr>
<tr>
<td>Seeding density (cells/cm²)</td>
<td>2500</td>
<td>2500, 25,000</td>
<td>3000, 30,000</td>
<td>30,000</td>
<td>30,000</td>
</tr>
<tr>
<td>Time points</td>
<td>2, 4 wks</td>
<td>4 wks</td>
<td>1, 2, 3, 4 wks</td>
<td>1, 3 wks</td>
<td>1, 2, 4 wks</td>
</tr>
<tr>
<td># of samples per group</td>
<td>s = 5</td>
<td>s = 2</td>
<td>s = 4</td>
<td>s = 4</td>
<td>s = 3</td>
</tr>
</tbody>
</table>

Table 10. Cell infiltration experiments. Experiments 6-10 used human dermal fibroblasts at passage 2-4, cultured in DMEM with 10% FBS. Abbreviations: PACM= pig ACM; HACM= human ACM; FD= freeze dried; r= number of times the experiment was performed; s= number of samples in each group.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Exp 11 (r = 1)</th>
<th>Exp 12 (r = 1)</th>
<th>Exp 13 (r = 2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACM type and batch tested</td>
<td>PACM 30-11-00, 10-8-00, HACM 23-11-00</td>
<td>PACM 10-8-00</td>
<td>PACM 21-8-00, FD-PACM 21-8-00, FD-HACM 24-11-00</td>
</tr>
<tr>
<td>Cell type</td>
<td>1°HFb-P#2</td>
<td>1°HFb-P#3</td>
<td>1°HFb-P#4</td>
</tr>
<tr>
<td>Seeding density (cells/cm²)</td>
<td>30,000, 50,000</td>
<td>5000, 50,000</td>
<td>50,000</td>
</tr>
<tr>
<td>Time points</td>
<td>4 wks</td>
<td>3 wks, 5 wks</td>
<td>4 wks</td>
</tr>
<tr>
<td># of samples per group</td>
<td>s=20 (s=4 for each level)</td>
<td>s=5 to 6</td>
<td>s=3</td>
</tr>
<tr>
<td>Media type</td>
<td>DMEM+FBS</td>
<td>DMEM+FBS</td>
<td>MCDB 131+ FBS</td>
</tr>
<tr>
<td>ACM diameter and setup</td>
<td>8 mm, 16 mm</td>
<td>16 mm ACM with stainless steel rings</td>
<td>16 mm</td>
</tr>
</tbody>
</table>

Table 11. Cell infiltration experiments (continued). Abbreviations: 1°HFb= human dermal fibroblasts; P#= passage number; FBS= 10% fetal bovine serum.
3.2.5.2 Seeding density
A range of cell seeding densities was used for seeding fibroblasts on the ACM. Seeding density values are calculated by the following equation:

\[
\text{Cell density (cells/cm}^2) = \frac{\text{[Volume of cell suspension seeded on ACM (ml) x Concentration of cell suspension (cells/ml)]}}{\text{ACM cell-seeding area (cm}^2)}
\]

\[
\text{ACM cell-seeding area (cm}^2) = \pi \times (\text{diameter}/2)^2
\]

3.2.5.3 Level of dermis
It was also of interest to determine whether the scaffold properties of the pig and human ACM were dependent on the level of dermis. In Experiment 11, ACM samples from each level of dermis were seeded with fibroblasts. The depth of dermal harvesting from the epidermal surface defined the dermal level of ACM. For example, level 1 consisted of the dermis 0.02 to 0.04 inches below the epidermal surface, level 2 was 0.04 to 0.06 inches below the surface and so on (Table 12). The pig dermis in this study was approximately 0.12 inches thick; five layers 0.02 inches in thickness were harvested. For human ACM, the levels were defined by the division of full thickness dermis (0.10 inches thick) into five layers of equal thickness (0.02 inches thick). Table 12 summarizes the levels of pig and human ACM which were compared.

<table>
<thead>
<tr>
<th>DEPTH FROM SURFACE (INCHES)</th>
<th>LEVEL</th>
<th>Pig</th>
<th>Human</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-0.02</td>
<td>Discarded (epidermis)</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>0.02-0.04</td>
<td>1</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>0.04-0.06</td>
<td>2</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>0.06-0.08</td>
<td>3</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>0.08-0.10</td>
<td>4</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>0.10-0.12</td>
<td>5</td>
<td>Discarded (fat)</td>
<td></td>
</tr>
</tbody>
</table>

Table 12. Levels of harvested dermis, defined by depth from surface.
3.2.5.4 Freeze-drying

Pig and human ACM samples were sterilized and transferred to sterile 50 ml polypropylene tubes. The tubes were without lids and wrapped in foil perforated with small holes. The tubes were immersed in liquid nitrogen for one minute to freeze the contained ACM. The tubes were placed in a freeze-dryer flask and connected to a vacuum freeze-dryer (Labconco Corp., Kansas City, MO) overnight. The ACM was rehydrated in sterile media overnight.

3.2.6 Myofibroblast Differentiation

Histological sections of fibroblast-seeded PACM, HACM and collagen gel were stained with antibodies to the myofibroblast marker α-smooth muscle actin. Samples were seeded with 30,000 cells/cm² and incubated until fixation of three samples per group at each of the following time points: 3 days, 1, 2, and 4 weeks (Experiment 14). These samples were cut in cross-section, stained for α-smooth muscle actin (SMA) and counterstained with hematoxylin. Each slide was analyzed for percentage of cells stained for SMA, with the total number of cells demonstrated by hematoxylin. Non-specific background staining was observed on some sections of pig ACM. These sections were excluded from analysis. Human fibroblasts and rat-tail fibroblasts cultured on coverslips were used as controls for SMA staining.

3.2.7 Contraction

The prepared ACM and collagen gel samples were seeded with human dermal fibroblasts, and incubated for the lengths of time indicated in Table 13. All samples were seeded with 25,000 fibroblasts/cm². Human fibroblasts between passages 2 and 3 were used. The collagen gel was detached from the sides and bottom of the plate 24 hours after seeding. This allowed the gel to float in the culture medium and contract freely. The medium was changed every 2 to 3 days. DMEM with 10% FBS and antibiotics was used in Experiment 15, while EBM-2 with 5% FBS was used in Experiments 16 and 17. In the latter two experiments, the fibroblast-seeded pig ACM samples were incubated in wells seeded with human microvascular endothelial cells. Once the time point was reached, the plate was photographed and the image imported into a GelDoc Multianalyst program. The surface area of the contracted sample was
measured using Scion Image software (Scion Corporation, Frederick, Maryland), and compared to the original surface area of the sample.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Experiment 15 (r = 2)</th>
<th>Experiment 16 (r = 2)</th>
<th>Experiment 17 (r = 2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group</td>
<td>PACM</td>
<td>Coll. Gel</td>
<td>PACM</td>
</tr>
<tr>
<td>Time points</td>
<td>1, 2, 3, 4 wks</td>
<td>4 wks</td>
<td>1, 3, 5 wks</td>
</tr>
<tr>
<td>PACM thickness</td>
<td>0.3-0.6 mm, 0.6-0.8 mm</td>
<td>0.2-0.4 mm, 0.5-0.6 mm</td>
<td>0.3-0.6 mm</td>
</tr>
<tr>
<td>Fibroblast density</td>
<td>25,000 cells/cm²</td>
<td>25,000 cells/cm²</td>
<td>25,000 cells/cm²</td>
</tr>
<tr>
<td>MEC density</td>
<td>0 cells/cm²</td>
<td>7500 cells/cm²</td>
<td>0, 7500 cells/cm²</td>
</tr>
<tr>
<td>Media type</td>
<td>2</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Controls</td>
<td>No fibroblasts</td>
<td>No fibroblasts</td>
<td>No MEC</td>
</tr>
<tr>
<td>Endpoints</td>
<td>% original surface area, histology</td>
<td>% original surface area, histology</td>
<td>% original surface area, histology</td>
</tr>
<tr>
<td># samples</td>
<td>s = 4</td>
<td>s = 3 or 4</td>
<td>s = 4 to 8</td>
</tr>
</tbody>
</table>

Table 13. Summary of contraction experiments. Abbreviations: PACM= pig ACM; Coll. Gel = collagen gel; MEC= microvascular endothelial cells.

3.3 Fibroplasia in a Glycosaminoglycan-Enriched Pig Acellular Dermal Matrix

3.3.1 Glycosaminoglycan Incorporation

3.3.1.1 Materials

Ultrafree-MC centrifuge filter tubes with 0.45 um pore size were used. These were purchased from Millipore Canada Ltd. The tubes were sterilized by soaking in 70% ethanol and rinsing in sterile PBS. For the localization and quantification experiments, fluorescently labeled glycosaminoglycans (GAGs) were used. FITC-labeled or dansyl-labeled hyaluronan (HA) from bovine trachea (MW_{avg} = 10^6 g/mol) and dansyl-labeled heparin (HP) from porcine intestinal mucosa (MW_{avg} = 10^4 g/mol) were obtained from Calbiochem Laboratories (Mississauga, Ont.). For the preparation of HA-ACM enriched samples used in cell-seeding experiments, non-labeled large molecular weight HA was used. Human umbilical cord HA was obtained from Seikagaku Corporation and rooster
comb HA was donated by Hyal Pharmaceuticals Ltd. (both $M_W^{avg} = 10^6$ g/mol). All chemicals and cell culture supplies were obtained from Sigma-Aldrich Canada Ltd. unless otherwise specified. An IEC Micromax microcentrifuge (International Equipment Company, Kansas City, MO) was used in all HA incorporation experiments.

### 3.3.1.2 Hyaluronan incorporation

Pig ACM was cut into 8 mm outer diameter discs using a biopunch. Care was taken to ensure consistent thickness. Sample disc thickness was measured using a micrometer; samples of greater than 1.5 mm or less than 0.7 mm in thickness were not used. The selected ACM samples were sterilized as previously described and all procedures were carried out in a biological cabinet. The inner diameter of the centrifuge filter tubes was 7 mm. The ACM discs were placed at the bottom of the filter insert (Figure 6) in a concave-down position. Each experimental sample disc was then covered with 100 ul of a sterile 5 mg/ml solution of HA in DMEM. The tubes were centrifuged at 1900 x g for 10 x 30 minute intervals. At the end of each 30 minute centrifugation, any HA solution which had passed into the bottom of the tube was collected and replaced above the ACM. This was done to prevent the centrifugation of dry ACM samples. The tubes were also rotated 180°C after each 30 minute interval (Lausman, 2000). The ACM samples enriched with HA are referred to as HA-ACM.

![Diagram of ACM and HA in centrifuge filter tube for HA incorporation.](image)

**Figure 6.** Diagram of ACM and HA in centrifuge filter tube for HA incorporation.
3.3.1.3 Heparin incorporation

ACM samples (8 mm o.d.) of comparable thicknesses were placed in the wells of a sterile 96 well plate. A 100 μl volume of a 5 mg/ml solution of heparin (HP) was added, the plate wrapped with parafilm and placed in a 37°C incubator for 48 hours. The ACM samples enriched with heparin are referred to as HP-ACM.

3.3.2 Glycosaminoglycan Localization & Quantification

In order to visualize the GAGs in tissues, samples cannot be formalin-fixed (Lin, Shuster et al., 1997). All HA-ACM and HP-ACM samples were therefore frozen at -70°C and embedded in O.C.T. compound® prior to sectioning. Frozen samples of the unmodified ACM, HA-ACM and HP-ACM were stained with alcian blue (at pH 1.0 and 2.5). While the histology did demonstrate some differential staining between the samples (Section 4.3.1), it did not adequately delineate the level of HA or HP incorporation. A further method was undertaken to facilitate GAG localization in the ACM. Solutions of 1, 5 and 10 mg/ml FITC-labeled HA were centrifuged into ACM discs in the same manner as described above. Dansyl-labeled HP was dissolved in PBS as a 5 mg/ml solution, and incorporated according to the method described (FITC-labeled HP was not available). The samples were frozen, and unstained cross-sections were obtained. A fluorescent Axiovert microscope (Zeiss, Germany) connected to a computer and a digital camera was used to demonstrate the areas of fluorescence in the ACM. A 450 nm blue light filter was used. The images were viewed by Spot Advanced version software (Diagnostic Instruments Inc, Sterling Heights, MI). A solution of fluorescein in water of equivalent concentration was centrifuged into control ACM samples, to demonstrate the specificity of the fluorescence in the ACM to the FITC-HA. All fluorescent solutions and tissues were kept from light during the experiment.

Existing methods to quantify GAGs in tissues are limited to some extent. The alcian blue assay has high error, and is too insensitive to detect microgram quantities. An antibody based on hyaluronan-binding protein was developed, but has been shown to cross-react with pig collagen (Tengblad, 1980). To overcome these problems, the fluorescently labeled GAGs incorporated into the ACM were quantified spectrofluorometrically. Once fluorescein-labeled HA or dansyl-labeled HP was
incorporated into ACM samples, the samples were rinsed off in PBS. They were then placed in a microplate, and 200 μl of 5 mg/ml type H collagenase were added to the wells. The ACM was digested for 48 hours in the dark. Equal volumes of each sample were transferred to separate wells for quantification. Calibration curves were prepared using equal volumes of standards containing digested ACM, and either fluorescein-HA or dansyl-HP. Calibration curves covering a wide range of concentrations were used, and prepared in duplicate or triplicate. Fluorescein-labeled HA was quantified using a spectrofluorometer with 485 nm excitation and 538 nm emission filters. Dansyl-labeled heparin samples were measured at 265 nm excitation and 510 nm emission wavelengths.

In this study, 100 μl of 1 mg/ml, 5 mg/ml and 10 mg/ml each of fluorescein-HA and dansyl-HP were incorporated into three ACM samples at each concentration. The amount of GAG incorporated into the ACM was quantified spectrofluorometrically using calibration curves of both FITC-HA and dansyl-HP in the presence of digested ACM. The volume of the digested GAG-enriched ACM was measured in order to calculate the mass of GAG in the ACM from the concentration. The percentage of available GAG incorporated into the ACM, at each concentration, was analyzed. The concentration of GAG in the ACM was estimated, by using an approximate ACM volume (Section 4.3.2).

In order to determine the longevity of the HA and HP in the ACM immersed in solution, HA-ACM and HP-ACM samples were quantified after soaking in HBSS for different times at 37°C (Table 14). The solution of HBSS was replenished every 3 days. The mass of GAG present in the ACM at each time point was compared to the mass of GAG in the ACM at day 0. A sample at each time point was frozen for histological analysis, to correlate with the quantitative data. Due to the cost of the fluorescently labeled GAGs, only a few repeats were possible.
### Table 14. Effect of diffusion on the quantity of HA and HP in ACM over time: qualitative and quantitative assessment.

