Functional Dissociation of SAPK and Apoptosis in Epithelial and Endothelial Cells: Implications for Anoikis

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A thesis submitted in conformity with the requirements for the degree of Master of Science
Graduate Department of Medical Biophysics
University of Toronto

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ABSTRACT

Adhesion to the extracellular matrix is a crucial survival signal for epithelial and endothelial cells. The stress caused by detachment from the solid substratum activates an endogenous death program termed ‘anoikis’ in both cell types. The question then arises: does stress kill? The signaling events that culminate in anoikis are still unclear. Although recent studies have implicated Stress Activated Protein Kinase (SAPK), also known as Jun-N-Terminal kinase, as a potentially crucial signal transducer and mediator of anoikis, the generality and the causal role of SAPK in anoikis remains unclear and controversial. The work described here does not dispute that SAPK is activated by the loss of cell-ECM interactions, but serves to provide further support for the lack of causative effects this activation has in the overall phenomenon of anoikis. Ongoing studies investigating other possible regulators of anoikis have pointed to a number of different gene families. Foremost among them is the bcl-2 family, although newly discovered apoptotic regulators, such as the IAP family, may be involved in the anoikis abrogation of transformed cells. Overall, it would appear that anoikis regulation is a complex process regulated by numerous and possibly redundant signaling pathways, which may further exhibit cell specific variations.
ACKNOWLEDGEMENTS

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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>cdk</td>
<td>cyclin dependent kinase</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
</tr>
<tr>
<td>INK4</td>
<td>inhibitor of cdk4</td>
</tr>
<tr>
<td>KIP</td>
<td>kinase inhibitor proteins</td>
</tr>
<tr>
<td>IEC</td>
<td>intestinal epithelial cell</td>
</tr>
<tr>
<td>TGFα</td>
<td>transforming growth factor α</td>
</tr>
<tr>
<td>TGFβ</td>
<td>transforming growth factor β</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine tri-phosphate</td>
</tr>
<tr>
<td>bFGF</td>
<td>basic fibroblast growth factor</td>
</tr>
<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
</tr>
<tr>
<td>IGF-1</td>
<td>insulin like growth factor – 1</td>
</tr>
<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
</tr>
<tr>
<td>PDGF</td>
<td>platelet derived growth factor</td>
</tr>
<tr>
<td>PARP</td>
<td>poly-ADP-ribose-polymerase</td>
</tr>
<tr>
<td>IAP</td>
<td>inhibitor of apoptosis</td>
</tr>
<tr>
<td>TNF</td>
<td>tumour necrosis factor</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen associated protein kinase</td>
</tr>
<tr>
<td>FAK</td>
<td>focal adhesion kinase</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
<tr>
<td>ERK</td>
<td>epidermal related kinase</td>
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<tr>
<td>SAPK</td>
<td>stress activated protein kinase</td>
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<tr>
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<td>Description</td>
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<td>--------------</td>
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</tr>
<tr>
<td>SKK</td>
<td>SAPK kinases</td>
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<tr>
<td>MKK</td>
<td>MAPK kinases</td>
</tr>
<tr>
<td>JNK</td>
<td>Jun-N-terminal kinase</td>
</tr>
<tr>
<td>MDCK</td>
<td>Madin Darby Canine Kidney</td>
</tr>
<tr>
<td>FTI</td>
<td>farnesyl transferase inhibitor</td>
</tr>
<tr>
<td>PI3K</td>
<td>phosphatidylinositol 3-kinase</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>α-MEM</td>
<td>alpha-minimal essential medium</td>
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Chapter 1: Introduction

1.1 The Genetic Progression of Cancer

Cancer occurs when several of the growth and death regulating processes become dysfunctional at the cellular and tissue level. In terms of the overall control of growth and development, cellular abilities to either propagate or differentiate along a distinct developmental pathway and perform a specific function are regulated either intrinsically, or by interactions with other cells or the surrounding environment. These complex behaviours require an intricate and precise governing of innumerable cellular processes, including responses to the extracellular environment, communication, and the tight regulation of the processing of genetic information.

No doubt existing since the evolution of multicellular organisms, the earliest available evidence of cancer comes from tumorous lesions found on dinosaur skeletal remains (Pitot, 1993). Even the derivation of the term cancer itself from the Greek karkinos, translating as crab or creeping ulcer in reference to the swollen veins surrounding tumors, serves to illustrate early knowledge of this disease pathology. Throughout human history there exist numerous examples detailing not only the presence of human cancer, but also attempts at treatment (Ladik and Forner, 1994). With the advent of modern medicine, it is now realized that the combination of aging population demographics and better detection techniques make cancer increasingly prevalent. The World Health Organization estimated in 1995 that cancer was the cause of death for approximately six million people (Rennie & Rusting, 1996). This increasing pervasiveness of cancer has prompted enormous growth in research at both the levels of basic science and medical therapeutics.
No longer thought of as solely a cellular proliferative phenomenon, cancer is finally recognized as the final consequence of multiple genetic alterations accumulating over many years during the course of the disease, ultimately resulting in the overall abnormal and inappropriate growth and survival of cells (Kinzler & Vogelstein, 1996; Weinberg, 1996; Vogelstein & Kinzler, 1993). Although only recently definitively proven by molecular genetic analysis, this multistep nature was first described almost fifty years ago.

