EPIDERMAL GROWTH FACTOR RECEPTOR OVEREXPRESSION
AS A TARGET FOR IMAGING AND RADIOTHERAPY
OF BREAST CANCER

by

Raymond Matthew Reilly

A thesis submitted in conformity with the requirements
for the Degree of Doctor of Philosophy,
Graduate Department of Medical Biophysics
University of Toronto

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ABSTRACT

Epidermal Growth Factor Receptor Overexpression as a Target for Imaging and Radiotherapy of Breast Cancer

Raymond Matthew Reilly
Doctor of Philosophy, 1999
Graduate Department of Medical Biophysics
University of Toronto

The overexpression of the epidermal growth factor receptor in 30-60% of human breast cancers was investigated as a target for radiopharmaceuticals specifically directed against the receptor for diagnostic imaging and radiotherapy of the disease. Using phantoms (models) of breast cancer lesions targeted in vitro with indium-111 labelled human epidermal growth factor (\textsuperscript{111}In-hEGF), it was demonstrated that diagnostic imaging through targeting EGFR overexpression in breast cancer could be a very sensitive technique able to detect as few as $5 \times 10^4$ to $10^5$ cancer cells under ideal conditions. The sensitivity was reduced up to 300-fold however by receptor heterogeneity or a low proportion of tumour cell targeting combined with tissue attenuation. A comparison of the tumour imaging properties of \textsuperscript{111}In-hEGF and \textsuperscript{111}In-labelled anti-EGFR monoclonal antibody 528 (\textsuperscript{111}In-mAb 528) in athymic mice with human breast cancer xenografts showed that macromolecules are more effective tumour targeting agents than peptide growth factors due to higher absolute tumour uptake at only slightly lower
tumour/blood ratios. The 10-fold higher tumour uptake of $^{111}\text{In-mAb 528}$ compared to $^{111}\text{In-hEGF}$ was likely due to its prolonged residence time in the blood. The internalization and nuclear translocation of $^{111}\text{In-hEGF}$ after binding to its receptor was exploited to selectively deliver $^{111}\text{In}$ into the cytoplasm and nucleus of breast cancer cells overexpressing the EGFR. Internalized $^{111}\text{In-hEGF}$ was radiotoxic in vitro to the breast cancer cells with <5% survival at <130 mBq/cell. The radiotoxicity was probably due to the emission of Auger electrons by $^{111}\text{In}$ at close proximity to the chromosomal DNA. There was no evidence of radiotoxicity in vivo in mice administered high doses of the $^{111}\text{In-hEGF}$ to normal tissues such as the liver and kidneys which express moderate amounts of EGFR. These results are encouraging for the application of radiopharmaceuticals specifically targeted to the EGFR for diagnostic imaging or radiotherapy of advanced forms of breast cancer in humans.
ACKNOWLEDGEMENTS

I would like to express my sincere appreciation to Dr. Richard Miller and the Department of Medical Biophysics who made it possible for me to enroll in a PhD program at the University of Toronto. A special thank you is extended to Dr. Jean Gariépy, who opened his lab to me and allowed me to work on my PhD under his auspices. It has been a great comfort to me to know that I could always count on Jean to support my ideas. It has also been a great pleasure to meet and discuss science with all of the post-docs, technicians and graduate students in Jean’s lab. My hope is that in the future, our respective labs will continue to have opportunities to work together. I would also like to thank the members of my supervisory committee, Dr. Richard Hill, Dr. Irene Andrulis and Dr. Martin Yaffe. Your insightful questions and comments at the meetings kept me on my toes and kept me thinking!

I would like to thank my employer, the Toronto Hospital for allowing me the time and opportunity to pursue my personal goals. I am also very grateful to the past and present members of my lab at the hospital who assisted me to conduct the experiments described in this thesis. In particular, I would like to thank Reza Kiarash, Rommel Domingo, Vincent Teo, Nicole Porlier and Ying Wai Lee. My appreciation is also extended to my scientific colleagues, particularly to the late Dr. Ronald Buick and to Dr. Jas Sandhu who provided breast cancer cell lines and to Dr. Ross Cameron who spared some of his time to perform the electron microscopy experiments. I am also grateful to DuPont Pharma and the Education and Research Foundation of the Society of Nuclear Medicine who provided much needed financial support for the research and to the U.S. Army Breast Cancer Research Program and the Susan G. Komen Breast Cancer Foundation which will provide funds to continue with the work in the future.

Finally, my deepest gratitude is extended to my wife, Anita who once again listened to my hopes and aspirations, comforted me in my fears and put up with my complaints. Thank you Anita. Without you, I would not have been able to accomplish this.
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<tr>
<td>$\bar{A}$</td>
<td>cumulative radioactivity</td>
</tr>
<tr>
<td>$E_{1cm}^{1%}$</td>
<td>extinction coefficient for a 1% solution over a path length of 1 cm</td>
</tr>
<tr>
<td>$\bar{D}$</td>
<td>radiation absorbed dose</td>
</tr>
<tr>
<td>% i.d./g</td>
<td>percent injected dose per gram</td>
</tr>
<tr>
<td>% i.d./mL</td>
<td>percent injected dose per milliliter</td>
</tr>
<tr>
<td>$^{125}$I-UdR</td>
<td>iodine-125 iododeoxyuridine</td>
</tr>
<tr>
<td>$^{18}$F-FDG</td>
<td>fluorine-18 2-fluorodeoxyglucose</td>
</tr>
<tr>
<td>$^{18}$F-FES</td>
<td>16-$\alpha$-[F]fluoro-17</td>
</tr>
<tr>
<td>$^{99m}$Tc-DTPA</td>
<td>technetium-99m diethylenetriaminepentaacetic acid</td>
</tr>
<tr>
<td>$^{99m}$Tc-MDP</td>
<td>technetium-99m methylene diphosphonate</td>
</tr>
<tr>
<td>ALT</td>
<td>alanine aminotransferase</td>
</tr>
<tr>
<td>AR</td>
<td>amphiregulin</td>
</tr>
<tr>
<td>AST</td>
<td>aspartate aminotransferase</td>
</tr>
<tr>
<td>ATCC</td>
<td>American type culture collection</td>
</tr>
<tr>
<td>AUC</td>
<td>area under the curve</td>
</tr>
<tr>
<td>Bmax</td>
<td>maximum number of receptors/cell</td>
</tr>
<tr>
<td>CAD</td>
<td>computer-assisted diagnosis</td>
</tr>
<tr>
<td>CAF</td>
<td>cyclophosphamide, Adriamycin®, 5-fluorouracil</td>
</tr>
<tr>
<td>CCAC</td>
<td>Canadian Council on Animal Care</td>
</tr>
<tr>
<td>CDAP</td>
<td>1-cyano-4-dimethylamino-pyridinium tetrafluoroborate</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary deoxyribose nucleic acid</td>
</tr>
<tr>
<td>cDTPAA</td>
<td>bicyclic anhydride of DTPA</td>
</tr>
<tr>
<td>CEA</td>
<td>carcinoembryonic antigen</td>
</tr>
<tr>
<td>$C_H$</td>
<td>complete constant domain of the heavy chain of an antibody</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>C11</td>
<td>constant domain-1 of the heavy chain of an antibody</td>
</tr>
<tr>
<td>C113</td>
<td>constant domain-3 of the heavy chain of an antibody</td>
</tr>
<tr>
<td>CL</td>
<td>clearance</td>
</tr>
<tr>
<td>CMF</td>
<td>cyclophosphamide, methotrexate, 5-fluorouracil</td>
</tr>
<tr>
<td>cpm</td>
<td>counts per minute</td>
</tr>
<tr>
<td>D0</td>
<td>radiation absorbed dose required to reduce the surviving fraction to 0.37</td>
</tr>
<tr>
<td>DAG</td>
<td>diacylglycerol</td>
</tr>
<tr>
<td>DAPI</td>
<td>4’6’-diamidino-2-phenylindole dihydrochloride</td>
</tr>
<tr>
<td>DNase</td>
<td>deoxyribose nucleic acid nuclease</td>
</tr>
<tr>
<td>dpm</td>
<td>disintegrations per minute</td>
</tr>
<tr>
<td>DTPA</td>
<td>diethylenetriaminepentaacetic acid</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGF-BP</td>
<td>epidermal growth factor-binding protein</td>
</tr>
<tr>
<td>EGFR</td>
<td>epidermal growth factor receptor</td>
</tr>
<tr>
<td>EGFRvIII</td>
<td>mutant form of epidermal growth factor receptor</td>
</tr>
<tr>
<td>ER</td>
<td>estrogen receptor</td>
</tr>
<tr>
<td>ERE</td>
<td>estrogen receptor response elements</td>
</tr>
<tr>
<td>ERK</td>
<td>extracellular regulated kinase</td>
</tr>
<tr>
<td>F(ab')2</td>
<td>divalent antigen-binding fragment of an antibody</td>
</tr>
<tr>
<td>Fab</td>
<td>monovalent antigen-binding fragment of an antibody</td>
</tr>
<tr>
<td>Fc</td>
<td>constant domain of an antibody</td>
</tr>
<tr>
<td>FCS</td>
<td>fetal calf serum</td>
</tr>
<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
</tr>
<tr>
<td>Fv</td>
<td>fragment consisting of variable region of heavy and light chains of an antibody</td>
</tr>
<tr>
<td>Gd-DTPA</td>
<td>gadolinium diethylenetriaminepentaacetic acid</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>GDP</td>
<td>guanosine diphosphate</td>
</tr>
<tr>
<td>GLUT1</td>
<td>glucose transporter-1</td>
</tr>
<tr>
<td>gp-30</td>
<td>glycoprotein-30 EGF-related protein</td>
</tr>
<tr>
<td>GRB2</td>
<td>growth factor receptor binding protein 2</td>
</tr>
<tr>
<td>GTP</td>
<td>guanosine triphosphate</td>
</tr>
<tr>
<td>Gy</td>
<td>Gray</td>
</tr>
<tr>
<td>HB-EGF</td>
<td>heparin-binding epidermal growth factor</td>
</tr>
<tr>
<td>hEGF</td>
<td>human epidermal growth factor</td>
</tr>
<tr>
<td>hEGF51</td>
<td>51-amino acid analogue of human EGF</td>
</tr>
<tr>
<td>hEGF53</td>
<td>53-amino acid analogue of human EGF</td>
</tr>
<tr>
<td>HMFG</td>
<td>human milk fat globule</td>
</tr>
<tr>
<td>HRG</td>
<td>heregulin</td>
</tr>
<tr>
<td>IgG</td>
<td>immunoglobulin-G</td>
</tr>
<tr>
<td>i.p.</td>
<td>intraperitoneal</td>
</tr>
<tr>
<td>i.v.</td>
<td>intravenous</td>
</tr>
<tr>
<td>ITP</td>
<td>inositol triphosphate</td>
</tr>
<tr>
<td>Ka</td>
<td>affinity constant</td>
</tr>
<tr>
<td>kb</td>
<td>kilobase</td>
</tr>
<tr>
<td>Kd</td>
<td>dissociation constant</td>
</tr>
<tr>
<td>kDa</td>
<td>kilo dalton</td>
</tr>
<tr>
<td>keV</td>
<td>kilo electron volt</td>
</tr>
<tr>
<td>LET</td>
<td>linear energy transfer</td>
</tr>
<tr>
<td>mAb</td>
<td>monoclonal antibody</td>
</tr>
<tr>
<td>mBq</td>
<td>milli becquerel</td>
</tr>
<tr>
<td>mEGF</td>
<td>murine epidermal growth factor</td>
</tr>
<tr>
<td>MEK</td>
<td>mitogen activated protein kinase</td>
</tr>
<tr>
<td>MEM</td>
<td>minimal essential medium</td>
</tr>
<tr>
<td>mGy</td>
<td>milli Gray</td>
</tr>
<tr>
<td>M,</td>
<td>molecular weight</td>
</tr>
<tr>
<td>MRI</td>
<td>magnetic resonance imaging</td>
</tr>
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</table>

xviii
mRNA  messenger ribose nucleic acid
MRS  magnetic resonance spectroscopy
NAF  neu activating factor
pCi  pico curie
PET  positron emission tomography
pgp-170  p-glycoprotein 170
PIP2  phosphatidylinositol
PLCγ  phospholipase C-γ
Pristane  2,6,10,14-tetramethylpentadecane
PTK  protein tyrosine kinase
RBF  receptor-binding fraction
ROI  region-of-interest
s.c.  subcutaneous
S1  subclone-1 of MDA-468 cells
SDS-PAGE  sodium dodecylsulfonate polyacrylamide gel electrophoresis
s.e.m.  standard error of the mean
SMSR  somatostatin receptor
SOS  son-of-sevenless
S-phase  DNA synthesis phase
T/B  tumour/blood
T/NT  tumour/non-target
$t_{1/2}^\beta$  half-life of elimination
$t_{1/2}^{\text{input}}$  half-life of first order input function
TAG-72  tumour-associated glycoprotein-72
TGFα  transforming growth factor-α
$V_d$  volume of distribution
$v-erb-B$  avian erythroblastosis virus oncogene

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Chapter 1

INTRODUCTION
1.1 Breast cancer

Breast cancer is a major health problem affecting Canadian women, with more than 18,000 new cases diagnosed each year and more than 5,000 deaths due to the disease annually (1). Breast cancer is also the most common type of female cancer and accounts for 30% of all new cases of cancer and 22% of all deaths due to cancer in women each year. Over a woman's lifetime, the probability of developing breast cancer is approximately 1 in 9 and the probability of dying from the disease is 1 in 24 (1). The incidence of breast cancer increases with advancing age. The disease is uncommon before age 30 (<5 cases/100,000 of population) but the incidence rises rapidly to 150 cases/100,000 by about age 50 and then increases more slowly to about 200 cases/100,000 by age 75 (2).

The etiology of breast cancer is currently unknown but nutritional, environmental, hormonal and genetic factors have been implicated as risk factors for the disease (3,4). A high fat diet is believed to contribute to the relatively high risk of developing breast cancer among North American women (3). Female hormones are also important risk factors for the disease. The likelihood of developing breast cancer is greater among women with early age menarche (<11 years) or late menopause (>55 years) and a higher risk is also associated with late age (>30 years) at first full-term pregnancy or nulliparity. The risk is also greater in obese women, perhaps due to increased metabolism of androstenedione to estradiol in adipose cells. Removal of the ovaries before age 35 reduces the risk of developing breast cancer. The majority of breast cancers are not hereditary, but genetic factors are nevertheless important in the development of the disease in some patients. For example, the risk of developing breast cancer increases 2-4 fold among women who have a first-degree relative who has previously developed breast cancer, particularly if the relative was diagnosed with the disease early in life (i.e. before age 40-45) (4). The discovery of the BRCA1 and BRCA2 genes (5) is an important development which should help to elucidate the possible mechanisms of hereditary forms of breast cancer. Mutated forms of these genes have been found in the germline of only about 5% of breast cancer patients (4) but women who carry BRCA1 mutations have a 70-75% lifetime risk of developing breast cancer (6) and also a higher probability than
the general population of developing ovarian cancer (6,7). It is hypothesised that BRCA1 and BRCA2 are tumour suppressor genes which are responsible for controlling cell growth (4).

Most breast cancers arise from the epithelial cells of the ducts of the breast. The most common histological type is infiltrating ductal carcinoma which accounts for 70-85% of cases, followed by lobular carcinoma which accounts for 5-10% of cases (2). Breast cancer is diagnosed through a combination of physical examination by a physician, mammography (see section 1.2) and histopathological analysis of a biopsy of the suspected lesion obtained by excisional methods or by a fine-needle aspiration. The prognosis of the patient is estimated by staging the disease at the time of initial surgical treatment. Patients with Stage 0 (carcinoma in situ) or Stage I breast cancer usually have small primary tumours and no involvement of the draining lymph nodes and therefore the best possibility for a complete cure. The prognosis is poorer in patients with Stage II or Stage III disease due to the presence of a larger sized primary lesion and involvement of the lymph nodes, both of which lead to a suspicion of possible dissemination of cancer cells. By definition, in these patients no metastatic disease has yet been detected using diagnostic imaging procedures (see section 1.2). The worst prognosis exists in patients with Stage IV disease due to the presence of known distant metastases.

Current treatment of breast cancer involves a combination of surgery, local radiotherapy and systemic chemotherapy or hormonal therapy (2,8). Surgical treatment may be limited to local excision of the primary lesion (lumpectomy) followed by local radiotherapy or involve complete removal of the affected breast and axillary lymph nodes (modified radical mastectomy). At the time of surgical treatment, an axillary lymph node dissection is performed in order to stage the disease. Patients treated conservatively with lumpectomy also receive 40-50 Gy of local external beam radiation in order to minimize the possibility of local or regional recurrence.

Systemic chemotherapy or hormonal therapy is used to treat known metastases or suspected micrometastatic disease (adjuvant treatment). Tamoxifen, an estrogen receptor (ER) antagonist is considered the first-line drug for hormonal therapy of breast cancer (8). The relatively low incidence and severity of side effects associated with tamoxifen
compared to that associated with systemic chemotherapy make tamoxifen the treatment of choice for post-menopausal ER-positive breast cancer patients. Pre-menopausal breast cancer patients are usually treated with chemotherapy but may also receive treatment with tamoxifen. The response rate to tamoxifen in ER-positive breast cancer patients can be as high as 60-85% but the response rate is <10% in patients with ER-negative tumours. Unfortunately less than half of breast cancer patients have tumours which express the ER and patients with advanced ER-negative breast cancer must therefore be treated instead with systemic chemotherapy. The most commonly used chemotherapeutic agents are cyclophosphamide, methotrexate, 5-fluorouracil and doxorubicin (Adriamycin®), which are usually given in combination (eg. CMF or CAF) (8). The response rate to combination systemic chemotherapy is approximately 50-75% but is limited by the dose-limiting toxicity to normal tissues and the rapid development of multi-drug resistance (9). New therapeutic approaches are therefore urgently needed to improve the prognosis in patients with advanced breast cancer, particularly in those patients whose tumours do not express the ER.

1.2 Diagnostic imaging modalities in breast cancer

Mammography, ultrasound, magnetic resonance imaging (MRI) and nuclear medicine imaging techniques may be utilized for the detection of primary breast cancer and also to detect recurrent or metastatic disease. Mammography, ultrasound and MRI may also be used to direct a fine-needle biopsy of a suspected lesion and to assist the surgeon in planning the extent of resection of the breast. The resolution of nuclear medicine imaging techniques is not sufficient for these purposes. A comprehensive discussion of the various diagnostic imaging modalities is beyond the scope of this thesis but a brief summary of the clinical utility of each technique in the management of breast cancer is provided in the following sections.

1.2.1 Mammography, ultrasound and magnetic resonance imaging

Mammography has a very high sensitivity (85-95%) for the detection of lesions in the breast and is the primary diagnostic imaging modality for the detection of primary
Mammography is also the most cost-effective method for screening for breast cancer in women most at risk for developing the disease (i.e. women >50 years old or those with a genetic predisposition to breast cancer). The detection of a lesion by mammography depends on differences in tissue density between the lesion and surrounding normal breast tissue, typically as a result of calcifications. Since many benign breast diseases may also result in calcifications, the specificity of mammography is very low (25-50%) and the technique cannot reliably distinguish between carcinomas and benign breast diseases (e.g. fibroadenomas or fibrocystic disease) (10). A lesion identified by mammography must therefore be sampled by biopsy and examined histopathologically in order to confirm malignancy. Although mammography is extremely useful in the early diagnosis of breast cancer, the high false-positive rate unfortunately leads to a large number of unnecessary biopsies. The sensitivity of mammography is also lower in women with radiographically dense breasts, women with scarring of breast tissue due to prior radiation or surgical treatment and in women with silicone breast implants (11). Research is currently in progress to improve the detectability of lesions by mammography using a combination of digital acquisition and image display methods (digital mammography) as well as computer-assisted analysis of the images (computer-assisted diagnosis, CAD) (10,12).

Ultrasound is a useful complementary technique to mammography for the detection of lesions on difficult to interpret mammograms (i.e. in dense breast tissue) or to differentiate between a cyst and a solid mass (12). Ultrasound can reduce the number of unnecessary biopsies by ruling out fibrocystic disease as a cause of a lesion on the mammogram. The mechanism of detection of a breast lesion by ultrasound is based on differences in the intensity of echoes reflected from the lesion compared to surrounding normal breast tissue (B-mode scanning) (12). Doppler ultrasound is a relatively new technique which detects echoes with a different frequency than the original ultrasound pulse caused by the reflection of a signal by moving substances (e.g. blood flow) (12). Doppler ultrasound is currently being investigated as a means of improving the specificity of ultrasound breast imaging since carcinomas have been found to exhibit increased vascularity compared to benign lesions.
Magnetic resonance imaging (MRI) measures the emission of radio waves emitted by tissues of the body caused by the relaxation of protons in biological molecules following exposure to a strong magnetic field (12). MRI is an extremely sensitive technique for the study of soft tissues and can detect lesions in the breast with a sensitivity of 90-100% (13) but it lacks specificity since carcinomas and benign breast disease exhibit similar MRI signals (12). The specificity of MRI for the detection of primary breast cancer has been reported to be <40% (14) but in some studies has been as low as 15% (13). Improvements in the specificity of MRI of the breast have recently been realized through the use of contrast agents such as gadolinium diethylenetriamine pentaacetic acid (Gd-DTPA). Gd-DTPA is a paramagnetic agent which enhances the signal from carcinomas but not from benign disease (12). A “flow study” can also be performed with Gd-DTPA by injecting the contrast agent and obtaining a series of images over a 5-10 minute period. In the flow studies carcinomas tend to enhance faster than benign lesions. Contrast-enhanced MRI using Gd-DTPA can also provide morphological information. Nevertheless, there are still problems with the specificity of MRI with some reported cases of benign lesions (eg. fibroadenomas) also enhancing rapidly with Gd-DTPA. Interestingly, nuclear medicine imaging techniques using 99mTc-DTPA (see section 1.2.2) have relatively high specificity (91%) for imaging primary breast cancer. MRI imaging of the breast is primarily indicated as a complementary technique to mammography which is useful for differentiating malignancy from scarring, detecting a lesion adjacent to a silicone implant, identifying a lesion in dense breast tissue or as a method for monitoring the response to chemotherapy.

Magnetic resonance techniques can also be used ex vivo to distinguish malignancy from benign disease in a fine-needle biopsy specimen through means of magnetic resonance spectroscopy (MRS). Mackinnon et al. (15) examined 218 fine-needle biopsy specimens by MRS and were able to identify malignant disease by the increased resonance intensity at 3.25 ppm compared to that at 3.05 ppm. The increased intensity at 3.25 ppm was attributed to an altered cellular chemistry in the breast cancer cells, particularly a high level of choline-containing metabolites. The sensitivity and specificity
of MRS for the detection of primary breast cancer in fine-needle biopsies were 95% and 96% respectively.

1.2.2 Nuclear medicine imaging in breast cancer

Nuclear medicine imaging utilizes radiopharmaceuticals which have properties allowing them to distribute differently in diseased tissues relative to normal ones. The radiopharmaceuticals are conjugated with gamma emitting radionuclides such as $^{123}$I, $^{131}$I, $^{99m}$Tc, $^{111}$In or $^{18}$F which permit their biodistribution to be visualized by external imaging. A γ-scintillation camera is used to detect single-photon emitting radionuclides (eg. $^{123}$I, $^{131}$I, $^{99m}$Tc or $^{111}$In) and a positron emission tomograph (or a γ-camera with dual-photon capability) is used to detect positron emitters (eg. $^{18}$F). While mammography, ultrasound and MRI of the breast rely predominantly on structural differences between tumour sites and normal surrounding tissues to detect a lesion, nuclear medicine imaging techniques exploit physiological differences in the tumour. Examples of physiological changes which can be detected by nuclear medicine imaging include increased vascularity, an increased metabolic rate (eg. glucose utilization), expression of steroid or peptide growth factor receptors, expression of cancer-associated antigens and upregulation of membrane transporter proteins involved in drug resistance (eg. pgp-170). Although there are situations where similar physiological changes also occur in benign processes, nuclear medicine imaging procedures in general offer a major advantage over other diagnostic imaging modalities by increasing the specificity of tumour imaging. Nuclear medicine imaging (and also MRI) allows a survey of the whole body to be performed in order to detect the dissemination of cancer cells to distant organs.

Nuclear medicine imaging techniques can be used to detect both primary and metastatic disease. The most commonly utilized nuclear medicine imaging procedure for detection of metastases in breast cancer patients is bone scanning using $^{99m}$Tc-methylene diphosphonate ($^{99m}$Tc-MDP). $^{99m}$Tc-MDP binds to hydroxyapatite crystals in the bone and accumulates avidly in the skeleton after an intravenous injection (16). The radiopharmaceutical exhibits intense localization in regions of the skeleton exhibiting increased bone turnover and repair and whole body images performed at 2-3 hours after
injection reveal metastases as focally increased uptake on the image. Bone scanning with \(^{99m}\)Tc-MDP is considered a very sensitive method for the detection of skeletal metastases in breast cancer (16) and can detect metastases as much as 12 months prior to radiographic changes (16). Nevertheless, a positive bone scan is not specific for cancer since increased uptake can also occur in non-malignant processes such as fractures, degenerative bone disease or osteomyelitis.

Interestingly, localization of \(^{99m}\)Tc-MDP in primary breast cancer has also been reported on early images obtained at 10-20 minutes after injection of the radiopharmaceutical. Piccolo et al. (17) showed that the sensitivity for detection of primary breast cancer in 200 patients evaluated with \(^{99m}\)Tc-MDP was 92% and the specificity was 95%. There were three false-positive studies in women with fibroadenomas. The mechanism of uptake of \(^{99m}\)Tc-MDP in primary breast cancer is not known but may be due to "non-specific" effects such as locally increased blood flow, inflammatory changes or calcification. Similar non-specific accumulation of \(^{99m}\)Tc-DTPA in primary breast cancer has been observed by den Outer et al. (18). The sensitivity for detection of primary breast cancer with \(^{99m}\)Tc-DTPA was 75% but the specificity was 91%.

Currently, \(^{99m}\)Tc-sestamibi is the only radiopharmaceutical approved in Canada for the detection of primary breast cancer. \(^{99m}\)Tc-sestamibi is a cationic, lipophilic radiopharmaceutical which was originally developed as a myocardial perfusion imaging agent and has been used for this purpose in nuclear medicine for the past 5-7 years. Khalkali et al. (19,20) subsequently showed that \(^{99m}\)Tc-sestamibi also localized effectively in breast cancer and could detect primary breast cancer lesions with a sensitivity of 94% and a specificity of 88%. The mechanism of uptake of \(^{99m}\)Tc-sestamibi by breast cancer is not known but it is believed that the radiopharmaceutical diffuses passively across the cell membrane and is then retained in the breast cancer cells due to the interaction of the positively-charged \(^{99m}\)Tc-sestamibi complex with the negatively-charged mitochondrial membrane (11,14). Since breast cancer cells are rapidly proliferating, they have an increased requirement for energy and therefore may possess an increased number of mitochondria compared to the normal epithelial cells of the breast (11). An alternative
explanation for the uptake of $^{99m}$Tc-sestamibi by breast cancer may be simply that the tumour exhibits increased vascularity compared to the surrounding normal breast tissue (21).

Numerous studies have evaluated the efficacy of $^{99m}$Tc-sestamibi imaging for the diagnosis of primary breast cancer (scintimammography), including a recent large multi-center trial involving a total of 673 patients (14). The results of the multi-center trial showed that the overall sensitivity and specificity of scintimammography with $^{99m}$Tc-sestamibi were 85% and 81% respectively. In women with palpable lesions, the sensitivity was 95% and the specificity was 74%, whereas in women with non-palpable lesions, the sensitivity was 72% and the specificity was 86%. An important finding was that scintimammography was not affected by radiographically dense breast tissue (sensitivity of 94% and specificity of 91%). Some false-positive studies in scintimammography have occurred in women with fibroadenomas or fibrocystic disease and some false-negative studies have been reported for small breast cancer lesions (<1 cm in diameter).

It is generally believed that the sensitivity of scintimammography with $^{99m}$Tc-sestamibi, particularly in women with non-palpable disease is not high enough to replace mammography (14). The technique is therefore indicated primarily as a complementary imaging procedure to mammography for the differentiation of benign from malignant lesions detected on a mammogram, especially in palpable disease. Since the likelihood of cancer with a positive $^{99m}$Tc-sestamibi image is high, scintimammography may be helpful to select patients with $^{99m}$Tc-sestamibi image for biopsy (11). Scintimammography is also very useful to detect breast cancer in cases where the mammogram is negative or not interpretable (eg. in women with dense breast tissue or where there is severe scarring due to prior radiation or surgery) (11).

An important recent discovery is that $^{99m}$Tc-sestamibi is a substrate for the p-glycoprotein multi-drug resistance membrane transporter (pgp-170) which is responsible for resistance to several diverse chemotherapeutic drugs used for the treatment of cancer including doxorubicin, vincristine and daunorubicin (22). The kinetics of uptake and washout of the radiopharmaceutical in a tumour could be used as a measure of the levels
of pgp-170 expression. Such "functional" tumour imaging could potentially predict the response of the tumour to chemotherapy or to drugs designed to inhibit pgp-170 (e.g. verapamil or PSC833, a non-immunosuppressive analog of cyclosporin). The concept of functional tumour imaging is that a particular property of the cancer cells can be detected by imaging, providing information which is not solely structural (i.e. size or location) but also reflects a particular physiological or biochemical characteristic of the tumour. Such information has clinical value in the management of the disease.

Positron emission tomography (PET) using $^{18}$F-2-fluoro-2-deoxyglucose ($^{18}$F-FDG) is also a very sensitive method for functional imaging of breast cancer. $^{18}$F-FDG is actively transported into cancer cells by the glucose transporter (GLUT1) and is then metabolized by hexokinase to $^{18}$F-FDG-6-phosphate (11,21). Since $^{18}$F-FDG-6-phosphate is not recognized by glucose-6-phosphatase and is not metabolized further in the glycolytic pathway, it becomes trapped within the cell. PET with $^{18}$F-FDG directly reflects the levels of GLUT1 and/or hexokinase in cancer cells and therefore assesses the metabolic activity of the tumour. The sensitivity of PET with $^{18}$F-FDG for the detection of primary breast cancer is very high (90-100%) and the specificity is 85-100% (23-25). PET with $^{18}$F-FDG is also very effective for the detection of axillary lymph node metastases from breast cancer (sensitivity of 85-100% and specificity of 90-100%) (23,25-27) and it has been suggested that the technique could eventually replace axillary lymph node dissection for the staging of breast cancer (11). $^{18}$F-FDG PET is also very useful for detecting distant metastases from breast cancer in bone and in soft tissues (11). Some false-positive studies can occur with $^{18}$F-FDG PET, primarily in inflammatory processes due to the local accumulation of macrophages which exhibit high glucose utilization (21). PET with $^{18}$F-FDG may also be a very sensitive method for monitoring the response of breast cancer to chemotherapy. Jansson et al. (28) observed a significantly decreased uptake of $^{18}$F-FDG in breast cancer lesions as early as 6-13 days after the first course of chemotherapy whereas conventional radiographic imaging techniques could only detect a response after 3-4 courses of chemotherapy.

Estrogen receptors (ER) have been successfully imaged on breast cancer cells in humans using 16-$\alpha$-[1$^{8}$F]fluoro-17$\beta$-estradiol ($^{18}$F-FES) (29) or 16-$\alpha$-[1$^{2}$I]-estradiol (30).
and progesterone receptors have been detected using 21-[\[^{18}\text{F}\]fluoro-16\alpha-ethyl-19-norprogesterone (31). Dehdashti et al. (29) observed a good agreement between ER expression detected by PET using \[^{18}\text{F}\]-FES and ER status evaluated in a biopsy of the lesion using a radioreceptor assay or by immunohistochemistry (concordance in 88% of cases). One of the major advantages of PET for imaging ER expression in breast cancer is that the primary lesion and also regional or distant metastases can be evaluated without the necessity to biopsy a lesion. PET also determines the ER expression of the entire lesion which could minimize the effects of receptor heterogeneity on the evaluation of ER status. Functional imaging of breast cancer by evaluating ER expression using \[^{18}\text{F}\]-FES PET may also be useful for predicting response to hormonal therapy with tamoxifen.

Finally, imaging of breast cancer can also be performed using radiolabelled monoclonal antibodies (mAbs) which specifically recognize tumour-associated antigens or growth factor receptors overexpressed on the surface of the cancer cells (32). For example, Nabi et al. (32) detected primary breast cancer with an anti-carcinoembryonic antigen mAb labelled with \[^{99}\text{m}\text{Tc}\} (\text{CEA-scan®}, \text{Immunomedics}) achieving a sensitivity of 97% and a specificity of 100%. Successful imaging of breast cancer has also been achieved with radiolabelled mAbs directed against mucin antigens such as human milk fat globule (HMFG) (33), sialyl-Tn (34) or the tumour-associated glycoprotein-72 (TAG-72) antigen (35). Imaging with \[^{99}\text{m}\text{Tc}\}-\text{mAb 170H.82} directed against the sialyl-Tn antigen detected 18/20 known primary breast cancer lesions, 36/40 lymph nodes metastases and 23/28 bone metastases (34). In a more recent study, Dessureault et al. (36) showed that the sensitivity and specificity for detecting axillary lymph node involvement in breast cancer with \[^{99}\text{m}\text{Tc}\}-\text{mAb 170H.82} was 71% and 89% respectively.