<table>
<thead>
<tr>
<th>Variables</th>
<th>FITC-HA</th>
<th>Dansyl-HP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amount available for incorporation (ug)</td>
<td>190</td>
<td>500</td>
</tr>
<tr>
<td>Time points</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0, 12, 24, 36, 48, 72, 96 hrs</td>
<td>0, 4, 7, 14, 28 days</td>
<td>0, 1, 3, 7, 14 days</td>
</tr>
<tr>
<td>Endpoints</td>
<td>Histology</td>
<td>Histology</td>
</tr>
<tr>
<td>Controls</td>
<td>FITC-ACM, at the same time points</td>
<td>ACM without FITC-HA</td>
</tr>
<tr>
<td># of samples per time point</td>
<td>1</td>
<td>Histology: 1 Quantification: 2</td>
</tr>
</tbody>
</table>

### 3.3.3 Cell Proliferation

Non-fluorescently labeled HA and HP were incorporated into sterile ACM samples in the manner described above. These were seeded with human dermal fibroblasts (passage 3 or 4) to determine their effects as components of a dermal matrix structure. After different lengths of times in culture, the cell-seeded samples were analysed for cell number by the CyQuant assay (Section 3.2.3.2). The relative proliferation rates of fibroblasts on the different ACM materials were determined in this way. Table 15 lists the experimental conditions of these cell proliferation experiments.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Experiment 18 (r = 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experimental groups</td>
<td>ACM</td>
</tr>
<tr>
<td></td>
<td>HA-ACM</td>
</tr>
<tr>
<td></td>
<td>HP-ACM</td>
</tr>
<tr>
<td></td>
<td>ACM without cells</td>
</tr>
<tr>
<td>Cell type</td>
<td>1st HFb-P#3 or 4</td>
</tr>
<tr>
<td>Seeding density (cells/cm²)</td>
<td>30,000</td>
</tr>
<tr>
<td>Time points</td>
<td>2 d, 1, 2, 4 wks</td>
</tr>
<tr>
<td># of samples per group</td>
<td>s = 3 or 4</td>
</tr>
</tbody>
</table>

### Table 15. Effect of HA or HP on fibroblast proliferation in PACM.

### 3.3.4 Collagen Synthesis

Samples of ACM, HA-enriched ACM and HP-enriched ACM were seeded with 30,000 cells/cm². Human dermal fibroblasts at passage 2 were used (Experiment 5). In each repeat, the samples were performed in quadruplicate, as were the cell-seeded samples to
be used for cell proliferation measurements. The experiment was repeated three times, leading a total sample size of 12 per group. The control groups consisted of cells cultured on the culture dish without ACM, and ACM cultured without cells. The cell-seeded samples were cultured in DMEM with 10% FBS and penicillin/streptomycin for 4 days, with a change of media on day 2. On day 4, the samples were washed with PBS and changed to pre-labeling medium. The pre-labeling medium consisted of the same medium with 50 μg/ml ascorbic acid and 50 μg/ml β-aminopropionitrile fumarate. The samples were incubated in pre-labelling medium for 7 hours, and then labeled with 200 ul of labeling medium for an additional 23 hours. This medium was identical to the pre-labeling medium, except that it contained 2 μCi of 2,3-3H-proline. The radiolabelled protein pellet was extracted and quantified using the same method as described in Section 3.2.4.

3.3.5 Cell Infiltration
ACM samples were freeze-dried and rehydrated prior to use. The samples were fixed and analyzed according to the methods outlined in Section 3.2.5. The experimental groups included ACM, HA-enriched ACM, HP-enriched ACM. Samples of 8 mm ACM were seeded with 30,000 cells/cm² and cultured for the following lengths of time: 1, 2, 4 wks. Three samples were fixed at each time point, with the experiment repeated three times (Experiment 19). The histology samples were analyzed to detect any effect the GAGs might have had on the rate of fibroblast migration below the ACM surface. The distance of the fibroblasts from the surface was also analyzed.

3.3.6 Myofibroblast Differentiation
Additional ACM samples were included in the three repeats of the above experiment to allow for fixation of three samples per group at each of the following time points: 3 days, 1, 2 and 4 weeks. These samples were cut in cross-section, and stained for α-smooth muscle actin and counterstained with hematoxylin. Each slide was analyzed for the percentage of cells stained for SMA, with the total number of cells demonstrated by hematoxylin staining.
3.3.7 Statistics

Cell viability, proliferation and infiltration were analyzed for differences between pig and human ACM. The statistical analyses for this study were performed using SSPS software. All data was tested for normality using the Shapiro-Wilk and Kolmogorov-Smirnov tests. Non-parametric data was analyzed for significance using non-parametric chi-squared, Kruskal-Wallis, Fisher’s exact test, Mann-Whitney U or Wilcoxon W tests. Normally distributed data was analyzed for significant differences by multivariate ANOVA and posthoc Tukey HSD test. Significance was defined as $p<0.05$. Differences between ACM acellularity and structure were determined qualitatively, and therefore were not analyzed statistically.
Chapter Four: RESULTS AND DISCUSSION

4.1 Pig Acellular Matrix Characterization

4.1.1 Acellularity
The acellularization protocol has not been applied to the acellularization of human dermis prior to this study. No previous immunohistochemistry has been done to determine its effectiveness in removing all cellular material from pig or human dermal tissue. Samples of freshly acellularized pig and human dermis were formalin-fixed for analysis of acellularity. Samples from each batch of acellularized dermis were stained with H&E, as well as with antibodies to vimentin, pankeratin and α-smooth muscle actin.

4.1.1.1 Pig ACM
Hematoxylin and eosin (H&E) staining of a sample from each batch of pig ACM revealed consistent removal of all nuclear material. Hematoxylin is a cationic dye; it binds to the negatively charged genetic material concentrated in the cell nucleus and stains it bluish-purple. Eosin, an anionic dye, imparts a pink to red color to the other dermal tissue components (Cormack, 1993). The presence of basophilic nuclear material can therefore be easily seen with H&E staining. Figure 7 shows the appearance of pig dermal ACM after staining with H&E. No basophilic (or blue) staining can be seen, indicating the removal of cellular nuclear material.
Figure 7. Pig dermal ACM, H&E, 10x.

Epithelial cells of the dermis (in the epidermis and dermal appendages) contain cytokeratins in their cytoplasm. The most superficial, epidermis-bearing layer of pig dermis was not acellularized in this study; after harvesting it with a dermatome, it was discarded. Only the deeper dermal layers were acellularized for cell seeding. Pankeratin staining, however, showed residual keratin proteins around some hair follicles in the ACM (Figure 8).

Figure 8. Pig ACM, stained for pankeratin, 10x. Positive (pink) staining around hair follicle indicates residual epithelial cellular material after acellularization.
Since the nuclei of these cells were not seen by hematoxylin staining, the nuclear material appears to have been successfully digested despite the incomplete removal of the cytoplasmic keratin. The presence of hairs likely prevents the complete removal of the follicular epithelial cells by this acellularization protocol.

A limitation of the acellularization protocol with respect to pig dermis lies in the failure of the acellularization process to solubilize hairs. These are only loosened, such that they might easily be removed with minimal friction at the end of the acellularization process. The hairs were most easily removed in very thin samples of ACM, where they separated from the matrix with mild shaking. It is believed that complete removal of proteins around the hair shaft, could be accomplished with a repeat of the last detergent step of the acellularization process after complete hair removal. This would allow greater access by the detergent to the proteins lining the hair follicle, and thus optimize removal of the proteins. This technique was not tested in this project however; the residual pig epithelial cellular material did not interfere with the ability to identify human fibroblasts in the pig ACM.

The most important stains for this project were the mesenchymal-cell cytoplasmic proteins, vimentin and α-smooth muscle actin. Complete removal of pig mesenchymal cells from the pig dermis is required for pig ACM to be used as an in vitro scaffold for human fibroblasts. This allows for the seeded fibroblasts within the matrix to be clearly identified. The degree of acellularity within ACM batches is demonstrated by immunohistochemistry using these antibodies in Figure 9 (vimentin) and Figure 10 (SMA). Negative vimentin staining demonstrates complete removal of this protein from the ACM; in contrast, the typical distribution of vimentin-staining in the dermis is shown in Figure 9 (b).
Figure 9. Pig dermal ACM and dermis stained for vimentin (brown).

Smooth muscle cells in the dermis stain positively for α-SMA. These surround blood vessels, hair follicles and dermal appendages, as shown in Figure 10 (b).

Figure 10. Pig ACM and dermis staining with smooth muscle actin (brown).

With the complete removal of native vimentin- and SMA-positive cells from the pig dermis, newly seeded human fibroblasts may be easily visualized on the pig ACM by vimentin or SMA staining.

SMA staining of ACM samples occasionally showed some irregular staining of the dermis, possibly due to residual protein adsorbed to the dermal matrix and/or to non-specific binding of the antibody to the matrix (Figure 11). This non-specific staining of
the ACM is distinguished from that of myofibroblasts by several features. Diffuse weak staining, seen in areas where the sectioned tissue is folded over, and not in association with a cell nucleus, was considered non-specific staining. Figure 12 shows a typical SMA-stained sample of fibroblast-seeded ACM, demonstrating the contrast between background SMA staining and seeded fibroblast staining.

Figure 11. Pig ACM stained for smooth muscle actin, showing non-specific background staining (brown), 20x.

Figure 12. Human fibroblasts (arrow) seeded on pig ACM stain for smooth muscle actin (red-brown) with hematoxylin counter-stain (blue-purple), 40x (left), 20x (right).

The presence of the background SMA staining in some ACM samples made the automated quantification of SMA-positive fibroblasts difficult. These sections were therefore excluded from cell counting analysis (Section 3.2.5).

4.1.1.2 Human ACM

Human dermis, obtained from two reduction mammaplasty donors, was acellularized for this study. Staining with H&E, vimentin and SMA revealed no background staining (Figure 13). In contrast to pig ACM preparation, the epidermal layer of the human
dermis was acellularized. Negative pankeratin staining demonstrates no residual epidermal cells in the human dermal ACM.

**Figure 13.** Human dermal ACM acellularity with different stains (cells indicated by arrows).
4.1.2 Acellular Matrix Structure

4.1.2.1 Pig dermal ACM versus pig dermis
Compared with whole dermis, the extracellular matrix of dermal ACM is devoid of cells. A thorough evaluation of the pig ACM matrix composition is beyond the scope of this project, and has not yet been done. A preliminary histological survey shows that most anionic matrix components (i.e. glycosaminoglycans) are removed in the acellularization process. The structure of the insoluble matrix is preserved, however. Figure 14 shows the preserved structure of the collagen and elastin matrix in the ACM.

![Figure 14](image)

Figure 14. Movats staining shows the preserved collagen (orange) and elastin (black) matrix structure of the pig dermal ACM (a), in comparison with that of pig dermis (b).

A comparison of the surface and cross-section of pig ACM and dermis by SEM, confirms the histological findings of preserved collagen bundle structure and residual hairs (Figure 15). In Figure 15 (a) and (b), the collagen bundles are seen in cross-section at high magnification. Both pig ACM and pig dermis show a tightly packed network of thick collagen bundles (circle).
Figure 15. A comparison of pig ACM and pig dermis by scanning electron microscopy at low magnification (a and b) and at high magnification (c and d). The circle delineates a single collagen bundle in cross-section. Arrows indicate hair follicles on surface of ACM and dermis.

Transmission electron microscopy (TEM) provides a means of examining the collagen ultra-structure of the ACM. Figure 16 shows the characteristic banding pattern of mature dermal collagen bundles in a sample of ACM.
Figure 16. Pig ACM by transmission electron microscopy (16,000x) showing the typical appearance of type I collagen fibrils.

Often experimental conditions can change the structure of a material under investigation, thereby introducing additional variables. The structure of pig dermal ACM however, appeared very stable. This can be hypothesized to be due to the tightly packing of the dense collagen network. It is known that collagen-based materials are more stable with a higher degree of crosslinking; the extent of crosslinking in pig dermis is not known however. Figure 17 shows how the ACM collagen matrix structure appears relatively unchanged histologically after freeze-drying, centrifuging, or after several weeks of soaking in culture media at 37°C. Preservation of pig ACM structure under these conditions was further confirmed by freeze-fracture SEM examination. These results suggest that, under the conditions used in this study, pig ACM may be a consistent model for studying fibroblast interactions in an intact dermal collagen matrix.
It was desirable as well to have a dermal matrix scaffold which could be selectively enriched with certain soluble matrix molecules. In this way, the specific effects of exogenous matrix molecules could more easily be determined. The presence of glycosaminoglycans in pig ACM was determined qualitatively by alcian blue staining of freeze-fixed sections. Figure 18 demonstrates alcian blue staining of the anionic components of pig dermis and the ACM. Alcian blue binds primarily to sulfated glycosaminoglycans such as heparan sulfate and chondroitin sulfate, but also binds to hyaluronan. These histology slides suggest that glycosaminoglycans appear to be removed in the acellularization process. For alcian blue staining, the tissue was embedded in O.C.T. compound without fixation, which prevented the extraction of glycosaminoglycans from the matrix that occurs with formalin fixation.
Figure 18. Pig ACM stained with alcian blue, showing minimal residual anionic matrix components after acellularization (compared with alcian blue staining of pig dermis (b)).

4.1.2.2 Pig ACM versus human ACM

Pigs are widely used as animal models of wound healing because of the many similarities between pig and human dermis. Grossly, pig and human ACM have several features in common (Figure 19). Both materials appear white, given the absence of cells, blood and fat in the tissue. Since pig and human ACM are both predominantly composed of collagen, they have similar handling properties.

Figure 19. Gross appearance of pig and human dermal ACM.

There are, however, differences in the physical properties of pig and human ACM. Pig ACM is qualitatively more rigid, and thus maintains its shape better than human ACM.
when cut. Human ACM appears more extensible than pig ACM, and exhibits more primary contraction, subjectively. Moreover, human ACM is more difficult to cut tangentially, than pig ACM; the extensible human dermal tissue does not provide as much resistance to the dermatome blade.

These differences may limit the relevance of pig ACM as a model of human dermal cell-matrix interactions. However, its ease of handling and processing make pig ACM an attractive material for studying certain aspects of dermal wound healing. For example, the study of regulatory cytokines in fibroplasia would likely require many experimental samples to investigate all possible concentrations of different cytokines. Pig ACM may be reliably harvested in large amounts to provide hundreds of samples of equal dimensions. Another example is the study of dermal contraction. This study shows that this phenomenon is highly dependent on the dimensions of the contracting material (Section 4.2.8). With pig ACM, it is much easier to control the dimensions of the ACM samples.

The histological similarities of pig and human dermal ACM include the interlacing network of collagen bundles of varying thickness, and the apparent spaces between the collagen bundles. These spaces appear to be large enough in both species to accommodate the migration of dermal cells such as fibroblasts into the matrix. These features can be seen in the H&E stained sections of pig and human dermal ACM in Figure 20.

![Figure 20. Histological comparison of pig and human dermal ACM collagen matrix structure by histology.](image-url)
Another difference is the relatively higher density of collagen in the pig ACM; the human collagen matrix appears to be less tightly packed. A discrepancy in the density of collagen in the dermal matrix may explain the apparent difference in “rigidity” between pig and human ACM. This tight packing of collagen may be a result of a greater proportion of collagen in the matrix, as well as more cross-linking between the collagen bundles, compared with human ACM. These aspects of pig dermis have not been investigated. The apparent difference in extensibility may be due to higher amounts of elastin in the human ACM (Meyer and Neurand, 1987). Furthermore, the human ACM samples used in this study were obtained from hypertrophic breasts requiring reduction mammaplasty. The dermal collagen fibres have therefore been previously stretched for long periods of time, which is known to result in lengthening of collagen fibres (Elder, Elenitsas et al., 1997), and thus loosening of the collagen network. In future work, it may be of interest to quantitatively compare the density of pig and human dermis, as well as to quantify the elastin-collagen ratio in the two materials.

Relatively minor structural differences may influence cell-matrix interactions (Yannas, 1997). For pig dermis to be used as an in vitro model of fibroplasia or as a dermal substitute, its dermal matrix structure must allow human fibroblast infiltration. Since histological processing can modify the dermal collagen matrix structure (Meyer and Neurand, 1987), scanning electron microscopy is believed to provide a more accurate representation of biological structures. SEM pictures were examined for any differences in dermal ACM structure between the two species. The surfaces of freshly acellularized pig and human ACM were compared by SEM in Figure 21. By freeze-fracture SEM, cross-sectional views were compared (Figure 22).
Figure 21. Pig (a) and human (b) ACM, scanning electron micrographs of tangentially cut surface, 500x. Collagen bundles (arrows) are loose and irregular on surface of human ACM (b).

Figure 22. Pig (a) and human (b) ACM, freeze-fracture scanning electron micrographs of ACM in cross-section, 100x. Pig and human ACM were taken from dermis 0.04-0.06" below the epidermal surface. Arrows point to cross-sectional aspect.

In both surface and cross-sectional SEM, the pig ACM appears denser than the human ACM. This difference in cross-sectional matrix structure may be significant in terms of the scaffold properties of pig ACM. Migrating cells may less easily infiltrate the pig matrix structure. As well, the surface of the human ACM appears more rough and
uneven than that of the pig ACM. This may be due to differences in (1) collagen matrix structure specific to anatomic location, (2) how the fibrillar collagen is altered by soaking in PBS for a few weeks, or (3) cutting techniques (i.e. electric dermatome for pig dermis, hand-held dermatome blade for human dermis). The slight differences between the surface characteristics of pig and human ACM may also affect the behaviour of fibroblasts on the surface. The effect of these discrepancies in matrix structure on in vitro fibroplasia will be investigated in Section 4.2. The structural properties of pig and human ACM found in this evaluation are summarized in Table 16.