Just as initially proposed by Foulds in 1949, tumour development can still be thought to occur in three stages; initiation, promotion and progression (Foulds, 1954; Rennie & Rusting, 1996). Inherited and somatic mutations of cellular genes are the first step (initiation) and provide a growth advantage to tumour cells and their progeny ensuring tumour promotion. Analysis of the age-dependent appearance of a variety of tumour-types in human populations has served to reinforce the multistep pathogenesis model of cancer (Vogelstein & Kinzler, 1993). Tumour frequency generally increases sharply with age. This is consistent with the theory that most tumours are the end result of four, five, or six independent events, each with a low probability of occurrence and all necessary for tumour progression (Budillon, 1995). One single mutation appears insufficient to confer a transformed phenotype to cells. It is the combined effects of several mutations over time that will provide cells the growth advantage they need, initially for clonal expansion (promotion) and finally for angiogenesis and metastatic spread (progression).

Most mutations involve genes controlling cellular growth. Although mutations can belong to more than one group, most of these genes can be considered to be one of three main types: (1) proto-oncogenes, (2) tumour suppressor genes and (3) DNA repair genes, the latter resulting in a 'mutator' phenotype. The actual mutations themselves include (1) chromosomal rearrangements, exemplified by the Philadelphia translocation between chromosomes 9 and 22 in
chronic myelogenous leukemia, (2) gene amplification, such as the n-myc gene in neuroblastoma, (3) gene mutation, as demonstrated by the point mutation in codon 12 of the Ha-ras gene in bladder carcinoma and (4) DNA aneuploidy, found in chronic lymphocytic leukemia (Barrett et al., 1992; Budillon, 1995).

The genes normally responsible for basic cellular functions governing proliferation and differentiation, which upon mutation result in a transformed phenotype, are known as proto-oncogenes. This complex cell behaviour is regulated at numerous functional levels. Cells respond to external stimuli, such as soluble growth factors, that bind to cell surface receptors. This generally results in the activation of a transmembrane molecule, initiating signaling cascades that are inevitably localized to the nucleus. Here, regulatory proteins influence gene expression.

Mutated forms of proto-oncogenes become transforming oncogenes, and act in an autosomal dominant manner as positive regulators of cell growth, thereby overriding instructions for normal growth and proliferation. The first oncogene to be discovered was src in 1976, where it was observed in avian tissue that viral activation of the gene resulted in an overexpression of the gene product that was responsible for cellular transformation (Stehelin et al., 1976). Oncogenes may be grouped by the functional and biochemical properties of their protein products in the following categories; growth factors, growth factor receptors, signal transducers, transcription factors and apoptosis regulators (Hunter, 1997). More than 100 oncogenes have been identified in animal systems, however only in a handful of cases has a significant role in the formation of human malignancies been documented to date (Pines et al., 1997).

During the study of certain heredity diseases it was discovered that, unlike the dominant behaviour of oncogenes, some cancer causing mutations are recessive in nature. Dubbed
“tumour suppressor genes”, these genes do not override normal function with one mutation, instead becoming inactivated after two genetic mutations (or “hits”), reflecting in some way the loss or altered function of both alleles of a gene (Bishop, 1991; Hansen & Cavenee, 1988). Study of retinoblastoma, a rare childhood syndrome causing aggressive tumours of the retinae, led to the “two hit” hypothesis and the subsequent identification of the Rb1 tumour suppressor gene (Knudson, 1971; Comings, 1973). In familial retinoblastoma, the initial hit is inherited as a germline mutation, while the second hit occurs as a somatic event (e.g. chromosome loss) or mutation. In the last decade the molecular proof has been provided for numerous additional tumour suppressor genes, among them p53, APC, WTI and VHL genes (Yokota & Sugimura, 1993), some of which require only one hit to function as a dominant negative (e.g. p53). Extensively studied familial cancers such as retinoblastoma and Wilms’ tumour (corresponding to the WTI gene), a childhood cancer of the kidneys, have revealed the loss of heterozygosity at the gene locus (Hansen et al., 1985; Haber et al., 1990; Ton et al., 1991). Moreover, the reintroduction of wildtype cDNA into cell lines derived from both tumour types was found to reverse the neoplastic phenotype (Huang et al., 1988; Weissman et al., 1987). Together with others, these results have provided the best evidence that inactivation of these genes is critical to tumour initiation.