Imaging of cancer by targeting the expression of tumour-associated antigens on cancer cells using radiolabelled mAbs cannot be considered as functional tumour imaging at the present time because the clinical significance of the expression of these antigens is not known. Targeting the overexpression of growth factor receptors such as the epidermal growth factor receptor (EGFR) or c-erbB-2 receptors (see section 1.5) on the cancer cells using radiolabelled mAbs is considered a type of functional tumour imaging however since overexpression of these receptors has been shown to be associated with a poor
prognosis in breast cancer and with a lack of response of the tumour to hormonal therapy with tamoxifen (see section 1.6). Although no clinical imaging studies have yet been carried out in breast cancer patients with radiolabelled mAbs directed against the EGFR or c-erbB-2 receptors, successful imaging of human breast cancer xenografts hosted in immunocompromised mice has been achieved using the anti-EGFR mAb 225 labelled with $^{111}$In (37) or the anti-c-erbB-2 mAb ICR12 labelled with $^{124}$I or $^{99}$mTc (38,39). In this thesis, anti-EGFR mAb 528 labelled with $^{111}$In was compared with $^{111}$In labelled epidermal growth factor for imaging EGFR-positive human breast cancer xenografts implanted in athymic mice (see Chapter 3).

1.3 Control of breast cancer growth by steroid hormones and growth factors

The growth regulatory pathways affecting breast cancer cells are thought to involve the interaction of two distinct types of growth signals: i) steroid hormones (eg. estrogen or progesterone) which bind to steroid nuclear receptors and ii) peptide growth factors (eg. epidermal growth factor) which bind to transmembrane tyrosine kinase receptors (40). The steroid hormone estrogen which is produced in the ovaries, is particularly involved in the growth regulation of breast cancer cells. Estrogen binds to the estrogen receptor (ER), a nuclear transcription factor which then interacts with specific sequences on DNA (estrogen receptor response elements, ERE) and upregulates the transcription of certain genes involved in cell growth. One of those genes codes for transforming growth factor-α (TGFα) (40). TGFα is a peptide growth factor (see section 1.4) produced and secreted by breast cancer cells which binds to the epidermal growth factor receptor (EGFR, see section 1.5) in an autocrine growth stimulatory loop. In fact, it is believed that the growth stimulatory properties of estrogen on breast cancer cell lines in vitro are mediated primarily by the TGFα autocrine pathway (41). The significance of this growth regulatory pathway in breast cancer patients is still controversial however (41), since numerous studies have shown that the level of EGFR expression is either undetectable or marginal in breast cancer biopsies which express the ER (see section 1.6). Nevertheless, a relatively large proportion (30-60%) of breast cancers exhibit high levels of EGF receptors on their surface, and as a result may receive excessive growth
stimulation by the TGFα autocrine pathway. EGFR overexpression by the breast cancer cells could result in the hypersensitivity of cells to the growth stimulatory effects of epidermal growth factor (EGF) or TGFα (see section 1.4). EGFR overexpression appears to confer a more aggressive phenotype to breast cancer cells and is associated with a poor prognosis in the disease, even in subgroups which would normally be expected to have a relatively good prognosis (eg. node-negative breast cancer). In contrast, ER-positive breast cancer cells exhibit a less aggressive phenotype and their growth can be controlled by blocking the binding of estrogen to its receptor using tamoxifen as previously discussed.

1.4 Epidermal growth factor and related growth factors

Human epidermal growth factor (hEGF) is a 53 amino acid peptide synthesized in the submaxillary gland which binds specifically to the epidermal growth factor receptor (EGFR), a 170 kDa transmembrane tyrosine kinase receptor present on the surface of epithelial cells (42). The affinity constant (Ka) for binding of EGF labelled with $^{125}$I to the EGFR on human fibroblasts is approximately $2 \times 10^9$ L/mol (43) but in some assays (44), two classes of binding sites have been detected: i) a high affinity but low capacity site and ii) a low affinity but high capacity site. The biological function of the two different classes of binding sites on the EGFR is currently unknown. EGF was originally isolated from the submaxillary glands of mice but the human analog was subsequently shown to be identical to urogastrone, a hormone found in the urine and which inhibited gastric acid secretion (45).

The primary structure of hEGF (Fig. 1.1 A) consists of a single polypeptide chain with arginine at its C-terminus and asparagine at its N-terminus which is folded into three distinct loops held in place by three disulfide bridges (46). Mouse EGF (mEGF) exhibits about a 70% sequence homology to hEGF and binds equally well to the human EGFR (45,47,48). One notable difference between mEGF and hEGF however, is that the former does not contain any lysine residues, whereas hEGF contains two lysine residues at positions 28 and 48. The corresponding residues in mEGF are serine-28 and arginine-48 (48). The ε-amino group on the two lysine amino acids in hEGF as well as the α-
Fig. 1.1: The primary structure of A. human epidermal growth factor (hEGF) and B. transforming growth factor-α (TGFα). Adapted from: Normanno N. et al. Breast Cancer Res. Treat. 29: 11-27, 1994. The lysine residues (numbered) in hEGF or TGFα are potential sites for conjugation with DTPA for radiolabelling with $^{111}$In. The tyrosine residues (numbered) are potential sites for radioiodination.
amino group are potential sites for derivatization of the EGF molecule, eg. attachment of a chelating moiety for a radionuclide (as described in Chapter 2) or a fluorescent probe such as fluorescein (as described in Chapter 4) through the formation of amide linkages with chemically activated forms of these probes. Both hEGF and mEGF also contain several tyrosine residues which allowed radioiodination of the growth factor (as discussed in Chapter 3). Human EGF is heat-stable, exhibits a pI of 4.6 and absorbs in the ultraviolet range at 280 nm with an extinction coefficient (\(E_{280}\)) of 30.9 (42).

The cDNA for mEGF or hEGF predicts a 1,217 or 1,207 amino acid precursor protein respectively (pro-EGF, \(M_r\) of 128 kDa) which is thought to be cleaved into mature EGF by an EGF binding protein (EGF-BP) with arginine esterase activity (45,49,50). The EGF-BP contains 237 amino acids and consists of three polypeptide chains linked by disulfide bridges (50). The EGF-BP interacts with the C-terminal arginine residue of EGF to form a complex containing two molecules of EGF and two molecules of EGF-BP (42,51). It is hypothesized that pro-EGF may be membrane bound since a putative transmembrane region has been identified at the C-terminus of the protein (50). Pro-EGF contains the mature 53 amino acid EGF sequence and also several other “EGF-like” domains located throughout the protein (52).

The tertiary structure of hEGF (Fig. 1.2 A) (53) consists of an N-terminal domain (residues 1 to 32) composed of a double-stranded, anti-parallel \(\beta\)-sheet linking residues 19 to 23 with residues 28 to 32. Residues 3 to 5 may also interact with residues 21 to 23 to form a triple-stranded \(\beta\)-sheet. The two strands of the \(\beta\)-sheet are linked by a turn (residues 24 to 27) and the second \(\beta\)-sheet then leads into the C-terminal domain. The C-terminal domain (residues 33 to 53) starts at cysteine-33 (the third disulfide bridge) with residues 34 to 37 forming a Type II \(\beta\)-turn followed by a short stretch of anti-parallel \(\beta\)-sheet pairing residues 37 and 38 with residues 44 and 45. Residues 39 to 43 form a turn which allows the third disulfide bridge to form and the minor \(\beta\)-sheet to fold.

The amino acids in the human EGF molecule which are thought to make direct contact with the EGFR are tyrosine-13, leucine-15, histidine-16, arginine-41, glutamine-43 and leucine-47 (53,54). These residues are all present on one face of the EGF molecule but it is hypothesized that EGF may in fact bind to two separate regions on the EGFR.
The major β-sheet (Fig. 1.2) is not directly involved in binding the receptor but forms a scaffold on which the binding site is constructed. The 6 cysteine residues are necessary for the formation of the three disulfide bridges which are required to maintain the proper folding of the EGF molecule. Reduction of the disulfide bridges has been shown to destroy the biological activity of EGF (55). Modification of the two lysine residues in hEGF (K$_{28}$ or K$_{48}$) by site-directed mutagenesis or by chemical derivatization does not affect its receptor binding property (46). Modification of the α-amino group of the hEGF molecule should also not affect receptor binding since the N-terminus is distant from the receptor-binding site (53). A wide variety of molecules such as biotin (56), dextran (57), horseradish peroxidase (58) or fluorescein (59) have been conjugated to the N-terminus of EGF with little or no effect on its receptor-binding ability. The resiliency of the EGF molecule to derivatization is evidenced by the observation that EGF fixed through its N-terminus to a solid glass surface remains biologically active (60). The final 6 residues at the C-terminus of hEGF are not required for receptor binding and hEGF$_{1-47}$ and hEGF$_{1-51}$ are biologically active forms of EGF (43,48).

Transforming growth factor-alpha (TGFα) is a 50 amino acid peptide (Fig. 1.1 B) harbouring a 40% sequence homology with EGF. It is synthesized by a variety of malignant cells and binds specifically to the EGFR in an autocrine growth stimulatory loop (as discussed in section 1.3). TGFα was originally named after its ability to transform normal rat kidney fibroblasts and is believed to be an evolutionary precursor of EGF (45,61). The tertiary structure of TGFα (Fig. 1.2 B) is also very similar to that of EGF (53). The residues on TGFα directly in contact with the EGFR are phenylalanine-15, phenylalanine-17, histidine-18, arginine-42, glutamine-44 and leucine-48 (54).

Amphiregulin (AR) and heparin-binding EGF-like growth factor (HB-EGF) are also ligands for the EGFR (61-63). AR is an EGF-related single chain, heparin-binding glycoprotein which is present in two forms (78 or 84 amino acids) with a $M_r$ of 9.2-9.7 kDa in its deglycosylated form and a $M_r$ of ~16.5 kDa in its glycosylated form. AR exhibits approximately 40% sequence homology with hEGF and specifically binds to the EGFR with a lower affinity than either EGF or TGFα (63). Interestingly, AR has a 43
Fig. 1.2: The tertiary structure of A. human epidermal growth factor (hEGF) and B. transforming growth factor-α (TGFα). The main β-sheet is indicated in black. Adapted from: Campbell I.D. et al. Prog. Growth Factor Res. 1:13-22, 1989.
amino acid N-terminal extension which precedes its “EGF-like” domain which contains two nuclear localization sequences at residues 26-29 and 40-43, consisting of a stretch of the basic amino acids, lysine and arginine (63). AR has also been detected in the nucleus of A431 epidermoid carcinoma cells and HTB 132 breast cancer cells (63). These findings suggest that AR has an intracellular site of action as well as activating the tyrosine kinase activity of the EGFR.

HB-EGF is an 86 amino acid glycoprotein (M, ∼19-23 kDa) with an “EGF-like” domain which is synthesized from a 208-amino acid transmembrane precursor (64). As in the case of AR, HB-EGF also contains two nuclear localization sequences in its N-terminal domain.

1.5 The type I growth factor receptor family

The EGFR (c-erbB-1, Fig. 1.3) is the first member of a family of structurally and functionally related growth factor receptors known as the Type I growth factor receptor family. Other members of the family include the c-erbB-2 (neu, HER-2), c-erbB-3 and c-erbB-4 receptors. They each consist of an extracellular ligand binding domain, a single short transmembrane region and an intracellular tyrosine kinase domain. There is a high degree of sequence homology in their tyrosine kinase domain but a lower level of homology in their ligand binding domain. For example, c-erbB-2 receptor exhibits an 82% sequence homology with EGFR in the tyrosine kinase region but only about a 40-45% homology in their ligand binding region (62,65).

The EGFR is a 170 kDa glycoprotein with an N-terminal extracellular ligand binding domain of 621 amino acids, a short membrane spanning region of 26 amino acids and a C-terminal tyrosine kinase domain of 542 amino acids (66). The C-terminal region of the receptor has a high sequence homology to the product of the v-erb-B (avian erythroblastosis virus) oncogene (Fig. 1.3). The v-erb-B oncogene codes for a mutated EGFR with truncation of the extracellular ligand-binding domain and partial deletion of the cytoplasmic C-terminal region which leads to the constitutive activation of the tyrosine kinase activity of the receptor (64). The EGFR gene which is located on chromosome 7p12 is relatively large (>50 kb) and contains 24 exons. The gene encodes
two mRNA transcripts: 10 kb and 6 kb. The 6 kb transcript is predominant; the 10 kb transcript contains a longer 3'-untranslated region. Several C-terminal tyrosines have been identified as phosphorylation sites on the EGFR including tyrosine-1068, tyrosine-1148 and tyrosine-1173. Binding of EGF or TGFα to the EGFR results in receptor dimerization and autophosphorylation of the C-terminal tyrosine residues. The phosphotyrosine residues then act as "docking" sites for intracellular signaling proteins which possess SH2 (src homology-2) domains.

The ras pathway is likely the most important signal transduction pathway following activation of the tyrosine kinase function of the EGFR (Fig. 1.4) but the receptor also acts on phosphatidylinositol bisphosphate to increase the intracellular levels of the secondary messengers inositol triphosphate and diacylglycerol (67). These secondary messengers then increase the concentration of Ca²⁺ in the cytoplasm and activate a calmodulin-dependent kinase (a serine-threonine kinase). Modulation of the signal following ligand binding is achieved through rapid internalization of the receptor-ligand complex into endosomes and lysosomes and degradation of the ligand by lysosomal proteases. A short segment of the C-terminal domain of the EGFR (residues 973-991) is believed to mediate receptor internalization (68). Intracellular phosphatases may also play a role in attenuating the signal from the activated EGFR (69).

Nuclear translocation of internalized EGF following binding to its receptor has been reported in several studies in both normal and malignant cells (59,70-74). The role of nuclear translocation is not well understood but it has been proposed that EGF may initiate two different types of signals within the cell: 1) a differentiation signal mediated by cell membrane binding and activation of the intracellular kinase signaling cascade and 2) a more potent mitogenic signal mediated by binding to nuclear receptors (67). It is interesting to note that nuclear translocation of EGF is enhanced considerably under growth stimulatory conditions (eg. in hepatocytes after partial hepatectomy) (70,73) or in the presence of lysosomal protease inhibitors (72). It is not known if EGF remains bound to its receptor during the nuclear translocation process but the EGFR contains a putative nuclear translocation sequence (RRRHIVRKRTLRR) at residues 645-657 which could
Fig. 1.3: A model of the epidermal growth factor receptor (EGFR), the product of the avian erythroblastosis virus oncogene (v-erbB) and a mutant form of the epidermal growth factor receptor (EGFRvIII) expressed by certain cancer cells, including malignant gliomas. The EGFR consists of an extracellular ligand binding domain (I-IV, 621 amino acids) with two cysteine-rich regions, a short transmembrane domain (TM, 26 amino acids) and an intracellular domain (542 amino acids) with protein kinase (PK), internalization (IN) and regulatory (REG) functions. The intracellular domain contains several C-terminal tyrosine residues which are potential sites for autophosphorylation. The v-erbB oncogene product consists of a truncated constitutively active form of the EGFR with deletion of most of the ligand binding domain. The mutant EGFRvIII gene has an in-frame deletion of codons 6-273 which results in a truncated receptor protein with a new glycine residue inserted at the deletion junction.
Fig. 1.4: A simplified schematic example of an intracellular signaling cascade following binding of a growth factor (eg. EGF or TGFα) to its receptor (eg. EGFR). Binding of EGF or TGFα to the EGFR receptor results in phosphorylation of several C-terminal tyrosine residues on the receptor which allows the “docking” of enzymes such as phospholipase Cγ (PLC-γ) as well as linker molecules such as growth factor receptor binding protein 2 (GRB2). PLC-γ catalyses the lysis of phosphatidylinositol (PIP2) into inositol triphosphate (ITP) which then increases cytoplasmic Ca"^2+" concentration and diacylglycerol (DAG) which activate the mitogenic protein kinase C (PKC). Binding of son-of-sevenless (SOS) to GRB2 occurs using a SH3 linkage. SOS catalyzes the exchange of ras-GDP for ras-GTP, which then phosphorylates the serine/threonine kinase raf-1. Raf-1 phosphorylates the kinase MEK (mitogen activated protein kinase) which then phosphorylates and activates ERK (extracellular regulated kinase). ERK targets include growth regulatory molecules such as transcription factors. Adapted from Zanke, B. Growth Factors and Intracellular Signaling. In: Tannock, I.F. and Hill, R.P. eds. The Basic Science of Oncology 3rd ed. Chapt. 6, Toronto: McGraw-Hill, 1998
mediate nuclear translocation (75). Specific binding sites for EGF on chromatin have also been detected (71).

A mutant form of the EGFR (EGFRvIII, Fig. 1.3) has been found in malignant gliomas, lung cancers and breast cancer (76). The EGFRvIII mutation results from an in-frame deletion of exons 2-7 of the EGFR gene with a new glycine codon inserted at the splice junction of exons 1 and 8. The resulting mRNA is translated into a truncated receptor protein (Mr of 140 kD) in which amino acids 6-273 of the extracellular ligand binding domain are replaced by a single glycine residue (77). The mutation results in constitutive activation of the tyrosine kinase function of the receptor (78) and so far, has only been detected in malignant cells.

The gene for the c-erbB-2 receptor was originally identified and cloned from a human breast and gastric carcinoma and is located on chromosome 17q21 (64,69). The c-erbB-2 gene encodes a 4.8 kb mRNA transcript which is translated into a 1,255 amino acid glycoprotein tyrosine kinase receptor (Mr of 185 kDa) (65). A related oncogene (neu) was identified in brain tumours of rat offsprings exposed to ethylnitrosourea during pregnancy and contains a single point mutation in the transmembrane region (Val→Glu) which results in constitutive activation of the tyrosine kinase activity of the receptor. The human c-erbB-2 homolog does not contain the point mutation. The c-erbB-2 receptor does not bind EGF but can form heterodimers with the EGFR following binding of EGF to its receptor (79). Heregulin (HRG), a 45 kDa glycoprotein originally thought to be the ligand for the c-erbB-2 receptor (80) has been identified instead as the ligand for c-erbB-4 receptor (81). Recently gp-30, an EGF-related protein and Neu Activating Factor (NAF) have been suggested as potential ligands for the c-erbB-2 receptor (69). The phosphorylation sites on the cytoplasmic tyrosine kinase domain of the c-erbB-2 receptor are tyrosine 1139, tyrosine 1222 and tyrosine 1248 (65).

The c-erbB-3 gene was cloned from human breast and epidermoid carcinoma cDNA libraries and is located on chromosome 12q13. The gene encodes a 6.2 kb mRNA transcript which is translated into a 160 kDa transmembrane receptor tyrosine kinase. The ligand for the c-erbB-3 receptor is not known. The c-erbB-4 receptor is a 180 kDa
transmembrane tyrosine kinase which specifically binds HRG (82). Members of the Type 1 growth factor receptor family may form homodimers or heterodimers upon ligand binding to one receptor. Heterodimers which have been detected include EGFR/c-erbB-2, c-erbB-2/c-erbB-3 and c-erbB-2/c-erbB-4 (69). Heterodimerization may allow for diversification of signals within the cell from the binding of a single ligand molecule to its receptor (83).

1.6 EGFR overexpression in breast cancer

Overexpression of the EGFR at levels up to 100-fold higher than that observed on normal epithelial tissues (~10^4 receptors/cell) has been detected in approximately 30-60% of human breast cancer biopsies (84-89). There is a wide range of EGFR expression detected ranging from as low as 1 fmol/mg to as high as 3,600 fmol/mg of membrane protein (~10^3 to 3 X 10^6 receptors/cell) (85). Clinically significant EGFR overexpression is considered to be ≥10 fmol/mg membrane protein (>10^4 receptors/cell) (86). ER expression is defined as ≥5 fmol/mg cytosolic protein and is exhibited by approximately 45-50% of the breast cancer biopsies. There is a strong inverse correlation between EGFR expression and ER expression (84,86,87,89). ER expression is not present in up to 83% of EGFR-positive breast cancers, whereas EGFR expression was absent in up to 85% of ER-positive tumours. Only a small proportion (6-13%) of breast cancers are found to express both the EGFR and ER. There is also a slightly higher expression of the EGFR in infiltrated lymph nodes and metastases compared to the primary tumour suggesting clonal selection of EGFR-positive malignant cells during the dissemination process (87,90-92).

The presence of high levels of EGFR in breast cancer is directly correlated with a lack of response to hormonal therapy with tamoxifen. In one study of 106 breast cancer patients treated with tamoxifen for locally advanced disease or distant metastases, 80% of EGFR-negative patients responded to the drug whereas only 7% of EGFR-positive patients responded (93). In another study of 61 post-menopausal women with breast cancer treated with tamoxifen, 77% of the patients responded to the drug but of those who were unresponsive, 85% had tumours which were EGFR-positive (94). In contrast, none of the patients who responded to tamoxifen had tumours which were EGFR-positive.
Tamoxifen is believed to exert its cytotoxic effect on breast cancer cells primarily by competitively blocking the ER and therefore it is anticipated that the response rate to the drug would be much higher in ER-positive compared to ER-negative tumours. However, tamoxifen has also been found to decrease the production of TGFα by ER-positive but not ER-negative breast cancer cells (95). Tumour growth may therefore also be inhibited by disrupting an autocrine growth stimulation loop involving binding of TGFα to the EGFR. Newby et al. (96) observed a response rate of 89% in ER-positive breast cancer patients treated with tamoxifen compared to only 10% in ER-negative breast cancer patients. Importantly, in the small proportion of patients whose tumours co-expressed both the EGFR and the ER, the presence of the EGFR was directly correlated with a decreased response rate to tamoxifen (84,97). Nicholson et al. (93) noted that 80% of patients whose tumours were negative for the EGFR by immunohistochemical staining responded to tamoxifen compared to only 43% of patients whose tumours exhibited moderate staining for the EGFR and <25% of patients whose tumours exhibited intense staining for the EGFR.

Loss of the ER is proposed as one mechanism whereby breast cancer cells may become resistant to tamoxifen (98,99). It has been hypothesized that treatment with tamoxifen could lead to the loss of an estrogen-dependent repressor protein for the EGFR gene, which would then result in increased synthesis of the receptor and hypersensitivity of the cancer cells to EGF or TGFα. The resulting autocrine signaling loop could then cause upregulation of the EGFR (100). A DNAse hypersensitive region at the exon-1/intron-1 boundary of the EGFR gene has been identified in ER-positive/EGFR-negative but not in ER-negative/EGFR-positive breast cancer cell lines and is believed to be a putative regulatory binding site for such an estrogen-dependent repressor protein (101). Interestingly, in one study almost two thirds of breast cancer metastases which expressed the EGFR originated from EGFR-negative primary tumours (91). Nevertheless, the upregulation of the EGFR in tamoxifen resistance is still controversial since Newby et al. (96) recently found no change in EGFR expression by immunohistochemistry in 155 breast cancer lesions unresponsive to tamoxifen compared to that observed in the pre-
treatment biopsy samples. Expression of the EGFR (or c-erbB-2) in the breast cancer lesions was associated however with a significantly decreased response rate to tamoxifen.

The strongest prognostic indicator in breast cancer patients is the involvement of the axillary lymph nodes (2). For example, Macias et al. (102) found that 60% of surgically treated breast cancer patients with involved lymph nodes relapsed over a 6-year follow-up period compared to only 8% in node-negative patients. The probability of survival from the disease also decreased proportionately with the extent of lymph node involvement. Only 8% of node-negative patients died within 6 years compared to 47% with 1-3 involved nodes and 77% when >4 nodes were affected. Most studies have also shown a direct correlation however between EGFR overexpression and a significantly decreased relapse-free survival and a decreased overall survival in breast cancer patients (84,86-88,93). In one study (88), the relapse-free survival at 2 years post-treatment was 3 times higher for breast cancer patients with EGFR-negative tumours compared to those with EGFR-positive tumours. Similarly, the overall survival at 2 years was 2-fold greater for patients with EGFR-negative tumours compared to those whose tumours expressed the EGFR. Nicholson et al. (93) observed that the survival of patients whose tumours exhibited intense staining for the EGFR by immunohistochemistry had an especially poor prognosis with a median survival of <1 year. Even in node-negative breast cancer, EGFR expression was associated with a poor prognosis (84). In some studies (85,102) only a weak correlation has been observed between EGFR expression and prognosis in breast cancer. The clinical prognostic significance of EGFR overexpression in breast cancer is therefore still controversial and it is not currently routinely utilized as a prognostic marker for the disease.

Overexpression of the c-erbB-2 receptor has been observed in about 15-45% of human breast cancers (69,96,103-105). There is also a direct correlation between a poor prognosis in breast cancer, and overexpression of the c-erbB-2 receptor (96,103,106) even in node-negative disease (107) but the relationship with resistance to tamoxifen is not clear (93,96). The prognostic value and the relationship to tamoxifen resistance of overexpression of the other Type I growth factor receptor members (c-erbB-3 and c-erbB-4) is not known at present.
1.7 Normal tissue expression of the EGFR.

The majority of normal tissues express <10⁴ EGFR/cell but hepatocytes (58,108,109) and renal tubular cells (110) exhibit moderate-high levels of EGFR expression (approximately 10² EGFR/cell). EGFR expression by hepatocytes is consistent with the major role of the liver in the sequestration and elimination of EGF from the blood (109). The explanation for EGFR expression in the kidneys is not known but the receptor may be involved in the binding and partial reabsorption of EGF which is secreted by renal tubular cells (111). Most importantly from a therapeutic target point of view, EGFR expression is present on <3% of the bone marrow stem cells, primarily of the monocytic lineage (112). The mutant form of the EGFR (EGFRvIII) has not been detected on normal tissues and appears to be tumour-specific (76).

1.8 EGFR overexpression as a target for the treatment of breast cancer

The overexpression of the EGFR in a high proportion of hormone-resistant and poor prognosis breast cancers makes it an ideal target for the development of new therapeutic approaches for the disease. Two different strategies have been explored to exploit the overexpression of the EGFR for the treatment of breast cancer: i) interfering with the mitogenic signal resulting from the binding of EGF or TGFα to the receptor and ii) designing cytotoxic agents which are selectively targeted to the cancer cells through specifically binding to the receptor.

The first approach has been pioneered by Mendelsohn et al. (113,114) who demonstrated that monoclonal antibodies directed against the extracellular ligand binding domain of the EGFR could block the binding of EGF and TGFα to the receptor as well as the mitogenic action of these growth factors on EGFR-positive cancer cells. Mendelsohn’s group (115) produced two murine monoclonal antibodies: mAb 225 (IgG₁) and mAb 528 (IgG₂a) which bind specifically to epitopes on the extracellular ligand binding domain of the human EGFR with affinity comparable to that of EGF itself (Ka of ~3 X 10⁸ L/mol). It is hypothesized that the antibodies do not bind directly to the ligand binding site but bind instead to epitopes which are sufficiently close to the ligand binding site to block the binding of EGF or TGFα to the receptor. The anti-EGFR mAbs were
strongly growth inhibitory *in vitro* to A431 epidermoid carcinoma cells or MDA-468 human breast cancer cells which overexpress the EGFR (116). Monoclonal antibodies 225 and 528 were also growth inhibitory *in vivo* against subcutaneous A431 or MDA-468 tumour xenografts hosted in athymic mice (116). The growth rate of the A431 or MDA-468 tumour xenografts was decreased at least 4-5 fold by treatment of the mice with 2 mg of the anti-EGFR mAbs by i.p. injection twice a week for 5-7 weeks. The anti-EGFR mAbs were cytostatic however and required continuous administration in order to control tumour growth. Nevertheless, more recent studies have shown that when mAb 225 or 528 were combined with the chemotherapeutic agents doxorubicin or cis-platinum, combination therapy was more effective than treatment with either the anti-EGFR mAb or the chemotherapeutic agent alone, and complete eradication of the tumours could be achieved in a high proportion of the animals (117). Clinical trials of anti-EGFR mAbs as well as mAbs directed against the c-erbB-2 receptor are currently in progress in several different types of malignancies including breast cancer (118).

Another potential strategy to interfere with the mitogenic signal following the binding of EGF to its receptor is the use of specific competitive inhibitors for the protein tyrosine kinase (PTK) function of the receptor (tyrphostins) (119). These compounds are typically hydroxylated benzylidene malononitriles which have structures which mimic the tyrosyl moiety of the receptor. Most tyrphostins inhibit preferentially the PTK activity of the EGFR compared to the insulin receptor by about 100-800 fold but recently a tyrphostin which specifically inhibited the EGFR was discovered (120).

The second approach to exploiting the overexpression of the EGFR in breast cancer for treatment of the disease has been focused towards the design of targeted cell toxin fusion proteins directed against the receptor. Highly potent plant (121) or bacterial (122,123) cell toxins have been fused to EGF or TGFα in order to selectively target these agents to EGFR-positive cancer cells. The rapid internalization of EGF or TGFα following binding to the EGFR effectively delivers the toxins to their site of action in the cytoplasm of the cell where they inhibit ribosomal protein synthesis. Arteaga et al. (123) demonstrated that *Pseudomonas* exotoxin fused to TGFα was highly toxic *in vitro* against MDA-468, MDA-231 and BT-20 breast cancer cells which overexpressed the EGFR (1.5-8 X 10⁵)
receptors/cell) but at least 10-fold less potent against MCF-7, T47D and ZR-75-1 breast cancer cells which expressed <10^4 EGFR/cell. In nude mice inoculated with breast cancer cells, treatment with the fusion protein for 7 days resulted in a 2-3 fold delay in tumour growth for MDA-468, MDA-231 and BT-20 cells but not for MCF-7 cells. Treatment of mixed breast cancer cell populations \textit{in vitro} or \textit{in vivo} with different EGFR expression levels resulted in selective killing of EGFR-overexpressing cells. Shaw et al. (122) showed that EGF fused to diptheria toxin was 100-fold more potent \textit{in vitro} against EGFR-overexpressing A549 squamous carcinoma cells, SK-BR-3 or ZR-75-1 breast cancer cells than against MCF-7 breast cancer cells.

\textit{Pseudomonas} exotoxin has also been chemically conjugated to murine monoclonal antibodies L8A4, H10 and Y10 directed against the mutant form of the EGFR (EGFRvIII) and the resulting immunotoxins tested for cytotoxicity against NR6 fibroblasts transfected with the mutant EGFRvIII gene (124). The gene transfected NR6 cells expressed approximately 5 \times 10^5 EGFRvIII receptors/cell. The immunotoxins were cytotoxic \textit{in vitro} against the NR6 cells expressing the mutant EGFRvIII receptor but not to A431 epidermoid carcinoma or MCF-7 breast cancer cells which expressed the wild type EGFR, suggesting that these agents may be able to discriminate between normal and malignant cells \textit{in vivo}. The results of these studies with anti-EGFR mAbs and EGF or TGF\alpha cell toxin fusion proteins suggest that novel therapeutic agents targeted to the EGFR could demonstrate selective toxicity against EGFR-overexpressing cancer cells while malignant (or normal) cells with low EGFR expression would be much less sensitive.

1.9 EGFR overexpression as a target for imaging and radiotherapy of breast cancer

EGFR and c-erbB-2 overexpression in breast cancer are also promising targets for the design of specific radiopharmaceuticals for the diagnostic imaging or radiotherapy of the disease. In this thesis, EGFR was chosen as the target because of the commercial availability of EGF, the ligand for the receptor whereas the ligand for the c-erbB-2 receptor was not known (or available) at the initiation of the thesis research. Radiopharmaceuticals that are specifically targeted to the EGFR and labelled with \(\gamma\)-
emitting radionuclides would allow "functional" imaging of breast cancer (as previously discussed for $^{18}$F-FDG and $^{18}$F-FES in section 1.2) by characterizing the EGFR expression status of the tumour. Information from such imaging studies would be valuable to decide on appropriate therapeutic strategies (i.e. chemotherapy versus hormonal therapy) or to select patients for treatment with new anti-cancer agents specifically targeted to the EGFR, eg. anti-EGFR mAbs, tyrphostins or EGF or TGFα-toxin fusion proteins (as discussed in section 1.8). Imaging EGFR expression on breast cancer cells would likely be most useful in the detection and characterization of metastatic disease rather than for the detection of primary breast cancer, since as previously discussed in section 1.2, primary breast cancer can currently be detected with high sensitivity by mammography. The clinical role of EGFR overexpression as a target for imaging of breast cancer is discussed further in Chapter 5 (section 5.3).