<table>
<thead>
<tr>
<th></th>
<th>Pig ACM</th>
<th>Human ACM</th>
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<tbody>
<tr>
<td><strong>Source</strong></td>
<td>Paravertebral area of back</td>
<td>Lateral poles of breast</td>
</tr>
<tr>
<td><strong>Processing</strong></td>
<td>Electric dermatome</td>
<td>Hand-held dermatome blade</td>
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<tr>
<td><strong>Gross</strong> (qualitative)</td>
<td>Conformable</td>
<td>Conformable</td>
</tr>
<tr>
<td></td>
<td>More rigid than human ACM</td>
<td>More clastic than pig ACM</td>
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<tr>
<td><strong>Histology</strong></td>
<td>Densely packed network of interwoven collagen bundles</td>
<td>Loosely packed network of interwoven collagen bundles</td>
</tr>
<tr>
<td><strong>Surface SEM</strong></td>
<td>Smooth, cleanly cut surface with residual hairs</td>
<td>Irregular surface with loose collagen bundles, no hair</td>
</tr>
<tr>
<td><strong>Cross-sectional SEM</strong></td>
<td>Densely packed network of interwoven collagen bundles</td>
<td>Loosely packed network of interwoven collagen bundles</td>
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Table 16. Structural properties of pig and human dermal ACM.

An obvious variable in this comparison of pig and human dermal ACM is their discrepant anatomic locations. One might think that a comparison of pig and human ACM structure should be performed on dermis from equivalent anatomic locations in each species. After all, back and breast dermis may differ in thickness, mechanical properties and ultrastructure. However, an adequate supply of human dermis for in vitro modeling could realistically only be obtained from abdominoplasty or reduction mammmaplasty surgical specimens. In addition, pig dermis can only be harvested efficiently from the rigid back and flank areas, where an electric dermatome can be used. As the objective of this investigation is to compare two potential materials for in vitro modeling, human breast dermis and pig back dermis were evaluated in this study. Moreover, the structure of pig back dermis is relevant to its potential application as a dermal substitute material as well.
4.1.3 Summary

A major challenge in the development of biomaterials is the modulation of the host inflammatory and ensuing fibrotic responses. An effective dermal replacement may be derived from non-antigenic, naturally occurring materials already present in the dermis. The restoration of the normal extracellular matrix architecture is believed to facilitate regeneration of the injured tissue. Despite ongoing research in this area, not enough is known to allow the reconstruction of a synthetic three-dimensional fibrous collagen matrix with sufficient resemblance to normal dermal collagen. Allograft and xenograft tissues are therefore the focus of considerable interest as starting materials for dermal replacement.

Our acellularization protocol appears to remove cells from pig dermis while preserving an intact dermal collagen and elastin matrix. This protocol has not been fully characterized in terms of its effect on basement membrane and specific soluble matrix components, and needs optimization for complete removal of all cellular proteins and hairs. However, the resulting three-dimensional scaffold has the potential to more closely replicate the structure of acellular human dermis, without the associated problems of limited availability and high cost. The ability of this scaffold to support human cell ingrowth will be critical to its use as a dermal substitute. For this in vitro evaluation of dermal fibroplasia on pig and human ACM, the current acellularization protocol was adequate. For future in vivo evaluations of acellularized pig dermis however, better removal of hairs and hair follicle proteins will be required.

It is essential in this study to produce a dermal matrix from which all pig fibroblasts have been removed, and preferably all pig hyaluronan and sulfated glycosaminoglycans as well. Vimentin staining clearly shows a complete removal of all pig mesenchymal cells, including fibroblasts, from the dermis (Figure 9). Alcian blue staining shows a decrease in staining for glycosaminoglycans in the matrix (Figure 18). This method however is not quantitative. In future studies, glycosaminoglycan extraction from the matrix, electrophoretic separation and quantification with alcian blue standards will be performed (Freund, Siebert et al., 1993).
The matrix structure of pig ACM was evaluated by histology, scanning electron microscopy and transmission electron microscopy. The structure of pig ACM was evaluated for several reasons: (1) To determine if the acellularization process or the experimental conditions modify the collagen and elastin matrix structure. (2) To determine if the structure of pig ACM is similar enough to that of human ACM, to be suitable for use as an in vitro model of human dermal fibroplasia. (3) To evaluate its suitability as a xenograft dermal substitute scaffold.

The collagen matrix structure appeared unchanged after acellularization, centrifugation, freeze-drying or culture with human fibroblasts. It remains to be investigated whether prolonged storage of pig ACM in sterile PBS at 4°C affects the tightness of the collagen bundle network. Grossly, the tissue did not appear changed, even after four months of storage.

Several differences were seen, subjectively, between the structure of pig and human ACM. It is not known whether these differences in collagen bundle packing, overall density of collagen, rigidity and extensibility are significant. Their significance will be determined by testing the relative effectiveness of pig and human ACM as scaffolds for human fibroblast infiltration. According to Yannas, the pore size of a scaffold for cell infiltration is important to the diffusion of nutrients through the scaffold’s pores to the infiltrated cells, as well as the provision of sufficient surface area for cell binding (Yannas, 1997). Both these parameters are critical to permit cell infiltration into a scaffold. The degradation properties of a scaffold can also influence its interactions with cells. It is possible that fibroblasts secrete collagenases to digest the ACM as they migrate into the collagen matrix (Mauch, Adelmann-Grill et al., 1989). The relatively tight collagen matrix of the pig ACM may therefore still be effective as a scaffold, provided it can be progressively digested by migrating fibroblasts.

The most compelling reason for testing pig ACM as a potential dermal substitute is the success of acellularized human dermis in vivo as a dermal substitute. Allograft human ACM becomes infiltrated with host cells and well-vascularized, supports overlying keratinocyte viability and decreases the extent of wound contraction in full thickness
wounds (Wainwright, 1995). A dermal substitute may fit in to one or more of the following designs. (1) They can be acellular but designed to become infiltrated by host cells. (2) They can be pre-seeded with cells normally found in the wounded tissue. (3) They can be fabricated with degradable biopolymers, into which infiltrating cells create a tissue specific extracellular matrix. (4) They can consist of an enriched scaffold designed specifically to influence cellular activities during the healing process. The rapid integration of human acellular dermal matrix into the wound, despite its acellularity, demonstrates the feasibility of using a dermal matrix as a dermal substitute.

4.2 Fibroplasia in an Acellular Pig Dermal Matrix

4.2.1 Cell Morphology
It is important to characterize the relative effects of pig ACM on fibroblast morphology, in order to determine the suitability of pig ACM as a dermal substitute, as well as an *in vitro* model of fibroplasia. The morphological appearance of firmly adherent cells differs from that of migrating cells as well as contracting cells (Ruoslhti, 1991). Cell morphology and behaviour are known to be influenced by a number of factors related to the extracellular environment. Certain materials (such as collagen) trigger the expression of surface receptors (such as integrins) to allow for specific material-receptor binding. The number and type of receptors expressed help to regulate the degree of adherence to and migration on the surface (Chevallay and Herbage, 2000). A difference in adherence is reflected in the cell morphology (Doillon, Silver et al., 1987). With the difference in collagen architecture and surface structure between the collagen gel and the ACM, it was hypothesized that fibroblasts may be differentially adherent to the two materials, and that the effects of the materials would be reflected in the cell morphology.

Confocal laser microscopy allows for the visualization of fluorescently-stained fibroblasts adherent to the ACM surface. While regular or phase-contrast microscopy is typically used to visualize cells cultured on coverslips or on the semi-translucent collagen gel, cells grown on the dense dermal ACM must be stained and viewed under fluorescence. Figures 23 and 24 show the appearance of primary human fibroblasts on pig ACM, human ACM, collagen gel and coverslips after five days in culture.
Figure 23. Confocal microscopy of passaged human dermal fibroblasts stained for phalloidin, cultured on different collagen substrata, at low magnification (10x).
The fibroblasts were clearly smaller on the collagen substrates, especially human ACM, relative to fibroblasts grown on coverslips. The spread-out, planar morphology of the coverslip fibroblasts contrasts starkly with the small, spindle-shaped morphological appearance of dermal fibroblasts in vivo (Grinnell and Lamke, 1984). On collagen gel, the fibroblasts are also large, but appear more linear, with a "bipolar" appearance (Nedelec, Ghahary et al., 2000). In general, fibroblasts on the pig and human ACM were smaller than those on collagen gel or polystyrene. The majority of fibroblasts on pig ACM were spindle-shaped (Figure 23c), while a wide range of morphologies was displayed on the human ACM (Figure 23d). This suggests perhaps a response of the
fibroblasts to the different material properties of pig and human ACM. Fibroblast morphology can be affected by surface roughness, cell surface receptor binding, collagen composition and mechanical tension (Guidry and Grinnell, 1985), (Nedelec, Ghahary et al., 2000). Pig ACM has both a smoother surface and is more rigid than human ACM, thereby possibly resulting in altered fibroblast morphology. The relevance of cell morphology to fibroblast behaviour on these materials in vitro will be discussed in later sections.

4.2.2 Cell Proliferation

It was not known prior to this study whether or not human fibroblasts would remain viable if cultured on the pig dermal ACM prepared in our laboratory. It was therefore necessary to develop methods for determining the viability of cells cultured on the ACM over time. Results from the MTT and CyQuant assay will be presented here. Other methods attempted included: trypsinization and cell counting with a hemocytometer, and digestion and spectrophotometric quantification of methylene blue-stained cells. These methods were unsuccessful for a number of reasons. The fibroblasts did not separate easily from the ACM with trypsinization. Moreover, given the small size of the ACM and the relatively few cells on its surface, the number of cells available for counting was below the sensitivity of the hemocytometer. There was also significant non-specific staining of the ACM tissue with methylene blue, despite multiple rinses in PBS.

4.2.2.1 MTT Assay

The MTT assay provided the first confirmation that fibroblasts seeded on the ACM remained viable under culture conditions. The yellow tetrazolium dye (MTT) is cleaved to a purple formazan by the succinate-tetrazolium reductase system, which belongs to the mitochondrial respiratory chain. Only viable cells are believed to be capable of this enzymatic cleavage. When the MTT solution is incubated with viable cells, the yellow solution becomes purple, in proportion to the number of cells present. However, serum enzymes are believed to interfere with this assay (Denizot and Lang, 1986). Serum components in the culture media adsorb to the ACM and may not be completely washed out prior to the addition of the MTT. It is therefore not surprising that the MTT is
converted to its purple product when placed on ACM samples even in the absence of cells (Figure 25).

![Image](image.png)

**Figure 25.** Pig ACM without cells, after four hour incubation with MTT and extraction with isopropanol. MTT is converted to purple formazan dye in absence of cells on the ACM. As well, the purple dye is incompletely extracted from the ACM with isopropanol.

Figure 26 shows a comparison of the MTT calibration curves with cells only and with cells cultured on the ACM. Background is increased in the presence of ACM due to non-specific conversion of MTT. It was therefore difficult to show a lack of cell viability for cells seeded on ACM, given the falsely elevated results in the presence of ACM.

![Image](image.png)

**Figure 26.** Calibration curves with the MTT assay of passaged human dermal fibroblasts in the presence and absence of ACM.

A preliminary assessment of cell viability over time on the pig ACM, as compared to cells grown directly on polystyrene wells, is shown in Figure 27. The results are difficult to interpret, due to the poor specificity of the MTT assay on the cell-seeded ACM. The rate of cell proliferation on the ACM appears to be low, accounting for the lack of increase in absorbance over time. It was observed that the cells floated off the culture dish with isopropanol extraction, but the cells seeded on the ACM did not. This may have resulted in a greater proportion of the formazan being measured in the cells only group. To clarify these potential errors with the use of the MTT assay with cells
grown on different substrates, the CyQuant proliferation assay was adapted for use on the ACM.

4.2.2.2 CyQuant Assay
The CyQuant assay was selected for reasons outlined in Section 3.2.3.2. A notable advantage of the CyQuant assay over the MTT assay was that the non-specifically

![Cell Viability by MTT Assay](image)

**Figure 27.** Passaged human dermal fibroblast viability on different substrates over time, by the MTT assay.

adsorbed dye does not affect the measured fluorescence. Fluorescence at 538 nm only occurs if the dye is bound to DNA. If anything, fluorescence would be falsely lowered in the presence of ACM because of the decreased availability of the dye for binding to DNA.

Figure 28 shows a comparison of the calibration curves performed in the absence and in the presence of digested ACM. The presence of digested ACM resulted in a higher fluorescence reading for blank samples, but did not significantly affect the slope of the graph or the $r^2$ values. It was found that collagenase digestion did not work as well in a
37°C incubator without CO₂ or humidification, and that calibration curves prepared in this manner had poor $r^2$ values (data not shown).

![CyQuant Calibration Curves](image)

**Figure 28.** Calibration curves with the CyQuant assay of human dermal fibroblasts in the presence and absence of digested ACM.

Given the reliability of the CyQuant assay in assessing cell numbers in the presence of ACM, this assay was used to quantitate fibroblast proliferation on pig ACM. Their proliferation on pig ACM was compared to that of fibroblasts cultured on human ACM, collagen gel and directly on the culture dish. In Figure 29, fluorescence is proportional to cell number. The percent change from day 0 fluorescence, for cell-seeded sample after different lengths of time in culture, is shown.
The error bars in Figure 29 represent standard deviations. As expected, the cells proliferated when cultured on the culture dish only (brown squares). The fibroblasts were also found to proliferate on the pig ACM (purple circles). A comparison of Figures 27 and 28 suggests that the MTT assay is unreliable in measuring cell viability on the ACM. The proliferation rate can be estimated by looking at the slope of the trendlines for each group. It can be seen that the proliferation rates for the fibroblasts are similar on the culture dish and on the pig ACM. However, the fibroblasts proliferated significantly more slowly on the collagen gel and human ACM, i.e. the two substrates which supported more cell infiltration (Section 4.2.4 and 4.2.6). These results suggest that human fibroblasts behave differently on pig ACM than on human ACM. Pig dermal ACM may therefore not be as similar to human dermal ACM as expected. This surprising difference between the pig and human ACM will be discussed more in later sections.

A source of error in the CyQuant assay was likely the incomplete digestion of the human ACM by collagenase. The thicker human ACM samples required more time to be digested than the pig ACM and the collagen gel samples, resulting in a higher
background reading for the human ACM. To account for this, the measured fluorescence for each sample was reduced by the average background reading for each substrate type.

4.2.3 Collagen Synthesis
New collagen synthesis by fibroblasts cultured on pig ACM was compared to that of fibroblasts cultured on collagen gel and in the absence of collagen. It was of interest to determine how the extracellular matrix environment influenced cellular behaviour in this aspect of fibroplasia. The amount of radiolabelled proline uptake by the cells corresponds to the total protein synthesis during the incubation time (23 hours). Figure 30 shows the results for the different groups. The figures for each sample are normalized for the cell number, as determined by the CyQuant assay.

![3H-Proline Incorporated](image)

**Figure 30.** Protein synthesis by human fibroblasts seeded on different substrates. * indicates significance < 0.05 between groups shown by bracket, as determined by the Mann-Whitney and Wilcoxon tests.

By comparing the total cpm with the cpm remaining after digestion with collagenase and removal of the collagenase-soluble radioactivity, it was possible to determine what percentage of total protein synthesis was devoted to collagen synthesis. Figure 31 shows the percentage of collagenase-soluble radiolabeled protein, i.e. newly synthesized collagen, by the fibroblasts in each group. The standard deviation in each group is indicated by error bars.
Collagen synthesis by human fibroblasts cultured on different substrates. Collagen synthesis by monolayer fibroblasts was significantly different than that of fibroblasts grown on the other two media (chi-squared test).