Activation of an oncogene or loss of a tumour suppressor gene in a cell will often result in altered growth characteristics. However, malignant cells are the end result of multiple genetic changes. Research in this area has uncovered increasingly diverse functions for tumour suppressor proteins and oncoproteins (Hunter, 1997). This diversity, in addition to the intricate interconnections between signaling, is indicative of the synergistic effect of several genetic changes as opposed to just one (Hunter, 1991). Burkitt’s lymphoma, a cancer of B cell
lymphocytes, is a well-characterized example of multiple genetic factors involved in malignancy. The primary genetic mutation is a chromosomal translocation resulting in activation of the c-myc proto-oncogene (Klein, 1989). Mutations of the p53 (Wiman et al., 1991; Farrell et al., 1991) and, to a lesser extent, the Rb1 (Adams & Cory, 1992) tumour suppressor genes have also been described in these tumours. Experiments in myc transgenic mice have further revealed that activation of c-myc alone is insufficient to induce B cell lymphoma (Adams et al., 1985), but that additional mutations are required. For example, it has been shown that myc and ras cooperate effectively together to accelerate tumour development in vivo (Alexander et al., 1989). Mutations in p53 have also been noted in B cell lymphomas from myc transgenic mice (Adams & Cory, 1992), demonstrating again that oncogenes can not only act cooperatively with other oncogenes, but also with tumour suppressor genes.

The complex networks between oncogenes and tumor suppressor genes govern cell growth and consequently, any mutations will have a direct effect. Conversely, DNA repair genes probably have only an indirect effect on growth. Problems in DNA repair will result in an increased rate of mutations in many other genes (Higgins et al., 1998). Whether a direct effector or an indirect facilitator, it is this accumulation of genetic alterations that is the driving force of an increasingly transformed phenotype.

It is within the context of the normal mechanisms of mitogenesis and survival that investigations can further identify and characterize the dysfunctions leading to cancer. Studies are focused not only on the individual cellular events of mitogenesis and survival, but also increasingly, on the cooperative behaviour of cell populations in tumour biology as a whole. There is much attention on the genes and their protein products, which regulate the malignant processes of angiogenesis, invasion, metastasis and drug resistance. The consequent scientific
and medical advances have already brought about declining mortality rates in the case of some, but not all of types of cancer, in most western nations (Gaudette et al., 1993; Wingo et al., 1998). However, further understanding of the biological processes that regulate cell behaviour is essential for both improving current treatment regimes, and for the creation of new cancer therapeutics.

1.2 Functional Cellular Changes Associated with Tumour Progression

1.2.1 Mitogenesis

In principle, cell numbers can be controlled during both development and adult life by events involving cell gain (mitosis) or cell loss (death), and in some organs even cell migration (Raff, 1996; Vaux & Korsmeyer, 1999). Any disruption in this careful regulation of population size will result in abnormal and potentially detrimental disease pathologies; either degenerative, as in the case of multiple sclerosis or amyelotrophic lateral sclerosis, or proliferative, exemplified by cancer.

Early descriptions of cancer were centered on the concept of uncontrolled cell proliferation. In fact, modern dictionaries remain coloured by what was once the predominant view in cancer research, defining cancer as the uncontrolled proliferation of cells. This initial focus on proliferation per se has clarified much of the normal cell growth regulation and provides the critical basis for comparison in studies of dysfunctional growth.

Cell growth, or at the most fundamental of levels – life or death decisions - is directed by six types of molecular regulators: growth factors (or hormones), growth factor receptors, intracellular signal transducers, nuclear transcription factors, cell-cycle control proteins and cell death effectors. Growth factors are usually extracellular polypeptides molecules that bind to
cell-surface receptors to initiate signal transduction pathways leading to proliferation, or in some cases, differentiation. Hormones, in many cases, bind to intracellular receptors which can migrate to the nucleus. There are signal transducers such as nonreceptor protein kinases (e.g. \textit{abl} and \textit{src}), GTP-binding proteins (e.g. the \textit{ras} family), adaptor proteins (e.g \textit{grb2} and DOKR) or phospho-tyrosine phosphatases. Transcription factors regulate the expression of genes by binding to regulatory DNA sequences in the target gene (e.g. \textit{c-myc}, \textit{E2F}, \textit{SP1}, \textit{jun} and \textit{fos}). Finally, cell cycle proteins, categorized into cyclins and cyclin dependent kinases (cdks), act as the effectors of proliferative changes regulating the cell cycle (Koepp et al., 1999).

As cells grow and divide, they proceed through a defined sequence of events composed of four phases: G1, S, G2, and M. In addition, mammalian cells can reversibly exit the cell cycle to a quiescent phase, known as G0. During G1 there is a critical period in which cells are sensitive to both growth stimulatory and growth inhibitory signals (such as growth factors and hormones). The restriction point defines the end of the period of sensitivity to growth factors, or the point at which a cell is irreversibly committed to entering the S phase. Prior to the restriction point, cells may either become committed to a new round of DNA replication, or may be stimulated to enter a reversible quiescent state, or withdraw permanently into a state of terminal differentiation. The S phase of the cell cycle represents the period in which cells undergo DNA replication. The integrity of this replication is verified during the G2 phase. Finally in M phase cells undergo mitosis and divide into two daughter cells.