A logical extension of this strategy would be to develop radiopharmaceuticals conjugated with radionuclides which emit particulate forms of radiation (eg. Auger electrons, α or β-particles) and are specifically targeted to the EGFR. Such radiopharmaceuticals could be used to treat EGFR-positive metastatic breast cancer. As previously described in section 1.6, EGFR-positive breast cancer is defined as >10 fmol EGFR/mg cell membrane protein (>10$^4$ EGFR/cell). The development of such targeted radiotherapeutic agents could potentially offer a novel strategy for the treatment of ER-negative and hormone-resistant advanced breast cancer which could significantly improve the poor prognosis in these patients (discussed further in section 5.3.2). Radiotherapeutic agents targeted to the EGFR could also be used in combination with chemotherapy or other anti-EGFR agents in order to achieve a higher rate of tumour remission than is currently possible.

In the research described in this thesis, the feasibility of imaging human breast cancer overexpressing the EGFR using either hEGF or anti-EGFR mAb 528 radiolabelled with the γ-emitting radionuclide, indium-111 ($^{111}$In) was explored (see Chapter 2 and 3). Since EGF is rapidly internalized following binding to its receptor and $^{111}$In also emits low energy Auger electrons which are radiotoxic to the cells when the radionuclide is
internalized, the potential for using $^{111}$In-hEGF for the treatment of EGFR-positive breast cancer cells was also investigated (see Chapter 4).

1.10 Somatostatin receptor expression as a target in breast cancer.

This thesis is focused on targeting the overexpression of the EGFR in breast cancer, but another cell-surface receptor which is also receiving considerable interest as a potential target for imaging and radiotherapy of cancer is the somatostatin receptor (SMSR). The SMSR specifically binds the 14-amino acid peptide inhibitory growth factor, somatostatin (Fig. 1.6 A). SMSR are found primarily on neuroendocrine malignancies (125) such as pancreatic cancer, medullary thyroid carcinoma, carcinoid tumours and small cell lung cancer but have also recently been detected in breast cancer (126-129). The explanation for the increased expression of a receptor for an inhibitory growth factor such as somatostatin on cancer cells is not entirely clear but it has been proposed that increased SMSR expression may be a compensatory mechanism by the cells intended to counteract the effects of increased mitogenic signaling mediated through other growth factors and their receptors (130). Interestingly, there is an inverse correlation between SMSR expression and EGFR expression on breast cancer cells (128).

Octreotide is an 8-amino acid synthetic analog of native somatostatin (Fig. 1.6 B) which retains the key receptor-binding residues but exhibits a prolonged serum half-life due to the replacement of two L-amino acid residues (L-Phe and L-Trp) by their D-isomers. Octreotide was originally developed as a pharmacological agent for the management of SMSR-positive malignancies. More recently, octreotide was derivatized with diethylenetriaminepentaacetic acid (DTPA) and labelled with the radiometal, $^{111}$In ($^{111}$In-pentetreotide, Fig. 1.6 C) for the diagnostic imaging of tumours which express SMSR. Krenning et al. (125) imaged more than 1000 patients with neuroendocrine tumours using $^{111}$In-pentetreotide and found that the sensitivity ranged from 61% for insulinomas to as high as 100% for pituitary tumours, glucagonomas, paragangliomas or small cell lung cancer. The sensitivity of detection of SMSR on cancer cells by γ-scintigraphy with $^{111}$In-pentetreotide may be higher than by immunohistochemical techniques, since imaging was positive in 75% of breast cancer patients in two different
studies (126,129) but immunohistochemistry only demonstrated SMSR in approximately 17% of breast cancer biopsies (131).

![Diagram showing the primary structure of somatostatin, octreotide, and pentetreotide.](image)

**A. Somatostatin**

Ala-Gly-Cys-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Ser-Cys

**B. Octreotide**

D-Phe-Cys-Phe-D-Trp-Lys-Thr-Cys-Thr(ol)

**C. Pentetreotide**

\[ ^{111}\text{In-DTPA-D-Phe-Cys-Phe-D-Trp-Lys-Thr-Cys-Thr(ol)} \]

Fig. 1.5. Primary structure of somatostatin, octreotide and pentetreotide. The residues required for receptor binding are indicated in bold.

Since \(^{111}\text{In-pentetreotide}\) is internalized into the cytoplasm and translocated to the nucleus of cancer cells following binding to its receptor (132), it is also currently being evaluated for targeted Auger electron radiotherapy (see Chapter 4) of SMSR-positive malignancies (133-135). The inverse correlation between EGFR and SMSR expression observed on breast cancer cells could be useful to circumvent the problem of heterogeneity of EGFR expression by combining \(^{111}\text{In-hEGF}\) and \(^{111}\text{In-pentetreotide}\) for targeted Auger electron radiotherapy of breast cancer (discussed in Chapter 5). The potency of \(^{111}\text{In-pentetreotide}\) for targeted Auger electron radiotherapy of breast cancer may be limited however by the relatively low level of SMSR expression on the cancer cells. In cell binding assays conducted with \(^{111}\text{In-pentetreotide}\), only relatively low levels of SMSR (<3 X 10^4 SMSR/cell, see Chapter 5, section 5.3.2) were detected on MDA-468, S1 or MCF-7 breast cancer cells. Since ER expression is inversely correlated with EGFR expression, it may also be possible to use estradiol analogues radiolabelled with Auger electron emitting radionuclides such as \(^{125}\text{I}\) (136) in combination with \(^{111}\text{In-hEGF}\) for targeted radiotherapy in order to target a higher proportion of breast cancer cells in the tumour.
1.11 Hypothesis of the thesis

The hypothesis explored in the thesis is that the overexpression of the EGFR on human breast cancer cells relative to that exhibited by normal epithelial tissues can be exploited to selectively target highly specific radiopharmaceuticals to the cancer cells for the diagnostic imaging or radiotherapy of the disease.

1.12 Goals and objectives

The overall goal of the research was to determine if certain selected radiopharmaceuticals which specifically bind to the EGFR could potentially be useful as diagnostic imaging or radiotherapeutic agents for human breast cancer. The specific objectives were:

i) To determine the factors controlling the sensitivity for detection of human breast cancer by γ-scintigraphy using indium-111 (\(^{111}\text{In}\)) labelled hEGF.

ii) To compare the properties of hEGF and mAb 528 labelled with \(^{111}\text{In}\) for the \textit{in vivo} detection of human breast cancer xenografts hosted in immunocompromised mice by γ-scintigraphy.

iii) To evaluate the radiotoxicity of hEGF labelled with the Auger electron emitter, \(^{111}\text{In}\) against human breast cancer cells \textit{in vitro} and the radiotoxicity \textit{in vivo} against normal tissues which express moderate-high levels of the EGFR.

The methodology, results and discussion of the research investigations addressing these three objectives are described in Chapters 2, 3 and 4 of the thesis. The conclusions of the thesis and a discussion of future research direction are provided in Chapter 5.
In addition to the research described in this thesis, I was also a joint author on a manuscript resulting from a separate but related project: Remy S, Reilly RM, Sheldon K, and Gariepy J. A new radioligand for the epidermal growth factor receptor: \(^{111}\)In labeled human epidermal growth factor derivatized with a bifunctional metal-chelating peptide. Bioconj. Chem. 6: 683-90, 1995.
Chapter 2

FACTORS INFLUENCING THE SENSITIVITY OF TUMOUR IMAGING WITH A RECEPTOR-BINDING RADIOPHARMACEUTICAL

2.0 ABSTRACT

The overexpressed cell surface receptors on cancer cells are potential targets for the design of receptor-binding radiopharmaceuticals for tumour imaging. The sensitivity of these agents depends on the interaction in vivo of factors such as the level and heterogeneity of receptor expression, the proportion of targeted cells, the tumour/non-target (T/NT) ratio and attenuation by overlying normal tissue. The relative importance of a single factor or combination of factors is unknown. The objective of this investigation was to evaluate under controlled experimental conditions the effect of these factors on the sensitivity for imaging breast cancer with a new receptor-binding radiopharmaceutical: human epidermal growth factor labelled with $^{111}$In ($^{111}$In-DTPA-hEGF51).

Methods: MDA-468, S1 or MCF-7 breast cancer cells expressing 1.3 X $10^6$, 3.3 X $10^4$ and 1.5 X $10^4$ epidermal growth factor receptors (EGFR)/cell were targeted in vitro with $^{111}$In-DTPA-hEGF51. Phantoms were constructed containing an internal well to simulate a lesion, surrounded by an outer well to simulate normal tissue. The effect of the level of receptor expression was studied with phantoms containing targeted MDA-468, S1 or MCF-7 cells. The effect of the proportion of cells targeted was evaluated using phantoms containing mixed targeted/non-targeted MDA-468 cells. Receptor heterogeneity was studied using phantoms containing mixed MDA-468 and S1 cells. The T/NT ratio was evaluated by varying the concentration of radioactivity in the outer well and tissue attenuation was simulated by overlaying the phantoms with water. Phantoms were imaged using a gamma camera fitted with a medium energy collimator interfaced to a computer.

Results: The sensitivity for detection of a lesion was directly proportional to the level of receptor expression or to the proportion of cells targeted and inversely proportional to the level of receptor heterogeneity. A tumour/non-target (T/NT) ratio $\geq 2:1$ was required for detection. Under ideal conditions with a single factor varied, as few as 5 X $10^4$ to $10^5$ MDA-468 cells with a high level of EGFR expression or 2.5 X $10^5$ to $10^6$ S1 or MCF-7 cells with a low level of EGFR expression were detected. When the receptor heterogeneity, the proportion of targeted cells and tissue attenuation were varied in combination with a T/NT ratio of 3:1, the sensitivity for detection approached that observed clinically with receptor-binding radiopharmaceuticals ($10^7$ cells).

Conclusion:
The results suggest that combinations of only four factors can account for the relatively low sensitivity for tumour imaging observed clinically with receptor-binding radiopharmaceuticals and in particular strategies aimed at minimizing the effects of receptor heterogeneity, a low proportion of cells targeted and tissue attenuation would improve the detection of small lesions.
2.1 INTRODUCTION

The targeting of radiopharmaceuticals to certain cell surface proteins overexpressed on tumour cells has been investigated over the past decade as a strategy for the diagnostic imaging of cancer (137,138). Such targeted radiopharmaceuticals have included monoclonal antibodies (mAbs) which recognize tumour-associated antigens (139-144) as well as peptide growth factors which recognize growth factor receptors (145). Octreotide, an octapeptide analogue of the growth hormone somatostatin represents a prototypic peptide radiopharmaceutical which has been radiolabelled with $^{111}$In and used successfully to image somatostatin receptor-positive tumours, primarily neuroendocrine malignancies (125) but also small cell lung cancer (145) and breast cancer (126). Another promising peptide-based radiopharmaceutical for imaging breast cancer is epidermal growth factor (EGF), a 53 amino acid peptide ligand for the epidermal growth factor receptor (EGFR). As previously discussed in Chapter 1, overexpression of the EGFR has been reported in 30-60% of breast cancer biopsies with up to a 100-fold higher level of expression detected on cancer cells compared to normal epithelial tissues (86,87,90,146). The following chapter describes an investigation of the feasibility of imaging EGFR-overexpressing breast cancer lesions with a novel 51 amino acid recombinant analog of human EGF (hEGF51) labelled with $^{111}$In.

The clinical application of radiolabelled EGF for imaging breast cancer will ultimately depend on the sensitivity of the radiopharmaceutical for detecting small numbers of EGFR-overexpressing tumour cells. The ability to detect a malignant lesion by imaging depends on the properties of i) the radiopharmaceutical, ii) the lesion (and surrounding normal tissue) and iii) the imaging system. The physical characteristics of the radionuclide ($\gamma$-energy, abundance and physical half-life) as well as the pharmacokinetic characteristics of the radiopharmaceutical (elimination rate from the blood, extent of distribution into tissues, tumour and normal tissue uptake and metabolism) are all important factors controlling the success of detecting a lesion. The vascularity of the tumour, the level and heterogeneity of receptor expression by the tumour cells as well as the lesion size and its location are also important parameters. Properties of the imaging system which can affect tumour imaging include collimator
resolution, the efficiency of the detection system, and the display of the final image. The combination of these factors determines the overall sensitivity of detection of a lesion with the radiopharmaceutical and it is difficult to ascertain from an in vivo study, the influence of any single factor. However, through the use of phantoms (models) of lesions targeted by the radiopharmaceutical in an in vitro situation, it is possible to independently vary one factor while maintaining others constant. This approach allows a systematic evaluation of the influence of single factors or known combinations of factors on the ability to detect a lesion with the radiopharmaceutical. In the following study, the effects of several potentially important factors were evaluated on the ability to image breast cancer lesions with $^{111}$In-DTPA-hEGF51. The factors investigated were: i) the number of tumour cells in the lesion, ii) the level of EGFR expression, iii) the receptor heterogeneity, iv) the proportion of targeted cells, v) the tumour/non-target (T/NT) ratio and vi) tissue attenuation. The results suggest that the effects of combinations of these factors can explain the relatively low sensitivity for tumour imaging observed in cancer patients with receptor-binding radiopharmaceuticals and strategies aimed at minimizing these effects would improve the sensitivity for detection of small lesions.

2.2 MATERIALS AND METHODS

2.2.1 Radiopharmaceutical

Human recombinant epidermal growth factor (hEGF51) was a generous gift from Dr. D. Maratea, Creative Biomolecules (Hopkinton, MA). hEGF51 (5 mg/mL in 50 mM bicarbonate buffer pH 7.5 in 150 mM sodium chloride) was reacted with the bicyclic anhydride of diethylenetriaminepentaacetic acid (cDTPAA, Sigma, St. Louis, MO) at a molar ratio (cDTPAA:hEGF51) of 5:1 for 30 minutes. The DTPA-conjugated hEGF51 was purified from free DTPA by size-exclusion chromatography on a P-2 mini-column (BioRad). Purified DTPA-hEGF51 was radiolabelled with $^{111}$In acetate to a specific activity of approximately 3.7-7.4 MBq/µg (22,200-44,400 MBq/µmol). $^{111}$In acetate was prepared by mixing equal volumes of $^{111}$In chloride (>7,400 MBq/mL, Nordion, Kanata, ON) and 1 M acetate buffer pH 6. $^{111}$In-DTPA-hEGF51 was purified from free $^{111}$In on a
P-2 column, then analysed for radiochemical purity by silica gel instant thin layer chromatography (ITLC-SG, Gelman) in 100 mM sodium citrate pH 5. The final radiochemical purity was 95-98%.

2.2.2 Breast cancer cells

MDA-468 breast cancer cells and this cell line's S1 subclone were obtained from Dr. R. Buick at the Ontario Cancer Institute. MCF-7 breast cancer cells were obtained from Dr. A. Marks at the Banting and Best Department of Medical Research, University of Toronto. MDA-468 and S1 cells were cultured in L-15 medium (Sigma) supplemented with 10% fetal calf serum (FCS). The medium for S1 cells was further supplemented with 10^8 M EGF (Sigma) since these cells have a growth requirement for EGF. MCF-7 cells were cultured in Minimal Essential Medium (MEM, Sigma) supplemented with 10% FCS, non-essential amino acids and glutamine (Gibco).

2.2.3 Measurement of receptor expression in vitro on breast cancer cells

"^{111}In-DTPA-hEGF51 (0.25-80 ng) was incubated with 1.5 X 10^6 MDA-468 cells or 4-7.5 X 10^6 S1 or MCF-7 cells in 1 mL of 0.1% human serum albumin in 35 mm multiwell culture dishes at 37 °C for 30 minutes. The cells were then transferred to a centrifuge tube and centrifuged. The cell pellet was separated from the supernatant and counted in a γ-scintillation counter to determine bound (B) and free (F) radioactivity. Non-specific binding was determined by conducting the assay in the presence of 100 nM hEGF51. The affinity constant (K_a = 1/K_d) and the number of receptors/cell (B_max) were determined from a non-linear fitting of the binding data (147) using a one-site binding model:

\[
Y = \frac{B_{\text{max}} \cdot X}{K_d + X}
\]

where, \(Y\) is the concentration of the radiopharmaceutical specifically bound (nM) and \(X\) is the concentration of the radiopharmaceutical (nM) incubated with the cells.
2.2.4 Radiolabelling of breast cancer cells

Breast cancer cells (MDA-468, S1 or MCF-7) were recovered from a 75 cm² tissue culture flask by replacing the culture medium with 25 mL of 150 mM sodium chloride and gently scraping the growth surface of the flask with a cell scraper. The cell suspension was transferred to a 50 mL centrifuge tube and an aliquot counted in a hemocytometer. The cells were then centrifuged at 1000 rpm (600 x g) for 5 minutes, the supernatant removed and the cells resuspended in fresh 150 mM sodium chloride to a final concentration of 5 X 10⁷ cells/mL. An aliquot of the resuspended cells (1 mL, 5 X 10⁷ cells) was transferred to a 35 mm plastic culture dish. ¹¹¹In-DTPA-hEGF51 (5 μg, 8.6 X 10⁴ μmol) was added to the dish, representing an 8-fold, 370-fold or 690-fold molar excess for MDA-468, S1 or MCF-7 cells respectively. The mixture was incubated at 37 °C for 30 minutes, transferred to a centrifuge tube and centrifuged at 1000 rpm for 5 minutes. The supernatant was removed and the radiolabelled cells were resuspended in 150 mM sodium chloride to a final concentration of 2.5 X 10⁷ cells/mL.

2.2.5 Phantom design and imaging

Phantoms were constructed by inserting a central internal cylindrical well (7 mm diameter X 12 mm) into each of six 35 mm diameter X 15 mm wells contained in a polystyrene culture dish (Falcon) (Fig. 2.1). Radiolabelled tumour cells were dispensed into the central well to simulate a lesion targeted by the radiopharmaceutical, whereas the surrounding background radioactivity was altered by dispensing solutions of different concentrations of radioactivity into the surrounding outer wells. The effect of attenuation was evaluated by overlaying the phantoms with identical multiwell dishes containing water. The phantoms were imaged using a Siemens ZLC-3700 γ-camera fitted with a medium energy parallel hole collimator set 4 cm from the top surface of the phantom and interfaced to a GE Star 4000i computer. Images were acquired for 10 minutes using a 20% window centered over the 172 and 247 keV photopeaks of ¹¹¹In.
Fig. 2.1: Design of phantom used for evaluating factors affecting sensitivity of tumour imaging.
2.2.6 Quantitation of phantom images

The relationship between the count intensity recorded on the images and the sensitivity for detection of a lesion was determined by manually drawing a region of interest (ROI) around the central well with the highest count rate in a phantom series. The same sized ROI was then manually placed over the other wells in the series and the counts per minute (cpm)/pixel for the simulated lesion obtained.

2.2.7 Effect of number of tumour cells and receptor expression on tumour imaging

The effect of the number of tumour cells present in the lesion and the level of receptor expression by the cells for $^{111}$In-DTPA-hEGF51 on tumour imaging was investigated by dispensing radiolabelled MDA-468, S1 or MCF-7 breast cancer cells ($10^2$ to $1.5 \times 10^7$ cells) into a central well of the phantom. MDA-468 cells expressed EGF receptors at a 39-fold higher level than S1 cells and at an 87-fold higher level than MCF-7 cells (see section 2.3-Results and Discussion). The volume of each central well was adjusted to 300 $\mu$L with 150 mM sodium chloride. No radioactivity was dispensed into the surrounding outer wells.

2.2.8 Effect of the proportion of targeted cells on tumour imaging

The effect of the percentage of tumour cells targeted by the radiopharmaceutical on tumour imaging was analyzed by mixing radiolabelled MDA-468 cells with unlabelled MDA-468 cells in different proportions (0.1% to 100% radiolabelled cells) and depositing a mixture of $1.5 \times 10^7$ cells into a central well of the phantom. The volume of the central well was adjusted as described above and no radioactivity was placed into the outer wells.

2.2.9 Effect of receptor heterogeneity on tumour imaging

The importance of heterogeneity in receptor expression for $^{111}$In-DTPA-hEGF51 was studied by mixing radiolabelled MDA-468 and S1 cells in different proportions (0% to 90% S1 cells) and dispensing mixtures totalling $1.5 \times 10^7$ radiolabelled cells into a central well of the phantom. The volume of the central well was adjusted to 300 $\mu$L with
150 mM sodium chloride. The phantoms were then imaged in the absence of radioactivity in the outer wells.

2.2.10 Effect of the tumour/non-target (T/NT) ratio on tumour imaging

The effect of the T/NT ratio on tumour imaging was investigated by placing $10^6$ radiolabelled MDA-468 or S1 cells into a central well of the phantom, adjusting the volume as described above and then dispensing a solution of known concentration of $^{111}$In radioactivity into the surrounding outer well to achieve T/NT ratios ranging from 0.5:1 to 20:1.

2.2.11 Effect of tissue attenuation on tumour imaging

The importance of attenuation by overlying normal tissues on tumour imaging was studied by depositing $5 \times 10^4$ to $1.5 \times 10^7$ radiolabelled MDA-468 cells into a central well of the phantom and adjusting the volume of each well as previously stated. No radioactivity was dispensed into the surrounding outer wells. The phantom was then incrementally overlayed with up to five identical multi-well dishes containing 12 mL of water in each well (depth of water was 1.3 cm in each well) to simulate attenuation of up to 6.5 cm of overlying normal tissue. After each overlay, the phantom was imaged with the collimator set 4 cm above the top dish.

2.2.12 Effect of combinations of factors on tumour imaging

The combined effect of varying the receptor heterogeneity or the proportion of tumour cells targeted, and tissue attenuation on the sensitivity for detection of a lesion was evaluated for phantoms containing $5 \times 10^4$ to $1.5 \times 10^7$ breast cancer cells targeted in vitro with $^{111}$In-DTPA-hEGF51. In one experiment, all tumour cells were targeted with $^{111}$In-DTPA-hEGF51. The level of receptor heterogeneity was varied by increasing the proportion of S1 cells (from 0% to 90%) while tissue attenuation was altered by overlaying the phantoms with increasing depths of water (0 cm to 10.4 cm). In a second experiment, the proportion of S1 cells was maintained at 25% (receptor heterogeneity) while the proportion of targeted cells was decreased (from 90% to 1%) by combining
mixtures of radiolabelled MDA-468 and S1 cells with unlabelled cells. In both experiments, the T/NT ratio was set at 3:1 by dispensing a solution of known concentration of $^{111}$In radioactivity into the outer well. The phantoms were then imaged as previously described.

2.3 RESULTS AND DISCUSSION

The effect of several potentially important factors on the sensitivity for imaging breast cancer with a new receptor-binding radiopharmaceutical, human epidermal growth factor labelled with indium-111 ($^{111}$In-DTPA-hEGF51) was investigated under controlled experimental conditions using phantoms. The factors investigated included the level and heterogeneity of receptor expression by the tumour cells, the proportion of tumour cells targeted by the radiopharmaceutical, the tumour/non-target (T/NT) ratio and the attenuation of the signal by overlying normal tissues. The relative importance of each factor in determining the sensitivity for tumour imaging (i.e. the minimum number of cancer cells detectable) was investigated both individually and in combination. The phantom results were then compared to that reported previously for other receptor-binding radiopharmaceuticals such as monoclonal antibodies (mAbs) and octreotide in cancer patients. Clinical imaging studies with radiolabelled mAbs have suggested that only lesions $\geq 0.5$-$1$ cm in diameter (i.e. $\geq 1.5 \times 10^7$ to $1.2 \times 10^8$ cells) are detectable. For example, in melanoma patients imaged with $^{111}$In-ZME-018 mAb, no lesions $<1$ cm in diameter could be detected compared to 100% of lesions $>5$ cm in diameter (143). Similarly, the smallest lesion visualized with $^{111}$In-170H.82 mAb in ovarian cancer patients was 1 cm in diameter with smaller peritoneal seedlings ($<1$ cm) observed as diffuse uptake on the image, rather than as discrete lesions (148). Collier et al. (149) observed a 2-fold decrease in the detection rate for colorectal cancer by imaging with $^{111}$In-B72.3 mAb for lesions $\leq 2$ cm in diameter (33% detected) compared to those $>2$ cm in diameter (62% detected). A small colorectal cancer lesion with dimensions of $0.8$ cm $\times$ $0.6$ cm was detected in one patient with $^{111}$In-ZCE025 mAb (150). The phantom imaging results suggest that combinations of four factors investigated can account for the
relatively low sensitivity for tumour imaging observed with these agents and strategies aimed at minimizing these effects would improve the detection of small lesions.

2.3.1 Characterization of \(^{111}\)In-DTPA-hEGF51

The specific activity of \(^{111}\)In-DTPA-hEGF51 achieved experimentally in this study was 22,200-44,400 MBq/µmol using an average substitution level of 1.5 mols of DTPA chelator/mol of hEGF51. The maximum theoretical specific activity for \(^{111}\)In-DTPA-hEGF51 based on chelation of one indium atom per molecule of DTPA-hEGF51 is 173,900 MBq/µmol. Cell binding assays were carried out to determine the level of receptor expression for \(^{111}\)In-DTPA-hEGF51 on the three breast cancer cell lines utilized in the study: MDA-468, S1 and MCF-7. The level of receptor expression for the MDA-468, S1 and MCF-7 cells was 1.3 ± 0.7 \(\times\) 10^6, 3.3 ± 1.1 \(\times\) 10^4 and 1.5 ± 0.7 \(\times\) 10^4 receptors/cell respectively (Fig. 2.2). There was a significant difference (t-test, \(p<0.05\)) in the affinity of binding for MDA-468 and S1 cells (7.5 ± 3.8 \(\times\) 10^8 and 2.1 ± 0.9 \(\times\) 10^8 L/mol respectively). The affinity constant for MCF-7 cells (3.9 ± 1.2 \(\times\) 10^9 L/mol) was significantly higher than that for MDA-468 or MCF-7 cells.

2.3.2 Effect of the number of cells and receptor expression on tumour imaging

A quantitative analysis of the images of phantoms containing breast cancer cells targeted with \(^{111}\)In-DTPA-hEGF51 demonstrated a linear decrease in the count intensity as the number of cells was decreased from 1.5 \(\times\) 10^7 to 10^2 cells (Table 2.1). The upper range of the number of cells studied with the phantom represents the approximate number of tumour cells which would be contained in a hypothetical spherical lesion with a diameter of 0.5 cm, assuming a diameter of 20 µm and a volume of 4 \(\times\) 10^9 cm^3 for an individual breast cancer cell. The count intensity in a lesion containing 1.5 \(\times\) 10^7 cells was 15-20 fold greater for MDA-468 cells which exhibited a high level of receptor expression compared to S1 or MCF-7 cells. A count intensity of \(\geq\)2-3 cpm/pixel was required to visualize the lesion (Table 2.1 and Fig. 2.3). In the phantom studies, this value was achieved by targeting a minimum of 5 \(\times\) 10^4 MDA-468 cells, 2.5 \(\times\) 10^5 S1 cells or 10^6 MCF-7 cells. Approximately 3.7 kBq of \(^{111}\)In was contained in a lesion containing
Fig. 2.1. Representative binding curve for $^{111}$In-DTPA-hEGF51 to MDA-468 human breast cancer cells. Total binding: (O); non-specific binding: (●); specific binding: (▲). The affinity constant ($K_a$) and the number of receptors/cell ($B_{max}$) were determined from a non-linear fitting of the binding data using a one-site binding model as described in section 2.2.3. Points represent single determinations. In this particular experiment, the $K_a$ for $^{111}$In-DTPA-hEGF was $5.4 \times 10^8$ L/mol and the number of receptors/cell ($B_{max}$) was $6.4 \times 10^5$. 
Fig. 2.3: Images of phantoms containing different numbers of MDA-468, S1 or MCF-7 cells targeted in vitro with $^{111}$In-DTPA-hEGF51. For MDA-468 cells, the number of cells (top left to bottom right) was $1.5 \times 10^7$, $10^7$, $5 \times 10^6$, $10^6$, $10^5$, and $5 \times 10^4$. For S1 and MCF-7 cells, the number of cells (top left to bottom right) was $1.5 \times 10^7$, $10^7$, $5 \times 10^6$, $10^6$, $2.5 \times 10^5$ and $10^5$. 
these numbers of targeted cells. Thus, under ideal conditions with all cells overexpressing the receptor and targeted to receptor saturation, and in the absence of surrounding background radioactivity or attenuation by overlying normal tissue, as few as 5 X 10⁴ breast cancer cells could be detected with ¹¹¹In-DTPA-hEGF51. This is a level of sensitivity approximately 300-fold higher than that reported clinically for other receptor-binding radiopharmaceuticals such as mAbs (143,149,150) or octreotide (151) and reflects the existence of non-ideal conditions in vivo. Such conditions include the heterogeneity in receptor expression by the tumour cells, a low proportion of tumour cells targeted by the radiopharmaceutical as well as the presence of background radioactivity surrounding the lesion and attenuation by overlying normal tissues.

2.3.3 Effect of receptor heterogeneity on tumour imaging

The level of receptor heterogeneity in a lesion is usually not known at the time of imaging. Nevertheless, heterogeneity in expression has been detected in in vitro studies for both the tumour-associated glycoprotein antigen, TAG-72 in colorectal cancer (152) and also for somatostatin receptors in breast cancer (128). Using phantoms, the effect of different levels of receptor heterogeneity on the sensitivity for imaging a breast cancer lesion with ¹¹¹In-DTPA-hEGF51 was evaluated. Receptor heterogeneity was simulated by constructing lesions composed of mixtures of MDA-468 breast cancer cells which expressed a high level of receptors and S1 breast cancer cells which exhibited a low level of receptor expression, targeted in vitro with ¹¹¹In-DTPA-hEGF51. The total number of cancer cells in the lesion was 1.5 X 10⁷. A linear decrease in the count intensity was observed as the proportion of S1 cells was increased from 0% to 90% (Table 2.2). There was a 4-fold lower count intensity for a lesion with only 10% MDA-468 cells, compared to a lesion containing 100% MDA-468 cells. The count intensity on the images of all lesions studied was ≥50 cpm/pixel and all lesions were detected. A lesion containing 1.5 X 10⁷ breast cancer cells targeted with ¹¹¹In-DTPA-hEGF51 with only 10% MDA-468 cells contained approximately 111 kBq of ¹¹¹In. As expected, receptor heterogeneity results in a lower number of binding sites for the radiopharmaceutical and consequently to a decrease in the amount of radioactivity which is targeted to the lesion and a loss of
### TABLE 2.1

Effect of the Number of Cells and Level of Receptor Expression on the Sensitivity for Detection of Breast Cancer Lesions

<table>
<thead>
<tr>
<th>Cell Line (^a):</th>
<th>MDA-468</th>
<th>S1</th>
<th>MCF-7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of Targeted Cells (^b)</td>
<td>Count Intensity in Lesion</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(X 10(^7))</td>
<td>(cpm/pixel)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.5</td>
<td>539</td>
<td>26</td>
<td>35</td>
</tr>
<tr>
<td>1.0</td>
<td>356</td>
<td>18</td>
<td>24</td>
</tr>
<tr>
<td>0.5</td>
<td>173</td>
<td>10</td>
<td>11</td>
</tr>
<tr>
<td>0.1</td>
<td>35</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>0.025</td>
<td>not determined</td>
<td>2</td>
<td>not determined</td>
</tr>
<tr>
<td>0.01</td>
<td>4</td>
<td>not visualized</td>
<td>not visualized</td>
</tr>
<tr>
<td>0.005</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.001</td>
<td>not visualized</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) MDA-468, S1 or MCF-7 cells express 1.3 ± 0.7 X 10\(^6\), 2.9 ± 1.2 X10\(^4\) and 1.5 ± 0.7 X 10\(^4\) receptors/cell respectively. \(^b\)Cells were targeted in vitro with \(^{111}\)In-DTPA-hEGF51. Values represent single determinations.
TABLE 2.2

Effect of Receptor Heterogeneity on the Sensitivity for Detection of a Breast Cancer Lesion $^a$

<table>
<thead>
<tr>
<th>Proportion of S1 Cells $^b$ (%)</th>
<th>Count Intensity in Lesion (cpm/pixel)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>216</td>
</tr>
<tr>
<td>10</td>
<td>196</td>
</tr>
<tr>
<td>25</td>
<td>174</td>
</tr>
<tr>
<td>50</td>
<td>117</td>
</tr>
<tr>
<td>75</td>
<td>72</td>
</tr>
<tr>
<td>90</td>
<td>50 $^c$</td>
</tr>
</tbody>
</table>

$^a$ Lesion contained 1.5 X 10$^7$ cells. $^b$Receptor heterogeneity was simulated by increasing the proportion of S1 cells (low level of receptor expression) in a lesion containing a mixture of S1 cells and MDA-468 cells (high level of receptor expression).

$^c$ All lesions were detected.