In gels of contracted collagen, the synthesis of collagen by fibroblasts is strongly inhibited (six to eight times less than in monolayer culture by both pre-transcriptional (Mauch, Hatamochi et al., 1988) and post-transcriptional mechanisms) (Eckes, Mauch et al., 1993). Reduction in collagen synthesis could be the result of negative feedback induced by biochemical confinement, as observed in contracted collagen gels containing fibroblasts (Nakagawa, Pawelek et al., 1989). Collagen synthesis in this study was measured four days after seeding the fibroblasts on the ACM and collagen gel; no infiltration would be expected at that point. The fibroblasts would be expected to have formed a monolayer on the ACM or gel surface, and to be in contact with the collagen matrix but not surrounded by it. The results suggest that collagen synthesis by fibroblasts is decreased even if the cells are not confined by collagen.

Dermal ACM and collagen were compared to determine whether the nature of the collagen in contact with the fibroblasts had any effect on their collagen biosynthesis. Collagen synthesis was less on the collagen gel than on the ACM. The inhibitory effect of extracellular collagen on fibroblast collagen synthesis may therefore be influenced by collagen structure. The percentage of new protein synthesis dedicated to newly synthesized collagen appeared consistent between the three groups. There was however considerable variability in these calculated values. The radiolabelled proline assay was
useful for measuring protein synthesis by cells cultured on ACM or collagen gel, but was less reliable at determining the collagenase-soluble portion of the total protein. An alternative collagen synthesis assay involves measuring radiolabelled hydroxyproline incorporation.

4.2.4 Cell Infiltration

The study of cell-matrix interactions in vitro allows for the isolation of specific wound healing events. In this study, the effect of the preserved pig dermal matrix structure on fibroblast infiltration is of interest for a variety of reasons. A potential dermal substitute must support rapid host cell infiltration to achieve incorporation into the wound bed and, in the case of fibroblasts, to begin matrix remodeling and wound closure. Previous studies demonstrate that acellular human dermis acts as an effective scaffold for cell infiltration in vivo. Acellular pig dermis however is not incorporated as effectively into full thickness wounds (Section 2.5.2.4). While the reason for this may be immune rejection, it is also possible that its structure is different enough from that of human dermis to prevent effective host cell infiltration.

Using the culture conditions described in the Methods section, minimal fibroblast infiltration was seen in the majority of pig ACM samples by H&E staining (Figure 32).

![Figure 32. Pig ACM seeded with human fibroblasts, after 4 weeks; H&E, 10x. There is considerable proliferation of the fibroblasts at the surface (arrow), with few cells present within the matrix.](image-url)
Vimentin staining of the cell-seeded ACM sections allowed greater visualization of the fibroblasts. The entire cytoplasm of the cells was stained, as opposed to just the nucleus. Even with vimentin staining however, very few cells were seen below the surface in the majority of pig ACM samples (Figure 33). A total of 75 cell-seeded ACM samples, cultured for over 3 weeks, were analyzed, histologically, representing six different batches of pig ACM and primary human dermal fibroblasts from five different donors. Of all the samples examined, 50/75 (67%) samples showed surface infiltration only, i.e. infiltration to only within 100 μm of the surface.

![Figure 33](image_url). Pig ACM seeded with human fibroblasts, after 4 weeks; vimentin, 10x.

In a minority of samples, (25/75; 33%) deeper fibroblast infiltration could be seen on at least one section. These results were initially difficult to interpret, without appropriate controls. The inconsistent fibroblast infiltration of pig ACM was hypothesized to be due to either (1) the in vitro culture conditions not favouring fibroblast migration, or to (2) poor scaffold properties of pig ACM. Since human ACM has been shown to support human fibroblast infiltration in vitro, this material was used in this study as a positive control for fibroblast infiltration in vitro.
4.2.5 Comparison of Pig ACM to Human ACM as a Scaffold

Acellular human dermis has been shown in previous studies to act as an effective scaffold for infiltrating fibroblasts in vitro (Ghosh, Boyce et al., 1997), (Ralston, Layton et al., 1997). Fibroblast infiltration in vitro is therefore a convenient basis of comparison for the structural similarity between pig and human dermis. The influence of various culture conditions on fibroblast infiltration of acellular dermis has not been studied previously. However it is known that fibroblasts do not infiltrate the dermal basement membrane in the absence of keratinocytes. All dermis used in this study was therefore second-cut dermis, without basement membrane.

Table 17 and Figure 34 compare pig and human ACM in terms of the percentage of samples with infiltration by human fibroblasts, and the number of cells per section, respectively. ACM samples in both groups were selected from all levels of dermis, freeze-dried, cut to fit the culture dish, seeded with 25,000 or 30,000 cells/cm² and incubated for 3 to 5 weeks. Equivalent freeze-dried and non-freeze-dried samples of pig ACM are included in the table for comparison.

<table>
<thead>
<tr>
<th>ACM</th>
<th>Total number of samples</th>
<th>Number of samples with cell infiltration in at least one section</th>
<th>% of samples</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pig</td>
<td>49</td>
<td>15</td>
<td>31%</td>
<td>*Human</td>
</tr>
<tr>
<td>FD-Pig</td>
<td>17</td>
<td>9</td>
<td>53%</td>
<td>*Human</td>
</tr>
<tr>
<td>FD-Human</td>
<td>24</td>
<td>20</td>
<td>83%</td>
<td>*Pig, FD-pig</td>
</tr>
</tbody>
</table>

Table 17. Comparison of human fibroblast infiltration in fresh pig ACM, freeze-dried pig ACM and freeze-dried human ACM. There was a significant difference between the groups indicated by *, by the chi-squared test.
Figure 34. Fibroblast infiltration of pig and human ACM at 4 weeks, determined by automated cell counting. The number of cells per section was normalized for section size, then averaged for 16 samples, with three sections each. The bars represent standard deviation.

The human ACM group had more samples with significant cell infiltration and more cells per section than did the pig ACM group. The substantial variability between samples is represented by the high standard deviation. It was also noted that the distribution of fibroblasts in the matrix differed considerably between pig and human ACM. The distance of each cell from the surface was therefore measured to quantitatively compare the cell distribution. In Table 18, the distance of the infiltrated cells from the cell-seeded surface is shown. Freeze-dried samples of cell-seeded pig and human ACM at 3 weeks were analyzed. To calculate the chi-squared statistic, the actual distance measurements for each cell were used to determine if the distribution of the cells in the two groups was statistically different.

<table>
<thead>
<tr>
<th>Range of cell distance from surface</th>
<th>Pig ACM (n = 5)</th>
<th>Human ACM (n = 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>15-434 μm</td>
<td>34-492 μm</td>
<td></td>
</tr>
<tr>
<td>% of infiltrated cells:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0-150 um</td>
<td>78.8 ± 8.3</td>
<td>38.3 ± 6.5</td>
</tr>
<tr>
<td>151-300 um</td>
<td>14.2 ± 7.9</td>
<td>29 ± 6.2</td>
</tr>
<tr>
<td>301-500 um</td>
<td>8 ± 1.2</td>
<td>33 ± 6.6</td>
</tr>
<tr>
<td>Chi-squared test on individual cell distance measurements (n = 300 per sample)</td>
<td>p &lt; 0.05</td>
<td></td>
</tr>
</tbody>
</table>

Table 18. Distance of cells from monolayer surface, in representative fields of vimentin-stained sections of cell-seeded ACM samples at 3 weeks. n = number of samples per group. Distances of over 100 cells per field were recorded.

Human ACM appeared to support fibroblast migration further from the surface. Although a few fibroblasts did migrate a considerable distance from the surface in pig ACM, the vast majority were within 150 μm from the surface. The cells in the human ACM appeared to have migrated more quickly throughout the matrix (Figure 35).
Figure 35. Typical appearance of human ACM seeded with fibroblasts after 3 weeks, vimentin, 10x. The cell-seeded surface is at the top of the image.

In pig ACM, the fibroblasts were observed to migrate below the cell-seeded surface along the empty hair follicle, but without migrating into the actual collagen matrix. This was not seen in the human ACM, due to the paucity of hair follicles. The successful infiltration of human ACM demonstrates the effectiveness of the in vitro methods at modeling in vivo host cell infiltration. It seems likely therefore that the poor infiltration of pig ACM by human fibroblasts is mainly attributable to its scaffold properties. The increased fibroblast infiltration in the human ACM suggests that human and pig dermis may differ in structure.

The structure of human and pig dermis were therefore compared, before and after fibroblast seeding. Figure 36 shows, by freeze-fracture SEM, a comparison of the structure of cell-free, and cell-seeded, pig and human ACM, after 4 weeks in culture. All samples were from dermis taken 0.02-0.04 inches below the epidermis, and freeze-dried prior to incubation.
Figure 36. Pig and human ACM at day 0 and at day 28 after cell seeding. Cross-sectional scanning electron micrographs of the matrix structure at the surface, 500x. Scale bar: 60 μm.

A greater change in matrix structure with cell seeding is seen in human ACM. The structure of pig ACM appears well preserved in the presence of human fibroblasts. In contrast, the collagen bundles in human ACM appear to be even more loosely packed after cell seeding. This change in matrix structure with cell culture may be attributable to the effect of matrix collagen digestion by fibroblasts, or to the effect of prolonged soaking in culture media at 37°C. It is not surprising that the looser matrix structure of the human ACM is more easily modified. The dense pig ACM, on the other hand, appeared more resistant to structural change under the culture conditions.

The mature collagen bundles and porous nature of pig ACM are favourable features in a potential dermal substitute. However, the results of fibroblast-seeding experiments show that pig ACM is not equivalent to human ACM, with respect to human fibroblast infiltration in vitro. The densely packed nature of pig dermal collagen matrix may delay its infiltration by host cells. Future work in this area should focus on the modification of the acellular pig dermal matrix structure, to improve its scaffold properties. This will be discussed in more detail in Section 5.1.2.
To determine whether the variability in fibroblast infiltration of pig ACM could be reduced, and infiltration optimized, each of the experimental variables hypothesized to be a factor in cell infiltration was analyzed for its effect. The conditions of samples with cell infiltration were examined to determine what factors influenced fibroblast infiltration into the pig ACM. The variables examined included fibroblast type, seeding density, cell culture setup, culture media, microvascular endothelial cell co-culture, time in culture, level of dermis and sample freeze-drying. Only time in culture showed a statistically significant effect, while a trend was seen towards greater infiltration with freeze-drying of samples.

### 4.2.5.1 Time in culture

<table>
<thead>
<tr>
<th>Time in culture</th>
<th>Number of infiltrated cells per section</th>
<th>Significance between groups</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Average</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>1 week (n = 8)</td>
<td>15.63</td>
<td>18.98</td>
</tr>
<tr>
<td>3 weeks (n = 8)</td>
<td>61.25</td>
<td>55.56</td>
</tr>
<tr>
<td>5 weeks (n = 16)</td>
<td>94.38</td>
<td>45.52</td>
</tr>
<tr>
<td>7 weeks (n = 8)</td>
<td>203.13</td>
<td>22.34</td>
</tr>
</tbody>
</table>

*Table 19. Effect of culture time on primary human fibroblast infiltration into pig ACM. ACM samples of 16 mm diameter and seeded with 25,000 cells/cm² only were compared. n = number of samples examined per time point. By one-way ANOVA, it was determined that at least one of the four groups was significantly different. Post-hoc Tukey HSD allowed pairwise comparisons of all the groups for significant differences.*

The length of time in culture had a clear effect on fibroblast infiltration of the pig ACM. Presumably the process of infiltration requires a period of time for fibroblast migration and possibly digestion of the matrix by secreted collagenolytic enzymes.

### 4.2.5.2 Level of dermis

It was hypothesized that the ultrastructure of the pig ACM varied with its depth from the epidermal surface. Histologically, only very minor structural differences were observed across a cross-section of full-thickness pig ACM (Figure 37).
Figure 37. Pig ACM for full-thickness pig dermis from 30-11-00 batch, H&E, 5x. Epidermal surface is at lower right-hand corner, and fat surface at upper left-hand corner.

Pig ACM from the 30-11-00 batch was cut into levels of equal thickness and seeded with the 25,000 cells/cm². There were only three samples of each level fixed for analysis at five weeks. The results are summarized in Table 20.

<table>
<thead>
<tr>
<th>Level of dermis</th>
<th>Total number of samples</th>
<th>Number of samples with cell infiltration in at least one section</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>2</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>4</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>5</td>
<td>3</td>
<td>2</td>
</tr>
</tbody>
</table>

Table 20. Effect of dermal level on fibroblast infiltration of pig ACM.

While the numbers are too small to analyze statistically, these results show that the level of dermis does not appear to have a major effect on the ACM's infiltration by fibroblasts. When comparing fibroblast infiltration between this and other experiments however, it was noted that the ACM prepared from this batch had the highest degree of infiltration (13/15, or 87%). Why fibroblast infiltration should vary between batches of
pig ACM, cannot be conclusively explained from this work. Possible reasons for this, which will be investigated for thoroughly in future work, include the effect of freeze-drying, the age of the pig, the length of time in storage in PBS, and the inter-experiment variability in cell adherence to the ACM.

4.2.5.3 Freeze-drying

Freeze-drying is a standard procedure for preparing biological materials for storage. Many potential biomaterials require freeze-drying before being stored and transported, and therefore before being used in the body. Previous studies suggest that the structure of a dermal collagen matrix is not significantly modified by freeze-drying (Ghosh, Boyce et al., 1997). However these studies employed histological methods for assessing the collagen matrix structure, which can in itself result in alteration in structure. Collagen may shrink slightly with freezing, but it is not known whether this significantly alters cellular responses to the material (Doillon, Brandwein et al., 1986). Freeze-dried samples were therefore compared to samples stored in PBS at 4°C for any effect on fibroblast infiltration. The use of freeze-drying was not required for fibroblast infiltration of the pig ACM to occur, but did not impair infiltration (Table 21). Freeze-dried samples were therefore compared to samples stored in PBS at 4°C for any effect on fibroblast infiltration. The use of freeze-drying was not required for fibroblast infiltration of the pig ACM to occur, but did not impair infiltration (Table 21). Freeze-dried samples were more convenient to store and handle, and easily rehydrated. In subsequent experiments, pig ACM samples were freeze-dried and rehydrated prior to cell seeding.

<table>
<thead>
<tr>
<th>Freeze-drying</th>
<th>Total number of samples</th>
<th>Number of samples with cell infiltration in at least one section</th>
<th>% of samples with cell infiltration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yes</td>
<td>17</td>
<td>9</td>
<td>53%</td>
</tr>
<tr>
<td>No</td>
<td>49</td>
<td>15</td>
<td>31%</td>
</tr>
</tbody>
</table>

Table 21. Effect of freeze-drying on fibroblast infiltration of pig ACM. ACM samples seeded with 25,000 or 30,000 cells/cm² and cultured for > 3 weeks. No significant association was found between freeze-drying and cell infiltration of pig ACM (two-sided Fisher’s exact test).

The examination of the experimental conditions in the cell infiltration experiments revealed some interesting information. Of all the variables studied in a controlled manner, only time had a statistically significant effect on cell infiltration. However, not all the ACM samples cultured for five weeks had fibroblast infiltration. There are
clearly additional variables at play which were not controlled for in this study. For example, it may be that the differences in the extent of cell infiltration among the pig ACM were due to differing lengths of storage time in PBS. Slight differences in cell seeding technique may also have occurred unknowingly. Alternatively, fibroblasts in culture may infiltrate the ACM scaffold to a greater degree after a certain number of passages than others. In future work, all ACM samples will be freeze-dried immediately after acellularization. In this way, the variable of storage time can be eliminated, and any variability of the pig ACM itself could be discerned.

4.2.6 Myofibroblast Differentiation
During wounding, fibroblasts at the wound edge receive signals from cytokines and matrix molecules to begin migrating into the wound clot. A proportion of these wound fibroblasts then differentiates to myofibroblasts. The forces generated by myofibroblasts adherent to the underlying matrix are believed to be responsible for wound contraction. Decreased wound contraction is associated with a more rapid disappearance of myofibroblasts from the wound (Rudolph, 1979), (Berry, Harding et al., 1998). It is known that wounds treated with intact dermal collagen contract significantly less than wound treated with denatured autograft collagen (Brown, Garner et al., 1990). Little is understood about the effect of dermal substitute structure on full thickness wound contraction. It was of interest therefore to determine whether the intact dermal collagen structure of allograft or xenograft dermis has an effect on myofibroblast differentiation.