Regulatory pathways commonly referred to as checkpoints regulate key proteins in order to coordinate the orderly progression of the events of the cycle. These checkpoints prevent catastrophes such as progression through S phase before repair of DNA damage or segregation of chromosomes prior to completion of chromosome replication. Cell cycle transitions are
governed by the action of cyclin-dependent kinases (cdks). Cdkp are positively regulated by cyclins, negatively regulated by cdk inhibitors, and are also regulated by phosphorylation. One of the key prerequisites for entrance into the S phase is phosphorylation of the retinoblastoma (Rb) protein, by cyclin D- and E-associated kinases. Hypophosphorylated Rb prevents entrance into S phase by sequestration of a series of transcription factors, including E2F, and active repression of genes whose expression is required for S phase (Adams & Kaelin, 1995).

Aberrant expression of cell cycle regulators has been frequently documented in numerous human cancers. Any change in the levels or function of one (or more) of the various cell-cycle control proteins in combination with other growth affecting proteins (i.e. growth factors, growth factor receptors, intracellular signal transducers, nuclear transcription factors) can result in an excessive proliferation of cells. In fact, changes in the levels of some cyclins, such as D1 and E or the loss of the cdk inhibitor p27 protein have been implicated as having prognostic significance in some human cancers (Porter et al., 1997; Nielsen et al., 1996).

1.2.2 Survival

Cell loss (death) is the opposite side of the balance to mitogenesis when thinking in terms of the operational control of (net) cell numbers. There are two main types of cell death; "apoptosis" and "necrosis". Necrosis is the commonly used term for nonapoptotic, but accidental cell death, such as death resulting from physical injuries and inflammatory responses. However, it does not actually indicate a cause of death, e.g. ischemic cell death, referring instead to secondary changes resulting from death induced by any mechanism. Whereas necrosis refers to "accidental cell death" (Majno & Joris, 1995), apoptosis refers to programmed cell death, or cellular suicide. Considered to be the counterpart to mitosis, apoptosis has been well
characterized on the basis of observed morphological as well as biochemical criteria. Loss of cell viability is accompanied by cell shrinkage, membrane blebbing, chromatin condensation, and DNA fragmentation (Wyllie et al., 1980). These changes are the result of several factors (oxidative damage, altered calcium homeostasis and abnormal mitochondrial function) which comprise what has been termed a deleterious network (Ying, 1998).

Subject to similar social controls that operate on cell proliferation, most cells are capable of killing themselves by apoptosis, which can be either activated or suppressed by signals from other cells (Raff, 1992). During the average human life span most normal cells (99.9%), undergo apoptosis when they are no longer needed for any one of a number of reasons (Vaux & Korsmeyer, 1999). Some cells are produced in excess, demonstrated by many vertebrate neurons (Kerr et al., 1974) or are eliminated due to abnormal locations, or development, such as lymphocytes that have failed to produce functional antigen-specific receptors (Golstein et al., 1991). Consequently, understanding the ability of cells to avoid apoptosis has become of paramount importance, particularly in the context of cancer.

The recognition that cancer can result not only from the excessive proliferation of cells, but also from alterations in the balance of life and death that normally exists to maintain tissue homeostasis has focused much of the current attention directed toward cell survival and death. First identified as part of the most common translocation in human follicular B cell lymphoma (Bakhshi et al., 1985; Tsujimoto et al., 1985) the bcl-2 gene was the first (proto)-Oncogene found to promote cell survival rather than stimulating cell proliferation. (Vaux et al., 1988; Hockenbery et al., 1990). This discovery served to focus attention on apoptosis, and studies correlating apoptosis resistance and cancer began to appear (Yawata et al., 1998; Bedi et al., 1995). More prevalent than originally anticipated, the relative failure of cancer cells to undergo
apoptosis can act as a contributing factor under a variety of circumstances to tumour
development and disease progression, including metastasis, as well as resistance to anti-cancer
drugs (Sen & D'Incalci, 1992; Kerr et al., 1994; Schmitt & Lowe, 1999). Accordingly,
elucidating the molecular and cellular factors, which govern tumour cell survival mechanisms,
has assumed a dominant place in both basic and clinical studies of cancer (Reed, 1999).