Values represent single determinations.
Nevertheless the phantom studies demonstrated that a lesion could still be detected with only 10% of the cells exhibiting a high level of receptor expression. Receptor heterogeneity therefore did not appear to be the single controlling factor for visualization of a lesion with 111In-DTPA-hEGF51, at least for lesions containing >1.5 X 10^7 cells (i.e. the equivalent to a clinically observed lesion >0.5 cm in diameter). Carrasquillo et al. (153) also showed in cancer patients that receptor expression is not the only factor controlling the detection of a lesion. 111In-9.2.27 mAb failed to image lesions in two melanoma patients despite expression of the antigen by the tumour. Similar results to those observed in the phantom studies have also been observed by Collier et al. (149) in colorectal cancer patients and by Gallup et al. (140) in ovarian cancer patients imaged with 111In-B72.3 mAb, which recognizes the panadenocarcinoma antigen TAG-72. Tumours were successfully imaged in 47% of colon cancer patients and 39% of ovarian cancer patients with 111In-B72.3 despite the fact that the tumours contained <5% TAG-72 (+) cells. However, a direct relationship was observed between the level of antigen expression and the detection rate, with 62% of images positive if the tumour expressed 5-39% TAG-72 (+) cells and 79% positive with 40-80% TAG-72 (+) cells.

2.3.4 Effect of the proportion of targeted cells on tumour imaging

The delivery of the radiopharmaceutical to tumour cells is a critical factor which can affect the ability to detect a lesion by imaging. The rate of elimination of the radiopharmaceutical from the blood, the rate of diffusion across the vascular endothelium into the tissues, the vascular supply of the tumour, the extent of sequestration by non-target organs and tissues and metabolic cleavage of the radiolabel from the ligand represent important factors controlling the delivery of a radiopharmaceutical to the lesion. Pharmacokinetic factors affecting the targeting of the radiopharmaceutical to a breast cancer lesion expressing the EGFR were investigated and are discussed further in Chapter 3. The proportion of tumour cells in a lesion which must be targeted for effective tumour imaging is currently not known. In the phantom imaging studies described in this chapter, the effect of different levels of cell targeting was investigated by constructing lesions.
composed of radiolabelled (targeted) and unlabelled (non-targeted) MDA-468 cells. The total number of cancer cells in the "model" lesion was maintained at $1.5 \times 10^7$. There was a linear decrease in the count intensity in the lesion as the proportion of targeted cells was decreased from 100% to 0.1% (Table 2.3). Visualization of a lesion required a minimum count intensity of 3 cpm/pixel which corresponded to $\geq 1\%$ of the cells targeted by $^{111}$In-DTPA-hEGF51. Approximately 11 kBq of $^{111}$In was contained in a lesion containing $1.5 \times 10^7$ cells with 1% of the cells targeted. Cuartero-Plaza et al. (154) recently demonstrated that squamous cell lung cancer could be successfully imaged in patients with 185-370 MBq (0.3-3 mg) of $^{131}$I-EGF. Assuming a similar injected dose (185-370 MBq) of $^{111}$In-DTPA-hEGF51 in breast cancer patients, targeting of 11kBq of $^{111}$In to a 0.5 cm diameter lesion would require a tumour uptake of 0.03-0.1 percent injected dose per gram (% i.d./g). This level of uptake is comparable to the tumour uptake of $^{111}$In-octreotide in patients with neuroendocrine malignancies, reported to range from 0.01-0.2 % i.d./g (125). Tumour uptake of $^{111}$In-mAbs typically ranges from 0.001-0.014 % i.d./g (153,155).

2.3.5 Effect of the tumour/background (T/NT) ratio on tumour imaging

The visualization of a lesion on the image depends on the contrast between the lesion and surrounding background (T/NT ratio). Background radioactivity can be due to binding of the radiopharmaceutical to surrounding normal tissues (cross-reactivity) or to circulating radiopharmaceutical in the blood. The phantom studies demonstrated that a T/NT ratio of 2:1 was sufficient for imaging even very small breast cancer lesions containing $10^6$ cells with $^{111}$In-DTPA-hEGF51 (Table 2.4). T/NT ratios of 5:1 to 20:1 improved the contrast and the quality of the image but were not absolutely required for detection. Interestingly, the T/NT ratio measured by ROI analysis of the images was less than the true T/NT ratio for ratios $>2:1$ (Table 2.4). The lower observed values may reflect the detection of scattered photons in the background area which originated in the lesion, resulting in an increase in the background count intensity and consequently, a decrease in the T/NT ratio (partial volume averaging effect). A decrease in contrast could also result from an increase in the thickness of the background region due to the two-
TABLE 2.3
Effect of the Proportion of Targeted Cells on the Sensitivity for Detection of a Breast Cancer Lesion

<table>
<thead>
<tr>
<th>Proportion of Targeted Cells (%)</th>
<th>Count Intensity in Lesion (cpm/pixel)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>213</td>
</tr>
<tr>
<td>50</td>
<td>104</td>
</tr>
<tr>
<td>25</td>
<td>52</td>
</tr>
<tr>
<td>10</td>
<td>23</td>
</tr>
<tr>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>0.1</td>
<td>not visualized</td>
</tr>
</tbody>
</table>

a Lesion contained 1.5 X 10^7 cells. b Lesion contained a mixture of MDA-468 cells targeted in vitro with ^111^In-DTPA-hEGF51 and non-targeted cells.

Values represent single determinations.
dimensional representation of the counts in the region. The thickness of the background region for all of the phantom studies was constant (0.5 cm). Lakshmanan et al. (156) using phantoms of brain lesions imaged with $^{99m}$Tc, showed that an "image contrast" of $\geq 1.3$ was required to visualize a small lesion (diameter of 0.9 cm). An image contrast value of 1.3 as defined by Lakshmanan et al. (156) corresponds to a T/NT ratio of 2.3:1. However, in a theoretical treatment of issues effecting tumour imaging, Rockoff et al. (157) have suggested that higher T/NT ratios ($\geq 5:1$) would be required to detect small lesions (0.6-1.2 cm in diameter), lesions deep within the patient (depth $\geq 5$ cm) or lesions with low count intensities.

T/NT ratios for receptor-binding radiopharmaceuticals in cancer patients usually exceed the minimum ratio of 2:1 required for detection of a lesion determined in our phantom studies. Murray et al. (143) observed T/NT ratios of 7:1 to 25:1 in patients with melanoma imaged with $^{111}$In-ZME018 mAb and Griffin et al. (155) observed T/NT ratios of 7-14:1 in patients with breast cancer imaged with $^{111}$In-260F9 mAb. Krenning et al. (125) noted that endocrine tumours could not be visualized with $^{111}$In-octreotide at 4 hours post-injection due to low T/NT ratios (1:1) but could be seen at 24 hours when T/NT ratios were higher ($>5:1$) due to excretion of radioactivity from the blood. Cuartero-Plaza et al. (154) successfully imaged squamous cell lung carcinoma lesions in patients with $^{131}$I-EGF at tumour/normal lung ratios of 1.2-1.7:1 at 24 hours post-injection. The pharmacokinetic properties of the radiopharmaceutical influence the T/NT ratio. High T/NT ratios would be associated with radiopharmaceuticals which diffuse readily across the vascular endothelium, exhibit high tumour penetration and retention and are rapidly eliminated from the blood and normal tissues. The pharmacokinetic characteristics of $^{111}$In-DTPA-hEGF51 in humans are not known, but previous animal (158,159) and human (154) studies with radioiodinated EGF have shown its rapid elimination from the blood into the urine. This rate of clearance should yield high T/NT ratios ($\geq 2:1$) for breast cancer lesions outside of the liver, in the skeleton, brain, lungs, ovaries and adrenal glands (2). Hepatic metastases however would likely exhibit relatively low T/NT ratios ($<2:1$) due to uptake of $^{111}$In-DTPA-hEGF51 by normal hepatocytes which have been observed to express moderate-high levels of the receptor (58).
TABLE 2.4

Effect of the Tumour/Non-Target (T/NT) Ratio on the Sensitivity for Detection of a Breast Cancer Lesion $^a$

<table>
<thead>
<tr>
<th>T/NT Ratio $^b$</th>
<th>Count Intensity (cpm/pixel)</th>
<th>T/NT Ratio Observed on Image</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lesion</td>
<td>Background</td>
</tr>
<tr>
<td>20.0</td>
<td>55</td>
<td>7</td>
</tr>
<tr>
<td>10.0</td>
<td>58</td>
<td>17</td>
</tr>
<tr>
<td>5.0</td>
<td>67</td>
<td>21</td>
</tr>
<tr>
<td>2.0</td>
<td>86</td>
<td>41</td>
</tr>
<tr>
<td>1.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^a$ Lesion contained $10^6$ cells. $^b$ T/NT ratio was varied by decreasing the concentration of $^{111}$In radioactivity surrounding a lesion containing MDA-468 breast cancer cells targeted with $^{111}$In-DTPA-hEGF51.

Values represent single determinations.
2.3.6 Effect of tissue attenuation on tumour imaging

Although a receptor-binding radiopharmaceutical may exhibit optimal tumour targeting properties, attenuation of the signal by overlying normal tissue can decrease the sensitivity for imaging a lesion. By overlaying phantoms of MDA-468 breast cancer lesions targeted in vitro with $^{111}$In-DTPA-hEGF51 with water, the effect of different amounts of tissue attenuation on tumour imaging was evaluated. Water was used as the attenuating substance due to its similar density to soft tissue. A 10-fold decrease in sensitivity for detection of a lesion (Table 2.5) was observed as the amount of attenuation was increased from 0 cm ($10^5$ cells detected) to 6.5 cm ($10^6$ cells detected). A minimum count intensity of 5-6 cpm/pixel was required for detection of a lesion. These results suggest that small superficial lesions would be readily detected by imaging with $^{111}$In-DTPA-hEGF51 but more deep-seated lesions would need to be up to 10 times larger to be detected.

2.3.7 Effect of combinations of factors on tumour imaging

In cancer patients, the factors investigated individually in our phantom studies act in combination to determine the overall sensitivity for tumour imaging with $^{111}$In-DTPA-hEGF51. Therefore two scenarios were investigated where factors were varied in combination. In the first scenario, the receptor heterogeneity and tissue attenuation were varied while maintaining the proportion of targeted cells at 100% and in the second scenario, the proportion of targeted cells and tissue attenuation were varied while maintaining the receptor heterogeneity at 25%. Certain factors such as the T/NT ratio and the proportion of targeted cells (first scenario) or receptor heterogeneity (second scenario) were maintained constant to reduce the experimental complexity of the phantoms. The T/NT ratio was set at 3:1, just slightly higher than the minimum T/NT ratio of 2:1 determined previously, to ensure that this factor would not confound tumour detection while varying the other factors. As previously described, T/NT ratios of $\geq3:1$ have been reported in cancer patients imaged with other receptor binding agents (125,143,155). The heterogeneity for EGFR expression in breast cancer lesions is not currently known but in the first scenario, it was varied over a wide range (0 to 90%) to include most
<table>
<thead>
<tr>
<th>Depth of Water $^a$ (cm):</th>
<th>0</th>
<th>1.3</th>
<th>2.6</th>
<th>3.9</th>
<th>5.2</th>
<th>6.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of Targeted Cells$^b$ (X 10$^7$)</td>
<td>1.5</td>
<td>165</td>
<td>166</td>
<td>144</td>
<td>122</td>
<td>102</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>128</td>
<td>118</td>
<td>100</td>
<td>84</td>
<td>71</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>67</td>
<td>57</td>
<td>50</td>
<td>41</td>
<td>34</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>17</td>
<td>16</td>
<td>15</td>
<td>13</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>0.01</td>
<td>5</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>not visualized</td>
</tr>
<tr>
<td></td>
<td>0.005</td>
<td>not visualized</td>
<td>not visualized</td>
<td>not visualized</td>
<td>not visualized</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ Tissue attenuation was simulated by overlaying phantoms with water. $^b$ MDA-468 cells were targeted in vitro with $^{111}$In-DTPA-hEGF51.

Values represent single determinations.
clinically relevant levels of receptor heterogeneity which may occur. In the second scenario, the receptor heterogeneity was maintained constant at a moderate level (25%) but the proportion of targeted cells was then varied over a wide range (1 to 90%) to include most clinically relevant levels of tumour targeting. In the case of $^{111}$In-DTPA-hEGF51 in breast cancer patients it is anticipated that approximately 1-2% of breast cancer cells in a lesion would be targeted, assuming an injected dose of 185 MBq of $^{111}$In-DTPA-hEGF51 (specific activity 22,200-44,400 MBq/μmol), a tumour uptake of 0.1% i.d./g and a receptor level of $10^6$ EGFR/cell. In both combined phantoms, the tissue attenuation was varied over a sufficiently wide range (up to 10.4 cm) to include both superficial and deep seated lesions in an average sized patient.

These studies showed that the sensitivity for tumour imaging was decreased more than 100-fold if receptor heterogeneity was high (i.e. <10% of cells exhibiting a high level of receptor expression, Table 2.6) or the proportion of targeted cells was very low (i.e. 1% targeted cells, Table 2.7) and the lesion was also attenuated by a moderate amount of overlying tissue (6.5-10.4 cm). In fact, the largest lesion evaluated using the phantom (1.5 X $10^7$ breast cancer cells) could not be detected if only 1% of cells were targeted by the radiopharmaceutical and the lesion was attenuated by 10.4 cm of simulated overlying tissue (Table 2.7). The count intensity in the lesions decreased exponentially as the percent heterogeneity was increased (Fig. 2.4) or the percent targeting was decreased (Fig. 5) while simultaneously increasing the tissue attenuation.

Although the phantom studies demonstrated that a minimum of $10^6$ cells could be detected under non-ideal conditions when a single factor was varied (i.e. low receptor expression or high tissue attenuation), combinations of the factors investigated further reduced the sensitivity to $\geq 10^7$ cells. Interestingly, this level of sensitivity ($10^7$ cells) is similar to the sensitivity previously reported for imaging lesions in cancer patients with other receptor-binding radiopharmaceuticals (i.e. 1.5 X $10^7$ cells contained in a 0.5 cm diameter lesion). The phantom study results therefore suggest that combinations of factors such as receptor heterogeneity, the proportion of tumour cells targeted and attenuation by overlying tissue can recreate the relatively low sensitivity observed with
TABLE 2.6
Effect of Receptor Heterogeneity Combined with Tissue Attenuation on the Sensitivity for Detection of Breast Cancer Lesions

<table>
<thead>
<tr>
<th>Depth of Water $^b$ (cm):</th>
<th>0</th>
<th>3.9</th>
<th>6.5</th>
<th>10.4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Minimum Sized Lesion Detected $^c$ (no. of cells X 10$^7$)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Proportion of S1 Cells $^a$ (%)</td>
<td>0</td>
<td>0.01</td>
<td>0.01</td>
<td>0.1</td>
</tr>
<tr>
<td>0</td>
<td>0.01</td>
<td>0.01</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>25</td>
<td>0.01</td>
<td>0.01</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>50</td>
<td>0.01</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>90</td>
<td>0.1</td>
<td>0.1</td>
<td>0.5</td>
<td>1.0</td>
</tr>
</tbody>
</table>

$^a$ Receptor heterogeneity was simulated by increasing the proportion of S1 cells (low level of receptor expression) in a lesion containing a mixture of S1 cells and MDA-468 cells (high level of receptor expression). The proportion of targeted cells was 100%. $^b$ Tissue attenuation was simulated by overlaying phantoms with water. $^c$ Cells were targeted in vitro with $^{111}$In-DTPA-hEGF51.

Values represent single determinations.
### TABLE 2.7
Effect of Proportion of Cells Targeted Combined with Tissue Attenuation on the Sensitivity of Detection of Breast Cancer Lesions

<table>
<thead>
<tr>
<th>Depth of Water $^b$ (cm):</th>
<th>0</th>
<th>3.9</th>
<th>6.5</th>
<th>10.4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Minimum Sized Lesion Detected $^c$ (no. of cells X 10$^7$)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Proportion of Targeted Cells $^a$ (%)</th>
<th>90</th>
<th>50</th>
<th>25</th>
<th>10</th>
<th>1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.01</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td>0.01</td>
<td>0.1</td>
<td>0.1</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>0.01</td>
<td>0.1</td>
<td>0.5</td>
<td>1.0</td>
<td>not visualized</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(&gt;1.5)</td>
</tr>
</tbody>
</table>

$a$ The proportion of targeted cells was varied by mixing targeted and non-targeted cells. Receptor heterogeneity was set at 25\% S1 cells. $b$ Tissue attenuation was simulated by overlaying phantoms with water. $c$ Cells were targeted $in vitro$ with $^{111}$In-hEGF51.

Values represent single determinations.
Fig. 2.4: Relationship between count intensity in a breast cancer lesion phantom containing $1.5 \times 10^7$ cells and the combination of receptor heterogeneity and tissue attenuation. Receptor heterogeneity was simulated by mixing MDA-MB-468 cells (high level of EGFR expression) with S1 cells (low level of EGFR expression). Symbols (○, •, □, ◆) indicate percentage of S1 cells. Tissue attenuation was simulated by overlaying phantom with different depths of water. Points represent single determinations.
Fig. 2.5: Relationship between count intensity in a breast cancer lesion phantom containing $1.5 \times 10^7$ cells and the combination of percent targeting and tissue attenuation. Percent targeting was varied by mixing radiolabelled (targeted) MDA-MB-468 cells with unlabelled (non-targeted) cells. Symbols ($\bigcirc$, $\bullet$, $\square$, $\blacklozenge$, $\triangle$) indicate percentage of targeted cells. Tissue attenuation was simulated by overlaying phantom with different depths of water. Points represent single determinations.
these agents *in vivo*. The effect of receptor heterogeneity could potentially be minimized by using a combination ("cocktail") of two different receptor-binding radiopharmaceuticals, each recognizing a unique receptor on the surface of the cancer cells. The proportion of tumour cells targeted by the radiopharmaceutical could be improved by using low molecular weight targeting agents such as peptides or single chain Fv fragments of mAbs, which exhibit improved tumour penetration (160). The effects of tissue attenuation could possibly be minimized by using tomographic imaging techniques or by using recently developed attenuation correction software. This software (161) has been utilized successfully to enhance the visualization of myocardial perfusion defects with $^{201}$Tl which are attenuated by overlying normal tissues.

2.4 CONCLUSION

Using phantoms of breast cancer lesions targeted by $^{111}$In-DTPA-hEGF51, several important factors which could affect the sensitivity of tumour imaging were evaluated under controlled experimental conditions. These factors included the level and heterogeneity of receptor (EGFR) expression, the proportion of tumour cells targeted by the radiopharmaceutical, the tumour/non-target ratio and attenuation by overlying tissues. The effects of each of these factors was evaluated individually and also in combination. The sensitivity for detection of a lesion ranged from $5 \times 10^4$ cells to $10^6$ cells when an individual factor was varied. However, when these factors were varied in combination, the sensitivity decreased to $\geq 10^7$ cells. This level of sensitivity is similar to the sensitivity observed clinically with other receptor-binding radiopharmaceuticals ($\geq 1.5 \times 10^7$ cells) suggesting that combinations of these factors, particularly receptor heterogeneity and the proportion of tumour cells targeted combined with tissue attenuation, may be responsible for the relatively low sensitivity observed with these agents.
Chapter 3

A COMPARISON OF EPIDERMAL GROWTH FACTOR AND MONOCLONAL ANTIBODY 528 LABELLED WITH INDIUM-111 FOR IMAGING HUMAN BREAST CANCER
3.0 ABSTRACT

Overexpression of the epidermal growth factor receptor (EGFR) occurs in about 30-60% of human breast cancers and is inversely correlated with estrogen receptor (ER) expression and directly correlated with a poor prognosis. The effectiveness of new drugs targeting the overexpression of the EGFR in breast cancer will depend on the ability to detect disseminated EGFR-positive lesions. One approach is gamma scintigraphy using highly specific radiopharmaceuticals. The objective of this investigation was to assess the properties of two receptor-binding radiopharmaceuticals, namely human epidermal growth factor (hEGF) and anti-EGFR monoclonal antibody (mAb) 528 labelled with indium-111 ($^{111}\text{In}$) for the detection of EGFR-positive breast cancer. 

**Methods:** hEGF and mAb 528 were derivatized with the bicyclic anhydride of diethylenetriaminepentaacetic acid (DTPA) and radiolabelled with $^{111}\text{In}$. Receptor binding assays were conducted against the human breast cancer cell line, MDA-468 (1.3 X $10^6$ EGFR/cell). Biodistribution, pharmacokinetic and tumour imaging studies were conducted following the i.v. injection of the radiopharmaceuticals in athymic mice bearing s.c. MCF-7, MDA-231 or MDA-468 human breast cancer xenografts or in scid mice implanted with a skeletal metastasis from a patient with advanced disease (JW-97 cells). MCF-7, MDA-231, JW-97 and MDA-468 cells exhibited 1.5 X $10^4$, 1.3 X $10^5$, 2.7 X $10^4$ and 1.3 X $10^6$ EGFR/cell respectively. 

**Results:** The two radiopharmaceuticals exhibited similar receptor binding properties. The affinity constant (Ka) for $^{111}\text{In}$-DTPA-hEGF was 7.5 X $10^8$ L/mol and for $^{111}\text{In}$-DTPA-mAb 528 was 1.2 X $10^8$ L/mol. $^{111}\text{In}$-DTPA-hEGF was gradually taken up by the MDA-468 xenografts reaching a maximum of 2.2 percent injected dose/g (% i.d./g) at 72 hours post injection (p.i.). Maximal uptake of $^{111}\text{In}$-DTPA-mAb 528 occurred at 24 hours p.i. and was almost 10-fold higher (21.5 % i.d./g) than that observed for $^{111}\text{In}$-DTPA-hEGF. The radiopharmaceuticals also accumulated in the liver and kidneys. Tumour/normal tissue ratios (except for the blood) were significantly higher at 72 hours p.i. for $^{111}\text{In}$-DTPA-mAb 528 (range 1.3:1 to 16:1) than for $^{111}\text{In}$-DTPA-hEGF (range 1.4:1 to 4:1). $^{111}\text{In}$-DTPA-hEGF was rapidly cleared from the blood with an elimination half-life ($t_{1/2}$ $\beta$) of 10.2 hours, whereas $^{111}\text{In}$-DTPA-mAb 528 was cleared more slowly ($t_{1/2}$ $\beta$ = 29.5 hours). The MDA-468 tumours were detected by gamma
scintigraphy using either radiopharmaceutical. Tumour masses were more discernable in images derived from the tissue localization of $^{111}$In-DTPA-mAb 528. The greater tumour uptake observed with $^{111}$In-DTPA-mAb 528 is probably due to its longer residence time in the blood which allowed a greater period of time for diffusion into the tissues and binding to the breast cancer cells. A direct quantitative relationship was not observed between EGFR expression on the different breast cancer cell lines measured in vitro and tumour uptake in vivo. However, in all cases the tumour uptake of $^{111}$In-DTPA-mAb 528 greatly exceeded that of $^{111}$In-DTPA-hEGF. Conclusion: The results suggest that the tumour uptake in vivo of receptor-binding radiopharmaceuticals is controlled to a greater extent by their pharmacokinetic properties than by the level of receptor expression on the cancer cells.
3.1 INTRODUCTION

Patients with disseminated, hormone-resistant breast cancer are currently candidates for systemic chemotherapy. As discussed in Chapter 1 (section 1.8), new drugs are currently under development which would specifically target the overexpression of the EGFR commonly observed in such malignancies. These drugs include mAbs which block the binding of epidermal growth factor (EGF) to its receptor (113), tyrosine kinase inhibitors (tyrphostins) which can interfere with the intracellular signaling pathways (120), and EGF-conjugated toxins (oncotoxins) which specifically deliver highly potent inhibitors of protein synthesis into the cytoplasm of the cancer cells (122). A further strategy may be to develop novel radiotherapeutic agents which could be used to deliver high doses of radiation specifically to EGFR-overexpressing tumours (162). The effectiveness of these new drugs will depend on the ability to detect and characterize EGFR-positive metastatic lesions throughout the body. ER status is commonly measured in biopsies of primary breast cancer lesions at the time of staging in order to select patients for hormonal therapy. EGFR expression in metastatic disease could possibly be inferred from the inverse correlation between ER and EGFR expression on breast cancer cells. However, this approach may be limited by potential differences in EGFR/ER positivity between the primary tumour and metastases, the heterogeneity in receptor expression by the tumour cells, temporal changes in ER/EGFR expression which can occur as a result of treatment (163) and the inability to directly evaluate EGFR expression in individual metastatic lesions. It would be helpful to devise a non-invasive diagnostic imaging procedure to survey the whole body of a patient to detect EGFR-overexpressing breast cancer and to select patients for novel anti-EGFR therapies.

Gamma scintigraphy using highly specific radiopharmaceuticals, such as radiolabelled mAbs against tumour-associated antigens (164) or radiolabelled growth factor receptor ligands (125) is one promising approach for the diagnostic imaging of cancer. Octreotide, an 8-amino acid analog of the inhibitory growth factor somatostatin, labelled with $^{111}\text{In}$ is a prototypic peptide radiopharmaceutical successfully utilized to image somatostatin receptor positive tumours in cancer patients (125). Epidermal growth factor labelled with $^{111}\text{In}$ ($^{111}\text{In}$-EGF) would represent a novel candidate peptide
radiopharmaceutical for imaging EGFR-positive human breast cancer. In Chapter 2, studies using phantoms (models) of breast cancer lesions targeted in vitro with $^{111}$In-EGF, demonstrated that targeting the overexpression of the EGFR on breast cancer cells for tumour imaging is potentially a very sensitive technique, able to detect as few as 50,000 breast cancer cells under ideal conditions. Under the non-ideal conditions which probably exist in vivo, such as a low proportion of tumour cells targeted by the radiopharmaceutical (i.e. <1% targeting), high levels of receptor heterogeneity (i.e. <10% of cells overexpressing the EGFR) and radioisotope attenuation by overlying normal tissues, the sensitivity of detection decreased to approximately $10^7$ cancer cells. Nevertheless, targeting of EGFR overexpression using specific radiopharmaceuticals is a promising approach for the diagnostic imaging of advanced breast cancer which could potentially detect small numbers of disseminated cancer cells at distant sites in the body.

It has been proposed (165) that peptide-based radiopharmaceuticals such as radiolabelled growth factors may be more effective for tumour imaging than radiolabelled monoclonal antibodies (mAbs) due to their different pharmacokinetic properties, particularly a more rapid elimination from the blood which produces higher tumour/blood (T/B) ratios at early time points. The objective of the study described in this chapter was therefore to directly compare $^{111}$In-EGF, a novel peptide radiopharmaceutical, with anti-EGFR mAb 528 (115) labelled with $^{111}$In for imaging human breast cancer. The results show that in certain situations, radiolabelled mAbs may be more effective targeting vehicles for receptor imaging of tumours than radiolabelled peptide growth factors, since they exhibit higher tumour uptake at only moderately lower T/B ratios.

### 3.2 MATERIALS AND METHODS

#### 3.2.1 Breast cancer cells

MDA-231 human breast cancer cells were obtained from ATCC (Rockville, MD) and were cultured in L-15 medium (Sigma, St. Louis, MO) supplemented with 10% FCS. MDA-468, S1 and MCF-7 breast cancer cells were obtained and cultured as previously described in Chapter 2 (section 2.2.2). JW-97 human breast cancer cells were obtained by
trypsinization of a skeletal metastasis from a patient with advanced disease which was passaged in scid mice. JW-97 cells were cultured in RPMI 1640 medium (Sigma, St. Louis, MO) supplemented with 10% FCS.

3.2.2 Radiopharmaceuticals

3.2.2.1 Radiolabelling of epidermal growth factor

hEGF (Upstate Biotechnology, Lake Placid, NY) was derivatized with DTPA using the bicyclic anhydride of DTPA (Sigma, St. Louis, MO) and radiolabelled with $^{111}$In to a specific activity of 3.7-7.4 MBq/μg (22,200-44,400 MBq/μmol) as previously described in Chapter 2 (section 2.2.1). The radiochemical purity of $^{111}$In-DTPA-hEGF by ITLC-SG (Gelman, Ann Arbor, MI) in 100 mM sodium citrate pH 5 was routinely between 95-98%.

hEGF was radioiodinated to a specific activity of 1.5-2.2 MBq/μg (8,880-13,320 MBq/μmol) by incubation of 10 μg of hEGF with 18.5-37 MBq of $^{125}$I sodium iodide (Nycomed-Amersham, Oakville, ON, Canada) and 20 μg of chloramine-T (Sigma, St. Louis, MO) for 30 seconds in a glass tube at room temperature. After addition of sodium metabisulfite (40 μg), the radioiodinated hEGF was purified by size-exclusion chromatography on a P-2 mini-column eluted with 150 mM sodium chloride. The radiochemical purity of $^{125}$I-hEGF was >95% as determined by paper chromatography (Whatman No. 1, Maidstone, England) in 85% methanol.

3.2.2.2 Production of monoclonal antibody 528

HB 8509 hybridoma cells secreting anti-EGFR monoclonal antibody (mAb) 528 (IgG2a) were obtained from ATCC (Rockville, MD) and were cultured in RPMI 1640 supplemented with 20% FCS. Balb/c mice were injected i.p. with 1 mL of Pristane (2,6,10,14-tetramethylpentadecane, Sigma, St Louis, MO) followed 3-4 days later with an i.p. injection of $10^7$ HB 8509 hybridoma cells in culture medium. After 2 weeks, the ascites fluid was removed from the peritoneal cavity and anti-EGFR mAb 528 was subsequently purified from the ascites fluid on a Protein G column (Pierce, Rockford, IL).
The purified mAb 528 was desalted on a Sephadex G-25 column (PD-10, Pharmacia, Uppsala, Sweden), concentrated on a Centricon-30 ultrafiltration device (Amicon, Beverly, MA) and diluted to a concentration of 10 mg/mL in trace-metal free 50 mM sodium bicarbonate buffer pH 7.5. The purity of the mAb 528 preparation was assessed by SDS-PAGE under non-reducing conditions on a 4-20% Tris-glycine gel (BioRad, Mississauga, ON, Canada). The protein preparation resulted in a single band migrating with an apparent molecular weight ($M_r$) of 150 kDa. Approximately 2 mg of mAb 528 was obtained per mL of ascites fluid.

3.2.2.3 Radiolabelling of monoclonal antibody 528

Monoclonal antibody 528 (0.5-1 mg), 10 mg/mL in trace-metal free 50 mM sodium bicarbonate buffer pH 7.5 was derivatized with DTPA using the bicyclic anhydride of DTPA (cDTPAA, Sigma, St. Louis, MO) at a molar ratio (cDTPAA:mAb 528) of 10:1 using a previously published method (166). DTPA-mAb 528 was purified by size-exclusion chromatography on a Sephadex G-50 (Pharmacia, Uppsala, Sweden) mini-column eluted with 50 mM sodium bicarbonate buffer pH 7.5 followed by ultrafiltration through a Centricon-30 device. DTPA-mAb 528 (250-500 µg) was radiolabelled to a specific activity of 74-148 kBq/µg (11,100-22,200 MBq/µmol) with $^{111}$In acetate (37 MBq) and purified from free $^{111}$In on a Sephadex G-50 mini-column eluted with 150 mM sodium chloride. The radiochemical purity of $^{111}$In-DTPA-mAb 528 was routinely $>95\%$ as determined by ITLC-SG developed in 100 mM sodium citrate pH 5. A control mouse IgG3, which does not specifically bind to the MDA-468 cells was obtained commercially (Sigma, Product No. M-9144), derivatized with DTPA and radiolabelled with $^{111}$In as described for mAb 528.

Monoclonal antibody 528 (25-50 µg) was radioiodinated to a specific activity of 185-370 kBq/µg (27,750-55,500 MBq/µmol) by incubation with 18.5 MBq $^{125}$I sodium iodide in a glass tube pre-coated with 20 µg of 1,3,4,6-tetrachloro-3α,6α-diphenylglycouril (Sigma, St. Louis, MO) at room temperature. Radioiodinated mAb 528 was purified on a Sephadex G-50 mini-column. The radiochemical purity of $^{125}$I-mAb 528 was $>95\%$ as determined by paper chromatography (Whatman No. 1) in 85% methanol.
3.2.3 Measurement of receptor binding in vitro

The binding of radiolabelled hEGF or mAb 528 to its receptor on MDA-468, S1, MDA-231, MCF-7 or JW-97 human breast cancer cells was measured using a binding assay as previously described in Chapter 2 (section 2.2.3). The affinity constant (Ka) and the number of receptors/cell (Bmax) were determined from a non-linear fitting of the binding data (147)

The receptor binding fraction (RBF) at infinite receptor excess was determined by incubating 0.5-1 ng of $^{111}$In-DTPA-hEGF or 10-20 ng of $^{111}$In-DTPA-mAb 528 with increasing concentrations of MDA-468 breast cancer cells (1-20 X $10^6$ cells/mL) for 30 minutes at 37 °C and determining the fraction of radioactivity bound. The RBF at infinite receptor excess was obtained from the intercept on the ordinate (1/RBF) of a plot of total/bound counts versus 1/cell concentration as previously described by Lindmo et al. (167).