Previous studies of the effect of acellular pig dermis on wound contraction suggest that the xenograft can reduce contraction, provided graft take is achieved quickly. (Wang, Chen et al., 1997), (Fang, Robb et al., 1990). It is therefore possible that pig dermal ACM may be associated with a reduced expression of myofibroblast markers by seeded fibroblasts, in comparison with fibroblasts cultured on the culture dish or a collagen gel. Another question that was addressed in this section was whether any differences in myofibroblast differentiation were seen between the pig and human ACM, to suggest a further evidence for a difference in the microenvironment of the two matrices. These experiments represent preliminary investigations into these questions, and are primarily
an evaluation of the methods used in this study. The results are shown in Tables 22 and 23.

**Figure 38.** Fibroblast-seeded pig ACM, stained for α-smooth muscle actin, demonstrating the cytoplasmic staining of the myofibroblasts (arrow). The nuclei of fibroblasts (asterisks) stain purple with the hematoxylin counter-stain, but their cytoplasm is not seen. 20x magnification.

<table>
<thead>
<tr>
<th>Time</th>
<th>3 days (n=12)</th>
<th>1 wk (n=12)</th>
<th>2 wks (n=15)</th>
<th>4 wks (n=14)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total # of cells per section</td>
<td>96.5 ± 15.1</td>
<td>77.0 ± 48.9</td>
<td>139.2 ± 78.3</td>
<td>226 ± 81.9</td>
</tr>
<tr>
<td>Total # of SMA(+) cells per section</td>
<td>47.3 ± 9.5</td>
<td>38.0 ± 38.2</td>
<td>45.1 ± 51.3</td>
<td>42.5 ± 10.3</td>
</tr>
<tr>
<td>% SMA(+) cells</td>
<td>48.7 ± 12.7</td>
<td>54.9 ± 27.4</td>
<td>32.3 ± 16.3</td>
<td>18.8 ± 9.7</td>
</tr>
<tr>
<td>Significance</td>
<td>* 2, 4 wk</td>
<td>* 2, 4 wk</td>
<td>* 3 d, 1, 4 wk</td>
<td>* 3 d, 1, 2 wk</td>
</tr>
</tbody>
</table>

**Table 22.** Expression of α-smooth muscle actin by fibroblasts cultured on pig ACM after different times in culture. SMA(+) = smooth muscle actin-positive. n = number of sections analyzed. Values represent average of all sections, with standard deviations. Significance determined by one-way ANOVA and post-hoc Tukey HSD.

The temporal expression of SMA by fibroblasts seeded on pig ACM reflects the transition as the fibroblasts migrate from the surface into the matrix; fibroblast expression of SMA was more marked by surface fibroblasts. However, at four weeks, fewer fibroblasts stained for SMA, even at the surface. It can be hypothesized that the surrounding collagen matrix eventually signals to the cell to cease SMA expression. Interestingly, these results can be correlated to the time frame for myofibroblast differentiation in wounds treated with full thickness skin grafts. Wounds covered with a
full thickness skin graft showed a more rapid decrease in myofibroblasts than untreated wounds. Myofibroblasts were found in untreated wounds from 4 to 42 days post-wounding, and from 4 to 21 days in wounds treated with full thickness grafts (Rudolph, 1979). This interesting correlation suggests the possibility that the effects of a dermal collagen matrix on fibroblast differentiation may, at least in part, be demonstrable in vitro.

The effect of pig ACM, human ACM and collagen gel on fibroblast SMA expression was then investigated using these methods. The results are summarized in Table 23.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Pig ACM (n=9)</th>
<th>Human ACM (n=9)</th>
<th>Collagen gel (n=9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total # of cells</td>
<td>139.2 ± 78.3</td>
<td>204.5 ± 97.6</td>
<td>172.6 ± 55.2</td>
</tr>
<tr>
<td>per section</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total # of</td>
<td>45.1 ± 51.3</td>
<td>21.2 ± 16.4</td>
<td>26.8 ± 19.7</td>
</tr>
<tr>
<td>SMA(+) cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% SMA(+) cells</td>
<td>32.3 ± 26.3</td>
<td>10.3 ± 8.9</td>
<td>15.5 ± 12.6</td>
</tr>
<tr>
<td>Significance</td>
<td>*HACM, coll gel</td>
<td>* PACM</td>
<td>*PACM</td>
</tr>
</tbody>
</table>

Table 23. Percentage of fibroblasts on each ACM type expressing α-smooth muscle actin at 2 weeks. SMA(+) = smooth muscle actin-positive. n= number of sections analysed. Values represent average of all sections. Significance determined by one-way ANOVA and post-hoc Tukey HSD.

Surprisingly, fewer fibroblasts expressed SMA when cultured on human ACM or on collagen gel, relative to those on pig ACM. It can be hypothesized that the human ACM and collagen gel are more quickly infiltrated by fibroblasts, thus dispersing the SMA-positive fibroblasts from the monolayer. On the pig ACM by comparison, multiple layers of fibroblasts are present around most samples at two weeks, in some ways resembling a fibrous capsule (Figure 39). A significant proportion of these monolayer fibroblasts expresses SMA.

Mechanical tension is believed to play a role in regulating myofibroblast differentiation. In vivo, the timing of the switch from fibroblast to myofibroblast phenotype appears to depend in part on the extent to which the wound resists contraction (Darby and Gabbiani, 1990). Wounds under tension are commonly observed to heal with more
scarring. Contraction is believed to be initiated by migrating fibroblasts at the wound margins. As contraction proceeds and resistance increases, migrating fibroblasts differentiate into myofibroblasts and the actin cytoskeleton becomes organized along the lines of greatest resistance (Grinnell, 1994). The same factors may be at play in initiating differentiation of fibroblasts on the substrates tested in vitro. The rigidity of the pig ACM, compared to the relative pliancy of the human ACM and collagen gel, may promote myofibroblast differentiation.

Figure 39. Pig ACM after 4 weeks, hematoxylin, 10x. Fibroblasts cultured on pig ACM often formed multiple layers (arrow) at the surface.

4.2.7 Contraction

Models of wound contraction are useful for studying the basic biologic mechanisms involved in wound contraction. Information may also be obtained on the potential of various drugs to modulate this event. Although wound contraction is a normal event in wound healing, excessive contraction can result in disfiguring scarring in certain predisposed individuals and in certain wounds. Identifying agents which modulate wound contraction offers an opportunity to develop treatments for affected patients.
Collagen gel as a model for fibroblast-mediated contraction has been well characterized. While the contraction of collagen gel occurs too quickly to be related to fibroblast migration into the gel matrix, the collagen solution can be mixed with cells prior to gelation, thus distributing the fibroblasts throughout the collagen matrix. In this study, fibroblasts were seeded on the surface of the gel only, to allow a better comparison with pig ACM.

As expected, in this study, the collagen gel contracted within 24 hours to 60% of its original surface area, and within 72 hours, to 15% of its original surface area (Figure 40). An interesting observation of the contracted gels was that the gels were not fixed in a contracted state; the curled up edges of the gels could be restored to their original position with the removal of the media. For thick gels (>3 mm), the cell-seeded surface and the lower portion of the gel contracted to different degrees. Because of the soft flexible nature of the gel, the thickness of the cell-free lower portion of the gel did not inhibit the upper surface of the gel from contracting. The rate of contraction of the cell-seeded surface was not decreased when thicker gels were used. However, the visualization of the outer diameter of the cell-seeded surface was difficult; the thicker gels appeared to have a greater diameter than the thinner gels.

![a) 1 mm thick gel at 24 hours b) 1 mm thick gel at 72 hours c) 3 mm thick gel at 72 hours](image)

**Figure 40.** Contracted collagen gels. All samples were originally the same diameter as the well (201 mm²). The degree of contraction was determined by measuring the change in surface of the cell-seeded surface (outlined in dotted line).

On cross-section histology, the extent of fibroblast infiltration into the collagen gel can be seen. Figure 41 shows minimal infiltration of human fibroblasts into a collagen gel 3 weeks after seeding.
Fibroblast-mediated contraction of collagen gels therefore occurred without minimal fibroblast infiltration below the surface. A similar phenomenon of contraction was seen with the fibroblast-seeded pig ACM. Over a variable period of time, depending on the thickness of the ACM, the cell-seeded surface slowly curled up, thereby reducing the surface area of the entire ACM. To confirm that this change in appearance of the ACM was in fact due to fibroblast-mediated contractile forces, ACM samples of equal thickness were incubated with or without cells and assessed for evidence of contraction. None of the 9 cell-free samples (0%) decreased in surface area, while 36 of 46 cell-seeded ACM (78%) decreased by more than a fifth of their original surface area. Figure 42 illustrates the degree of contraction noted in the cell-seeded ACM samples. The percent change in surface area measurements are shown in Table 24.

The same samples were examined histologically, and found to have minimal fibroblast infiltration into the dermal matrix (Figure 43). It was surprising that fibroblast-mediated
contraction of pig ACM, like the collagen gel, occurred in the absence of significant cell infiltration.

**Figure 43.** Fibroblast-seeded pig ACM at 4 weeks, hematoxylin, 10x. This sample contracted down to 40% of its original surface area, presumably by the forces generated by the monolayer of fibroblasts at the surface (dark blue cell layer on top surface). Very few cells infiltrated below the surface; a few are indicated by red circles.

Fibroblast-mediated contraction of collagen materials has been hypothesized to occur by two possible mechanisms (Ehrlich and Rajaratnam, 1990). The fibroblasts may attach to the collagen surface and subsequently contract, causing a reduction in the distance between collagen bundles. Alternatively, the fibroblasts may migrate a certain distance along the collagen bundles and in doing so, bring the different collagen bundles closer together. Both of these mechanisms seem plausible for the contraction of pig ACM. Regardless of the mechanism, the resulting contraction is restricted to one surface only, causing the ACM to curl up towards the cell-seeded surface as illustrated in Figure 44.

**Figure 44.** Schematic of the mechanism of fibroblast-mediated contraction of pig ACM.
It should be noted that no contraction at all was noted in ACM above a thickness of 0.7 mm. Table 24 shows the relationship between ACM thickness and contraction.

<table>
<thead>
<tr>
<th>Approximate Thickness*</th>
<th>Number of samples</th>
<th>Number of samples with contraction†</th>
<th>Average % original surface area in contracted samples*</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.2 mm</td>
<td>6</td>
<td>6</td>
<td>59% ± 9%</td>
</tr>
<tr>
<td>0.3 mm</td>
<td>6</td>
<td>6</td>
<td>65% ± 6%</td>
</tr>
<tr>
<td>0.4 mm</td>
<td>8</td>
<td>8</td>
<td>71% ± 9%</td>
</tr>
<tr>
<td>0.5 mm</td>
<td>10</td>
<td>7</td>
<td>78% ± 10%</td>
</tr>
<tr>
<td>0.6 mm</td>
<td>16</td>
<td>9</td>
<td>79% ± 6%</td>
</tr>
<tr>
<td>&gt;0.7 mm</td>
<td>40</td>
<td>0</td>
<td>100% ± 4%</td>
</tr>
</tbody>
</table>

Table 24. Effect of ACM thickness on fibroblast-mediated contraction. †Contraction defined as greater than 15% decrease in surface area. All samples were seeded with 25,000 cells/cm², incubated for 4 weeks in EBM-2 with 5% FBS and co-cultured with MEC. Initial surface area: 201 mm². *There was a significant effect of thickness on contraction, by the chi-squared test.

This study attempted to study the effects of the co-culture of MECs on contraction. Fibroblast-seeded samples of equal thickness were either co-cultured with MECs or cultured alone, both in EBM-2 media. A representative set of samples and controls are shown in Figure 45.

Figure 45. Fibroblast-mediated contraction of pig ACM (0.6 mm in thickness) after 4 weeks in culture (Experiment 17). Scale: each well is 16 mm in diameter.

There was no clear difference in contraction associated with the co-culture of MEC. These results were not convincing however; the small degree of contraction seen in the thicker 0.6 mm samples did not leave much room for differences between groups. The effect of even minor differences in thickness, among samples in the same group, was far greater than any stimulatory effect by endothelial cells.
The finding that fibroblast-mediated contraction of a cross-linked dermal collagen matrix can be seen in vitro is quite surprising. A previous study, in which no contraction of fibroblast-seeded human acellular dermis was seen, suggested even that the role of fibroblasts in contraction was over-emphasized (Ralston, Layton et al., 1997). The same study did however demonstrate contraction of the human dermal matrix by keratinocytes, which did not infiltrate below the surface. Our results confirm their findings in one respect; contraction of the dermal matrix, whether by keratinocytes or fibroblasts, does not require infiltration of the matrix. Moreover, these results suggest new possibilities as to the mechanism of fibroblast-mediated contraction of skin grafts.

The dermal component of skin grafts appears to exert the main influence on wound contraction. One study of rat wound healing found that grafts with an intact collagen matrix produce lasting inhibition of wound contraction. In contrast, grafts with a denatured collagen matrix limit the process to a lesser degree (Brown, Garner et al., 1990). This, and other data in the study, suggests that the structure of collagen is important in regulating contraction. The exact mechanism by which this inhibition is expressed is as yet unknown. Wounds covered with full-thickness skin grafts had a minimum of contraction with a very rapid decrease in the number of myofibroblasts. In wounds treated with split thickness skin grafts, myofibroblasts remained in the wound for an average of 40 days, compared with 21 days with full thickness grafts (Rudolph and Ballantyne, 1990). Full-thickness skin grafts thus appeared to influence contracting wounds not by preventing myofibroblast differentiation, but by speeding up completion of their life cycle.

The direct effect of thickness on ACM contraction by fibroblasts correlates well with clinical observations of contraction in grafted wounds. Thin split-thickness grafts contract more quickly and to a greater degree than thicker grafts. The effect of thickness on contraction may be mediated by a greater mechanical resistance of thicker grafts to the contractile forces of the fibroblasts. Several researchers have found that it is not the absolute thickness of the graft which affects contraction, but rather the relative thickness of the dermis that is included (Rudolph, 1979). An interesting topic for further research may be to examine whether ACM thickness has any effect on myofibroblast
differentiation \textit{in vitro}. The reduced proportion of myofibroblasts on human ACM, relative to pig ACM, may have contributed to the lack of observed contraction of human ACM \textit{in vitro}. Given the very thin samples that are required to demonstrate contraction, pig ACM may be more suitable than human ACM as a model of fibroblast-mediated dermal contraction. Human ACM exhibits considerable primary contraction, which is associated with a marked change in observed thickness and diameter after cutting. Pig ACM has practically no primary contraction, and therefore maintains its desired dimensions after cutting into small pieces.

The fact that contraction of collagen gel occurs rapidly without significant SMA expression by fibroblasts suggests that collagen gel models the early contraction of wound healing by wound margin fibroblasts. Pig ACM may enable the study of myofibroblast-mediated contraction that occurs later in wound healing, such as in full thickness wounds treated with split-thickness skin grafts. It has been suggested that collagen gels are too simple in structure to mimic the mature extracellular matrix structure of the dermis, and that there may be problems in extrapolating from the ability of fibroblasts to contract collagen gels \textit{in vitro} to the clinical situation (Ralston, Layton et al., 1997). Pig ACM may provide an alternative model in which to study this aspect of wound healing.

\textbf{4.2.8 Summary}

In this section, pig ACM was evaluated as a model for dermal fibroplasia \textit{in vitro}. Fibroblast morphology, proliferation, collagen synthesis, cell infiltration, expression of myofibroblast smooth muscle actin and contraction were studied. In all these aspects of fibroplasia, pig ACM was compared to human ACM. Several differences were noted in the interactions of human fibroblasts with pig ACM as opposed to with human ACM.