One system where cell survival by 'resistance to apoptosis' is recognized as a significant
participant in tumour development is colorectal cancer (Bedi et al., 1995; Morin et al., 1996;
Hawkins et al., 1997; Yawata et al., 1998). Normal colorectal epithelial homeostasis is highly
dependent on both cell proliferation and apoptosis due to its high rate of turnover. Normal
epithelial cells both in vitro (Frisch & Francis, 1994; Rak et al., 1995b) and in vivo (Hermiston &
Gordon, 1995) are so highly susceptible to 'death by detachment', that a new term, anoikis, has
been coined to describe the apoptosis that results from the lack of attachment (Frisch & Francis,
1994). It becomes logical to assume that a progressive loss of cell death capacity corresponds to
transformation and cellular abilities to survive in what would normally be unacceptably stressful
conditions. Together these will ultimately select for those cells with the greatest survival
advantages, and hence highest tumorigenicity.

1.2.3 Angiogenesis

Once established, the continued expansion of transformed cells now becomes dependent
on their access to vasculature (Folkman, 1971). Colonies less than 1 mm in diameter can rely on
diffusion to effectively import oxygen and essential nutrients and remove metabolic wastes and
carbon dioxide. However, angiogenesis - the formation of new vessels from existing vessels,
must exist for any clinically apparent growth (Folkman, 1995). Tumour cells must therefore
acquire the ability to recruit or "coopt" capillaries (Holash et al., 1999) from the surrounding normal tissue by releasing angiogenic factors, notably basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF) and about a dozen known others (Asahara et al., 1995; Yoshida et al., 1996; Cao et al., 1998; Seghezzi et al., 1998).

The ability of a tumour to initiate angiogenesis is determined by the balance between stimulating and inhibiting molecules (Folkman, 1986; Folkman & Klagsbrun, 1987; Hanahan & Folkman, 1996). This concept of positive and negative regulators for angiogenesis is known as the balance hypothesis (D'Amore, 1999). The success of tumorigenic cells is achieved by 'tipping the scale' to either overcome the normal inhibitors of vessel formation, or to increase the number, or response to, stimulating molecules. Once this 'angiogenic switch' is accomplished, tumour cell populations are no longer kept in check by death from hypoxia, starvation and metabolic toxicity, but instead can form an aggressively expanding tumour (Hanahan et al., 1996; Weinberg, 1996). Studies are ongoing to determine if the cells of the vasculature itself play a role in tumorigenesis through the release of mitogenic factors (Rak et al., 1995a), what correlations exist between vessel density and subsequent tumour growth and metastasis (Weidner et al., 1991; Takahashi et al., 1996; Takahashi et al., 1998) and most recently the targeting of angiogenesis as an anti-cancer therapeutic (Folkman, 1971; Kerbel, 1991).

The discovery of endogenous angiogenesis inhibitors which prevent new vessels from forming and thus not only prevent tumour growth, but in some cases even cause regression, has created intense interest in both the efficacy of the substances themselves and their potential therapeutic value. Angiostatin and endostatin, both proteolytic cleavage products from plasminogen and collagen XVIII respectively, are the latest of a growing list of experimental products targeting endothelial cells (O'Reilly et al., 1994; O'Reilly et al., 1997; Cao et al., 1998).
Although presently the signaling cascades and genes regulating the angiogenesis induced by tumours are poorly understood, the potentially high therapeutic index of anti-angiogenic therapy should ensure that research in this field will continue to rapidly evolve and result in the elucidation of the mechanisms of control. It is now understood that operationally speaking, angiogenesis acts as an indirect and 'communal' growth, as well as, survival factor for tumour cells and is a necessary facilitator for tumour growth invasion and metastasis (Fidler & Ellis, 1994).

1.2.4 Invasion and Metastasis

Invasion and metastasis remain the greatest clinical challenge for successful cancer therapies. Once a tumour is no longer localized to its tissue of origin medical problems result when (1) sheer bulk becomes a factor in normal physiologic functioning, or (2) there is an excess secretion of biologically active substances e.g. hormones, or mitogenic growth factors. Metastatic cells overcome normal tissue architectural constraints in a 'chain reaction' process of malignant dissemination. After a period of progressive growth at the primary tumour site, metastatic cells penetrate basement membranes. Following vascularization of a tumour, local invasion (requiring penetration of the subendothelial matrix) and intravasation of cells (requiring adhesion to endothelial cells with their subsequent reaction) allows entry of cells into circulation through either blood vessels or lymphatics. There, homotypic (tumour cell-tumour cell adhesion i.e. microemboli) or heterotypic aggregates (e.g. involving platelets and leukocytes) may facilitate successful cell migration and survival, before cells finally arrest in a distant capillary bed. Cells then exit the vasculature and migrate to a distant, foreign site where metastatic spread
is finally accomplished, once growth is successfully established from one or more clonogenic cells.