3.2.4 Biodistribution and tumour imaging studies

Four to six weeks old female, Swiss athymic mice (nu/nu) were obtained from Charles River Laboratories (Montreal, PQ, Canada) and housed in sterile filter cages with autoclaved food and water. The scid mice were bred at the Samuel Lunenfeld Research Institute. The athymic mice were injected s.c. in the right hind leg with 5 X $10^6$ to $10^7$ MDA-468, MDA-231 or MCF-7 human breast cancer cells in growth medium. Mice inoculated with MCF-7 cells also received bi-weekly s.c. injections of 0.5 mg of conjugated estrogens (Premarin®, Wyeth-Ayerst, St. Laurent, PQ, Canada) required for MCF-7 cells to form tumour xenografts. A freshly obtained biopsy of a skeletal metastasis from a patient with advanced breast cancer (JW-97 cells) was implanted in the left hind leg of the scid mice. When the tumours reached a diameter of 0.25-0.5 cm, the mice were injected with 1.85-3.7 MBq of $^{111}$In-DTPA-hEGF (0.5-1 µg) or $^{111}$In-DTPA-mAb 528 (25-50 µg). Control mice were injected with 1.85-3.7 MBq of $^{111}$In-DTPA-hEGF pre-mixed with 400 µg of unlabelled hEGF (to competitively inhibit the binding of $^{111}$In-DTPA-hEGF to the receptor on tumour cells in vivo), $^{111}$In-DTPA (DraxImage, Dorval, PQ) or $^{111}$In labelled non-specific mouse IgG$_{2a}$. At 1, 2, 4, 24, 48 and 72 hours
post-injection, groups of mice were sacrificed by cervical dislocation and the tumour and samples of normal tissues were obtained to measure levels of radioactivity. Tissue samples were weighed and counted along with a sample of the injectate in a γ-counter (Packard Auto Gamma 5650, Packard Instruments, Downer's Grove, IL) using a window (150-270 keV) to include the two γ-photopeaks of 111In (172, 247 keV). Tumour and normal tissue uptake of the radiopharmaceuticals was expressed as percent injected dose per gram (% i.d./g) of tissue. At 24, 48 and 72 hours post-injection, posterior images of the mice were obtained on a Siemens ZLC-3700 γ-camera (Siemens, Knoxville, TN) fitted with a medium energy pinhole collimator and interfaced to a GE Star 4000i computer (General Electric, Milwaukee, WI). Images were acquired for 10 minutes using a 20% window centered over the 172 and 247 keV photopeaks of 111In. Animal studies were conducted under an approved Animal Care Protocol (# 94-036) at The Toronto Hospital and following the Canadian Council on Animal Care (CCAC) guidelines.

3.2.5 Pharmacokinetic and statistical analysis

The mean blood radioactivity values expressed as percent injected radioactive dose per mL of blood (% i.d./mL) versus time post-injection were fitted to a one compartment pharmacokinetic model with first order input using PCNONLIN software (168) as follows:

\[ C(t) = \frac{C_0 K_{01}}{K_{01} - K_1} \left( e^{-K_{10} \tau} - e^{-K_{01} \tau} \right) \]

where \( C(t) \) is the concentration of 111In-DTPA-hEGF in the blood at time \( t \), \( C_0 \) is the concentration of 111In-DTPA-hEGF in the blood at time \( t = 0 \) hours, \( K_{01} \) is the first order input rate constant from the injection site into the blood and \( K_{10} \) is the elimination rate constant from the blood. The first order input function was required to take into account absorption of a relatively high proportion of the injected dose which was inadvertently deposited interstitially during the tail vein injection. The model used assumes that the entire dose was administered interstitially. Pharmacokinetic parameters were calculated and compared for 111In-DTPA-hEGF and 111In-DTPA-mAb 528. The mean values for
localization in the different breast cancer xenografts and normal tissues and tumour/normal tissue ratios were compared for $^{111}$In-DTPA-hEGF and $^{111}$In-DTPA-mAb. Statistical analyses were performed by ANOVA (F-test, $p<0.05$) and Student’s t-test ($p<0.05$).

### 3.3 RESULTS

#### 3.3.1 Binding of radiolabelled hEGF and mAb 528 to breast cancer cells in vitro

$^{111}$In-DTPA-hEGF and $^{111}$In-DTPA-mAb 528 (Fig. 3.1) bound with high affinity and specificity in vitro to the MDA-468 human breast cancer cells. The affinity constant ($K_a$) was approximately 6-fold higher for $^{111}$In-DTPA-hEGF than for $^{111}$In-DTPA-mAb 528 (Table 3.1). There was no significant difference in binding affinity between the $^{111}$In and corresponding $^{125}$I labelled analogs (Table 3.1), suggesting that the conjugation of the DTPA chelator to amino groups and their radiolabelling with $^{111}$In did not adversely effect the binding of the resulting radiopharmaceutical to the EGFR. The number of binding sites recognized on the MDA-468 cells ($B_{max}$, Table 3.1) was similar for all four radiolabelled ligands. There was no significant difference ($p = 0.0847$) in the fraction of radiolabelled ligand molecules which could theoretically bind to the MDA-468 cells at infinite receptor excess (RBF). The RBF was $0.73 \pm 0.17$ for $^{111}$In-DTPA-hEGF ($n = 3$) and $0.50 \pm 0.04$ for $^{111}$In-DTPA-mAb 528 ($n = 3$).

EGFR expression varied considerably among the five breast cancer cell lines tested (Fig. 3.2). The highest expression was observed on MDA-468 cells which have an amplified EGFR gene ($>10^6$ EGFR/cell) (169). MCF-7 and S1 cells exhibited the lowest expression ($<10^4$ EGFR/cell). MCF-7 is an ER-positive cell line, expected to have low EGFR expression and S1 cells are a subclone of the MDA-468 cell line, where expression of the EGFR gene is down-regulated (169). JW-97 cells, originally obtained from a biopsy of a skeletal metastasis in a patient with advanced disease, exhibited intermediate levels of EGFR expression, similar to the levels on the MDA-231 breast cancer cell line.
### TABLE 3.1

Comparison of binding of $^{111}$In and $^{125}$I labelled hEGF or mAb 528 to MDA-468 human breast cancer cells.

<table>
<thead>
<tr>
<th></th>
<th>$^{111}$In-DTPA-hEGF</th>
<th>$^{125}$I-hEGF</th>
<th>$^{111}$In-DTPA-mAb 528</th>
<th>$^{125}$I-mAb 528</th>
</tr>
</thead>
<tbody>
<tr>
<td>$n$</td>
<td>6</td>
<td>10</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>$K_a$ (L/mol) $^a$</td>
<td>(b) $7.5 \pm 1.6 \times 10^8$</td>
<td>(c) $7.3 \pm 1.1 \times 10^8$</td>
<td>(d) $1.2 \pm 0.3 \times 10^8$</td>
<td>(e) $9.4 \pm 1.0 \times 10^7$</td>
</tr>
<tr>
<td>$B_{max}$ (Sites/cell) $^a$</td>
<td>$1.3 \pm 0.1 \times 10^6$</td>
<td>$7.2 \pm 0.1 \times 10^5$</td>
<td>$9.0 \pm 2.0 \times 10^5$</td>
<td>$7.0 \pm 1.9 \times 10^5$</td>
</tr>
</tbody>
</table>

$^a$ Mean value ± s.e.m. for binding parameter

$^b-c$, $d-e$ Not significantly different.

$^b-d$, $c-e$ Significantly different.
Fig. 3.1. Representative curves for binding of $^{111}$In-DTPA-hEGF and $^{111}$In-DTPA-mAb 528 to MDA-468 human breast cancer cells. Total binding: (●); non-specific binding: (□); specific binding: (○). In this particular experiment, the $K_a$ for $^{111}$In-DTPA-hEGF was $6 \times 10^8 \text{ L/mol}$ and the number of receptors/cell (Bmax) was $4.9 \times 10^5$. The $K_a$ for $^{111}$In-DTPA-mAb 528 was $9 \times 10^7 \text{ L/mol}$ and the Bmax was $4.6 \times 10^5$ receptors/cell.
Fig. 3.2: EGFR expression on different human breast cancer cell lines as measured in a binding assay using $^{111}$In-DTPA-hEGF. MDA-468 and MDA-231 are EGFR-overexpressing cell lines. S1 is a clone of the MDA-468 cell line with down-regulation of the EGFR gene. MCF-7 is an estrogen receptor expressing cell line. JW-97 cells were cultured from a skeletal metastasis obtained from a breast cancer patient with advanced disease. Error bars indicate one s.e.m.
(1-3 X 10^5 receptors/cell) but almost 30-fold higher than on most normal epithelial tissues (<10^4 EGFR/cell).

### 3.3.2 Biodistribution studies

The biodistribution of ^111^In-DTPA-hEGF and ^111^In-DTPA-mAb 528 at selected times after tail vein injection in athymic mice bearing subcutaneous MDA-468 human breast cancer xenografts is shown in Tables 3.2 and 3.3 respectively. Difficulty in obtaining a good bolus tail vein injection of the radiopharmaceuticals was encountered and a relatively high proportion of the injected dose was inadvertently deposited interstitially. Blood levels of ^111^In-DTPA-hEGF therefore increased up to 2 hours post-injection. The blood levels then rapidly declined (~20-fold) by 72 hours (Fig. 3.3). In contrast, levels of ^111^In-DTPA-mAb 528 in the blood (Fig. 3.3) increased more slowly reaching a maximum at 24 hours, then declined only about 2-fold by 72 hours post-injection. ^111^In-DTPA-hEGF was rapidly accumulated by the liver and kidneys reaching maximum uptake values at 2 hours post-injection, then decreasing only slightly by 72 hours (Table 3.2). ^111^In-DTPA-mAb 528 also accumulated in the liver and kidneys but maximum values were reached only at 48 hours post-injection then declined slightly (Table 3.3). The apparently lower liver uptake of ^111^In-DTPA-mAb 528 at 72 hours post-injection compared to that observed for ^111^In-DTPA-hEGF (6% i.d./g versus 10% i.d./g respectively) was not statistically significant (p = 0.149). There was also no significant difference in the level of radioactivity in the kidneys at 72 hours post-injection between ^111^In-DTPA-hEGF and ^111^In-DTPA-mAb 528.

^111^In-DTPA-hEGF accumulated gradually in the MDA-468 breast cancer xenografts reaching a maximum of 2.2 % i.d./g at 72 hours post injection (Table 3.2). The level of ^111^In-DTPA-mAb 528 in the MDA-468 breast cancer xenografts increased up to 24 hours post-injection, then decreased slightly by 72 hours (Table 3.3). The maximum tumour uptake was almost 10-fold higher however for ^111^In-DTPA-mAb 528 than for ^111^In-DTPA-hEGF (21.6 vs. 2.2 % i.d./g). The mean uptake of ^111^In-DTPA-hEGF in the MDA-468 breast cancer xenografts at 72 hours post-injection was decreased more than 5-fold by co-administering 400 µg of unlabelled hEGF (0.40 ± 0.15 % i.d./g) suggesting
that tumour uptake was receptor-mediated. The uptake of the control $^{111}$In-DTPA-IgG$_2$, in the MDA-468 breast cancer xenografts at 72 hours post-injection (9.13 ± 1.92 % i.d./g) was approximately 2-fold lower than that observed for $^{111}$In-DTPA-mAb 528. The mean tumour uptake of $^{111}$In-DTPA at 72 hours post-injection was 0.07 ± 0.01 % i.d./g.

The tumour/normal tissue (T/NT) ratios for $^{111}$In-DTPA-hEGF and $^{111}$In-DTPA-mAb 528 as a function of the time after injection are shown in Tables 3.4 and 3.5 respectively. The rapid decrease in blood levels of $^{111}$In-DTPA-hEGF yielded tumour/blood (T/B) ratios >2:1 as early as 24 hours post-injection and as high as 12:1 at 72 hours post-injection (Table 3.4). T/NT ratios for $^{111}$In-DTPA-hEGF were moderately high for the heart, lungs, stomach and intestine ranging from 1.5:1 to 4.5:1, but were very low for the liver and kidneys (<<1:1) due to the high uptake and retention of $^{111}$In radioactivity in these organs. The higher levels of $^{111}$In-DTPA-mAb 528 in the blood compared to those observed for $^{111}$In-DTPA-hEGF resulted in significantly lower maximal T/B ratios at 72 hours post-injection (5:1 versus 12:1, Tables 3.3 and 3.4) despite the fact that the tumour uptake was considerably higher for $^{111}$In-DTPA-mAb 528. Nevertheless, T/B ratios for $^{111}$In-DTPA-mAb 528 were also >2:1 as early as 24 hours post-injection. With the exception of the blood, T/NT ratios for all other tissues were significantly higher at 72 hours post-injection for $^{111}$In-DTPA-mAb 528 than for $^{111}$In-DTPA-hEGF, due to a combination of greater tumour uptake and slightly lower normal tissue accumulation. In particular, the tumour/liver and tumour/kidney ratios for $^{111}$In-DTPA-mAb 528 (Table 3.5) were 7-10 times higher than those for $^{111}$In-DTPA-hEGF (Table 3.4). The tumour/liver ratio for $^{111}$In-DTPA-mAb 528 approached 3:1 at 72 hours post-injection. Although the tumour/kidney ratio was significantly higher for $^{111}$In-DTPA-mAb 528 than for $^{111}$In-DTPA-hEGF it did not exceed 2:1.

Unexpectedly, the results of the study did not show a direct quantitative relationship between EGFR expression measured in vitro on the different human breast cancer cell lines and the tumour uptake of the radiopharmaceuticals in vivo in the corresponding breast cancer xenografts hosted in athymic or scid mice (Table 3.6). For example, there were no significant differences in the accumulation of $^{111}$In-DTPA-hEGF (or $^{111}$In-DTPA-mAb 528) in MCF-7 or MDA-468 breast cancer xenografts despite a 100-
<table>
<thead>
<tr>
<th>Tissue</th>
<th>1 hour</th>
<th>2 hours</th>
<th>4 hours</th>
<th>24 hours</th>
<th>48 hours</th>
<th>72 hours</th>
<th>Blood</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood</td>
<td>2.01 ± 0.79</td>
<td>3.38 ± 0.60</td>
<td>2.80 ± 0.27</td>
<td>0.74 ± 0.24</td>
<td>0.33 ± 0.04</td>
<td>0.19 ± 0.03</td>
<td>0.75 ± 0.03</td>
</tr>
<tr>
<td>Liver</td>
<td>9.47 ± 0.82</td>
<td>6.20 ± 0.15</td>
<td>1.49 ± 0.08</td>
<td>1.00 ± 0.02</td>
<td>1.23 ± 0.09</td>
<td>1.54 ± 0.15</td>
<td>1.81 ± 0.64</td>
</tr>
<tr>
<td>Kidneys</td>
<td>1.23 ± 0.25</td>
<td>1.08 ± 0.22</td>
<td>0.75 ± 0.09</td>
<td>0.63 ± 0.08</td>
<td>0.76 ± 0.09</td>
<td>0.73 ± 0.09</td>
<td>0.77 ± 0.09</td>
</tr>
<tr>
<td>Heart</td>
<td>1.73 ± 0.32</td>
<td>1.09 ± 0.25</td>
<td>0.84 ± 0.10</td>
<td>0.60 ± 0.15</td>
<td>0.84 ± 0.18</td>
<td>0.57 ± 0.03</td>
<td>0.57 ± 0.03</td>
</tr>
<tr>
<td>Lungs</td>
<td>14.90 ± 2.54</td>
<td>16.02 ± 1.33</td>
<td>2.41 ± 0.53</td>
<td>1.23 ± 0.25</td>
<td>2.13 ± 0.29</td>
<td>2.13 ± 0.29</td>
<td>2.13 ± 0.29</td>
</tr>
<tr>
<td>Stomach</td>
<td>1.05 ± 0.40</td>
<td>1.33 ± 0.22</td>
<td>1.00 ± 0.22</td>
<td>1.23 ± 0.41</td>
<td>1.23 ± 0.41</td>
<td>1.00 ± 0.22</td>
<td>1.00 ± 0.22</td>
</tr>
<tr>
<td>Spleen</td>
<td>1.08 ± 0.40</td>
<td>1.33 ± 0.22</td>
<td>1.00 ± 0.22</td>
<td>1.23 ± 0.41</td>
<td>1.23 ± 0.41</td>
<td>1.00 ± 0.22</td>
<td>1.00 ± 0.22</td>
</tr>
<tr>
<td>Intestine</td>
<td>0.65 ± 0.21</td>
<td>1.11 ± 0.36</td>
<td>1.54 ± 0.17</td>
<td>0.63 ± 0.08</td>
<td>0.63 ± 0.08</td>
<td>0.63 ± 0.08</td>
<td>0.63 ± 0.08</td>
</tr>
<tr>
<td>Tumour</td>
<td>0.65 ± 0.21</td>
<td>1.11 ± 0.36</td>
<td>1.54 ± 0.17</td>
<td>0.63 ± 0.08</td>
<td>0.63 ± 0.08</td>
<td>0.63 ± 0.08</td>
<td>0.63 ± 0.08</td>
</tr>
</tbody>
</table>

*Mean ± s.e.m. of 3-5 animals per time point.*
Biodistribution of \( ^{111}\text{In-}	ext{DTPA-mAb 528} \) in athymic mice bearing subcutaneous MDA-468 human breast cancer xenografts.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>1 hour</th>
<th>2 hours</th>
<th>4 hours</th>
<th>24 hours</th>
<th>48 hours</th>
<th>72 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood</td>
<td>2.21 ± 0.49</td>
<td>3.12 ± 0.80</td>
<td>3.86 ± 1.38</td>
<td>8.75 ± 1.96</td>
<td>5.65 ± 0.02</td>
<td>3.22 ± 0.98</td>
</tr>
<tr>
<td>Heart</td>
<td>0.40 ± 0.07</td>
<td>0.78 ± 0.30</td>
<td>0.63 ± 0.26</td>
<td>3.24 ± 0.92</td>
<td>2.92 ± 0.43</td>
<td>2.12 ± 0.53</td>
</tr>
<tr>
<td>Lungs</td>
<td>0.63 ± 0.06</td>
<td>1.03 ± 0.26</td>
<td>1.10 ± 0.24</td>
<td>5.42 ± 1.91</td>
<td>4.82 ± 0.86</td>
<td>3.68 ± 0.95</td>
</tr>
<tr>
<td>Liver</td>
<td>0.80 ± 0.21</td>
<td>0.90 ± 0.26</td>
<td>1.03 ± 0.33</td>
<td>5.94 ± 1.22</td>
<td>7.72 ± 1.62</td>
<td>6.35 ± 1.15</td>
</tr>
<tr>
<td>Kidneys</td>
<td>0.70 ± 0.18</td>
<td>1.54 ± 0.23</td>
<td>1.45 ± 0.25</td>
<td>12.25 ± 3.12</td>
<td>17.36 ± 3.29</td>
<td>13.76 ± 2.85</td>
</tr>
<tr>
<td>Spleen</td>
<td>0.63 ± 0.07</td>
<td>0.75 ± 0.20</td>
<td>0.89 ± 0.25</td>
<td>9.88 ± 4.02</td>
<td>10.85 ± 3.81</td>
<td>6.21 ± 1.87</td>
</tr>
<tr>
<td>Stomach</td>
<td>0.09 ± 0.05</td>
<td>0.13 ± 0.03</td>
<td>0.13 ± 0.04</td>
<td>1.34 ± 0.28</td>
<td>1.15 ± 0.30</td>
<td>1.09 ± 0.22</td>
</tr>
<tr>
<td>Intestine</td>
<td>0.23 ± 0.09</td>
<td>0.19 ± 0.05</td>
<td>0.51 ± 0.24</td>
<td>2.54 ± 0.44</td>
<td>3.21 ± 0.65</td>
<td>2.85 ± 0.62</td>
</tr>
<tr>
<td>Tumour</td>
<td>0.60 ± 0.23</td>
<td>0.39 ± 0.05</td>
<td>0.93 ± 0.31</td>
<td>21.55 ± 6.98</td>
<td>11.16 ± 3.00</td>
<td>15.35 ± 2.49</td>
</tr>
</tbody>
</table>

\( ^{111}\text{In} \) (Percent injected radioactive dose/g) \( ^a \)

\( ^a \) Mean ± s.e.m. of 3-6 animals per time point
TABLE 3.4

Tumour/normal tissue ratios for $^{111}$In-DTPA-hEGF in athymic mice bearing subcutaneous
MDA-468 human breast cancer xenografts.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Time post-injection</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 hour</td>
</tr>
<tr>
<td>Blood</td>
<td>0.39 ± 0.10</td>
</tr>
<tr>
<td>Heart</td>
<td>1.29 ± 0.38</td>
</tr>
<tr>
<td>Lungs</td>
<td>0.74 ± 0.19</td>
</tr>
<tr>
<td>Liver</td>
<td>0.07 ± 0.01</td>
</tr>
<tr>
<td>Kidneys</td>
<td>0.10 ± 0.02</td>
</tr>
<tr>
<td>Spleen</td>
<td>0.47 ± 0.12</td>
</tr>
<tr>
<td>Stomach</td>
<td>0.63 ± 0.03</td>
</tr>
<tr>
<td>Intestine</td>
<td>0.63 ± 0.15</td>
</tr>
</tbody>
</table>

$a$ Mean ± s.e.m. of 3-5 animals per time point
### TABLE 3.5

Tumour/normal tissue ratios for \(^{111}\)In-DTPA-mAb 528 in athymic mice bearing subcutaneous MDA-468 human breast cancer xenografts.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>1 hour</th>
<th>2 hours</th>
<th>4 hours</th>
<th>24 hours</th>
<th>48 hours</th>
<th>72 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood</td>
<td>0.22 ± 0.13</td>
<td>0.14 ± 0.03</td>
<td>0.19 ± 0.01</td>
<td>2.49 ± 0.57</td>
<td>3.72 ± 1.60</td>
<td>5.50 ± 1.16</td>
</tr>
<tr>
<td>Heart</td>
<td>1.17 ± 0.70</td>
<td>0.61 ± 0.16</td>
<td>1.25 ± 0.16</td>
<td>6.92 ± 1.61</td>
<td>4.21 ± 0.96</td>
<td>8.67 ± 1.45</td>
</tr>
<tr>
<td>Lungs</td>
<td>0.70 ± 0.38</td>
<td>0.42 ± 0.10</td>
<td>0.70 ± 0.11</td>
<td>4.51 ± 1.16</td>
<td>2.73 ± 0.52</td>
<td>5.28 ± 0.95</td>
</tr>
<tr>
<td>Liver</td>
<td>0.64 ± 0.39</td>
<td>0.47 ± 0.08</td>
<td>0.68 ± 0.10</td>
<td>3.32 ± 0.67</td>
<td>1.42 ± 0.19</td>
<td>2.96 ± 0.36</td>
</tr>
<tr>
<td>Kidneys</td>
<td>0.71 ± 0.45</td>
<td>0.26 ± 0.03</td>
<td>0.54 ± 0.11</td>
<td>1.75 ± 0.39</td>
<td>0.65 ± 0.07</td>
<td>1.34 ± 0.20</td>
</tr>
<tr>
<td>Spleen</td>
<td>0.72 ± 0.40</td>
<td>0.56 ± 0.10</td>
<td>0.84 ± 0.31</td>
<td>2.56 ± 0.69</td>
<td>1.12 ± 0.16</td>
<td>2.82 ± 0.47</td>
</tr>
<tr>
<td>Stomach</td>
<td>5.60 ± 1.23</td>
<td>3.14 ± 0.32</td>
<td>5.79 ± 0.15</td>
<td>15.76 ± 3.80</td>
<td>10.48 ± 0.69</td>
<td>16.33 ± 3.30</td>
</tr>
<tr>
<td>Intestine</td>
<td>2.58 ± 1.68</td>
<td>2.18 ± 0.31</td>
<td>1.31 ± 0.02</td>
<td>8.28 ± 2.13</td>
<td>3.51 ± 0.34</td>
<td>7.13 ± 1.07</td>
</tr>
</tbody>
</table>

\(^{a}\) Mean ± s.e.m. of 3-6 animals per time point.
TABLE 3.6

Tumour localization of $^{111}$In-DTPA-hEGF and $^{111}$In-DTPA-mAb 528 at 72 hours post-injection as a function of EGFR expression.

<table>
<thead>
<tr>
<th>Breast cancer xenograft</th>
<th>EGFR expression (receptors/cell × 10$^5$)</th>
<th>$^{111}$In-DTPA-hEGF</th>
<th>$^{111}$In-DTPA-mAb 528</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCF-7</td>
<td>0.15 ± 0.04</td>
<td>$d$ 1.95 ± 0.56</td>
<td>$e$ 8.40 ± 1.60</td>
</tr>
<tr>
<td>MDA-231</td>
<td>1.33 ± 0.50</td>
<td>$f$ 1.46 ± 0.89</td>
<td>$g$ 18.03 ± 7.87</td>
</tr>
<tr>
<td>JW-97</td>
<td>2.71 ± 0.48</td>
<td>$h$ 0.70 ± 0.07</td>
<td>$i$ 4.90 ± 1.08</td>
</tr>
<tr>
<td>MDA-468</td>
<td>12.80 ± 0.94</td>
<td>$j$ 2.24 ± 0.32</td>
<td>$k$ 15.35 ± 2.49</td>
</tr>
</tbody>
</table>

$^{a}$ Mean ± s.e.m. of 3-6 animals per experiment.

$^{b}$ MCF-7, MDA-231 and MDA-468 xenografts were hosted in athymic mice. JW-97 xenografts were hosted in scid mice.

$^{c}$ Determined in vitro with $^{111}$In-DTPA-hEGF. Mean ± s.e.m. of 3-10 experiments. The data for MDA-468 and MCF-7 cells were taken from Chapter 2.

$^{d-j}$, $^{e-k}$, $^{f-j}$, $^{g-k}$, $^{f-h}$, $^{g-i}$ Not significantly different.

$^{d-e}$, $^{h-j}$, $^{i-k}$, $^{f-g}$, $^{h-i}$, $^{j-k}$ Significantly different.
fold difference in receptor expression (p = 0.6408 and p = 0.0957 respectively). Similarly, there were no significant differences in the accumulation of $^{111}$In-DTPA-hEGF (or $^{111}$In-DTPA-mAb 528) in MDA-231 and MDA-468 breast cancer xenografts despite a 10-fold difference in receptor expression (p = 0.3955 and p = 0.6838 respectively). However, tumour uptake values for $^{111}$In-DTPA-mAb 528 were 4-12 fold higher than those for $^{111}$In-DTPA-hEGF in all cases. The accumulation of either $^{111}$In-DTPA-hEGF or $^{111}$In-DTPA-mAb 528 was significantly lower in JW-97 tumours than in the MDA-468 breast cancer xenografts (p = 0.0041 and p = 0.0260 respectively). There was no significant difference in the tumour uptake of $^{111}$In-DTPA-hEGF in MDA-231 breast cancer xenografts as compared to the JW-97 tumours (p = 0.427). Similarly, there was no significant difference in the tumour uptake of $^{111}$In-DTPA-mAb 528 between the MDA-231 or JW-97 tumour xenografts (p = 0.684).

3.3.3 Pharmacokinetic studies

The concentration versus time profiles for $^{111}$In-DTPA-hEGF and $^{111}$In-DTPA-mAb 528 in the blood are shown in Fig. 3.3. The profiles were fitted to a one compartment pharmacokinetic model with first order input to account for absorption of a high proportion of the injected dose of the radiopharmaceuticals which was deposited interstitially during the tail vein injections (168). The model fitting assumes that all of the injected dose was actually deposited interstitially. The values for the calculated pharmacokinetic parameters are shown in Table 3.7.

3.3.4 Tumour imaging studies

The MDA-468 (Fig. 3.4), MDA-231 and JW-97 breast cancer xenografts (not shown) were successfully imaged with either $^{111}$In-DTPA-hEGF or $^{111}$In-DTPA-mAb 528 at 72 hours post-injection. MCF-7 tumours could not be visualized by imaging due to their very small size (approximately 2 mm in diameter). However, the 10-fold higher tumour uptake of $^{111}$In-DTPA-mAb 528 compared to $^{111}$In-DTPA-hEGF resulted in an enhanced definition of the MDA-468 xenografts. For both radiopharmaceuticals, the liver and the kidneys were the major normal organs observed on the images. Excretion of
Fig. 3.3: Concentration of radiopharmaceutical versus time in the blood of athymic mice bearing a subcutaneous MDA-468 human breast cancer xenograft following tail vein injection of $^{111}$In-DTPA-hEGF (○) or $^{111}$In-DTPA-mAb 528 (●). The initial increase in blood radioactivity levels was due to a relatively high proportion of the injected dose which was deposited interstitially. The fitted lines through the data points represent the data fitted to a one compartment pharmacokinetic model with first order input to account for the fraction of interstitially deposited radiopharmaceutical. The error bars indicate one s.e.m.
### TABLE 3.7

Pharmacokinetic parameters for $^{111}$In-DTPA-hEGF and $^{111}$In-DTPA-mAb 528 $^a$.

<table>
<thead>
<tr>
<th></th>
<th>$t_{1/2}$ input</th>
<th>$t_{1/2}$ $\beta$</th>
<th>$V_d$</th>
<th>$^b$ AUC$_{0-72h}$</th>
<th>CL</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^{111}$In-DTPA-hEGF</td>
<td>0.7</td>
<td>10.2</td>
<td>26.6</td>
<td>64.4</td>
<td>1.6</td>
</tr>
<tr>
<td>$^{111}$In-DTPA-mAb 528</td>
<td>6.6</td>
<td>29.5</td>
<td>7.2</td>
<td>416.1</td>
<td>0.2</td>
</tr>
</tbody>
</table>

$a$ Symbols are: $t_{1/2}$ input: half-life of first order input function; $t_{1/2} \beta$: elimination half-life; $V_d$: volume of distribution; AUC$_{0-72h}$: area under the blood concentration versus time curve from 0 to 72 hours post-injection; % i.d.: percent injected radioactive dose; CL: systemic clearance.

$b$ Calculated using the Trapezoidal Rule.
Fig. 3.4: Posterior whole body image of an athymic mouse bearing a subcutaneous MDA-468 human breast cancer xenograft (0.25-0.5 cm in diameter) at 72 hours post-injection with A. $^{111}$In-DTPA-hEGF or B. $^{111}$In-DTPA-mAb 528. The MDA-468 tumour was visualized using either radiopharmaceutical with the localization of the tumour being more obvious with $^{111}$In-DTPA-mAb 528. The liver and kidneys were the major normal organs visualized on the images.
radioactivity into the bladder was also observed, particularly on the early images with $^{111}$In-DTPA-hEGF and to a lesser extent with $^{111}$In-DTPA-mAb 528. In addition, there appeared to be some uptake of radioactivity in the region of the submaxillary glands for both radiopharmaceuticals. The levels of circulating background submaxillary glands for both radiopharmaceuticals. The levels of circulating background radioactivity and whole body radioactivity were considerably lower on the images obtained using $^{111}$In-DTPA-hEGF than those obtained with $^{111}$In-DTPA-mAb 528.

3.4 DISCUSSION

The results of this study demonstrated that human breast cancer xenografts overexpressing the EGFR implanted in immunocompromised mice can be successfully imaged using human EGF or anti-EGFR mAb 528 labelled with the $\gamma$-emitting radioisotope, $^{111}$In. The tumour/blood ratios were higher for $^{111}$In-DTPA-hEGF than for $^{111}$In-DTPA-mAb 528 (12:1 versus 5:1 at 72 hours post-injection, Tables 3.4 and 3.5) but the 10-fold higher tumour uptake observed with $^{111}$In-DTPA-mAb 528 compared to $^{111}$In-DTPA-hEGF (21.6 versus 2.2 % i.d./g, Tables 3.2 and 3.3) yielded much clearer images of the breast cancer xenografts and suggests that in certain instances mAbs may be more appropriate targeting agents than peptide growth factors for tumour imaging.

The high tumour/blood ratios observed for $^{111}$In-DTPA-hEGF were due to rapid elimination of the radiopharmaceutical from the blood in the animals. Its elimination half-life was 3 times shorter than that observed for $^{111}$In-DTPA-mAb 528 (10 versus 29 hours respectively, Table 3.7). The rapid elimination from the blood was likely due to filtration of $^{111}$In-DTPA-hEGF by the kidneys and excretion into the urine. As discussed in Chapter 1 (section 1.4), EGF was previously known as urogastrone (a hormone found in the urine and which stimulated gastric acid secretion). It has been shown that EGF radiolabelled with $^{125}$I is cleared by glomerular filtration and is also secreted by the proximal renal tubules following binding to receptors on renal tubular cells (158,170,171). $^{125}$I-EGF is not reabsorbed however by the renal tubules (171). The large molecular size of $^{111}$In-DTPA-mAb 528 (M, ~150 kDa) would prevent its filtration by the kidneys, a process which is generally restricted to proteins with a M, <60 kDa. Interestingly, the elimination
half-life observed for $^{111}$In-DTPA-hEGF in this study (10 hours) was actually 5-10 times longer than that reported for radioiodinated EGF in animals (half-life of 1-2 hours) \((159,172)\). Since the half-life is determined based on the elimination of the radioisotope from the blood rather than the elimination of the EGF molecule, the results suggest that EGF conjugated with $^{111}$In may be more stable \textit{in vivo} than the radioiodinated analog. The very short elimination half-life previously reported for $^{125}$I-EGF was likely affected by extensive \textit{in vivo} deiodination and rapid elimination of radioactive catabolites (e.g., free radiiodine and radioiodotyrosine) into the urine. Nevertheless, it is also possible that the slow absorption of interstitially deposited $^{111}$In-DTPA-hEGF may have contributed to the apparent longer half-life of the radiopharmaceutical in the blood and its retention by normal tissues.