Fibroblasts adhered well to the pig ACM surface, as evidenced by the fibroblast morphology. Fibroblasts on the human ACM appeared rounder and less spindle-shaped, consistent with the greater infiltration of human ACM by the fibroblasts. Fibroblasts proliferated on pig ACM almost as much as they proliferated on the culture dish, but synthesized less collagen on pig ACM. These results are only partially consistent with
those of studies on fibroplasia in a collagen gel. In an unattached collagen gel, proliferation of the fibroblasts seeded within the gel is inhibited by contraction of the gel (Bell, Ivarsson et al., 1978), (Ehrlich and Rajaratnam, 1990), (Nakagawa, Pawelek et al., 1989). It has been shown that growth is not inhibited among the fibroblasts grown on the surface of the gel (Tornasek, Hay et al., 1982). It appears therefore that cell growth inhibition is a result of the number and distribution of contact sites between collagen and cells rather than an inhibition by cell-to-cell contact. In Guidry and Grinnell’s attached gel model however, fibroblasts do multiply within the collagen matrix (Guidry and Grinnell, 1985). An explanation for this has not yet been determined. In our study, collagen synthesis seemed to be inhibited by the adherence of the fibroblasts to a collagen substrate, whereas proliferation rates were minimally affected, even after four weeks in culture.

Pig ACM may be a suitable model for studying certain dermal cell-matrix interactions only, such as contraction. Fewer fibroblasts infiltrated the pig ACM compared to the human ACM, suggesting that pig ACM may not be equivalent to human ACM as a scaffold for human cells. However, this may provide an opportunity to demonstrate the pro-migratory effects of incorporated matrix molecules, such as hyaluronan. The influence of culture conditions on fibroblast infiltration were studied to determine how to best achieve fibroblast infiltration into pig ACM in vitro. Of all the variables studied, only time showed a significant effect on infiltration into pig ACM. However, the considerable variability among samples suggests that there are still unrecognized experimental variables influencing fibroblast infiltration.

Pig ACM may be a useful model of fibroblast-mediated, dermal contraction. The results of this investigation clearly demonstrate the ability of fibroblasts to contract a dermal collagen matrix in vitro. For a number of practical reasons, pig ACM has several advantages over human ACM for this application. Improved harvesting techniques however would be required to obtain pig ACM samples of standard dimensions. This would help ensure greater reliability of this model. Fibroblast-mediated contraction in this model occurred independently of fibroblast infiltration below the ACM surface.
This was consistent with observations of collagen gel contraction without fibroblast infiltration.

The results of this section are also pertinent to the evaluation of pig ACM as a potential xenograft dermal substitute. Successful infiltration of human ACM demonstrated that the culture conditions used are effective in allowing fibroblast infiltration of a matrix. Samples of pig ACM which had been stored in PBS for long periods of time and were then freeze-dried, appeared to have a greater degree of fibroblast infiltration. The reason for this is not known. Storage time was not controlled for in this study. These results suggest however, that the dense pig dermal matrix supports more cell infiltration after it may have been slightly altered. The variability in fibroblast infiltration between pig ACM samples indicates that the structure of pig ACM must be modified to ensure consistent and rapid cell infiltration. It is particular important *in vivo*, that pig ACM as a scaffold does not require degradation by macrophages before it is infiltrated by cells. Such a response would promote inflammation and scarring in a cutaneous wound.

4.3 Fibroplasia in a Glycosaminoglycan-Enriched Acellular Pig Dermal Matrix

4.3.1 Glycosaminoglycan Incorporation

In most studies on the effects of these matrix molecules on cells *in vitro*, glycosaminoglycans (GAGs) are either coated on the culture dish or added to the culture medium bathing the cells. In these ways, the effects of GAGs are determined on cells grown in two dimensions. By using collagen gels or sponges or an even more natural material such as acellular dermal matrix, as scaffolds for 3D cell culture, it is possible to study the effects of GAGs on fibroblasts in a more "life-like" environment. GAGs can be easily incorporated into a collagen gel by mixing the GAGs with the denatured collagen solution prior to gelation or freeze-drying. This however altered the structure of the collagen gel or sponge, confounding any effect attributable to the presence of the GAG itself. The incorporation of solubilized GAGs into dermal ACM must of course employ a different method. It was therefore necessary to develop methods for
incorporating GAGs into the acellular dermal matrix without modifying the tissue architecture.

In order to evaluate the effectiveness of the incorporation, fluorescently-labeled HA and HP were incorporated into the pig ACM. Figure 46 (a) shows the distribution of fluorescein-HA and dansyl-HP in the pig ACM after incorporation, viewed with a UV filter of 450 nm. This allowed for optimal visualization of fluorescein-HA. However, the dansyl-labelled HP was not as visible, as it excites at 265 nm and emits at 510 nm. The samples enriched with HP were therefore also stained for alcian blue (pH 2.5) to confirm the presence of HP in the ACM (Figure 47).

![Figure 46](image)

**Figure 46.** Distribution of fluorescently-labeled HA and HP in pig ACM, frozen unstained sections, 5x.

![Figure 47](image)

**Figure 47.** Distribution of heparin in pig ACM, as seen by alcian blue staining, pH 2.5, 5x. Background alcian blue staining of pig ACM (b) is shown for comparison.

This model allows a unique opportunity to study the effects of matrix glycosaminoglycans on fibroblasts cultured within a natural dermal collagen environment. Moreover, the incorporation process does not appear to modify the collagen matrix structure (Section 4.1.2.1). Previous studies incorporated GAGs into a
collagen gel or sponge to study their effects on cells. However their incorporation affected the relative proportion of collagen in the matrix and the three-dimensional architecture of the collagen matrix. It is therefore difficult to extrapolate any findings to the in vivo effects of GAGs in the dermis. The current study also has limitations in terms of the artificially uniform distribution of GAGs throughout the dermal matrix. In human dermis GAGs are predominantly found in the papillary dermis, and in lesser quantities, in the reticular dermis (Poggi, Klein et al., 1999).

4.3.2 Glycosaminoglycan Quantification
The effects of GAGs on cells are believed to be highly dependent on their molecular weight and concentration in the matrix. In this study therefore, it was important to be able to quantify the GAG incorporated into the ACM. Moreover, this would allow the efficiency of the incorporation methods to be determined. To evaluate the approximate concentration of HA and HP incorporated into the ACM, fluorescently-labeled GAGs were again used. Standards of fluorescein-HA and dansyl-HP, in solutions of digested ACM, were quantified by spectrofluorometry, to create calibration curves for GAG quantification. A fixed volume of digested ACM was included in the standards to better approximate the background of the experimental samples. All three calibration curves shown represent fluorescence readings for standards in the presence of digested ACM. Figures 49 and 50 show the calibration curves (of solubilized FITC-HA and dansyl-HP in the presence of digested ACM) that were used in the quantification.

![FITC-HA Calibration Curve](image)

**Figure 48.** Calibration curve of FITC-HA at high concentrations.
At higher FITC-HA concentrations, as Figure 48 shows, the fluorescence was no longer proportional to concentration. Maximal fluorescence appeared to be reached at 1500 μg/ml FITC-HA. For quantification of experimental samples, a calibration curve of lower concentrations was used to obtain a linear equation (Figure 49).

![FITC-HA Calibration Curve](image)

**Figure 49.** Calibration curve of FITC-HA at low concentrations.

The fluorescent marker of heparin, dansyl, does not produce as much fluorescence as the fluorescein. A calibration curve at high concentrations therefore shows a linear relationship between fluorescence and dansyl-HP concentration (Figure 50).

![Dansyl-HP Calibration Curve](image)

**Figure 50.** Calibration curve of dansyl-HP (blue), with trendline indicated in black.

The amount of HA that was be incorporated into the ACM using our centrifugation method is listed in Table 25. The amount of HP incorporated into the ACM by 48 hours of soaking is shown as well.
Table 25. Evaluation of GAG incorporation methods. †Volume of ACM assumed to be πr²h = π(0.4 cm)²(0.1 cm) = 0.05 cm³ or 0.05 ml.

Given the preliminary nature of this study, it was decided to err on the side of excess when choosing a target concentration of HA and HP in the ACM. The concentration of HA in normal human dermis is 0.508 mg/ml (Ueno, Chakrabarti et al., 1992). In early wound healing, the concentration of HA increases to between 4 and 10 mg/ml, and to 12 to 20 mg/ml in developing fetal tissues, where scarless wound healing can occur (Forrester and Lackie, 1981). The most predominant GAGs of human dermis are derived from the heparin family, namely heparan sulfate and heparin, and have been quantified as between 0.9 and 1.3 mg/ml (Poggi, Klein et al., 1999). The concentrations of HA and HP incorporated into the cell-seeded ACM samples were 2.26 mg/ml HA and 0.72 mg/ml HP. The effects of HA at this concentration would be expected to be similar to that of HA’s effects during wound healing. The effects of HP at this concentration are not known, but roughly estimate the quantity of heparan sulfate present in normal dermis.

The small number of samples in each group limits the accuracy of the quantification methods used here. A larger sample size would be needed to adequately evaluate the consistency of the incorporation method. It is not known from the results of this study, whether the incorporation methods are effective at enriching each ACM sample by an equal amount of HA or HP.

Biomaterials composed of GAGs are generally prepared by covalently cross-linking the GAG to other components in the material to other GAG molecules. It was not known
how long the non-crosslinked GAGs such as HA and HP would remain in the dermal matrix after incorporation. This was a factor in the decision to incorporate more rather than fewer GAGs into the ACM. In order to evaluate HA or HP's effects on long term aspects of fibroplasia such as cell infiltration, it was hoped that the GAGs would remain in the ACM for as long as possible. It was expected that the higher molecular weight HA (1,000,000 Da versus 10,000 Da for HP) would diffuse out of the ACM more slowly. HA-enriched ACM was therefore soaked in HBSS for various time points, up to 28 days, while the HP-enriched ACM was soaked for up to 14 days. The effect of diffusion on GAGs in the ACM was assessed qualitatively and quantitatively. Figure 51 shows the histological appearance of fluorescently labeled HA-ACM and HP-ACM after soaking for 28 and 14 days, respectively. All photographs are taken with the same exposure times and gain.

![Figure 51](image)

**Figure 51.** Qualitative histological assessment of effects of diffusion on GAG-enriched ACM. Frozen, unstained sections of pig ACM are viewed under fluorescence at 450 nm, 5x.

Once it was established that some HA and HP was present in the ACM at these later time points, samples of ACM with 113.1 μg of HA, or with 35.8 μg of HP, were quantified spectrophotometrically after soaking for different lengths of time. Fresh calibration curves were prepared at each time point, of both FITC-HA and dansyl-HP. Figure 52 shows that even after 28 days, HA maintained its original mass. For HP, 20 ug have diffused out, leaving 44% of the original mass at day 14 (Figure 53).
As expected, the amount of HP in the ACM decreased significantly due to rapid diffusion of the small molecule. The lack of diffusion of HA out of the ACM was very surprising. Previous reports found 60% of GAG leaching within 1 week of immersion in isotonic crystalloid (Poggi, Klein et al., 1999), as well as a half-life of one day for newly synthesized HA in dermal tissue, in the presence of cells (Tammi, Tuhanen-Martikainen et al., 1996). It is possible that the values in Figure 52 were not accurately quantified by spectrofluorometry, given the high concentration of HA (>2000 µg/ml). As shown in Figure 48, fluorescence is not linearly proportional to concentration above 1500 µg/ml. This was not a problem for the dansyl-labeled HP, which was less fluorescent. It is likely however that a significant portion of the HA did remain in the ACM despite some diffusion, given the very large amount of HA originally incorporated. A second experiment was therefore performed, in which lower
concentrations of FITC-HA were incorporated into the ACM (0.001 to 1 mg/ml). Samples in this experiment were only soaked for 1 week. Even with smaller concentrations of HA, at least 50% of the original HA mass remained in the ACM after a week in HBSS (results not shown).

There are a number of possible explanations for the longevity of FITC-HA in the ACM. The HA may adsorb to the collagen matrix, despite soaking in aqueous solution. Alternatively, HA may self-aggregate at the relatively high concentration present in the matrix; these aggregates may not be as soluble in water. These results can only estimate the duration of HA’s presence in the ACM under actual cell culture conditions, given the absence of cells in this experiment.

4.3.3 Cell Proliferation
No significant difference was seen between the number of cells cultured on ACM, HA-ACM or HP-ACM (Experiment 18- Figure 54).

![Fibroblast Proliferation](image)

**Figure 54.** Effect of HA and HP on fibroblast proliferation on pig ACM, by the CyQuant assay. There was no significant difference between groups at each time point, by the Mann-Whitney test. For all groups, Day 2 values were significantly different from Day 28 values by the Mann-Whitney test.

These results were encouraging in terms of the low toxicity of the GAG concentrations selected. HA has been previously shown to inhibit fibroblast proliferation at high concentrations (Huang-Lee, Wu et al., 1994), but has also been reported by other investigators to increase as well as to have no effect on proliferation (Pieper, van
A trend of decreased cell numbers at Day 2 and 7 was observed for the HP-enriched ACM. This is in keeping with other reports of heparin’s ability to inhibit cell-collagen attachment (San Antonio, Lander et al., 1992). The number of attached cells was clearly adequate to proliferate to that of the other groups by Day 28.

4.3.4 Collagen Synthesis

The effect of HA and HP on collagen synthesis by fibroblasts cultured on pig ACM is described in Figure 55. The number of cells in each well was determined by the CyQuant assay, at the time of the radiolabelled proline incorporation.

![Collagen Synthesis (mean + SD)](image)

**Figure 55.** Effect of HA and HP in ACM on collagen synthesis by fibroblasts on pig ACM. Error bars represent standard deviation. There was no significant difference between groups by the Tukey HSD test.

For all groups, approximately 10% of incorporated proline was found in the collagenase-soluble fraction. There did not appear to be a significant effect of HA or HP in the ACM on collagen synthesis in this model. The large standard deviation seen in this experiment suggests possible variability in the amount of GAG incorporated into the ACM. Future work may focus on examining the effect of GAGs on collagen synthesis once the incorporation method has been standardized.

HA is found in decreased levels in hypertrophic scars and keloids, which are associated with increased collagen production (Bertheim and Hellstrom, 1994). However, fibroblasts in HA-rich provisional wound matrices produce high levels of collagen
(Clark, 1996). HA’s influence on collagen synthesis is therefore not clear. A direct influence of HA on fibroblast synthesis of collagen remains to be shown. Little is likewise known about HP’s effects on collagen synthesis. The concentration of HP in the matrix at the time collagen synthesis was measured (day 4), may not have been high enough to demonstrate an effect. Future experiments may require a higher concentration of HP at day 0.

4.3.5 Cell Infiltration
In view of the variability seen in terms of cell infiltration into pig ACM, care was taken to ensure homogeneity in ACM characteristics and consistent seeding techniques between groups. Groups (ACM, HA-ACM, HP-ACM) were only compared within each experiment repeat, not between repeats. Freeze-dried ACM samples were rehydrated and seeded with second passage human dermal fibroblasts at a density of 30,000 cells/cm². Samples of ACM 8 mm in diameter were fitted into the wells of a 96 well plate for seeding. Figure 56 shows representative histological sections of each group after four weeks in culture, at 20x magnification.
Qualitatively, the presence of HA or HP did not appear to have any effect in terms of preventing or facilitating fibroblast infiltration into the pig ACM. Infiltration occurred in the majority of Repeat 1 and 2 samples (4/6 for ACM and HA-ACM, 5/6 for HP-ACM). Minimal infiltration was noted in repeat 3 samples (1/3 for ACM and HP-ACM, 0/3 for HA-ACM). The number of cells per section were counted to see if the presence of HA or HP quantitatively influenced the ability of the cells to infiltrate the pig ACM. Samples from Repeats 1 and 2 only were used. Figure 57 shows the average number of cells seen per section in each group, after 4 weeks in culture. The cells on the surface of the ACM (seen as a monolayer in sections) were not included in the count. The presence of HA or HP in the matrix did not significantly change the number of infiltrated fibroblasts. In fact, the presence of HA appeared to have a slight inhibitory effect on fibroblast infiltration into the pig ACM.
Figure 57. Effect of HA and HP on fibroblast infiltration into pig ACM after 4 weeks. Results represent mean of Repeat 1 and 2 samples (18 sections per group). There was no significant difference between the three groups by the Kruskal-Wallis test.