Normally cells are constantly remodeling the extracellular matrix, in part by proteases released from growing cells. For example, one controlling enzyme in this process is the urokinase plasminogen activator which activates plasminogen to plasmin, in turn activating a series of collagenases that degrade components of the ECM (Liotta & Stetler-Stevenson, 1991). Transformed cells possessing an invasive phenotype acquire the ability to constitutively activate the ECM remodeling system. This is often accomplished by the production of elevated levels of both the receptors for the basal lamina proteins and enzymes that digest collagen and other basal lamina proteins (predominantly integrins), such as proteoglycans and glycosaminoglycans. Once cells have penetrated the lamina they can further adhere to endothelial cells to aid in their migration, before escaping and ultimately exiting the vasculature to establish growth in a foreign cellular environment (Fidler & Hart, 1981). It should also be noted, that some recent reports have implicated the endothelial cells themselves, under certain circumstances, can act as a facilitator in tumour invasion (Brooks et al., 1998; Skobe & Fusenig, 1998).

Tumour progression produces malignant cells which have attained a significant survival advantage (such as an increased resistance to undergoing apoptotic cell death), first over normal cells and later over the parent tumour clone (Nowell, 1976), finally acquiring the competence for metastatic spread (Kerbel, 1990). Although not the sole requirement for metastasis, growth advantages, together with 'best survival', i.e. apoptosis resistance, are primary attributes of metastatic cells. Moreover, it is these fundamental survival characteristics, outlined in section 1.3, that are probably necessary, though not sufficient, for metastasis. The concept of the selective nature of the metastatic phenotype assumes that within each 'survival enhanced' clonal
expansion, new mutants will sequentially arise and a small fraction will possess an additional advantage and displace the parent clone which spawned the subclone.

The studies that followed the initial convincing evidence (Fidler, 1973) for the clonal selection of a metastatic phenotype, were comprised of cloning experiments designed to examine variations in metastatic potential (Fidler & Kripke, 1977; Kerbel, 1979; Chambers et al., 1981). It was observed by some investigators that clones were not phenotypically stable. This provided the basis for a dynamic heterogeneity model of metastatic cells. As the term implies, it was expected that a dynamic equilibrium would develop where the frequency of metastatic variants in a particular clonal population would be controlled by the rates of generation and loss of the variants (Ling et al., 1985; Hill, 1986).

However, conflicting observations regarding the lack of obvious differences between metastases and primary tumours required a revised theory to explain the events resulting in malignancy. Now widely accepted, the clonal dominance theory proposed by Kerbel (1990) is based on the central idea that sequential genetic changes provide metastatically competent cells with such significant growth advantages so that even within the primary tumour these cells will come to fully dominate it over time (Kerbel et al., 1988; Kerbel, 1990).

1.3 Cell Survival as a Critical Determinant of Malignancy

Transformed cells exhibit numerous alterations endowing them with formidable survival advantages over normal cells. Examining properties of in vitro growth provides an easily accessible manner for investigations of mechanisms used to prolong life and ultimately attain clonal dominance during tumour formation. Enhanced survival can be manifested by: (1) decreased growth factor requirements, (2) loss of capacity for growth arrest, (3) ability to grow
anchorage independently, (4) loss of contact inhibition, (5) changed cell morphology and (6) survival after exposure to cytotoxic agents such as chemotherapeutic drugs. Each of these characteristics is supported by phenomenological evidence obtained from studies of cancer cells both in vitro and in vivo.

The release of transforming or mitogenic growth factors by tumour cells acting in either an autocrine, paracrine or endocrine manner and the loss of response to certain inhibitory growth factors provide still more survival advantages (Baserga, 1994). Growth factor autonomy, autocrine stimulation and even enhanced response to stimulatory growth factors such as insulin, insulin-like growth factor-I (IGF-1), basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF) all contribute to cell survival and ultimately the selection of the most autonomous and hence, stalwart cells. For example, TGFα expressed in intestinal tumours (Filmus et al., 1993), or interleukin 6 (IL-6) in advanced stage human melanoma cells (Lu et al., 1996) can function as endogenous in vivo growth stimulatory factors.

Conversely, one must consider that just as increased pro-growth factors improve survival, so does a decreased response to growth inhibitory factors. Interestingly, in vitro analysis has demonstrated that IL-6, in addition to a later role in growth stimulation, initially acts in a growth inhibitory manner in cell lines derived from early stage melanoma, with this response later lost in cells derived from advanced stage lesions (Lu et al., 1996). TGFβ is another such paradigm for such a loss of response resulting in an increased survival and proliferation (Roberts & Sporn, 1988; Akhurst & Balmain, 1999).

Numerous studies have established that survival signals for many types of cells are supplied by the cell matrix and cell-cell adhesion (Meredith, Jr. et al., 1993; Bates et al., 1994; Frisch & Francis, 1994; Peluso et al., 1996; Kantak & Kramer, 1998). Compared to normal
cells, transformed cells are less inhibited by cell-cell contact and succeed to varying extents in overriding normal limitations on tissue architecture. In fact, neoplastic cells can survive and flourish in anchorage independent conditions in the absence of the normal survival signals (see section 1.5.1). One example of this behaviour is the lack of death by ras-transformed IEC-18 cells when forced to grow independent of anchorage.