The prolonged retention (up to 72 hours post injection) of $^{111}$In radioactivity in the kidneys of the animals (Table 3.2) may also be due to the greater stability \textit{in vivo} of $^{111}$In-DTPA-hEGF compared to $^{125}$I-hEGF (or partially due to slow absorption of interstitially deposited radiopharmaceutical). Although accumulation of radioactivity in the kidneys has also been reported for $^{125}$I-EGF, it was rapidly cleared within a few hours (172-174). The mechanism of "wash-out" of $^{125}$I-EGF from the kidneys was thought to be due to binding to renal tubular cells followed by internalization and degradation to free $^{125}$I and $^{125}$I-iodotyrosine, which were then exported from the cells and subsequently eliminated (111,175). $^{111}$In-DTPA-hEGF likely follows a similar biological pathway involving binding and internalization by renal tubular cells and degradation by intracellular proteases. However, in the case of $^{111}$In-DTPA-hEGF, the final catabolite is probably $^{111}$In-DTPA covalently linked to one of the two lysine residues (K28 or K48) or to the N-terminal asparagine residue. These terminal catabolites are not recognized by amino acid transporters and are therefore retained within the cells (176). The accumulation and retention of $^{111}$In-DTPA-mAb 528 in the kidneys was almost identical to that observed with $^{111}$In-DTPA-hEGF suggesting that a similar biological mechanism may also be responsible. It is not clear if the receptor on renal tubular cells involved in binding and internalizing $^{111}$In-DTPA-mAb 528 (and possibly also $^{111}$In-DTPA-hEGF) is actually the EGFR however, since mAb 528 has been reported to bind to an epitope which is unique
to the human EGFR and is also not thought to be internalized after binding to the receptor (113). Nevertheless, specific receptors for $^{125}$I-EGF have been detected in vitro in rat kidney homogenates (177) and on renal tubular cells (110).

As previously discussed in Chapter 1 (section 1.7), normal hepatocytes exhibit moderate to high levels of EGFR expression ($8 \times 10^4$ to $10^5$ EGFR/cell) (58,108). EGFR expression combined with the large proportion of cardiac output perfusing the liver were likely responsible for the high hepatic uptake of $^{111}$In-DTPA-hEGF (up to 16% i.d./g, Table 3.2). The liver has been shown to have a high capacity to extract $^{125}$I-EGF from the circulation (108,109). $^{125}$I-EGF taken up by hepatocytes was primarily internalized into lysosomes and degraded, but a fraction of internalized EGF molecules were transported by a non-lysosomal pathway and secreted into the bile (109,178). Similar to the situation with renal uptake of $^{111}$In-DTPA-hEGF, radioactivity in the liver in the animals decreased only slowly over a 72 hour period. The slow clearance of $^{111}$In-DTPA-hEGF from the liver again differs from that previously observed with $^{125}$I-EGF, where $>90\%$ of liver associated radioactivity was cleared in as little as 90 minutes (109). The mechanism of retention of $^{111}$In-DTPA-hEGF in the liver may also be due to binding to the EGFR on hepatocytes followed by internalization and degradation to a radioactive catabolite which is retained by the cells. There was a suggestion of lower accumulation of $^{111}$In-DTPA-mAb 528 in the liver (Table 3.3) compared to that observed with $^{111}$In-DTPA-hEGF (Table 3.2) but the difference was not statistically significant. Liver uptake and retention of $^{111}$In-DTPA-mAb 528 could be mediated by binding to the EGFR or by interaction of the Fc portion of the mAb with Fc receptors on hepatocytes (179). Despite the normal tissue accumulation of radioactivity observed in the liver and kidneys, both $^{111}$In-DTPA-hEGF and $^{111}$In-DTPA-mAb 528 localized to a sufficient extent in the MDA-468 human breast cancer xenografts to allow visualization of the tumour at 72 hours post-injection by $\gamma$-scintigraphy (Fig. 3.4). The normal tissues observed on the images were primarily the liver and kidneys for the reasons previously discussed.

One of the most important findings in this study was the approximately 10-fold greater tumour uptake observed with radiolabelled mAb 528 compared to EGF. The greater tumour uptake observed with $^{111}$In-DTPA-mAb 528 may be due to the much
longer period of time spent by the radiopharmaceutical in the blood compared to $^{111}$In-DTPA-hEGF. The area under the blood concentration versus time curve (AUC) provides an indication of the length of time that a radiopharmaceutical spends in the blood pool. The AUC$_{0-72}$h was 6.5 times greater for $^{111}$In-DTPA-mAb 528 than for $^{111}$In-DTPA-hEGF (Table 3.6). The higher levels of radioactivity in the MDA-468 breast cancer xenografts observed in vivo with $^{111}$In-DTPA-mAb 528 compared to $^{111}$In-DTPA-hEGF were not due to a higher binding affinity for the EGFR, since cell binding assays demonstrated that the $K_a$ value for $^{111}$In-DTPA-mAb 528 was actually 6-fold lower than that for $^{111}$In-DTPA-hEGF ($1.2 \times 10^8$ versus $7.5 \times 10^8$ L/mol respectively, Table 3.1). The prolonged residence time in the blood of $^{111}$In-DTPA-mAb 528 compared to $^{111}$In-DTPA-hEGF probably allowed a greater period of time for diffusion of the radiopharmaceutical into the tissues (despite its poorer tissue penetration properties) and binding to the breast cancer cells. It has been recognized that the tumour uptake of radiolabelled mAbs and their fragments is decreased as their elimination rate from the blood is increased (179). For example, the elimination rate from the blood of the pancarcinoma TAG-72 mAb CC49 and its fragments radiolabelled with $^{131}$I was $F_v > F_ab > F(ab')_2 > IgG$ but the tumour uptake in LS174T human colon cancer xenografts hosted in athymic mice was $IgG > F(ab')_2 > F_ab > F_v$ (170).

In the phantom studies described in Chapter 2, it was determined that a minimum tumour/background ratio of 2:1 would be required for successful tumour imaging. The tumour/normal tissue ratios for $^{111}$In-DTPA-hEGF at 72 hours post-injection exceeded this minimum value for the blood, heart, lungs and stomach but not for the liver, kidneys, spleen and intestine. The tumour/blood ratios (12:1) observed for $^{111}$In-DTPA-hEGF in mice bearing subcutaneous MDA-468 human breast cancer xenografts however compared quite favourably with those recently reported for $^{99m}$Tc nerve growth factor analogs which successfully imaged nerve growth factor receptor-expressing tumours in animals (tumour/blood ratios of 13:1 to 26:1) (180). In the case of $^{111}$In-DTPA-mAb 528, almost all normal tissues including the liver, exhibited a tumour/normal tissue ratio of $\geq$ 2:1. For $^{111}$In-DTPA-mAb 528. The kidneys were the only normal tissue which exhibited a tumour/normal tissue ratio < 2:1. The tumour/normal tissue and tumour/blood ratios
observed for $^{111}$In-DTPA-hEGF and $^{111}$In-DTPA-mAb 528 should be sufficient for imaging breast cancer metastases in the lungs, brain or bone but would not be high enough for detection of metastases in the adrenal glands or in the liver (due to high uptake of the radiopharmaceuticals by the kidneys and liver).

Other forms of radiolabelled EGF have also recently been shown to localize in EGFR-positive tumours. Capala et al. (181) showed that $^{99m}$Tc-EGF was selectively retained in the brains of rats inoculated with glioma cells transfected with the EGFR gene but not in normal rats. Rusckowski et al. (182) imaged A431 squamous cell carcinoma xenografts (2 x $10^6$ EGFR/cell) implanted in athymic mice with $^{99m}$Tc-EGF achieving tumour/blood ratios of 4:1 at 12 hours post injection. Cuartero-Plaza et al. (154) detected squamous cell lung carcinoma in 6/9 cancer patients by $\gamma$-scintigraphy using a dose of 185-370 MBq of $^{131}$I-EGF (0.3-3 mg). The study described in this chapter however is the first investigation of imaging of human breast cancer which overexpresses the EGFR using radiolabelled EGF or anti-EGFR mAb 528. MDA-468 human breast cancer xenografts implanted in athymic mice have been previously imaged by Goldenberg et al. (37) using the anti-EGFR mAb 225 (IgG2a) labelled with $^{111}$In, but the tumour uptake was more than 5-fold lower than was observed in this study with $^{111}$In-DTPA-mAb 528 (4 versus 22 % i.d./g). Since $^{111}$In-DTPA-mAb 225 has already been shown to successfully image squamous cell lung carcinomas in cancer patients (183), the higher tumour uptake of $^{111}$In-DTPA-mAb 528 in the MDA-468 human breast cancer xenografts, is encouraging for the ultimate clinical application of this new radiopharmaceutical for the diagnostic imaging of EGFR-positive breast cancer in humans.

It is interesting to speculate why there was no direct quantitative relationship in the study between the level of receptors on the breast cancer cell lines measured in vitro and the accumulation of either radiopharmaceutical in the corresponding breast cancer xenografts in vivo. This finding was not due to the inactivation of either hEGF or mAb 528 upon radiolabelling with $^{111}$In, since cell binding assays demonstrated that both radiopharmaceuticals exhibited their expected receptor binding properties (Table 3.1). One possible explanation is that in the context of tumour-bearing mice, only very small concentrations of the radiopharmaceuticals actually reached the extracellular fluid bathing
the cancer cells. Under these conditions, the concentration of receptors on the breast cancer cells may have been in excess and the amount of radioligand was therefore the limiting factor which controlled tumour uptake. Based on a tumour uptake of ~2% i.d./g (Table 3.2) and an injected dose of 1 µg of $^{111}$In-DTPA-hEGF, there would be approximately $2 \times 10^{12}$ molecules of the radiopharmaceutical (0.02 µg) delivered to $2.5 \times 10^{8}$ MDA-468 cells contained in 1 g of a breast cancer xenograft (assuming a breast cancer cell with a diameter of 20 µm). The cells would express a total of $2.5 \times 10^{14}$ EGFR at an expression level of ~$10^6$ EGFR/cell (Table 3.1) and therefore there would be approximately a 100-fold excess of receptors present on the tumour compared to the radioligand ($2.5 \times 10^{14}$ molecules of EGFR versus $2 \times 10^{12}$ molecules of $^{111}$In-DTPA-hEGF). Similarly, for $^{111}$In-DTPA-mAb 528, assuming an injected dose of 50 µg and a tumour uptake of 15% i.d./g, there would be approximately $3 \times 10^{13}$ molecules (7.5 µg) of monoclonal antibody delivered to the tumour xenograft. In this case, there would be about a 10-fold excess of EGFR compared to radioligand. In contrast, in vitro the receptor level on the breast cancer cells was measured by increasing the concentration of radioligand until the concentration of receptors on the cells was the limiting factor. In the in vitro situation therefore, breast cancer cells with a lower level of receptor expression (eg. MCF-7 cells) bound less radioligand than those with a higher level of receptor expression (eg. MDA-468 cells). Rusckowski et al. (182) also recently obtained similar results using $^{99}$mTc-EGF in athymic mice bearing either A431 squamous cell carcinoma or LS174T colon cancer xenografts. Despite a 20-fold difference in EGFR expression in vitro between the A431 and LS174T cells ($2 \times 10^6$ versus $3.6 \times 10^5$ EGFR/cell respectively), there was no statistically significant difference in tumour uptake in vivo (0.4 ± 0.09 versus 0.32 ± 0.06 % i.d./g respectively). Since the level of EGFR expression on the breast cancer xenografts was not measured in this study, it is nevertheless possible that changes in receptor expression in vivo could also account for the inability to observe a direct correlation between tumour uptake of the radiopharmaceuticals and EGFR expression levels.
3.5 CONCLUSIONS

Human breast cancer xenografts overexpressing the EGFR implanted in immunocompromised mice were successfully imaged with $^{111}$In-DTPA-hEGF or $^{111}$In-DTPA-mAb 528. Tumour/blood ratios were moderately lower for $^{111}$In-DTPA-mAb 528 than for $^{111}$In-DTPA-hEGF but absolute tumour uptake was much higher for $^{111}$In-DTPA-mAb 528. The higher tumour uptake of $^{111}$In-DTPA-mAb 528 resulted in better definition of the breast cancer xenografts on the images. The much longer residence time of $^{111}$In-DTPA-mAb 528 in the blood may be responsible for the high tumour uptake observed with the radiopharmaceutical. A correlation was not observed between the level of EGFR expression on breast cancer cell lines and the uptake of the radiopharmaceuticals in the corresponding breast cancer xenografts in vivo. These results suggest that the level of tumour localization of receptor-binding radiopharmaceuticals in vivo is controlled to a greater extent by their pharmacokinetic properties rather than by the level of receptor expression on the cancer cells, provided that the radiopharmaceutical retains receptor binding capability and a minimal level of receptors is available for binding. Although this observation suggests that a direct quantitation of receptor expression on cancer cells in vivo by $\gamma$-scintigraphy may not be feasible, the results suggest that breast cancer cells overexpressing the EGFR can be qualitatively imaged using radiopharmaceuticals which bind specifically to the receptor. Radiolabelled anti-EGFR mAbs may be more effective for tumour imaging than EGF in light of their longer residence time in the blood leading to higher tumour uptake at only moderately lower tumour/blood ratios. The inadvertent partial interstitial injection of $^{111}$In-DTPA-hEGF may also have partially enhanced the accumulation in the breast cancer xenografts by providing a depot of the radiopharmaceutical which was slowly absorbed suggesting that slow infusion of radiolabelled peptide growth factors may be one means of prolonging the residence time in the blood which could promote tumour uptake.
INDIUM-111 LABELLED EPIDERMAL GROWTH FACTOR IS SELECTIVELY RADIOTOXIC TO HUMAN BREAST CANCER CELLS OVEREXPRESSING THE EPIDERMAL GROWTH FACTOR RECEPTOR
4.0 ABSTRACT

Auger electron emitting radionuclides are unique in that they are only radiotoxic to cells which are able to internalize the radionuclide, due to the extremely short, subcellular range of the electrons. In situations where the Auger electron emitter is internalized into the cytoplasm of the cell or particularly when localized in the cell nucleus however, they deliver very high linear energy transfer (LET) radiation to the DNA and cause radiotoxicity comparable to that of α-emitters. The objective of this study was to evaluate the radiotoxicity of a novel radiopharmaceutical: human epidermal growth factor (hEGF) radiolabelled with the Auger electron emitter, indium-111 (\(^{111}\text{In-DTPA-hEGF}\)) against human breast cancer cells which overexpress the epidermal growth factor receptor (EGFR). **Methods:** The kinetics of receptor binding, internalization and nuclear translocation was studied for \(^{111}\text{In-DTPA-hEGF}\) using MDA-468 human breast cancer cells (>\(10^6\) EGFR/cell). The radiotoxicity of the radiopharmaceutical against MDA-468 or MCF-7 (~\(10^4\) EGFR/cell) breast cancer cells was evaluated in a growth inhibition assay and by clonogenic survival of the cells. The radiotoxicity against normal tissues such as the liver and kidneys was evaluated by administering escalating doses of \(^{111}\text{In-DTPA-hEGF}\) to mice and measuring the levels of alanine aminotransferase (ALT) and creatinine in the plasma and by histopathological examination of the liver and kidneys at necropsy (3 days or 7 weeks post administration of the radiopharmaceutical). **Results:** \(^{111}\text{In-DTPA-hEGF}\) was rapidly bound by the MDA-468 cells, internalized into the cytoplasm and partially translocated to the cell nucleus. A fraction of the internalized \(^{111}\text{In-DTPA-hEGF}\) (up to 10%) was also associated with the chromatin. \(^{111}\text{In-DTPA-hEGF}\) (37 mBq/cell) reduced the growth of the MDA-468 cells in culture by 60-70% but had no effect on the growth of MCF-7 cells. The growth of the MDA-468 cells was also reduced by 30% by treatment with unlabelled hEGF but was unaffected by treatment with \(^{111}\text{In-DTPA}\), a radiopharmaceutical which does not bind or internalize into the cells. In a clonogenic assay, the surviving fraction of the MDA-468 cells was reduced to <3% at only 130 mBq of \(^{111}\text{In-DTPA-hEGF}\) targeted per cell. There was no radiotoxicity against MCF-7 human breast cancer cells at up to 133 mBq of \(^{111}\text{In-DTPA-hEGF}\) per cell, suggesting that the radiopharmaceutical exhibits selective radiotoxicity against breast
cancer cells which overexpress the EGFR. Cellular dosimetry predicted that 24.7 Gy would be delivered to each MDA-468 cell at receptor saturation with $^{111}$In-DTPA-hEGF (specific activity of 37 MBq/µg) and 19.3 Gy to the cell nucleus. Although only 15% of the radiopharmaceutical localized in the cell nucleus, this accounted for 80% of the total radiation dose to the cell nucleus (15.2 Gy). The cytoplasmic fraction of $^{111}$In-DTPA-hEGF accounted for 18% of the radiation dose to the nucleus (3.5 Gy) and the membrane-bound fraction accounted for only 3% (0.6 Gy). No detectable radiotoxicity to normal tissues (liver or kidneys) which exhibit moderate-high levels of the EGFR was observed over a 7 week time period in mice administered high amounts of $^{111}$In-DTPA-hEGF (equivalent on a MBq/m² basis to human amounts of up to 14,208 MBq). **Conclusion:** These findings are promising for the ultimate application of $^{111}$In-DTPA-hEGF in humans for targeted Auger electron radiotherapy of hormone-resistant, advanced forms of breast cancer which overexpress the EGFR.
4.1 INTRODUCTION

Over the past decade, numerous studies have investigated the potential for targeted radiotherapy of human malignancies using highly specific monoclonal antibodies (mAbs) directed against tumour-associated antigens conjugated with β-particle emitting radioisotopes (radioimmunotherapy) (184). Although some success has been achieved clinically in radioimmunotherapy of blood-borne tumours such as B-cell lymphoma and leukemias, the major solid tumours such as breast, colon, ovarian and prostate cancer remain refractory to this form of treatment. One of the major reasons for the failure of radioimmunotherapy to achieve significant tumour regressions in patients with solid tumours was the inability to escalate the administered dose of the radiolabelled mAb to a therapeutically effective level, due to the dose-limiting radiotoxicity to the bone marrow (179). Myelotoxicity in radioimmunotherapy was primarily due to non-specific irradiation of the bone marrow stem cell population by the persistently high levels of circulating radiolabelled mAb in the blood, combined with the relatively long-range (2-10 mm) of the β-particles in tissues.

An intriguing alternative to β-particle emitters for targeted radiotherapy of cancer are Auger electron emitting radioisotopes, such as $^{125}$I and $^{111}$In (185). Auger electrons, first discovered in 1925 by Pierre Auger (186), are very low energy (<30 keV) outer shell electrons released by radioisotopes which decay by electron capture. A unique property of Auger electrons is that they exhibit an extremely short, subcellular range (2-12 μm) in tissues. Due to their subcellular range, Auger electron emitters exert their radiotoxic effects on the cell only when internalized into the cytoplasm and particularly when localized in the cell nucleus (187,188). The very high linear energy transfer (LET) radiation associated with the Auger electrons in the cell is comparable to that of α-emitters (189). The high doses of radiation delivered to the cell nucleus are able to cause DNA fragmentation and ultimately cell death (190-192). Decay of an Auger electron emitting radioisotope outside the cell or even on the cell membrane delivers an insignificant radiation dose to the cell nucleus (193,194) and is not radiotoxic. The selective radiotoxicity of Auger electron emitters only for cells which can bind and internalize the radioisotope is of particular interest for targeted radiotherapy of cancer.
since this unique property could in theory minimize or even eliminate the non-specific radiotoxicity against bone marrow stem cells which was previously observed with the use of β-emitters in radioimmunotherapy.

The most extensively studied Auger electron emitting radiopharmaceutical is iodine-125 iododeoxyuridine (\(^{125}\text{I}-\text{UdR}\)), a radiolabelled thymidine analog which is imported into cells using nucleoside transporters (195) and is incorporated directly into DNA during S-phase (185,188). Chan et al. (196) demonstrated that \(^{125}\text{I}-\text{UdR}\) was highly radiotoxic in vitro to Chinese hamster V79 fibroblasts with only very small amounts (<3 pCi/cell; <111 mBq/cell) required to reduce the surviving fraction in vitro to <1%. The survival curve also had no shoulder, a characteristic of high LET radiation. \(^{125}\text{I}-\text{UdR}\) has limitations as a radiotherapeutic agent however due to its relative lack of specificity for tumour cells, targeting of cells only in S-phase and its extensive deiodination in the liver. Nevertheless, \(^{125}\text{I}-\text{UdR}\) is currently being investigated for the treatment of bladder cancer (197), gliomas (198) and hepatic metastases (199), where normal tissue uptake can be minimized by local administration.

The Auger electron emitter, \(^{111}\text{In}\) has also been shown to be radiotoxic to cells when internalized (200). \(^{111}\text{In-oxine}\) is a lipophilic chelate which diffuses non-specifically into cells and is used in nuclear medicine for radiolabelling of leukocytes for imaging of infection. The use of \(^{111}\text{In-oxine}\) for this purpose is controversial however since the radiopharmaceutical has been shown to cause DNA damage to lymphocytes (eg. gaps, breaks and exchanges in chromosomes) and is potentially radiotoxic and mutagenic to the cells (201,202). Ten Berge et al. (201) demonstrated that <1 pCi/cell (<37 mBq/cell) of \(^{111}\text{In-oxine}\) decreased the proliferation of lymphocytes in response to several different mitogens by 70-95%. \(^{111}\text{In-oxine}\) has also been shown to be radiotoxic at very low amounts (<5-10 pCi/cell; <185-370 mBq/cell) to hematopoietic stem cells (200), fibroblasts (194,203), sperm heads (204) and cervical carcinoma cells (205). \(^{111}\text{In-oxine}\) cannot be used as a radiotherapeutic agent however because it internalizes non-specifically into both normal and malignant cells. Nevertheless, these observations suggest that \(^{111}\text{In}\) could be a very effective Auger electron emitter for the treatment of
cancer if the radioisotope could be specifically targeted and internalized into malignant cells.

As previously described in Chapter 1 (section 1.6), overexpression of the EGFR at levels up to 100 times higher than that observed on normal epithelial cells has been observed in approximately 30-60% of human breast cancers (84,88,90-92). EGFR overexpression is an attractive target for the design of novel anti-cancer therapies because it is present in almost all ER-negative and hormone-resistant, advanced forms of the disease (88,90,97) and is also associated with a poor prognosis (84,104) (see Chapter 1, section 1.8). Such patients are candidates for systemic chemotherapy but response rates to current regimens are inadequate and long term survival is poor (8). There is an urgent need to develop new alternative treatment strategies for these patients. The study described in this chapter demonstrates for the first time that a novel radiopharmaceutical, namely human epidermal growth factor (hEGF) radiolabelled with the Auger electron emitter $^{111}$In, is selectively radiotoxic to human breast cancer cells overexpressing the EGFR. The radiopharmaceutical specifically binds to the breast cancer cells, internalizes into the cytoplasm and is partially translocated to the cell nucleus. These properties combined with the emission of the Auger electrons rendered the radiopharmaceutical lethal to the breast cancer cells. The findings are promising for the application of targeted Auger electron radiotherapy using $^{111}$In-hEGF for the treatment of hormone-resistant, advanced forms of breast cancer overexpressing the EGFR.

4.2 MATERIALS AND METHODS

4.2.1 Radiolabelling of epidermal growth factor and binding to breast cancer cells

hEGF (Upstate Biotechnology, Lake Placid, NY) was derivatized with DTPA and radiolabelled with $^{111}$In to a specific activity of 3.7-11.1 MBq/µg (22,200-66,600 MBq/µmol) as described in Chapter 2 (section 2.2.1). The radiochemical purity of $^{111}$In-DTPA-hEGF was 95-98%. MDA-468 and MCF-7 human breast cancer cells were obtained and cultured as previously described in Chapter 2 (section 2.2.2). $^{111}$In-DTPA-hEGF was shown (section 2.3.1) to bind specifically and with high affinity to the MDA-
468 and MCF-7 cells (Ka of 7.5 X 10^8 and 3.9 X 10^9 L/mol respectively). There were 1.3 ± 0.7 X 10^6 receptors/cell for ^111^In-DTPA-hEGF on the MDA-468 cells and 1.5 ± 0.7 X 10^4 receptors/cell on the MCF-7 cells (see Chapter 2 section 2.3.1).

4.2.2 Derivatization of hEGF with fluorescein

hEGF was derivatized with fluorescein using a previously published method (59). Briefly, hEGF (100 μg, 10 μL) in 100 mM sodium bicarbonate buffer pH 9 was reacted with 80 μL of a 1 mg/mL solution of fluorescein isothiocyanate (FITC, Pierce Chemical Co., Rockford, IL) in 100 mM sodium bicarbonate buffer pH 9 in a glass Reacti-Vial (Pierce Chemical Co., Rockford, IL). The molar ratio of FITC:hEGF in the reaction was 12:1. The reaction mixture was incubated at room temperature in the dark for 1 hour, then the fluorescein-hEGF was purified from excess fluorescein by size-exclusion chromatography on a P-2 (Bio-Rad, Mississauga, ON, Canada) mini-column eluted with 150 mM sodium chloride. The absorbance of the eluted fractions was measured at 495 nm and the fractions eluting in the void volume of the column containing the pure fluorescein-hEGF were pooled. Purified fluorescein-hEGF was stored in amber polypropylene tubes at -10 °C.

4.2.3 Fluorescence microscopy of breast cancer cells

The intracellular localization of hEGF in the MDA-468 human breast cancer cells was qualitatively evaluated by fluorescence microscopy using fluorescein-hEGF and the fluorescent nuclear stain, 4’6’-diamidino-2-phenylindole dihydrochloride (DAPI, Boehringer-Mannheim, Laval, PQ, Canada). Approximately 3 X 10^4 MDA-468 cells were seeded onto a chamber slide (Nunc, Life Technologies, Burlington, ON, Canada) and cultured for 48 hours. The adherent cells were then washed 3 times with 150 mM sodium chloride and the slide was incubated in 100 nM fluorescein-hEGF for 1 hour at 37 °C. The fluorescein-hEGF solution was then removed and the slide was washed an additional 3 times with 150 mM sodium chloride. In order to visualize the cell nucleus, the slide was then incubated with 100 nM DAPI for 10 minutes at 37 °C, followed by washing 3 times with 150 mM sodium chloride. The slides were fixed in 0.8% glutaraldehyde (Sigma, St.
Louis, MO) and examined using a fluorescence microscope with excitation in the ultraviolet range for DAPI (340-380 nm) or the blue visible range for fluorescein (470-490 nm).

4.2.4 Intracellular localization of $^{111}$In-DTPA-hEGF in breast cancer cells

The intracellular localization of $^{111}$In-DTPA-hEGF was quantitatively determined at selected times up to 24 hours of incubation with MDA-468 human breast cancer cells. $^{111}$In-DTPA-hEGF (5 ng) was dispensed into 35 mm culture dishes containing 3 X $10^6$ MDA-468 cells in 1 mL of 0.1% w/v HSA in 150 mM sodium chloride. The dishes were incubated for 0.25, 0.5, 1, 2, 3, 4 or 24 hours at 37 °C. The cells were then transferred to tubes and centrifuged at 9,000 rpm (8,600 x g) for 5 minutes. The proportion of $^{111}$In radioactivity which was bound to the cells at the selected times was determined by separating the cell pellet from the supernatant and counting each in a γ-scintillation counter (Auto Gamma model 5650, Packard Instruments, Downer’s Grove, IL). The proportion of $^{111}$In radioactivity which was internalized by the cells was determined as previously described for $^{125}$I-EGF and dextran-conjugated $^{125}$I-EGF by Olsson et al. (206) by re-suspending and incubating the cell pellet in 1 mL of 200 mM sodium acetate/500 mM sodium chloride pH 2.5 at 4 °C for 5 minutes. The tubes were then centrifuged again to separate the internalized $^{111}$In radioactivity (cell pellet) from the non-internalized $^{111}$In radioactivity (supernatant).

Nuclear localization of $^{111}$In-DTPA-hEGF in the MDA-468 human breast cancer cells at the selected times was determined as previously described for $^{125}$I-EGF by Rakowicz-Szulczynska et al (207). The MDA-468 cells were first incubated with $^{111}$In-DTPA-hEGF as described above, then the cells were transferred to tubes and centrifuged at 600 x g for 10 minutes to separate the cell pellet from the supernatant. The cell pellet was resuspended in a buffer containing 350 mM sucrose/10 mM KCl/1.5 mM MgCl$_2$/10 mM Tris hydrochloride pH 7.6 with 0.2% Triton X-100 (BioRad, Mississauga, ON, Canada) and 12 mM 2-mercaptoethanol and the cells were disrupted by ultrasonication for 5 minutes in an ultrasonic bath (EMC Model 150, RAI Research). The cell nuclei were then isolated by centrifugation of the cell suspension at 2,700 rpm (900 x g) for 10
minutes. The quality of the procedure for isolation of the cell nuclei was evaluated in a separate experiment by conducting the procedure using MDA-468 cells pre-stained with the fluorescent nuclear stain, DAPI (Boehringer-Mannheim, Laval, PQ, Canada) and examining the nuclear preparation by fluorescence microscopy. Well-defined, intact nuclei without contamination by cytoplasmic organelles or portions of the cell membrane were observed by fluorescence microscopy.

Chromatin was isolated from the nuclei of the MDA-468 cells as previously described by Rakowicz-Szulcynska et al. (207) by first washing the nuclear pellet with a buffer containing 200 mM sucrose/3 mM calcium chloride/50 mM Tris hydrochloride pH 7.6, then with a buffer containing 140 mM sodium chloride/10 mM Tris hydrochloride pH 8.3 and centrifuging at 2,700 rpm (900 x g) for 10 minutes. The supernatant containing the nucleoplasmic proteins was separated from the nuclear pellet. The nuclei were then swelled in a small amount of 1 mM Tris hydrochloride pH 7.9 and centrifuged at 4,000 rpm (1,700 x g) for 10 minutes to separate the chromatin fraction from the supernatant which contained the nucleoplasm and nuclear membranes.

### 4.2.5 Growth inhibition of human breast cancer cells in vitro by \(^{111}\text{In-DTPA-hEGF}\)

The effect of treatment \textit{in vitro} with \(^{111}\text{In-DTPA-hEGF}\) on the growth in culture of MDA-468 or MCF-7 human breast cancer cells was determined in a growth inhibition assay. The breast cancer cells were recovered from a 75 cm\(^2\) culture flask by trypsinization with trypsin/EDTA (Gibco-BRL, Life Technologies, Burlington, ON, Canada) and then approximately 2 X 10\(^7\) breast cancer cells in 1 mL of 150 mM sodium chloride were dispensed into a sterile 35 mm culture dish. \(^{111}\text{In-DTPA-hEGF}\) (5 µg, 11.1 MBq) representing a 20-fold or 2,500-fold molar excess for MDA-468 or MCF-7 cells respectively, was then added to the dish and the dish was incubated at 37 °C for 30 minutes. The treated breast cancer cells were then separated from the incubation medium by transferring the cells to a sterile polystyrene culture tube and centrifuging at 1,000 rpm (600 x g) for 5 minutes. The amount of \(^{111}\text{In}\) radioactivity associated with the cell pellet was assayed in a radioisotope calibrator (Model CRC-12, Capintec, Montvale, NJ) and divided by the number of treated cells to calculate the average amount of \(^{111}\text{In}\)
radioactivity targeted to each cell (mBq/cell). The cells were then resuspended in growth medium and 5 X 10^5 cells were seeded into six replicate 60 mm culture dishes and the cells were cultured for a period of 7 days. Control dishes contained untreated cells, cells treated with unlabelled DTPA-hEGF (5 μg) or cells incubated with growth medium containing a concentration of ^111^In-DTPA (11 kBq/mL, DraxImage, Dorval, PQ, Canada) equivalent to the amount of ^111^In-DTPA-hEGF bound to the treated cells. At 1 and 7 days, cells were recovered from dishes using trypsin/EDTA and counted in a hemocytometer. The average number of cells recovered at the two time points and the average growth index was determined and compared for treated and untreated cells. The growth index was defined as the number of cells recovered at day 7 divided by the number of cells recovered at day 1. Both the treated and control dishes contained cells which were subconfluent throughout the 7 day experiment.