The distance of each cell from the surface was also analysed to see if HA or HP had any effect on cell migration. The results are shown in Table 26.

<table>
<thead>
<tr>
<th>Distance from surface</th>
<th>% of total cells per section</th>
<th>ACM (n = 9)</th>
<th>HA-ACM (n = 9)</th>
<th>HP-ACM (n = 9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-150 µm</td>
<td>89.7 ± 7.4%</td>
<td>89.0 ± 6.7%</td>
<td>88.3 ± 5.3%</td>
<td></td>
</tr>
<tr>
<td>151-300 µm</td>
<td>8.2 ± 7.3%</td>
<td>9.9 ± 6.5%</td>
<td>8.5 ± 5.3%</td>
<td></td>
</tr>
<tr>
<td>301-500 µm</td>
<td>2.1 ± 0.2%</td>
<td>1.1 ± 0.2%</td>
<td>3.2 ± 0.5%</td>
<td></td>
</tr>
</tbody>
</table>

Table 26. Effect of HA and HP on distance of fibroblast migration from the cell-seeded surface into the pig ACM (Repeat 1 samples). The number of sections analyzed in each group is indicated by n. There was no significant effect of HA or HP on the distance of the cells from the surface, by the chi-squared test.

The results for this section demonstrate how the effect of the ACM batch was much greater than the effect of the matrix GAGs, on fibroblast infiltration. For these batches of ACM, infiltration was independent of the presence of HA or HP. It was surprising that HA in particular did not facilitate fibroblast infiltration into the matrix. HA is known for its stimulatory effects on cell migration during wound healing and morphogenesis. Its presence in a collagen sponge dramatically improved fibroblast infiltration in vitro (Doillon and Silver, 1986). In this study, embryonic chick tendon fibroblasts were cultured on collagen sponges for 9 days. By day 9, only about 25% of
the collagen sponge was infiltrated with fibroblasts based on histological observation. In collagen sponges prepared with 5% (w/w) hyaluronic acid, fibroblasts infiltrated the entire width of the sponge in 9 days. The effect of heparin could not be tested in this model because the resulting collagen-heparin mixture failed to form a sponge structure. Another study (Reid and Newman, 1991) showed that the incorporation of HA and HP into collagen gels also stimulated infiltration by leukocytes. However, this pro-migratory effect may arise from the altered collagen sponge structure, rather than from any direct effect of HA on the cells. In contrast, the structure of HA-enriched ACM appears unchanged from that of ACM, by histology and SEM.

Neither HA nor HP had any significant effect on modulating fibroblast infiltration into the pig ACM. The lack of effect by HP on fibroblast infiltration is not surprising given the rapid diffusion of incorporated HP out of the ACM, which occurs long before the fibroblasts begin infiltrating the matrix. In the case of HA however, significant levels were shown to remain, despite diffusion, up to four weeks following incorporation. It may be that HA’s lack of effect was particular to the molecular weight and concentration chosen in this study. High molecular weight HA, such as that used in this study, has been shown to stimulate cell migration in other models (Laurent and Fraser, 1992), (Hayen, Goebeler et al., 1999). There is controversy however regarding the relationship between the concentration of HA and its effects on cell migration (Laurent, Hellstrom et al., 1988).

The most surprising finding in this section was the degree of infiltration which occurred into the pig ACM samples in general. An interesting observation is that these experiments were done later, using pig ACM samples which had been stored in PBS for a relatively longer time, prior to freeze-drying and cell-seeding. The ACM batch used for Repeats 1 and 2 in particular, had been stored for several months prior to use. Unfortunately these samples were not evaluated by SEM to determine the effect of prolonged storage on the ACM structure. The histology results suggest that the ACM structure is loose enough for fibroblasts to migrate from the surface into the matrix. Clearly more work needs to be done on this model, to optimize the pig ACM structure and allow for rapid cell infiltration in all samples.
4.3.6 Myofibroblast Differentiation

Human fibroblasts were found to express SMA when seeded on pig ACM, as shown in Section 4.2.7. To determine the effect of HA and HP on fibroblast differentiation to myofibroblasts, all SMA-positive cells per section, included those at the monolayer, were counted. Table 27 shows the percentage of SMA-positive cells per section at the different time points examined.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>ACM (n = 12)</th>
<th>HA-ACM (n = 9)</th>
<th>HP-ACM (n = 9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total # of cells per section</td>
<td>90.6 ± 31.7</td>
<td>95.1 ± 23.5</td>
<td>110.1 ± 27.0</td>
</tr>
<tr>
<td>Total # of SMA positive cells per section</td>
<td>28.3 ± 19.3</td>
<td>16.4 ± 23.2</td>
<td>48.5 ± 17.1</td>
</tr>
<tr>
<td>% SMA positive cells</td>
<td>31.2 ± 12.5%</td>
<td>17.2 ± 15.7%</td>
<td>44.1 ± 21.2%</td>
</tr>
<tr>
<td>Significance</td>
<td>*HA</td>
<td>*Pig ACM, HP</td>
<td>*HA</td>
</tr>
</tbody>
</table>

Table 27. Effect of HA and HP on fibroblast smooth muscle actin expression at 2 weeks. Significance determined by one-way ANOVA and post-hoc Tukey HSD.

Heparin stimulates SMA expression by fibroblasts in vitro and in vivo, by selecting for SMA-positive cells among replicating fibroblasts (Desmouliere, Rubbia-Brandt et al., 1992). The results of the CyQuant assay clearly show continued proliferation of the fibroblasts on the ACM. The mechanism of HA's effects on myofibroblast differentiation is less clear. Research on the mechanisms of fetal scarless wound healing in sheep shows that the transition from scarless tissue repair (at 75 days gestation) to healing with scar formation (at 120 days gestation) coincides with the expression of SMA by myofibroblasts (Desmouliere and Gabbiani, 1996). Since fetal repair is also characterized by persistently elevated levels of hyaluronan in cutaneous wounds, it could be hypothesized that HA may play a regulatory role in fibroblast-mediated contraction, and possibly myofibroblast differentiation. Another interesting observation is that the timing of HA's disappearance from adult wounds (around 3 to 5 days, through the degradative action of hyaluronidase) corresponds somewhat to the timing of the emergence of myofibroblasts in wounds (Weigel, Fuller et al., 1986). HA's role in regulating myofibroblast differentiation has otherwise not been directly studied.
An objective for future work in this area should include the development of methods to assess SMA expression by confocal microscopy, rather than by cross-sectional microscopy. With phalloidin counterstaining, the percentage of SMA-positive cells on the surface of the ACM could be determined. This method would likely address the large standard deviation caused by significant variation in the number of cells seen on each section.

4.3.7 Summary

Although a prototypic dermal replacement consisting of bovine collagen and a single xenogenic GAG (chondroitin sulfate) was described more than 15 years ago, this construct has not achieved widespread use in the field of burn care. This may suggest that collagen alone, or even in combination with a single matrix GAG, is probably insufficient to replace missing dermis. With an increasing recognition of the role of GAGs in the modulation of cytokine signalling and other wound healing events, it is reasonable to hypothesize that an acellular dermal substitute with a GAG composition that more closely resembles that of normal dermis might function more effectively. Acellular dermal matrix may be processed to specifically conserve the GAGs of the dermal matrix. An objective of this study was to determine the value of preserving dermal GAGs in the acellularization process, in terms of their influence on fibroplasia. The re-incorporation of specific GAGs (HA and HP) attempted to demonstrate the effect of these molecules in the ACM.

Methods of GAG quantification in bovine and porcine tissues are limited by practical difficulties in GAG extraction techniques and GAG visualization (Robinson-Seurig, 1999). For large tissue samples, alcian blue staining of electrophoretically separated GAGs with standards, allows for accurate quantification (Freund, Siebert et al., 1993). In the current study, this method was unsuitable for quantification of small ACM samples with nanogram amounts of GAGs. A more thorough evaluation of the GAG incorporation methods developed in this project should include a scaling-up of the sample volume, to allow more accurate quantification of matrix GAGs.
It was expected that the incorporated GAGs in the ACM would quickly diffuse out under culture conditions, even without cells. As expected, the mass of HP in the ACM tended to decrease over time. It was therefore not surprising that HP only had an effect on fibroblast proliferation early on. This inhibitory effect on fibroblast infiltration was not long lasting. HP-ACM was associated with SMA expression by 44% of seeded fibroblasts, compared with only 31% of fibroblasts on the ACM at 2 weeks. The role of cell-matrix interactions in regulating myofibroblast differentiation may therefore not require prolonged contact between the matrix and the cells. However, HP had no effect on fibroblast infiltration into pig ACM, nor on collagen synthesis.

Surprisingly, HA appeared to remain in the ACM, despite soaking, for up to 28 days. Pig ACM therefore seems to be a promising model for studying the effects of HA on fibroblast proliferation, collagen synthesis and SMA expression. HA did not influence fibroblast proliferation, infiltration or collagen synthesis. At the high concentration tested in this study, HA would be expected to increase fibroblast infiltration into the ACM. It may be that HA's role in facilitating fibroblast migration in dermis may not be as significant as previously believed. HA did significantly decrease SMA expression by fibroblast cultured on pig ACM for 2 weeks. Hyaluronan may be a desirable additive for many reasons, including its inhibitory effect on myofibroblast differentiation seen in this study. However, to more accurately evaluate their role as dermal substitute components, a range of GAG molecular weights and concentrations should be tested in the ACM. The results of this preliminary investigation show only that hyaluronan and heparin, at the concentrations tested here, had minimal effects on fibroplasia in an acellular pig dermal matrix.
Chapter Five:
CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

5.1.1 Acellular pig dermal matrix as a dermal substitute
Xenograft materials are an attractive alternative to human-derived materials, which are limited by their high cost and short supply. The acellular pig dermal matrix has considerable potential as a dermal substitute for use in burns and chronic wounds. Pig ACM consists of a mature dermal collagen network, which has been shown in allografts, to decrease contraction and improve keratinocyte take as an allograft (Livesay, Herndon et al., 1995). Grossly, pig dermis has many similarities to human dermis, and was hypothesized to interact with human cells in a beneficial way. Collagen as a xenograft is relatively non-immunogenic; the complex conformational structure of collagen demands a certain amount of conservation of its primary sequence across species. The acellularization process further reduces the immunogenicity of pig ACM, by removing the cellular targets of xenograft rejection responses.

Pig ACM has been extensively tested as an allo- and xenograft dermal substitute in animal models, with varying results. However, pig ACM has been tested in human wounds without success. Possible reasons for its failure include inadequate acellularization, clinically significant immunogenicity of pig collagen and/or unsuccessful infiltration by host cells. A major objective of this project was to investigate the third possibility. By seeding pig ACM with human fibroblasts in an in vitro environment, the scaffold properties of the material could be tested in isolation, in the absence of any immunological issues.

Pig and human ACM have many similarities in their structure and composition, as well as several differences. Both consist of an interwoven network of cross-linked collagen bundles. However, pig ACM appears denser and more resistant to structural alteration
under culture conditions. *In vitro*, pig ACM supported human fibroblast proliferation and enabled fibroblast infiltration under certain conditions. In comparison to human ACM, pig ACM was infiltrated more slowly and in fewer samples. In the presence of pig ACM, fibroblasts synthesized less collagen than controls grown directly on the culture dish. Above a thickness of 0.7 mm, contraction of the pig ACM did not occur. A percentage of fibroblasts cultured on the ACM expressed the myofibroblast marker smooth muscle actin, in a time-dependent manner; by four weeks, only 19% of fibroblasts expressed SMA on pig ACM. However, fewer myofibroblasts were found among cells seeded on human ACM at all time points. The results of this *in vitro* investigation suggest that as a dermal substitute, pig ACM may have some beneficial effects on fibroplasia but would likely produce results inferior to that of human ACM. Pig ACM was not found to be equivalent to human ACM as a scaffold for human fibroblasts. Its structure should be modified to ensure adequate infiltration by host cells as a dermal substitute.

5.1.2 *Acellular pig dermal matrix as a model of cell-matrix interactions and contraction*

Interactions between cells and their environment play an important role in many biological processes. Three-dimensional culture systems have been developed to mimic natural interactions between cells and the extracellular matrix. These models recreate the natural extracellular matrix environment more closely than the traditional monolayer culture can. Acellular pig dermal matrix was tested as a convenient scaffold for the three-dimensional study of human fibroplasia. As most soluble matrix components are removed in the acellularization process, specific matrix molecules can be re-incorporated and studied in a dermal matrix. The original tissue architecture of acellular dermis offers a unique opportunity to study fibroblast-mediated dermal contraction.

Pig ACM was compared to human ACM with respect to its effects on certain aspects of fibroplasia. Pig ACM is easily obtained from the paravertebral areas of the animal and can be processed to yield many experimental samples. With the complete removal of pig fibroblasts from the matrix, seeded human fibroblasts can be visualized histologically. Subjectively, pig ACM feels more rigid and is less extensible than human
ACM. Its ultrastructure is denser, with less elastin than human ACM. Human fibroblasts behave similarly on pig and human ACM in terms of cell morphology. However, the differences in ACM structure do seem to have an influence on other aspects of fibroplasia in vitro. Specifically, fibroblasts proliferate more on pig ACM than on human ACM. Fibroblasts on pig ACM express smooth muscle actin in greater proportions than on human ACM. Fibroblasts form a multi-layered monolayer on the surface of pig ACM with some infiltration below the surface, whereas they form a minimal monolayer on and quickly infiltrate into human ACM. Fibroblasts can contract thin samples of pig ACM (<0.7 mm thin) in two to four weeks, whereas human ACM is not contracted by fibroblasts in vitro.

Contraction, as a normal feature of wound healing, is an important element in cutaneous repair. Pathologic or excessive wound contraction is a major factor leading to loss of function as a result of hypertrophic scarring, scleroderma, Dupuytren’s disease and other systemic disorders. Pig ACM was found to be effective as an in vitro model of dermal contraction, and offers several advantages to the collagen gel contraction model. Pig ACM provides a three-dimensional substrate in which the interaction of dermal fibroblasts with a mature dermal collagen matrix can be examined and, hopefully, the effects of potential treatments quantified. Also, with optimization and standardization of its processing and structure, pig ACM may be developed into a representative model of human dermis for studying cell-matrix interactions. Table 28 summarizes the unique features of pig ACM as an in vitro model of fibroplasia, in comparison with other proposed models.
Table 28. Comparison of pig ACM with existing *in vitro* models of fibroplasia. Symbols refer to qualitative representation of the quantitative results presented in previous sections.

### 5.1.3 Hyaluronan and heparin as dermal substitute components

Glycosaminoglycans are common constituents of cell surfaces and extracellular matrices. Biological functions of GAGs include the facilitation of cell migration, proliferation and differentiation, the binding and modulation of enzymes, protease inhibitors and cytokines as well as tissue organization. The presence of GAGs in biomaterials offers an opportunity to exploit the many biocharacteristics of these polysaccharides and possibly to modulate the healing responses elicited by the biomaterial. As components of an acellular dermal matrix, it was hypothesized that hyaluronan and heparin may influence fibroblast responses to the matrix. Other desirable effects of hyaluronan and heparin on dermal substitute healing may include stimulation of angiogenesis, modulation of inflammation and delivery of covalently-linked substances (such as antibiotics, analgesics or growth factors) to the wound.

Hyaluronan and heparin were successfully incorporated into the acellular pig dermal matrix. Each GAG was distributed throughout the matrix. In this study, a near-physiological concentration of HP and a supra-physiological concentration of HA in the matrix were achieved. This facilitated an evaluation of the incorporation methods and to allow for leaching of the GAGs from the matrix over the course of the experiment. Most
of the incorporated HA remained in the matrix despite soaking for 28 days, while most of the HP diffused out of the ACM in 3 days. The presence of the GAGs did not interfere with fibroblast proliferation on the ACM, and did not alter collagen synthesis. Fibroblast infiltration into the GAG-enriched ACM was equivalent to that of the unmodified ACM. A greater proportion of fibroblasts expressed α-smooth muscle actin on the pig ACM in the presence of HP, while HA was found to have an opposite effect. The effects of heparin on fibroblast SMA expression and contraction should be further elucidated. Hyaluronan does not appear to interfere with normal healing as a dermal substitute component, at the concentration and molecular weight evaluated in this study.