Changes in cell morphology are also likely to play a role in better overall survival. The loss of actin microfilaments, which become diffusely distributed or concentrated under the cell surface, are probably the basis for the altered morphology of transformed cells and may explain in part their enhanced mobility (Prasad et al., 1999).

Thus, it is any one, or combination of the above characteristics, which allow a cell to survive noxious conditions more effectively than those surrounding it. This facilitation of the physical process of cancer cell invasion and metastasis by survival is a critical determinant of malignancy.

1.4 Molecular Control of Life and Death Signaling

Molecular mechanisms controlling cellular life and death signaling in tumour cells are gradually becoming uncovered. Defects in death mechanisms extend cellular lifespans and alter population size independently of proliferation. This, in addition to the permissive environment created for genetic instability and the accumulation of gene mutations, is a significant contributor to carcinogenesis (Reed, 1999). Understanding apoptotic mechanisms will undoubtedly create novel strategies to battle cancer through the selective restoration of death signaling in cancer cells.
Apoptosis induced by any type of stimuli, exhibits a series of well characterized changes, suggesting that regardless of the initial source, all signals eventually converge in a common death pathway (Majno & Joris, 1995; White, 1996). Recent discoveries have clarified some of the molecular mechanisms involved in apoptosis execution.

Playing a central role in the ‘cell-death machine’ is the intracellular caspase family of cysteine proteases. Highly conserved across animal evolution, caspases are produced as inactive zymogens with their activation by specific stimuli an absolute requirement of apoptosis. Caspase activation results in an irreversible proteolytic cascade culminating in specific substrate cleavage. This is accountable, either directly or indirectly, for the biochemical and morphological changes that have become well documented as “apoptosis” (see section 1.2.2).

The initial observation of proteolytic involvement in apoptosis came from chemotherapy studies in leukemic cells where the nuclear protein poly-ADP-ribose-polymerase (PARP) was cleaved as an early event (Patel et al., 1996). The ability of caspases to cleave substrates, and their own activation by cleavage (both at Asp residues) creates the potential for proteolytic cascades. Some caspases act as upstream initiators, possessing large prodomains capable of protein binding, and others, activated predominantly by upstream caspases, as downstream effectors (Patel et al., 1996; Salvesen & Dixit, 1997; Alnemri, 1997).

A novel class of apoptosis inhibitors initially identified in baculoviruses (Crook et al., 1993; Birnbaum et al., 1994) has been receiving increased attention. The inhibitor of apoptosis (IAP) family is widely expressed and the diversity of triggers against which IAPs suppresses apoptosis is greater than any other family of apoptosis inhibitors (including the bcl-2 family) (LaCasse et al., 1998). The primary mechanism of IAP activity is through both direct caspase and pro-caspase inhibition and modulation of the transcription factor NF-κB (Chu et al., 1997;
IAPs bind directly to caspases 3, 7 and procaspase 9 in the proteolytic cascade (Deveraux et al., 1998; LaCasse et al., 1998; Reed, 1999).

The IAP family includes neuronal apoptosis inhibitory protein (NIAP), X-chromosome linked IAP (XIAP) (homologous to RIAP3 in rodents), and HIAP1 and HIAP2 (human IAP-1 and -2; homologous to RIAP1 and -2 in rodents). Also included in this family is the newly discovered "survivin" gene (Ambrosini et al., 1997; Ambrosini et al., 1998). The IAPs have in common two defining structural motifs, one being the so-called "BIR" (Baculovirus Inhibitor of apoptosis Repeat) domains (Miller, 1999) and the second being a RING zinc finger domain near the carboxy terminus (Deveraux & Reed, 1999; LaCasse et al., 1998; Miller, 1999). Little is presently known about IAP expression and functionality in cancer, although survivin is reported to be overexpressed in fetal tissues and numerous human malignancies, but not in normal adult cells (Ambrosini et al., 1997).

Bcl-2 and its homologues represent the other major gene family involved in apoptotic regulation. Several bcl-2 family proteins, including bcl-2, bcl-XL, Mcl-1 and A1/Bfl-1 suppress apoptosis, while others facilitate/induce apoptosis. Members that facilitate apoptosis fall into two groups: (1) those that dimerize with bcl-2 or bcl-2 like proteins to act as transdominant inhibitors (BAD, Bik, Bid, Bim, Bim/Bod, Hrk) and, (2) those that induce death independently of dimerization (Bax, Bak, Bo/Mtd) (Zha & Reed, 1997; Inohara et al., 1998; Simonian et al., 1997). The latter still possess the capability to dimerize with anti-apoptotic bcl-2 members and thus have two potential modes of action (Kelekar & Thompson, 1998).