4.2.6 Radiotoxicity of ^111^In-DTPA-hEGF against human breast cancer cells in vitro

The radiotoxicity of ^111^In-DTPA-hEGF against MDA-468 or MCF-7 human breast cancer cells was determined in a clonogenic assay. Increasing amounts of ^111^In-DTPA-hEGF (37 kBq-2.6 MBq, 0.01-70 μg) were dispensed into sterile 35 mm culture dishes containing 5 X 10^6 MDA-468 or MCF-7 cells in 1 mL of 150 mM sodium chloride. The dishes were incubated for 30 minutes at 37 °C then the cells were recovered and assayed for the average amount of ^111^In-DTPA-hEGF targeted to each cell (mBq/cell) as previously described in section 4.2.5. Sufficient cells were then seeded in triplicate into 60 mm sterile culture dishes (Nunclon, Life Technologies, Burlington, ON) to obtain a measureable number of colonies after culturing for a period of 10 days at 37 °C. Control dishes contained cells incubated with growth medium alone. At the end of the culture period, the growth medium was removed, the dishes were washed 3 times with 150 mM sodium chloride and the colonies were stained with methylene blue (1% in a 1:1 mixture of ethanol and water). After staining, the dishes were washed twice with 150 mM sodium chloride, air dried and the number of colonies in each dish were counted using a manual colony counter (Manostat Corp., New York, NY). The plating efficiency was calculated by dividing the number of colonies observed in each dish by the number of cells seeded.
The surviving fraction was calculated by dividing the plating efficiency for the dishes containing treated cells by the plating efficiency for the control dishes.

The survival curves for the treated MDA-468 or MCF-7 breast cancer cells were obtained by plotting the log of the mean surviving fraction for the triplicate dishes versus the amount of $^{111}$In radioactivity targeted to the entire cell, internalized into the cytoplasm or associated with the chromatin fraction (mBq/cell). The amount of $^{111}$In radioactivity internalized into the cytoplasm or associated with the chromatin was estimated as 70% and 10% of the total $^{111}$In radioactivity bound to the cells respectively (see Results section. Table 4.2). A straight line was then fitted through each of the survival curves by log-linear regression analysis. The intercept on the ordinate n, the slope and the amount of cell-bound radioactivity corresponding to a radiation absorbed dose which reduced the surviving fraction to 0.37 ($D_0$ values) were estimated from the regression lines.

4.2.8 Evaluation of the hepatotoxicity and renal toxicity of $^{111}$In-DTPA-hEGF

To evaluate the potential for acute hepatotoxicity and renal toxicity of $^{111}$In-DTPA-hEGF, groups of normal Balb/c mice were injected in the tail vein with 3.7, 9.25, 18.5 or 44 MBq of $^{111}$In-DTPA-hEGF in 100 μL of 150 mM sodium chloride. The administered amount of $^{111}$In-DTPA-hEGF corresponded on a MBq/m² basis to human amounts of 740-8,510 MBq (surface area of a mouse is 0.009 m² compared to 1.73 m² in a human (208)). Control mice received a tail vein injection of 150 mM sodium chloride. Prior to injection of $^{111}$In-DTPA-hEGF and at 24, 48 and 72 hours post-injection, blood samples were obtained by knicking the tails of the mice with a sterile scalpel blade and collecting 50 μL samples into heparinized capillary tubes (Microcaps, Fisher Scientific Co., Nepean, ON). The blood samples were transferred to microcentrifuge tubes and centrifuged at 10,000 rpm (10,600 x g) for 5 minutes to separate the plasma. The plasma samples were transferred to microcentrifuge tubes and stored at -10 °C for subsequent measurement of alanine aminotransferase (ALT) concentrations by standard clinical biochemistry techniques. At 72 hours post-injection, the mice were sacrificed by cervical dislocation and samples of the liver and kidneys were removed, sectioned and examined by light and electron microscopy for morphological evidence of hepatotoxicity or renal
toxicity. The time period selected for measurement of the ALT levels was based on that previously found to be informative for drug toxicity to the liver (eg. acetaminophen toxicity) (209) but Wordsworth et al. (210) have also shown that increases in serum ALT levels occur within a few days in rats administered amounts of $^{198}$Au colloid calculated to deliver 30-110 Gy to the liver. Maximum increases in serum ALT following administration of $^{198}$Au colloid occurred at about 6-8 weeks post-injection (210) and ALT was a more sensitive indicator of radiotoxicity to the liver from $^{198}$Au colloid than aspartate aminotransferase (AST) levels (210).

The potential for hepatotoxicity and renal toxicity of $^{111}$In-DTPA-hEGF over a longer time period was investigated by administering two separate amounts of the radiopharmaceutical to athymic mice (37 and 74 MBq) by tail vein injection separated by an interval of 4 weeks. The administered radioactivity corresponded on a MBq/m² basis to human amounts of 7,104 MBq and 14,208 MBq of $^{111}$In-DTPA-hEGF. Blood samples were collected prior to the first administration of radioactivity, then every 3-4 days and the plasma was separated and analysed for ALT and creatinine concentrations as previously described. The mice were sacrificed at 7 weeks after the first administration of the radiopharmaceutical and the liver and kidneys were removed, sectioned and examined by light and electron microscopy for morphological evidence of hepatotoxicity or renal toxicity. The observation period in this study should have been sufficient to detect at least early evidence of morphological damage to the liver since Hebard et al. (211) found that the hepatic injury following administration of high amounts of $^{198}$Au colloid to rats occurred in two phases: an early phase occurring within the first 2-12 weeks involving fibrosis and infiltration of inflammatory cells into the liver and a late phase occurring at 16-40 weeks manifested by severe vasculature damage. Animal studies were conducted under an approved Animal Care Protocol (# 94-036) at the Toronto Hospital and following the Canadian Council on Animal Care (CCAC) guidelines.

4.2.9 Statistical analysis. Statistical comparisons were made using Student’s t-test (p<0.05).
4.3 RESULTS

4.3.2 Fluorescence microscopy of breast cancer cells

Fluorescence microscopy of MDA-468 human breast cancer cells incubated with fluorescein-hEGF showed binding to the cell surface, internalization into cytoplasmic vesicles and partial translocation to the cell nucleus. After 1 hour of incubation with fluorescein-hEGF at 37 °C, a relatively low level of fluorescence was observed diffusely throughout the cytoplasm of the cells with a more intense ring of fluorescence surrounding the cell nucleus (Fig. 4.1 A). The cell nucleus was also clearly visualized using the fluorescent nuclear stain, DAPI (Fig. 4.1 B) which diffuses non-specifically into the cells and binds by intercalation to double-stranded DNA (212).

4.3.3 Intracellular localization of $^{111}$In-DTPA-hEGF in breast cancer cells

$^{111}$In-DTPA-hEGF was rapidly bound and internalized by the MDA-468 breast cancer cells in vitro at 37 °C (Table 4.1). About 42% of the $^{111}$In-DTPA-hEGF was bound by the cells within 15 minutes of incubation but this increased to about 56% at 4 hours. At 15 minutes, approximately 70% of the $^{111}$In-DTPA-hEGF remained bound to the cells after acid-washing with a pH 2.5 buffer and was presumed to be internalized. The fraction of internalized $^{111}$In-DTPA-hEGF increased slightly to about 80% by 24 hours. A small proportion of the $^{111}$In-DTPA-hEGF which was bound to the MDA-468 breast cancer cells was also localized in the cell nucleus and associated with the chromatin fraction. After 30 minutes of incubation, approximately 7% of the $^{111}$In-DTPA-hEGF was localized in the cell nucleus and 2.5% was associated with the chromatin fraction (Table 4.2). After 24 hours, more than 15% of the $^{111}$In-DTPA-hEGF was localized in the cell nucleus and almost 10% was associated with the chromatin fraction.

4.3.4 Growth inhibition of human breast cancer cells in vitro by $^{111}$In-DTPA-hEGF

Incubation of approximately 2 X $10^7$ MDA-468 or MCF-7 human breast cancer cells in vitro with $^{111}$In-DTPA-hEGF (5 μg, 11.1 MBq) resulted in targeting of an average
Fig. 4.1. Fluorescence microscopy of MDA-468 human breast cancer cells incubated with fluorescein-hEGF for 1 hour at 37 °C (A) and with the nuclear stain, DAPI (B). Fluorescein-hEGF was rapidly internalized into the cytoplasm of the MDA-468 cells and formed a ring surrounding the cell nucleus. The cell nucleus was well visualized using DAPI.
## TABLE 4.1

Kinetics of binding and internalization of $^{111}$In-DTPA-hEGF in MDA-MB-468 human breast cancer cells.

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>$^{111}$In-DTPA-hEGF bound to MDA-MB-468 cells (%) $^a$</th>
<th>Proportion of cell-bound $^{111}$In-DTPA-hEGF internalized by MDA-MB-468 cells (%) $^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.25</td>
<td>41.6 ± 6.8</td>
<td>66.9 ± 2.9</td>
</tr>
<tr>
<td>0.5</td>
<td>42.9 ± 7.6</td>
<td>66.2 ± 3.7</td>
</tr>
<tr>
<td>1</td>
<td>45.6 ± 8.8</td>
<td>67.4 ± 2.4</td>
</tr>
<tr>
<td>2</td>
<td>46.2 ± 9.1</td>
<td>73.1 ± 1.5</td>
</tr>
<tr>
<td>3</td>
<td>48.1 ± 10.0</td>
<td>73.0 ± 2.7</td>
</tr>
<tr>
<td>4</td>
<td>42.8 ± 8.4</td>
<td>78.3 ± 2.2</td>
</tr>
<tr>
<td>24</td>
<td>56.1 ± 7.4</td>
<td>78.6 ± 7.5</td>
</tr>
</tbody>
</table>

$^a$ Mean ± s.e.m. of 3-6 experiments.
TABLE 4.2

Kinetics of nuclear localization of $^{111}$In-DTPA-hEGF in MDA-468 breast cancer cells.

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Proportion of cell-bound $^{111}$In-DTPA-hEGF bound to the cell nucleus (%)$^a$</th>
<th>Proportion of cell-bound $^{111}$In-DTPA-hEGF associated with the chromatin (%)$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>7.2 ± 0.8</td>
<td>2.5 ± 1.4</td>
</tr>
<tr>
<td>1</td>
<td>8.2 ± 0.5</td>
<td>2.9 ± 0.9</td>
</tr>
<tr>
<td>2</td>
<td>7.1 ± 2.2</td>
<td>3.4 ± 1.1</td>
</tr>
<tr>
<td>4</td>
<td>8.2 ± 1.0</td>
<td>3.6 ± 0.6</td>
</tr>
<tr>
<td>24</td>
<td>15.5 ± 2.1</td>
<td>9.6 ± 1.3</td>
</tr>
</tbody>
</table>

$^a$ Mean ± s.e.m. of 3 experiments.
of 45-60 mBq of $^{111}$In-DTPA-hEGF to each cell. The majority of the $^{111}$In-DTPA-hEGF targeted to the MCF-7 cells was likely non-specifically bound since a maximum of 3.7 mBq of $^{111}$In-DTPA-hEGF could in theory be targeted to the cells at the receptor saturation conditions employed taking into account the specific activity of the radiopharmaceutical (22,200 MBq/μmol) and the receptor expression level of the cells ($1.5 \times 10^4$ EGFR/cell). Non-specifically bound $^{111}$In-DTPA-hEGF would not be expected to undergo receptor-mediated internalization and therefore should not be radiotoxic to the cells.

There was a significant decrease in the growth index of the MDA-468 cells treated with $^{111}$In-DTPA-hEGF compared to the untreated cells (1.3 ± 0.1 versus 3.7 ± 0.2 respectively, p<0.0001, Table 4.3). MDA-468 cells treated with unlabelled DTPA-hEGF also exhibited a significantly lower growth index compared to the untreated cells but the magnitude of the decrease was smaller (2.5 ± 0.2 versus 3.7 ± 0.2 respectively, p<0.01). It has been previously shown that MDA-468 cells are growth inhibited by concentrations of EGF >$10^{-9}$ M (213). The concentration of unlabelled DTPA-hEGF in the incubation medium in the growth inhibition assay was approximately $8 \times 10^{-7}$ M (i.e. 5 μg in 1 mL) but the cells were only exposed to this concentration for a very brief period of time (30 minutes). Nevertheless, this may explain the decreased growth index for the MDA-468 cells treated with unlabelled DTPA-hEGF. The growth index of MDA-468 cells treated with $^{111}$In-DTPA, a radiopharmaceutical which does not bind to the cells or internalize non-specifically, was not significantly different from the growth index of the untreated cells (3.1 ± 0.1 versus 3.7 ± 0.6 respectively, p = 0.169). There was also no significant change in the growth index of MCF-7 cells treated with $^{111}$In-DTPA-hEGF compared to untreated cells (1.7 ± 0.7 versus 2.5 ± 0.5 respectively, p = 0.193).

4.3.5 Radiotoxicity of $^{111}$In-DTPA-hEGF against human breast cancer cells in vitro.

The surviving fraction of the MDA-468 breast cancer cells treated in vitro with $^{111}$In-DTPA-hEGF was reduced to <3% when the cells were targeted with up to 130 mBq/cell of $^{111}$In-DTPA-hEGF (Fig. 4.2). Log-linear regression analysis of the survival
TABLE 4.3

Inhibition of the growth of human breast cancer cells by treatment in vitro with $^{111}$In-DTPA-hEGF.

<table>
<thead>
<tr>
<th>Treatment:</th>
<th>MDA-468 Cells</th>
<th>MCF-7 Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>None</td>
<td>DTPA-hEGF</td>
</tr>
<tr>
<td>1 day</td>
<td>2.9 ± 0.2</td>
<td>3.5 ± 0.2</td>
</tr>
<tr>
<td>7 days</td>
<td>10.6 ± 0.9</td>
<td>8.6 ± 0.3</td>
</tr>
<tr>
<td>Growth Index</td>
<td>3.7 ± 0.2</td>
<td>2.5 ± 0.2</td>
</tr>
</tbody>
</table>

$a$ Mean ± s.e.m. of 3-6 replicates.

$b$ Number of cells recovered at day-7 divided by number of cells recovered at day-1.

$c$ Significantly different from untreated cells ($p = 0.008$).

$d$ Not significantly different from untreated cells.

$e$ Significantly different from untreated cells ($p < 0.0001$).
Survival measured in a clonogenic assay for MDA-468 (●) or MCF-7 (○) human breast cancer cells treated in vitro with $^{111}$In-DTPA-hEGF. Targeting of an average of 130 mBq of $^{111}$In-DTPA-hEGF to each MDA-468 cell reduced the surviving fraction to <3%. There was no significant decrease in the surviving fraction of MCF-7 cells maximally targeted with $^{111}$In-DTPA-hEGF (133 mBq/cell). Also shown is the survival curve for MDA-468 cells adjusted for the proportion of $^{111}$In-DTPA-hEGF present in the cytoplasm (●) or bound to chromatin (■). The error bars represent the s.e.m. of the surviving fraction in triplicate dishes which were plated (error bars not visualized are due to a small s.e.m. which is obscured by the data point).
curve for radioactivity targeted to the entire cell exhibited a straight line ($r = 0.946$) with an intercept on the ordinate ($n$) of 1.06, a slope of -0.0114 cell.mBq$^{-1}$ and an amount of radioactivity (40 mBq/cell) corresponding to $D_0$. The survival curve adjusted for radioactivity associated with the chromatin (i.e. 10% of the total radioactivity bound to the cells) as expected exhibited a straight line with a slope of -0.114 cell.mBq$^{-1}$ and an amount of radioactivity (4 mBq/cell) corresponding to $D_0$. The survival curve adjusted for the proportion of radioactivity localized in the cytoplasm (i.e. 70% of total radioactivity bound to the cells) exhibited a straight line with a slope of -0.0163 cell.mBq$^{-1}$ and an amount of radioactivity (28 mBq/cell) corresponding to $D_0$.

There was no significant decrease in the surviving fraction of MCF-7 cells when up to 133 mBq/cell of $^{111}$In-DTPA-hEGF was bound to the cells (Fig. 4.2). At low amounts of $^{111}$In-DTPA-hEGF bound to the MCF-7 cells (<20 mBq/cell), there actually appeared to be a slight growth stimulatory effect. As previously described in the growth inhibition assay, the majority of the $^{111}$In-DTPA-hEGF bound to the MCF-7 cells was probably non-specifically bound since a maximum of 3.7 mBq of $^{111}$In-DTPA-hEGF could in theory be specifically targeted to each cell at receptor saturation given the level of EGFR expression on the cells (1.5 X $10^4$ EGFR/cell) and the specific activity of the radiopharmaceutical used in the experiment (22,200 MBq/μmol). Non-specifically bound $^{111}$In-DTPA-hEGF would not be expected to undergo receptor-mediated internalization and should not be radiotoxic.

4.3.6 Evaluation of the hepatotoxicity and renal toxicity of $^{111}$In-DTPA-hEGF

The ALT concentration in the plasma of Balb/c mice administered increasing amounts of $^{111}$In-DTPA-hEGF (3.7-44 MBq) at selected times after injection of the radiopharmaceutical is shown in Fig. 4.3. Only at the highest amount of radioactivity administered (44 MBq) was there a slight rise in ALT concentration compared to pre-administration values. The increase in plasma ALT concentration at this amount of radioactivity was most evident at 72 hours post-injection. However, at the lower amounts of administered radioactivity (3.7-18.5 MBq), there was no significant difference in plasma ALT concentration compared to the pre-administration values at any of the time
Fig. 4.3. ALT concentrations in the plasma of Balb/c mice prior to tail vein injection (PRE) and at different times after the injection of increasing amounts of $^{111}$In-DTPA-hEGF (3.7-44 MBq). Only at the highest administered amount of radioactivity was there a slight rise in ALT concentrations at 72 hours post-injection of the radiopharmaceutical.
Fig. 4.4. Electron micrographs of the liver (A) and kidneys (B) at 7 weeks after tail vein injection to athymic mice of two separate amounts of $^{111}$In-DTPA-hEGF (37 and 74 MBq) separated by a four week time interval. There was no evidence of morphological damage to the liver such as mitochondrial changes, proliferation of smooth endoplasmic reticulum or lysosomes or steatosis. Similarly in the kidney there was no apparent morphological damage to the renal tubular structures.
points evaluated. Light and electron microscopy of the liver and kidneys at 72 hours after administration of $^{111}$In-DTPA-hEGF also did not reveal any acute morphological damage caused by the radiopharmaceutical at any of the doses studied (not shown). The hepatocytes did not exhibit any morphological features commonly associated with acute hepatocellular injury such as irregularly shaped mitochondria, fatty infiltration or proliferation of smooth endoplasmic reticulum or lysosomes (209).

There was also no significant change in ALT or plasma creatinine concentration compared to the pre-treatment values over the course of a 7 week period in athymic mice administered two high amounts of $^{111}$In-DTPA-hEGF (37 and 74 MBq) separated by a 4 week time interval. The plasma ALT concentration ranged from 18-46 U/L for treated mice and from 16-40 U/L for control mice (normal values are <40 U/L in humans). The plasma creatinine concentration ranged from 60-78 μmol/L for treated mice and 60-66 μmol/L for control mice (normal values are <105 μmol/L in humans). Light and electron microscopy (Fig. 4.4) of the liver and kidneys at 7 weeks also did not reveal any morphological damage over the time interval evaluated caused by the administration of high amounts of the radiopharmaceutical.

4.4 DISCUSSION

The results of this study demonstrated for the first time that hEGF conjugated with DTPA and radiolabelled with the Auger electron emitting radionuclide, $^{111}$In was selectively radiotoxic to human breast cancer cells which overexpress the EGFR. $^{111}$In-DTPA-hEGF was rapidly bound by the breast cancer cells, internalized into the cytoplasm and partially translocated to the cell nucleus. It is hypothesized that the internalization and nuclear translocation of $^{111}$In-DTPA-hEGF delivered the radionuclide to a proximity with the chromosomal DNA at which the emitted Auger electrons were lethal to the cells. The radiotoxicity of $^{111}$In-DTPA-hEGF in vitro was manifested by a significantly decreased growth rate of MDA-468 human breast cancer cells ($>10^6$ EGFR/cell) at relatively low amounts of radioactivity targeted to the cells (45-60 mBq/cell) and almost a two-log decrease in the survival of the cells in a clonogenic assay.
at higher amounts (130 mBq/cell). No radiotoxicity was observed against MCF-7 human breast cancer cells which exhibited a 100-fold lower level of EGFR expression (approximately $10^4$ receptors/cell) suggesting that the radiopharmaceutical was selectively radiotoxic to cancer cells which overexpress the EGFR. Over a 7 week observation period in mice administered high amounts of $^{111}$In-DTPA-hEGF, there was no evidence of radiotoxicity to normal tissues such as the liver and kidneys which exhibit moderate-high levels of EGFR expression (58,110). These results are promising for the ultimate application of targeted Auger electron radiotherapy using $^{111}$In-DTPA-hEGF for the treatment of EGFR-overexpressing breast cancer in humans.

The internalization and nuclear translocation of hEGF in MDA-468 human breast cancer cells was evaluated by fluorescence microscopy using fluorescein-hEGF. Rapid binding of fluorescein-hEGF to the MDA-468 cells was observed followed by internalization into the cytoplasm of the cells and translocation of the hEGF to form a ring of fluorescence surrounding the cell nucleus. As previously discussed in Chapter 1 (section 1.5), the internalization of the EGF/EGFR complex after binding of EGF to its receptor is well known (214) and is believed to play a role in modulating the effects of receptor activation through down-regulation of receptor expression and by intracellular degradation of internalized EGF in lysosomes. Nuclear translocation of internalized EGF has also been observed by fluorescence microscopy and by other techniques in A431 epidermoid carcinoma cells (59), SW948 human colon cancer cells (207), MDA-231 human breast cancer cells (74), fibroblasts (70), hepatocytes (73) and rat pituitary cells (72). The proportion of internalized EGF molecules which translocated to the cell nucleus normally ranged from 1-10% (71,72,215) but this increased to 14-40% under certain growth stimulatory conditions (70,73) or in the presence of lysosomal protease inhibitors (72). It is not known if EGF remains bound to its receptor during the nuclear translocation process but it has been recognized that the EGFR contains a putative nuclear localization sequence (RRRHIVRKRRTLRR) at residues 645-657 which could mediate nuclear translocation (75). Specific EGFR binding sites on chromatin have also been detected (71). The role of nuclear translocation of EGF is not currently understood
since as previously described in Chapter 1, EGF is thought to affect gene expression indirectly by activation of the \textit{ras} intracellular signaling pathway (61).

$^{111}$In-DTPA-hEGF was rapidly bound and internalized by the MDA-468 human breast cancer cells \textit{in vitro} at 37 °C, with more than 70% of the radiopharmaceutical internalized within 2 hours (Table 4.1). There was also a relatively high localization of $^{111}$In-DTPA-hEGF in the cell nucleus ranging from approximately 7% at 2 hours to >15% at 24 hours. Interestingly, up to 10% of the radioactivity bound to the cells (almost two thirds of the radioactivity found in the cell nucleus) was directly associated with the chromatin fraction, suggesting that internalized $^{111}$In-DTPA-hEGF (perhaps bound to the EGFR) may interact directly with the nuclear DNA. Similar results have been previously observed for $^{125}$I-EGF by Rakowicz-Smleczynska et al. (207) in SW948 human colorectal carcinoma cells which express the EGFR. After incubation of the SW948 cells with $^{125}$I-EGF for 24 hours at 37 °C, >94% of the internalized radioactivity was localized in the cytoplasm and about 6% was present in the cell nucleus. The majority of the $^{125}$I radioactivity which was localized in the cell nucleus was directly associated with the chromatin fraction.

The decay of $^{111}$In results in the emission of two \textit{\textgamma}-photons (172 and 247 keV) and a cascade of Auger and conversion electrons (216). Approximately 14-15 electrons are released with each decay of $^{111}$In and almost all of the electrons (>99%) are characterized by an average energy of <30 keV and a maximum range in tissue of <9-12 \textmu m (<1 cell diameter) (204,216). The extremely short, subcellular range of the electrons requires internalization of the radionuclide into the cytoplasm and ideally translocation to the cell nucleus in order to deposit sufficient energy in the nucleus to cause DNA damage and radiotoxicity to the cell. Cellular dosimetry models (193,217) of Auger electron emitting radionuclides deposited in mammalian cells have estimated that the energy deposited in the cell nucleus is approximately 9-fold greater when $^{111}$In is localized in the cytoplasm compared to a situation where the radionuclide is localized on the cell membrane and 14-fold higher when the radionuclide is localized in the nucleus itself.

Chromosomal DNA is the radiosensitive target in the cell for the radiotoxic effects of the Auger electrons emitted by $^{111}$In. The cellular distribution of $^{111}$In-DTPA-
hEGF, i.e. the amount localizing in the cell membrane, cytoplasm and nucleus will control the radiation dose delivered to the nucleus and consequently the radiotoxicity of the radiopharmaceutical. Based on the distribution of $^{111}$In-DTPA-hEGF in the MDA-468 breast cancer cells at 24 hours after incubation with the radiopharmaceutical, i.e. 20% bound to the cell membrane, 70% internalized into the cytoplasm and 10% localized in the cell nucleus (Table 4.2), the radiation dose to the entire cell and to the cell nucleus was estimated using a previously described cellular dosimetry model (193). The estimated radiation absorbed dose to each MDA-468 cell when targeted to receptor saturation with $^{111}$In-DTPA-hEGF (specific activity of 3.7 MBq/μg) was 24.7 Gy with 14.5 Gy delivered to the cell nucleus (Table 4.4). Interestingly, although only 10% of the $^{111}$In-DTPA-hEGF was localized in the cell nucleus, this fraction accounted for >70% of the total radiation dose to the nucleus. The $^{111}$In-DTPA-hEGF in the cytoplasm was responsible for an additional 25% of the radiation dose to the nucleus but the 20% of the radiopharmaceutical remaining bound to the cell membrane accounted for <5% of the radiation dose to the cell nucleus. This highlights the importance of the extremely short, subcellular range of the Auger electrons emitted by $^{111}$In, and the necessity to deliver the radiopharmaceutical as close as possible to its biological target, i.e. the chromosomal DNA, in order to manifest a radiotoxic effect on the cell. Targeting of the MDA-468 cells to receptor saturation with $^{111}$In-DTPA-hEGF (specific activity of 3.7 MBq/μg) would deliver approximately $1.7 \times 10^5$ atoms of $^{111}$In to each cell based on a receptor expression of $1.3 \times 10^6$ EGFR/cell. The estimated radiation absorbed dose to a single MDA-468 human breast cancer cell from the decay of each $^{111}$In atom would therefore be 0.145 mGy/decay.

The relative radiotoxicity against the MDA-468 cells of $^{111}$In-DTPA-hEGF localized in different cellular compartments could not be measured directly since the radiopharmaceutical is distributed into multiple compartments (i.e. cell membrane, cytoplasm and nucleus). Nevertheless, by adjusting the survival curves for the proportion of the radiopharmaceutical bound to chromatin, present in the cytoplasm or distributed evenly throughout the entire cell, a comparison could be made of the potential differences in radiosensitivity of the cells which may occur when the radiopharmaceutical is localized
TABLE 4.4

Radiation absorbed dose estimates \(^a\) to the cell nucleus from \(^{111}\text{In-DTPA-hEGF}\) localized in various compartments in a MDA-468 human breast cancer cell \(^b\).

<table>
<thead>
<tr>
<th>Cell compartment</th>
<th>(\bar{\lambda}) (Bq.sec) (^c)</th>
<th>(S) (Gy/Bq.sec (\times 10^{-4}))</th>
<th>(\bar{D}) (Gy)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Membrane</td>
<td>3,357</td>
<td>1.78</td>
<td>0.60</td>
</tr>
<tr>
<td>Cytoplasm</td>
<td>10,917</td>
<td>3.18</td>
<td>3.47</td>
</tr>
<tr>
<td>Nucleus</td>
<td>2,522</td>
<td>60.30</td>
<td>15.21</td>
</tr>
<tr>
<td></td>
<td><strong>Total:</strong></td>
<td></td>
<td><strong>19.28</strong></td>
</tr>
</tbody>
</table>

\(^a\) The cellular radiation dosimetry model of Goddu et al. J. Nucl. Med. 35: 303-316, 1994 was used to estimate the radiation absorbed dose (\(\bar{D}\)) to the cell nucleus: \(\bar{D} = \bar{\lambda} \cdot S\) where \(S\) is the radiation absorbed dose in the nucleus (Gy) per unit of cumulated radioactivity in the source compartment, \(\bar{\lambda}\) (Bq.sec).

\(^b\) Based on targeting a single MDA-MB-468 human breast cancer cell with a diameter of 10 \(\mu\)m and a nucleus with a diameter of 6 \(\mu\)m to receptor saturation with \(^{111}\text{In-DTPA-hEGF}\). At concentrations of the radioligand leading to receptor saturation, approximately 48 mBq of \(^{111}\text{In-DTPA-hEGF}\) would be bound to each MDA-MB-468 cell at a specific activity of 3.7 MBq/\(\mu\)g.
Assumes the rapid localization of $^{111}$In-DTPA-hEGF in a compartment and a rate of elimination corresponding to the radioactive decay of the radionuclide, indium-111.

$\tilde{A} = A_0 / \lambda$, where $\lambda$ is the radioactive decay constant for $^{111}$In ($2.83 \times 10^{-6}$ sec$^{-1}$).
in these compartments. The adjusted survival curves showed that there could be a 6-10 fold greater radiosensitivity of the MDA-468 cells when \(^{111}\text{In-DTPA-hEGF}\) is bound to the chromatin (amount of radioactivity corresponding to \(D_0\) of 3 mBq/cell, Fig. 4.2) compared to a situation where the radiopharmaceutical is localized in the cytoplasm (amount of radioactivity corresponding to \(D_0\) of 18 mBq/cell) or evenly distributed in the entire cell (amount of radioactivity corresponding to \(D_0\) of 30 mBq/cell). The projected radiosensitivity of the MDA-468 cells to \(^{111}\text{In-DTPA-hEGF}\) bound to chromatin was also very similar to that previously reported for Chinese hamster V79 fibroblasts treated with \(^{125}\text{I-UdR}\), i.e. amount of radioactivity corresponding to \(D_0\) of 1.3 mBq/cell (188). This finding suggests that \(^{111}\text{In-DTPA-hEGF}\) could exhibit radiotoxicity which is comparable to \(^{125}\text{I-UdR}\) against mammalian cells when bound to DNA, but unlike \(^{125}\text{I-UdR}\) the radiopharmaceutical is selectively targeted to cancer cells which overexpress the EGFR.

Other forms of radiolabelled EGF have also been shown to be radiotoxic to cancer cells which overexpress the EGFR. Capala et al. (218) demonstrated that the surviving fraction of a subclone of the U-343-MG human glioma cell line could be reduced almost 60% by targeting of approximately 1.3 dpm/cell (~37 mBq/cell) of \(^{131}\text{I-EGF}\) to the cells in vitro. However, almost half of the radiotoxicity observed for the U-343-MG cells was not due to the \(^{131}\text{I-EGF}\) directly bound to the cells but rather due to the non-specific effects from the excess \(^{131}\text{I-EGF}\) present in the incubation medium due to the relatively long range (2 mm) of the \(\beta\)-particles. A similar decrease in the surviving fraction of the U-343-MG cells could also be obtained using \(^{131}\text{I-bovine serum albumin}\) or by incubating the cells in \(^{131}\text{I-EGF}\) after first saturating the receptors on the cells with non-radioactive EGF. The relatively long-range of the \(\beta\)-particles from \(^{131}\text{I-EGF}\) could potentially cause radiotoxicity to normal cells which are not targeted by the radiopharmaceutical, analogous to the situation previously observed for \(^{131}\text{I-mAbs}\) in radioimmunotherapy (219). \(^{111}\text{In-DTPA-hEGF}\) would therefore offer a major advantage over \(^{131}\text{I-EGF}\) for targeted radiotherapy of breast cancer since the extremely short, subcellular range of the Auger electrons emitted by \(^{111}\text{In}\) would render the radiopharmaceutical exquisitely selective only for cells which express the EGFR and are able to specifically bind and internalize the radiopharmaceutical.
Most normal tissues express very low levels of the EGFR ($<10^4$ receptors/cell) and should not be adversely effected by targeted radiotherapy of breast cancer using $^{111}$In-DTPA-hEGF. The radiopharmaceutical could be radiotoxic however to the liver (58) and the kidneys (110) which exhibit moderate-high levels of the EGFR ($\sim 10^5$ receptors/cell). Over a 7-week observation period there was no evidence of radiotoxicity to the liver or kidneys in mice administered high amounts of $^{111}$In-DTPA-hEGF (equivalent to human amounts up to 14,208 MBq). There were no significant increases in the concentration of ALT (Fig. 4.3) or creatinine in the plasma and there was no necrosis in the liver or kidneys by histopathological examination of the tissues by light and electron microscopy (Fig. 4.4). Histologically, hepatotoxicity would be manifested by subtle mitochondrial changes or proliferation of the smooth endoplasmic reticulum or lysosomes (209) and renal toxicity would be manifested by renal tubular degeneration (220). These morphological changes were not observed on the electron micrographs of the liver or kidneys at either the early (3 days) or later (7 weeks) time points. It is nevertheless possible that very late radiation injury to the liver or kidneys could become evident at time points beyond 7 weeks. It is also possible that more subtle forms of liver radiotoxicity resulting from administration of $^{111}$In-DTPA-hEGF may not be seen until the hepatocytes attempt cell division, a process which could require a long period of time for a normally quiescent tissue such as the liver. The bone marrow is the most radiosensitive and dose-limiting normal tissue in radioimmunotherapy of cancer using mAbs labelled with $\beta$-emitters such as $^{131}$I or $^{90}$Y (219). The radiotoxicity of $^{111}$In-DTPA-hEGF against bone marrow stem cells was not measured in this study but myelotoxicity is not anticipated since $<$3% of the bone marrow stem cell population has been found to express the EGFR (112) and the radiopharmaceutical must specifically bind and internalize into the cells in order to exert a radiotoxic effect.