5.2 Recommendations
A number of possible projects related to this study may be pursued. These relate to the application of pig ACM as either a dermal substitute or an in vitro model material.

5.2.1 Dermal substitute development

(1) Modification of xenograft dermal collagen matrix structure
The results of this study suggest that the effectiveness of pig ACM as a dermal substitute scaffold may be limited by its structural characteristics. The major difference between the structure of pig and human ACM lies in the relative tightness of the collagen bundle network. The collagen matrix of pig ACM could be modified therefore, through mechanical, enzymatic or physico-chemical methods. The in vitro methods developed in this study of fibroplasia may be applied to a modified pig ACM substrate, in order to determine the effect of the structural modification on its suitability as a scaffold for human cells.

(2) Evaluation of acellular pig dermal matrix from different anatomic locations
It is known that the structure of the dermis varies according to its anatomic location (Elder, Elenitsas et al., 1997). In this study, only pig dermis taken from the donor's back was evaluated as an in vitro scaffold. In the future, improved techniques may allow for convenient harvesting of pig dermis from other anatomic locations. It may be worthwhile therefore to evaluate samples of acellularized pig dermis from different
anatomic locations as a scaffold for human cells. It is suggested that abdominal dermis may be a desirable option.

(3) Hyaluronan for drug delivery in acellular allograft dermal substitutes
Hyaluronan was shown in this study to become incorporated into the pig ACM, and to remain in the matrix at high levels despite predicted outward diffusion. The presence of HA in pig ACM was not associated with fibroplasia characteristic of excessive scarring. HA could therefore hold promise as a component in an acellular dermal matrix dermal substitute. The HA incorporation methods used in this study could be applied to the incorporation of HA bound to other therapeutic substances, in order to develop a kind of drug delivery system for the wound. Furthermore, an evaluation of HA-enriched ACM in vivo may also demonstrate the known water-absorbing and anti-inflammatory properties of HA.

5.2.2 In vitro modelling

(1) Correlation of findings with in vivo fibroplasia (including contraction)
An effective in vitro model should generate results in keeping with the in vivo situation. Some in vitro aspects of fibroplasia on pig ACM could be correlated with in vivo experimentation of pig ACM grafted onto full thickness cutaneous wounds. There are many challenges in finding a xenogenic animal model for studying the effects of pig ACM on wound contraction. While pigs are a good model of human wound contraction, the use of domestic pigs in this study would constitute an allogenic, and not xenogenic, animal model. Loose-skinned animals such as rats or guinea pigs, while xenogenic, bear less resemblance to humans with respect to wound contraction. These smaller animals however have been used and validated in several studies. Although caution must be used in extrapolating information about human wound healing from this model, future in vivo testing would likely employ guinea pigs.

(2) Effect of glycosaminoglycans on contraction of ACM
It was shown using this model that HA and HP have effects on myofibroblast differentiation. The next logical step is to correlate these findings with the effects of matrix HA and HP on contraction of pig ACM in vitro. Pig ACM would offer a
considerable advantage over other *in vitro* models of contraction in this area: its matrix structure is not grossly modified by the incorporation of HA or HP. Any effect on matrix contraction would therefore be specific to the GAGs’ effects on cells. Additional work is needed to adapt HA and HP incorporation methods to very thin ACM samples, as well as to improve the longevity of HP in the matrix.

(3) Human ACM as a model of cell-matrix interactions

In this study, effective methods of GAG incorporation and quantification in an acellular dermal matrix were developed. These methods could be applied to the use of human ACM instead of pig ACM, as a three-dimensional substrate for modeling cell-matrix interactions in fibroplasia *in vitro*. Human ACM may be a suitable model for studying dermal fibroblast proliferation, smooth muscle actin expression, collagen synthesis and fibroblast infiltration. A study of fibroblast-mediated contraction with human ACM would not be feasible, since this material is difficult to cut to the required dimensions.

(4) Pig ACM as a model of contraction

Fibroblast-mediated contraction of a mature dermal matrix has not been previously demonstrated *in vitro*. It was shown in this study that this type of contraction is dependent on fibroblasts, but the mechanism is unclear. The effect of cell seeding density on contraction would be of interest, as well as the effect of selective inhibitory agents on ACM contraction. It would be useful to characterize whether the contraction of the matrix is reversible with cell removal, or is dependent on particular serum components.

5.3 Summary

This thesis provides an evaluation of acellular pig dermal matrix as a biomaterial. Acellular pig dermal matrix was investigated for use as a possible xenograft dermal substitute material as well as an *in vitro* model of dermal cell-matrix interaction. The extracellular matrix components, hyaluronan and heparin, were evaluated for their effects on fibroplasia *in vitro* using pig ACM as a model scaffold. The results of this thesis demonstrate the benefits and limitations of pig ACM in terms of the following parameters:
(1) similarity in structure to human dermis
(2) ability to be infiltrated by human cells
(3) effects on the behaviour of seeded fibroblasts
(4) ability to be processed and enriched by glycosaminoglycans without gross modification of its structural integrity.

This project did not address any quality control issues, i.e. the variability in structure between samples. The structural analysis of the dermal matrix was not quantitative. Only one concentration and molecular weight of each glycosaminoglycan was tested. Each of these methodological aspects could be improved in future research. The most valuable contributions of this thesis are (1) the characterization and application of an in vitro model of fibroplasia in a dermal matrix and (2) the comparison of pig to human dermal matrix as a scaffold for human fibroblasts.
REFERENCES


Appendix: CULTURE OF HUMAN DERMAL MICROVASCULAR ENDOTHELIAL CELLS (MEC) ON PIG ACM

Introduction

Angiogenesis, the formation of new blood vessels, is an important component of various physiological and pathological processes, including wound healing. Little is known about the specific conditions under which wound healing angiogenesis may be stimulated. Increased knowledge of these mechanisms would certainly prove useful in the treatment of chronic wounds, the inflammation of rheumatoid arthritis, capillary hemangiomas as well as malignant transformation, tumour growth and metastasis (as in melanoma). Research has been limited by the lack of reliable, reproducible assays of the angiogenic response. In view of both the ethical and cost issues inherent in in vivo angiogenesis models, several groups have established microvascular endothelial cell culture techniques. The important role played by microvascular endothelial cells (MECs) in a wide range of normal and pathological processes has stimulated the development of in vitro models for capillary formation.

MECs tend to grow as monolayers on two-dimensional substrata, but have been shown to form capillaries in artificial type 1 collagen and fibrin matrices under certain conditions (Folkman and Haudenschild, 1980). The lack of correlation between two-dimensional and three-dimensional cultures establishes the need to understand why cells behave differently in a more natural three-dimensional matrix configuration. The challenge in designing an in vitro model of dermal capillary formation lies in recreating the structure and composition of the connective tissue matrix which surrounds blood vessels in the body. We propose to develop a three-dimensional model of capillary formation based on an acellularized dermal matrix. This model would allow us to study the effects of the interactions between MECs and the mature collagen and elastin network of the dermis. Our acellularization protocol removes most of the soluble matrix molecules of the dermis. We have developed methods to reincorporate these molecules individually, in order to study their effects on capillary formation in this model.
Hypotheses

(I) Capillary formation by human dermal microvascular endothelial cells may be induced \textit{in vitro} in acellularized dermal matrix from human and porcine dermis.

(II) This three-dimensional model may be used to study the effects of hyaluronan and heparin on capillary formation.

Methods

Protocols for harvesting and acellularizing pig dermis conformed to the requirements of our institution's Research Ethics Board and have been extensively used in our laboratory. Primary human microvascular endothelial cells (Clonetics, San Diego, USA) were seeded on samples of acellularized matrix (ACM) in 96 well plates. The variables examined were: (i) cell seeding density (1000, 10,000, 50,000 cells/sample), (ii) the presence of a primary human fibroblast feeder layer, (iii) the presence of a second layer of matrix over the cell-seeded sample, and (iv) the time of incubation (2, 7, 14 days). For all experiments, EBM-2 media (Clonetics) was used with the additives listed in Table A-1.

<table>
<thead>
<tr>
<th>Media</th>
<th>Additives</th>
</tr>
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<tbody>
<tr>
<td>EBM-2</td>
<td>5% FBS, gentamycin/amphotericin, 2 ml human basic fibroblast growth factor, 0.5 ml vascular endothelial growth factor, 0.5 ml insulin-like growth factor-1, 0.2 ml hydrocortisone, 0.5 ml ascorbic acid, 0.5 ml human-epidermal growth factor</td>
</tr>
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Table A-1. Culture media used in fibroblast infiltration experiments.

Bilayer collagen gels served as positive controls. 108 porcine ACM samples, 8 mm in diameter, were seeded with human MEC, with a fibroblast feeder layer and a second ACM layer. Cell viability of the MEC on ACM, as well as any infiltration of the MEC, was determined by cross-sectional histology. The samples were fixed in a 2.5% glutaraldehyde, 2% paraformaldehyde solution. Subsequently, 5 x 10 micron thick central cross-sections of paraffinized samples were stained with toluidine blue or hematoxylin. The number of microvessel fragments seen per high power field was counted for each section.
In the second set of experiments, samples of pig ACM enriched with hyaluronan or heparin were seeded with MEC (50,000 cells/sample, for 14 days, in a bilayer model). Control ACM samples contained no hyaluronan or heparin. Histology was performed on triplicate samples in each group in three separate experiments.

Results
Cross-sectional histology of MEC-seeded collagen gels and ACM samples showed the presence of adherent MEC at different time points (Figure A-1).

Figure A-1. Adherent MEC on (a) collagen gel and (b) pig ACM at 2 days, hematoxylin, 20x. MEC are stained dark blue, and are indicated by arrows.

Under every condition examined, including the collagen gel samples intended as positive controls, no capillary formation below the surface was observed. However, the MEC survived at the surface, but were rarely seen on cross-sectional histology below the matrix surface (Figure A-2).
Figure A-2. Lack of MEC infiltration below the surface of pig ACM at (a) 7 days and (b) 14 days, hematoxylin, 20x. MEC are stained dark blue, and are indicated by arrows.
Figure A-3. Surface MEC (arrows) on pig ACM at 14 days, toluidine blue, 40x.

Some infiltration of MEC was seen in the collagen gel samples, but no capillary formation was observed.
Figure A-4. MEC on surface of collagen gel at 14 days, hematoxylin, 20x.

Although not all the histological results are presented here, the variables examined (cell seeding density, unilayer or bilayer, fibroblast co-culture, duration of culture) had no effect on cell infiltration or capillary formation. Moreover, neither hyaluronan- nor heparin-enriched pig ACM samples showed any MEC infiltration.

**Discussion**

The ability to model angiogenesis in tissues *in vitro* would provide an alternative to animal experimentation. Also, the use of simplified experimental conditions *in vitro* may allow for more control over experimental variables. In this study, acellular pig dermal matrix was investigated as a possible scaffold for human dermal microvascular endothelial cells. These cells were selected based on their tissue of origin, given that dermal endothelial cells are responsible for capillary formation in dermal wounds. Unfortunately, no previous studies have demonstrated *in vitro* capillary formation with these cells. In future work, the more commonly used human umbilical vein endothelial cells will be studied in this model.
Capillary formation is a complex, multifactorial process which is tightly regulated in vivo. For the phenomenon of angiogenesis to occur in an ex vivo environment, certain cues must be provided to the cells artificially. The content of the culture media is therefore supplemented with pro-angiogenic factors. As well, the cells require a three-dimensional framework for actual capillaries to take shape. The extracellular matrix must also provide an appropriate stimulus to the endothelial cell. The use of Matrigel is an example of how a matrix can be specifically designed to stimulate angiogenesis. Matrigel is derived from the basement membrane matrix of Englebreth-Holm-Swarm tumors taken from lathrytic mice. The major components of this material are laminin, collagen IV, entactin/nidogen, heparan sulfate proteoglycan and growth factors (Cockerill, Gamble et al., 1995). This model demonstrates the importance of the basement membrane matrix as a biological mediator of angiogenesis. Within 18 hours of culture on Matrigel human umbilical endothelial cells form a network of capillary-like structures with well-defined lumens (Cockerill, Gamble et al., 1995).

As with Matrigel, capillary-like networks in collagen gels occur mostly on the surface of the material (Montesano, 1992). As the collagen gel is semi-transparent, the cells can be visualized in the culture dish under microscopy. The difficulty with using a more dense tissue, such as pig ACM, arises during its removal from the culture dish and histological analysis. This process tends to disrupt delicate cellular arrangements at the surface; only cells which have infiltrated below the matrix surface can be visualized in this model. Unfortunately, the endothelial cells did not infiltrate into the pig ACM. In future work, a tissue which supports more cell infiltration would be more advisable. An obvious choice for a material for this purpose is acellular human dermal matrix, which appeared to be a better in vitro scaffold for human fibroblast infiltration than pig ACM. This material may therefore be more suitable for histological analysis of capillary formation within the matrix. Cells present below the surface to be identified histologically. Another long-term objective of this study is to develop a better xenograft-derived scaffold material. Structural modifications to pig ACM may improve its effectiveness at supporting MEC ingrowth, which could then be determined histologically.
Endothelial cell migration into acellular tissue \textit{in vitro} is not well understood. This process is stimulated \textit{in vivo} by specific cytokines and local matrix changes, and is therefore highly dependent on cell-to-cell communication. In this study, MEC-seeded ACM samples in one group were co-cultured with human fibroblasts at the bottom of the well. This co-culture arrangement proved insufficient to stimulate MEC infiltration into the ACM. Perhaps the MEC might be seeded on the same side of the ACM as the fibroblasts, in order to better reproduce multicellular \textit{in vivo} conditions. In this study design, it would be necessary to use cell-specific stains to distinguish cell types (i.e. von Willebrand factor immunohistochemistry).

Hyaluronan- and heparin-enriched ACM samples were also investigated for MEC infiltration. Unfortunately, one cannot conclude any effect of HA or HP on MEC behaviour, given the lack of infiltration in the control group. To evaluate the effect of HA and HP on capillary formation within a dermal matrix, different experimental conditions would be required:

1. Human umbilical vein endothelial cells (HUVECs) might be used on human ACM in order to determine whether endothelial cells can infiltrate a dermal matrix scaffold \textit{in vitro}.

2. HA and HP incorporation techniques developed for pig ACM may be applied to human ACM. The HA- or HP-enriched human ACM may then be seeded with MECs.

3. Alternatively, pig ACM enriched with HA or HP may be tested as a potential model using HUVECs.

4. Finally, in an effort to develop a xenograft material as a potential dermal substitute, the structure of pig ACM might be specifically modified to allow for easier cellular infiltration. The modified xenograft material might then be enriched with HA or HP and seeded with MECs.

It would be expected that as HA is degraded to HA oligosaccharides, a possible pro-angiogenic effect might be seen. HP's role in delivering bFGF (present in the media) may also result in a pro-angiogenic effect, although HP would be without its usual proteoglycan configuration. HP alone has been shown to stimulate endothelial cell proliferation \textit{in vitro}.
(Thornton, Mueller et al., 1983). However this effect on angiogenesis has not been studied with HP as a matrix component.

**Conclusions**

We proposed an *in vitro* model of human capillary formation in a mature porcine dermal matrix. The goal here was to develop a model which more closely reproduced the cell-matrix interactions of angiogenesis in the dermis. This preliminary study employed a new dermal material, a new MEC line, and new *in vitro* conditions. Since the cells did not form capillary structures, even on the established collagen gel model, the cell type is clearly not suitable for studying angiogenesis *in vitro*. As such, pig ACM may not be a suitable material for studying capillary formation, given that fibroblasts take several weeks to infiltrate below the surface. Without infiltration, the dense ACM material, which requires histological processing to visualize capillary formation, becomes impractical as a model. There were clearly too many variables for this study to be conclusive within its existing methodology. Due to time restrictions, the other approaches proposed here were not pursued in this thesis. Further methodological research is encouraged to develop an *in vitro* model of dermal angiogenesis.

**References**