Since $^{111}$In also emits two $\gamma$-photons (172 and 247 keV) in addition to the Auger and conversion electrons, the radiopharmaceutical can also be used to image and characterize breast cancer lesions with respect to EGFR expression, as previously described in Chapters 2 and 3. The results of such imaging procedures performed with relatively low doses of the radiopharmaceutical could be used to select patients for
targeted Auger electron radiotherapy with much higher doses. However, as previously pointed out in Chapter 3, the uptake of $^{111}$In-hEGF in human breast cancer xenografts overexpressing the EGFR in athymic mice was very low and modifications to the hEGF molecule to improve its tumour uptake would be required for both imaging and radiotherapeutic applications (discussed further in Chapter 5). Based on the accumulation of the radiopharmaceutical observed in MDA-468 human breast cancer xenografts implanted in athymic mice (i.e. ~2% i.d./g, Chapter 3, Table 3.2) administration of the maximum dose of $^{111}$In-DTPA-hEGF evaluated in this study for hepatotoxicity or renal toxicity (i.e. 74 MBq) would deliver approximately 6-12 mBq of $^{111}$In to each tumour cell at a specific activity of 3.7-7.4 MBq/µg. This amount of radioactivity would only reduce the surviving fraction of the MDA-468 cells in vivo by about 30% based on the survival curve for the cells treated in vitro with the radiopharmaceutical (Fig. 4.2). Improvements in the tumour uptake of $^{111}$In-DTPA-hEGF in vivo would therefore be required for effective radiotherapy of breast cancer.

4.5 CONCLUSIONS

In conclusion, it was shown that the novel radiopharmaceutical, $^{111}$In-DTPA-hEGF, was selectively radiotoxic in vitro to human breast cancer cells which overexpress the EGFR. There was no evidence of radiotoxicity in vivo against normal tissues such as the liver or kidneys which exhibit moderate-high levels of EGFR expression over a 7-week observation period in mice administered very high amounts of the radiopharmaceutical. Although, there is still the possibility that a longer observation period may demonstrate radiotoxicity against these normal tissues, the results are nevertheless encouraging for the application of $^{111}$In-DTPA-hEGF for targeted Auger electron radiotherapy of advanced breast cancer overexpressing the EGFR in humans.
Chapter 5

SUMMARY AND FUTURE DIRECTIONS
5.1 Thesis conclusions

The overall conclusions of the research described in this thesis are:

1) Targeting the overexpression of the EGFR on breast cancer cells using highly specific radiopharmaceuticals is potentially a very sensitive means for the diagnostic imaging of breast cancer.

2) Radiopharmaceuticals targeted to the EGFR which exhibit a longer residence time in the blood than EGF accumulate to a greater extent in breast cancer nodules and would be advantageous for imaging and radiotherapeutic applications.

3) The normal biological processing of EGF after binding to its receptor can be effectively exploited to deliver the Auger electron emitter, $^{111}$In into the cytoplasm and nucleus of breast cancer cells for targeted radiotherapy of the disease.

In Chapter 2, using phantoms of breast cancer lesions, it was shown that as few as 50,000 breast cancer cells could be detected by imaging using hEGF radiolabelled with $^{111}$In. It was also determined that the sensitivity of tumour imaging with $^{111}$In-hEGF was reduced by factors such as the level of receptor expression by the breast cancer cells, the extent of receptor heterogeneity, attenuation by overlying normal tissues and the proportion of cancer cells targeted by the radiopharmaceutical. Nevertheless, the results from the phantom studies argue that under optimized conditions, imaging of breast cancer using radiopharmaceuticals specifically targeted to the EGFR would be able to detect small numbers of EGFR-positive breast cancer cells in humans.

The critical factor for imaging breast cancer however appears to be the extent of delivery of the radiopharmaceutical from the blood to the tumour nodule, which is in turn controlled by its pharmacokinetic properties. In Chapter 3, it was shown that the localization of anti-EGFR mAb 528 radiolabelled with $^{111}$In in subcutaneous human breast cancer xenografts implanted in athymic mice was 10-times greater than that
observed for $^{111}$In-hEGF, despite a slightly lower receptor binding affinity in vitro. The images of the breast cancer xenografts were also much clearer using $^{111}$In-mAb 528 than with $^{111}$In-hEGF. Evaluation of the tumour uptake of the two radiopharmaceuticals in breast cancer xenografts with widely different levels of EGFR expression showed that tumour uptake was controlled to a greater extent by their pharmacokinetic properties than by the level of receptor expression by the breast cancer cells. In all cases, the accumulation of $^{111}$In-mAb 528 in the breast cancer xenografts was much greater than that of $^{111}$In-hEGF. These results illustrate that in certain situations, radiolabelled macromolecules such as mAbs would be more effective tumour imaging agents than peptide growth factors. As previously proposed in Chapter 3, one hypothesis to explain the greater tumour uptake of $^{111}$In-mAb 528 is that the longer residence time of macromolecules in the blood compared to peptide growth factors allows a greater period of time for diffusion of the radiopharmaceutical from the blood into the tissues and binding to the cancer cells in the tumour nodule.

The rapid internalization of hEGF into breast cancer cells after binding to its receptor and its partial translocation to the cell nucleus were exploited in the study described in Chapter 4 to deliver $^{111}$In into the cytoplasm and nucleus of the cancer cells using $^{111}$In-hEGF. The emission of Auger electrons by $^{111}$In at close proximity to the nuclear DNA was likely responsible for the high radiotoxicity of the radiopharmaceutical observed against the breast cancer cells. The radiopharmaceutical was selectively radiotoxic to MDA-468 human breast cancer cells which have high levels of EGFR expression compared to MCF-7 breast cancer cells with a low level of EGFR expression. In mice administered high doses of the radiopharmaceutical, there was no evidence of radiotoxicity over a 7-week observation period against normal tissues such as the liver and kidneys which exhibit moderate to high levels of EGFR expression. Late toxicity to these tissues is still possible but these results are nevertheless encouraging for the application of $^{111}$In-hEGF for targeted Auger electron radiotherapy of EGFR-positive advanced forms of breast cancer in humans. Improvements in the tumour uptake of the radiopharmaceutical will be necessary however to increase the delivery of radiation to the cancer cells and achieve a satisfactory therapeutic response in vivo.
5.2 Improving the tumour uptake of hEGF in breast cancer

Since a macromolecule such as mAb 528 (M_r ~ 150 kDa) exhibited much greater accumulation in human breast cancer xenografts hosted in athymic mice than a peptide such as hEGF (M_r ~ 6 kDa), one approach to improving the tumour uptake of \( ^{111} \text{In-hEGF} \) may be to prolong its residence time in the blood through increasing its molecular size. Increasing the molecular size of hEGF would decrease its renal elimination rate and prolong the residence time in the blood. A longer residence time in the blood was previously identified in Chapter 3 as a major factor responsible for the higher tumour uptake of \(^{111} \text{In-mAb 528} \) compared to \(^{111} \text{In-hEGF} \). Several approaches are possible for increasing the molecular size of hEGF including chemical conjugation with a macromolecule such as dextran (discussed in section 5.2.1) or production of an hEGF fusion protein by recombinant DNA technology (discussed in section 5.2.2).

5.2.1 Dextran-EGF

Westermark et al. (57) described a method for conjugating mEGF to dextran by first activating the dextran molecule with 1-cyano-4-dimethylamino-pyridinium tetrafluoroborate (CDAP) and then reacting the activated dextran with mEGF. A pilot study was conducted to evaluate this approach for increasing the molecular size of EGF and to determine the influence of conjugation with dextran on the elimination rate from the blood and on tumour and normal tissue accumulation. In order to minimize the possibility of cross-linking of multiple activated dextran molecules with a single EGF molecule, murine EGF (mEGF) which has only a single site for conjugation of the activated dextran (i.e. the \( \alpha \)-amino group) was selected for derivatization instead of hEGF. Dextran with an average nominal molecular weight of approximately 20 kDa (Sigma Chemical Co., St. Louis, MO) was activated and conjugated to \(^{125} \text{I-mEGF} \) as previously described by Westermark et al. (57) to produce a high molecular weight dextran-\(^{125} \text{I-EGF} \) species. The dextran-\(^{125} \text{I-mEGF} \) conjugate was purified from free \(^{125} \text{I-mEGF} \) by size-exclusion chromatography on a Sephadex G-25 mini-column. The EGF concentration was then determined by measuring the absorbance of the dextran-\(^{125} \text{I-mEGF} \) preparation at 280 nm (\( E_{1%}^{1%} = 30.9 \)). An ultraviolet spectrum of unconjugated
dextran did not show any absorbance at a wavelength of 280 nm (not shown) and the dextran moiety therefore did not interfere with measuring the mEGF concentration in dextran-mEGF. SDS-PAGE of the dextran-\textsuperscript{125}I-mEGF (Fig. 5.1) demonstrated a broad, diffuse band on the gel suggesting that several dextran-mEGF species with different molecular sizes (~14-100 kDa) were present in the preparation. The heterogeneous size distribution of dextran-mEGF was unexpected and prompted an analysis of the dextran raw material used to derivatize the mEGF. The dextran was radiolabelled with \textsuperscript{99m}Tc according to Dass et al. (221) and analysed by filtration through ultrafiltration devices (Centricon-3, -10, -50 and -100, Amicon, Beverly, MA) with molecular weight cut-offs ranging from 3 kDa to 100 kDa. This analysis (not shown) revealed that the dextran raw material with a nominal average $M_t$ of 20 kDa contained mostly molecules in the range of 30-100 kDa but a small proportion of molecules were also outside this size range. The heterogeneity in size distribution of the dextran-mEGF could therefore be due to the heterogeneous size distribution of the dextran raw material used for conjugation or to reaction of multiple mEGF molecules with several activated sites on a single dextran molecule. The presence of multiple dextran-EGF species would be a problem for the ultimate application of this approach for modifying the properties of hEGF since each species could conceivably exhibit a different binding affinity for the receptor and also considerably different pharmacokinetic properties. Nevertheless, the dextran-\textsuperscript{125}I-mEGF preparation competed specifically with \textsuperscript{111}In-hEGF for binding \textit{in vitro} to MDA-468 breast cancer cells with an estimated $K_d$ of $4 \times 10^4$ mols/L compared to a $K_d$ of $2 \times 10^8$ for mEGF (Fig. 5.2).

A comparative biodistribution study of the dextran-\textsuperscript{125}I-mEGF conjugate preparation with \textsuperscript{125}I-mEGF in athymic mice bearing subcutaneous MDA-468 breast cancer xenografts (Table 5.1) showed that the tumour and normal tissue uptake of both radiopharmaceuticals at 72 hours post-injection were much lower than that previously observed for \textsuperscript{111}In-hEGF (Table 3.6). As discussed earlier in Chapter 3, radioiodinated EGF is metabolized \textit{in vivo} to free radioiodine and radioiodotyrosine which are then rapidly excreted from the body by the kidneys. The conjugation of dextran to EGF has been reported to stabilize \textsuperscript{125}I-EGF to intracellular lysosomal degradation (206), but
extensive \textit{in vivo} deiodination is nevertheless one possible explanation for the very low tissue retention of dextran-$^{125}\text{I}$-mEGF and $^{125}\text{I}$-mEGF in the animals. If the dextran-EGF conjugate could be radiolabelled with a radiometal such as $^{111}\text{In}$, the resulting radiopharmaceutical would be more stable \textit{in vivo} (previously discussed in Chapter 3), and a longer residence time in the blood and improved tumour uptake compared to EGF may be observed. Radiolabelling of the dextran-mEGF conjugate with $^{111}\text{In}$ was not possible since the DTPA chelator for $^{111}\text{In}$ must be attached at the same site as the dextran molecule, i.e. the $\alpha$-amino group of mEGF. Another limitation for conjugation of EGF with dextran is that it appeared to result in a 20-fold increase in liver uptake compared to EGF (Table 5.1). The increased liver uptake of dextran-$^{125}\text{I}$-mEGF may be due to interaction of the dextran moiety with Fc receptors on hepatocytes, since $^{14}\text{C}$-dextran has also been previously shown to exhibit receptor-mediated uptake by the liver (222).

![Figure 5.1](image.png)

\textbf{Fig. 5.1.} SDS-PAGE of dextran-$^{125}\text{I}$-mEGF (lanes 1 and 2), mEGF (lane 3) and molecular weight markers (lane 4) on a 4-20% Tris/glycine gel stained with coomassie blue G-250. $^{125}\text{I}$-mEGF migrates as a single band with an apparent $M_r$ of $\approx 6$ kDa. Dextran-$^{125}\text{I}$-mEGF migrates as a broad band with a $M_r$ range of 14-100 kDa.
Fig. 5.2. Competitive displacement of the binding of $^{115}$In-DTPA-hEGF to MDA-468 human breast cancer cells by mEGF (●) or dextran-mEGF (○). The abscissa for dextran-mEGF is the concentration of mEGF (nM) in the preparation. The Kd for mEGF was $2 \times 10^8$ mols/L and for dextran-mEGF was $4 \times 10^8$ mols/L.
TABLE 5.1

Comparison of the biodistribution of $^{125}$I-mEGF and dextran-$^{125}$I-mEGF in athymic mice bearing subcutaneous MDA-468 human breast cancer xenografts at 72 hours post-injection.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>$^{125}$I-mEGF</th>
<th>Dextran-$^{125}$I-mEGF</th>
<th>$^{125}$I-mEGF</th>
<th>Dextran-$^{125}$I-mEGF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood</td>
<td>0.05 ± 0.01</td>
<td>0.04 ± 0.01</td>
<td>2.57 ± 0.64</td>
<td>2.13 ± 0.32</td>
</tr>
<tr>
<td>Heart</td>
<td>0.03 ± 0.04</td>
<td>0.04 ± 0.01</td>
<td>3.94 ± 1.32</td>
<td>2.18 ± 0.23</td>
</tr>
<tr>
<td>Lungs</td>
<td>0.06 ± 0.01</td>
<td>0.06 ± 0.02</td>
<td>2.81 ± 0.25</td>
<td>2.27 ± 1.21</td>
</tr>
<tr>
<td>Liver</td>
<td>0.07 ± 0.01</td>
<td>1.32 ± 0.36</td>
<td>1.93 ± 0.54</td>
<td>0.07 ± 0.02</td>
</tr>
<tr>
<td>Kidneys</td>
<td>0.11 ± 0.01</td>
<td>0.30 ± 0.02</td>
<td>1.19 ± 0.36</td>
<td>0.27 ± 0.05</td>
</tr>
<tr>
<td>Spleen</td>
<td>0.04 ± 0.02</td>
<td>0.16 ± 0.03</td>
<td>1.62 ± 0.17</td>
<td>0.56 ± 0.18</td>
</tr>
<tr>
<td>Stomach</td>
<td>0.15 ± 0.04</td>
<td>0.13 ± 0.03</td>
<td>0.65 ± 0.17</td>
<td>0.66 ± 0.18</td>
</tr>
<tr>
<td>Intestine</td>
<td>0.10 ± 0.04</td>
<td>0.10 ± 0.02</td>
<td>1.51 ± 0.30</td>
<td>0.89 ± 0.31</td>
</tr>
<tr>
<td>Tumour</td>
<td>0.13 ± 0.04</td>
<td>0.08 ± 0.01</td>
<td>$b$ na</td>
<td>na</td>
</tr>
</tbody>
</table>

$^a$ Mean ± s.e.m. of 3 animals per experiment. $^b$ na: not applicable.
5.2.2 An EGF-immunoglobulin fusion protein

An alternate strategy for increasing the molecular size of hEGF would be to construct a hEGF-immunoglobulin fusion protein using recombinant DNA technology. The elimination rate of immunoglobulins from the blood is controlled by their molecular size. For example, the relative rates of elimination from the blood of an intact IgG mAb and its F(ab')2, Fab or Fv fragments (molecular weights of 150, 100, 50 and 30 kDa respectively) are Fv, Fab >> F(ab')2 >> IgG. Therefore, fusion of hEGF with an appropriately sized immunoglobulin sequence may be one means of slowing the elimination of hEGF from the blood and potentially improving its tumour uptake. This concept has been previously applied to other targeting molecules which are also known to be cleared rapidly from the blood. For example, Capon et al. (223) fused soluble CD4 receptor (Mr ~41 kDa) to the complete constant (Cn) domain of IgG1 (Mr ~36 kDa) to create a dimeric fusion protein with a Mr of ~112 kDa. The CD4-Cn receptor fusion protein retained its affinity for binding to the ligand (gp120) but exhibited a much longer plasma half-life in rabbits than the native, soluble CD4 receptor (48 h versus 15 minutes respectively). Similarly, Hu et al. (224) fused two anti-CEA mAb scFv fragments (Mr of ~30 kDa) through a flexible peptide linker to a single constant domain of IgG1 (Cn3, Mr ~12 kDa) to create a novel antibody fusion protein ("minibody") with a Mr of ~80 kDa. The minibody radiolabelled with 125I exhibited a prolonged residence time in the blood compared to the scFv form and the tumour uptake in athymic mice bearing subcutaneous LS174T colon cancer xenografts was increased more than 7-fold (29 vs. 4 % i.d./g).

In the ultimate design of an hEGF fusion protein, it is important to achieve a reasonable balance between prolonging the residence time of hEGF in the blood and preserving its tumour penetration properties. Since hEGF is a peptide (Mr ~6 kDa), it would be expected to cross the vascular endothelial barrier easily and exhibit very good penetration into a tumour nodule. However, as previously described in Chapter 3, the relatively small molecular size of hEGF results in rapid elimination from the blood into the urine and low tumour uptake. A macromolecule (eg. an IgG mAb, Mr ~150 kDa) would exhibit a much longer residence time in the blood and higher tumour uptake but its poor tissue penetration properties would restrict its diffusion into the tumour nodule.
Inadequate delivery to the deeper regions of the tumour nodule would present a potential difficulty, particularly for the use of such a delivery vehicle for targeted Auger electron radiotherapy of EGFR-positive breast cancer, since the mechanism of radiotoxicity requires that each cell be specifically targeted by the radiopharmaceutical. The optimum size for an hEGF-immunoglobulin fusion protein is therefore one which would prolong the residence time in the blood sufficiently to lead to increased tumour uptake but would also preserve its ability to penetrate deeply into tumour nodules. The optimum molecular size is not known but likely lies in the range of ~30-100 kDa. For example, Milenic et al. (225) demonstrated that the tumour uptake of radioiodinated CC49 mAb fragments in athymic mice bearing LS174T colon cancer xenografts was increased 20-fold using an F(\(\text{ab}^\prime\))\(_2\) fragment (\(M_r \sim 100\) kDa) compared to an Fv fragment (\(M_r \sim 30\) kDa). However, the Fv fragment exhibited deeper penetration than the F(\(\text{ab}^\prime\))\(_2\) fragment into the LS174T tumour nodules (226).

One approach to creating such an hEGF-immunoglobulin fusion protein would be to fuse the gene for hEGF to the gene for one or more C\(_\text{H}1\) domains of IgG (\(M_r \sim 12\) kDa). If a single C\(_\text{H}1\) domain were fused, the resulting fusion protein would have a molecular weight of ~\(18\) kDa in its monomeric form and \(36\) kDa in its dimeric form. If two C\(_\text{H}1\) domains were fused to the hEGF gene, the fusion protein would have a molecular weight of \(30\) kDa as a monomer and \(60\) kDa as a dimer. The two different hEGF fusion proteins could be produced, radiolabelled and their elimination rates from the blood and tumour uptake compared in mice bearing human breast cancer xenografts in order to select the optimum tumour targeting agent. The tumour penetration properties of the radiolabelled hEGF fusion proteins could also be evaluated and compared by performing microautoradiography of the breast cancer xenografts recovered from the animals.

Two other important issues in the production of an hEGF fusion protein are: i) the ability of the recombinant protein to specifically bind to the EGFR and ii) its ability to internalize into EGFR-positive breast cancer cells. The latter issue is critical if the hEGF fusion protein is intended for targeted Auger electron radiotherapy of breast cancer. These properties would likely be preserved after fusion of hEGF to an appropriately sized macromolecule since EGF and TGF\(\alpha\) have already been successfully fused with cell
toxins (M, ~40 kDa) (121,227) or a subunit of streptavidin (M, ~15 kDa) (228) without significant decreases in receptor binding affinity and these fusion proteins readily internalize into cancer cells expressing the EGFR. Furthermore, as previously described in section 5.2.1, conjugation of mEGF with dextran (M, ~20 kDa) resulted in only a 2-fold decrease in binding affinity for the EGFR on MDA-468 breast cancer cells (Fig. 5.3). Nevertheless, it may be necessary to insert a flexible peptide linker between the EGF molecule and the immunoglobulin sequence in order to preserve binding affinity for the EGFR. For example, Ohno et al. (228) found that an EGF-streptavidin fusion protein could only bind biotin when a flexible peptide linker was inserted between the EGF and streptavidin moieties.

One of the advantages of the fusion protein approach to improving the pharmacokinetic properties of hEGF is that the C,1 domain could also function as a carrier for multiple 111In atoms. Each C,1 domain contains approximately 10 lysine residues which are potential sites for attachment of multiple DTPA chelating moieties for 111In, whereas hEGF contains only 3 such sites (K28, K48 and the α-amino group). In order to avoid substitution of DTPA onto the hEGF domain of the fusion protein (and therefore potential decreased receptor binding affinity) it may also be possible to perform site-directed mutagenesis on the hEGF gene to convert the two lysine residues to the corresponding residues in mEGF (i.e. K28→S28 and K48→R48). Mutation of these residues should not affect receptor binding since as previously described in Chapter 1 (section 1.4), both hEGF and mEGF bind equally to the human EGFR. Furthermore, Campion et al (45) observed that chemical modification of lysine residues in hEGF (K28/48→HomoCit) did not diminish receptor binding and site-directed mutagenesis of K28→L28 caused only a 25% reduction. Radiolabelling of the hEGF fusion protein with multiple 111In atoms would increase the specific activity of the radiopharmaceutical considerably and therefore deliver more radioactivity to each breast cancer cell. This should increase the sensitivity of the radiopharmaceutical for the detection of breast cancer lesions by γ-scintigraphy and also improve its efficacy for targeted Auger electron radiotherapy of the disease.
5.3 What is the clinical role of radiopharmaceuticals targeted to the EGFR in breast cancer?

The pre-clinical studies described in this thesis demonstrated that radiopharmaceuticals specifically targeted to the EGFR could potentially be very useful in breast cancer patients for: i) the detection and characterization of malignant lesions and ii) targeted Auger electron radiotherapy of advanced forms of the disease.

5.3.1 What is the clinical role of imaging for EGFR expression in breast cancer?

When the sensitivity of a tumour imaging procedure is defined as the minimum number of cancer cells detectable, imaging of the overexpression of the EGFR on breast cancer cells using specific radiopharmaceuticals is potentially a very sensitive means for the detection of malignancy, as previously described in Chapter 2. However, the clinical definition of sensitivity for a diagnostic test is the number of true positive studies divided by the number of individuals who present with the disease (i.e. true-positive + false-negative). Since EGFR overexpression has been detected on only 30-60% of human breast cancer biopsies (discussed in Chapter 1), the maximum theoretical sensitivity of an imaging procedure which targets EGFR overexpression in the general population of breast cancer patients would therefore be 30-60%. There would be a high proportion of “false-negative” studies in breast cancers which do not express the EGFR. This level of diagnostic sensitivity would not be sufficient for such an imaging procedure to be used as a primary diagnostic tool for breast cancer, i.e. to initially diagnose the disease or to stage the extent of disease in a patient. Mammography as well as several other nuclear medicine imaging procedures such as scintimammography with $^{99m}$Tc-sestamibi, bone scanning with $^{99m}$Tc-MDP, or PET with $^{18}$F-FDG have a much higher sensitivity (85-100%) for the detection of primary or metastatic breast cancer and would therefore be more appropriate in the diagnostic work-up of the patient. Nevertheless, imaging of EGFR overexpression in breast cancer could be a very useful complementary “functional” imaging procedure which would provide a method for the in vivo assessment of the EGFR expression status of a lesion.
Imaging of EGFR expression in breast cancer would likely be most useful for the assessment of metastatic disease since the EGFR expression of primary breast cancer can normally be relatively easily assessed in vitro by immunohistochemistry or by radioassay methods on a biopsy obtained by surgical or fine-needle methods. Nevertheless, imaging of primary lesions using radiopharmaceuticals targeted to the EGFR may have some advantages over biopsy techniques in that it evaluates the EGFR expression of the entire lesion rather than a sample of the lesion. This may circumvent problems associated with determining the EGFR expression in the context of receptor heterogeneity of the breast cancer cells.

Nuclear medicine imaging of primary breast cancer has been previously found to be helpful, particularly in cases where mammograms are difficult to interpret (e.g. women with radiographically dense breasts) but the sensitivity for imaging EGFR expression (maximum of 30-60%) would not be high enough to detect malignant lesions reliably. Nevertheless, since EGFR overexpression is inversely correlated with ER-expression and directly correlated with a lack of response to hormonal therapy with tamoxifen and a poor long-term survival (discussed in Chapter 1), “functional” imaging of EGFR overexpression could have prognostic value in the management of advanced breast cancer and may also be useful to decide on appropriate therapeutic strategies (i.e. chemotherapy vs. hormonal therapy) or to select patients for treatment with novel anti-EGFR agents. As previously discussed in Chapter 1, these novel anti-EGFR agents include anti-EGFR mAbs and EGF or TGFα-toxin fusion proteins as well as therapeutic radiopharmaceuticals specifically targeted to the EGFR (the subject of Chapter 4).

One could envision several different types of functional imaging procedures which could be performed on a breast cancer lesion after its initial detection by a high sensitivity primary diagnostic imaging test. Such functional imaging procedures would characterize the biochemical and physiological properties of the lesion and might include the evaluation of: i) the EGFR status of the lesion using a radiolabelled anti-EGFR mAb or a radiolabelled EGF fusion protein as proposed in section 5.2.2, ii) estrogen receptor status using 18F-FES, iii) glucose utilization using 18F-FDG or iv) pgp-170 expression using 99mTc-sestamibi. A knowledge of the functional properties of the tumour may assist
the oncologist in separating those patients with poor-prognosis breast cancer requiring aggressive treatment or treatment with novel agents from those whose tumours can be managed more conservatively or with traditional therapeutic agents.

5.3.2 What is the clinical role of targeted Auger electron radiotherapy of breast cancer?

Breast cancer patients with ER-negative forms of the disease do not respond to hormonal therapy with tamoxifen, have a particularly poor prognosis and need to be treated with systemic chemotherapy. The initial tumour response rate ranges from 50-75% but the efficacy of chemotherapy is ultimately limited by the high degree of toxicity to normal tissues and by the rapid development of the multi-drug resistance phenotype by the tumour. New therapeutic strategies are therefore urgently needed to improve the long-term survival and quality of life in these patients. As previously proposed in Chapter 1, one possible therapeutic approach is to target the relatively high expression of the EGFR on the surface of the cancer cells observed in ER-negative breast cancers. In Chapter 4, a novel method was described to deliver high doses of radiation selectively to EGFR-overexpressing breast cancer cells using EGF radiolabelled with the Auger electron emitting radionuclide, $^{111}$In. There are still issues to be resolved with respect to improving the tumour uptake of $^{111}$In-EGF in vivo (as discussed in section 5.2) and the potential radiotoxicity of the radiopharmaceutical to normal tissues such as the liver and kidneys which express moderate-high levels of EGFR. Nevertheless, targeted Auger electron radiotherapy using $^{111}$In-hEGF (or more likely an $^{111}$In-hEGF-immunoglobulin fusion protein as proposed in section 5.2.2) represents a promising novel therapeutic strategy for the treatment of ER-negative and hormone-resistant advanced forms of breast cancer which could improve the prognosis in this group of patients. It is likely that targeted Auger electron radiotherapy of EGFR-positive breast cancer would ultimately be used in combination with other therapeutic modalities (i.e. conventional chemotherapy, other novel anti-EGFR agents or other therapeutic radiopharmaceuticals) in order to achieve a greater rate of response and more durable remissions. Combination therapy may be particularly necessary since not all cancer cells may be targeted by the
radiopharmaceutical due to delivery problems or to receptor heterogeneity. As previously described in Chapter 1 (section 1.10) SMSR expression on breast cancer cells is inversely correlated with EGFR expression. It may be possible to combine $^{111}$In-DTPA-hEGF and $^{111}$In-pentetreotide for targeted Auger electron radiotherapy of breast cancer in order to address the issue of receptor heterogeneity and target a greater fraction of the tumour cells in the lesion. Krenning et al. (134) treated 20 patients with neuroendocrine tumours with up to 12 doses of 6,000-7,000 MBq of $^{111}$In-pentetreotide at 2-3 week intervals (total cumulative dose of 25,000 MBq). A temporary improvement in the clinical condition of the patients and in serum hormone levels and other tumour markers was noted as well as a stabilization of disease progression or in some cases a decrease in the size of the tumour was observed. Treatment with high dose $^{111}$In-pentetreotide was associated with thrombocytopenia and decreased lymphocyte counts however (134,135), suggesting that SMSR may be present on certain bone marrow stem cells. The clinical response observed in patients with neuroendocrine tumours treated with $^{111}$In-pentetreotide suggests that the radiopharmaceutical may also be effective in SMSR-positive breast cancer. However, in cell binding assays with $^{111}$In-pentetreotide, I found that the level of expression of SMSR on breast cancer cell lines (MDA-468, S1 and MCF-7) was relatively low (<3 X $10^4$ receptors/cell). This may limit the amount of radioactivity delivered to breast cancer cells in vivo and the effectiveness of targeted Auger electron radiotherapy using $^{111}$In-pentetreotide.

One of the requirements for targeted Auger electron radiotherapy is that the radiopharmaceutical must bind and internalize into a cancer cell in order to deliver a cytotoxic dose of radiation to the cell nucleus. Inadequate delivery of the radiopharmaceutical to all cancer cells in the tumour nodule could diminish the effectiveness of targeted Auger electron radiotherapy. One strategy to overcome this problem may be to combine Auger electron radiotherapy with other targeted radiopharmaceuticals radiolabelled with higher energy $\beta$-emitters such as $^{131}$I, $^{186}$Re, $^{188}$Re or $^{90}$Y (eg. $^{90}$Y-DOTA-octreotide (229)). The longer range of these $\beta$-emitting radionuclides (2-10 mm) delivers radiation to cancer cells which have not been directly targeted by the radiopharmaceutical but which are nevertheless in sufficient proximity to
targeted cells to absorb radiation from decays which occur on targeted cells ("cross-fire effect"). However, it should be recognized that the cross-fire effect can also result in non-specific irradiation of normal cells, including bone marrow stem cells as discussed in Chapter 4.

5.4 Future directions

Future research in this project will be to: i) produce an hEGF-immunoglobulin fusion protein by recombinant DNA techniques (as previously described in section 5.2.2), ii) radiolabel the fusion protein with multiple $^{111}$In atoms and test its binding, internalization and nuclear translocation in human breast cancer cells, iii) evaluate the pharmacokinetics and tumour and normal tissue localization of the $^{111}$In-labelled fusion protein in athymic mice bearing human breast cancer xenografts and iv) evaluate its radiotoxicity against breast cancer cells and normal tissues both in vitro and in vivo. The animal xenograft models of breast cancer also need to be improved to take into account the heterogeneity in EGFR expression commonly observed in human tumours and the metastatic nature of the disease.

This is the end of the thesis but.....

......only the beginning of the search for the answer.

REFERENCES