Regulation of apoptosis by the bcl-2 family remains controversial due to the multifunctionality of members (Reed, 1997). Two types of function have been described; (1) ion-channel activity (in intracellular membranes); and (2) binding to other proteins. Although
effects have been reported on the endoplasmic reticulum and the nuclear envelope (Reed, 1994; Schendel et al., 1998) recent attention has been primarily focused on the mitochondria. Initially implicated in ischemic-reperfusion injuries resulting in necrotic death, mitochondria are now known to play an important role in apoptosis (Green & Reed, 1998). For example, apoptotic stimuli cause the translocation of pro-apoptotic proteins such as Bax, from the cytosol to the mitochondrion (Wolter et al., 1997). Here, the caspase activating protein cytochrome c is released from within the organelle (Rosse et al., 1998; Jurgensmeier et al., 1998). The precise mechanism utilized by bcl-2 proteins for this pore forming process remains unclear. Once cytochrome c is released, it activates Apaf-1, which in turn activates pro-caspase 9 thereby initiating the entire caspase proteolytic cascade. The current information regarding bcl-2/bcl-X \textsubscript{L} suggests two methods of suppressing apoptosis: (1) preventing cytochrome c release; and (2) interfering with caspase activation by cytochrome c and Apaf-1 (Hengartner, 1998). This functional redundancy may serve to ensure that death does not occur inadvertently. Moreover, it may explain the ability of bcl-2 and bcl-X \textsubscript{L} to suppress not only caspase-dependent apoptosis, but also caspase-independent necrosis (resulting from mitochondrial rupture) (Reed, 1999).

Although bcl-2 clearly has an influence over cell death upstream of caspase activation, the existence of bcl-2-independent death mechanisms have been reported through the tumour necrosis factor (TNF) family of "death receptors" pathways (e.g. Fas, TNFR1, DR4 (Trail-R1) and CAR) (Nagata et al., 1996; Vaux & Strasser, 1996). All TNF members contain "death domains" in their cytosolic tails, which induce recruitment of cytosolic pro-caspases capable of interacting with specific adaptor proteins (e.g. Fadd, Tradd) to directly induce caspase activation. The death receptor pathways that result in apoptosis are well characterized. However, the mechanisms controlling entry to the apoptotic pathway by other stimuli are not as clear, with
numerous signaling cascades implicated, based on both stress stimuli and cell type (Anderson, 1997). Some of the known stimulators and potential regulators involved in apoptosis execution described above are summarized in Figure 1.1.

1.5 **Adhesion as a Survival Mechanism: “Anoikis”**

1.5.1 **Adhesion Dependent Survival**

Adhesive interactions regulate diverse cellular processes such as cell motility, growth, differentiation gene expression, and apoptosis. Numerous cell surface protein families including selectins, the CD44 family, members of the immunoglobulin superfamily of cell adhesion molecules, integrins and cadherins, mediate adhesive interactions with components of the ECM and each other (cell-cell adhesion) (Joseph-Silverstein & Silverstein, 1998).

One view regarding cell survival is the concept of 'death by default'. Initially proposed by Raff, it states that cells require signals to proliferate, and in their absence initiate apoptosis (Raff, 1992). This concept is exemplified by the particular manifestation of apoptosis known as “anoikis”. Named in reference to the Greek work for homelessness (Frisch & Francis, 1994; Frisch & Ruoslahti, 1997), anoikis refers to the phenomenon of detachment-induced cell death. Here, normal (or immortalized, but non-cancerous) epithelial or endothelial cells initiate a cellular suicide program when they become physically separated from the ECM or basement membrane with which they are normally in close contact. First reported by Meredith in 1993, anoikis presumably serves as a critical safeguard to maintain normal tissue architecture. The survival signals provided by contact with the ECM are thought to be mainly mediated by integrin receptors, which bind to various components of the ECM (Meredith, Jr. et al., 1993; Schwartz, 1997). Additional reports of integrin-dependent survival in a number of epithelial and
Figure 1.1 Simplified model of the apoptotic death pathway

Various stimuli can trigger apoptosis. At least two pathways have been identified: a pathway involving caspase activation, of which the upstream caspases contain death effector domains (DEDs) and which are involved in Fas and TNF signaling, and a mitochondrial-dependent pathway governed by members of the bcl-2 family. Extensive cross-talk probably exists between the pathways. Anti-apoptotic death effector domain (AEDD)-family proteins bind to and prevent activation of initiator DED-containing caspases. IAP family proteins bind to and inhibit active effector caspases. (Adapted from Reed, 1999)
endothelial cell lines soon followed (Re et al., 1994; Frisch & Francis, 1994; Boudreau et al., 1995; Ishizaki et al., 1995; Rak et al., 1995b). Subsequent studies have further reported anoikis-like death in many other cell types including fibroblasts, and myoblastic cells (Ishizaki et al., 1995; Vachon et al., 1996).

It is thus thought that anoikis occurs, at least in part, by the disruption of integrin mediated survival signals (Meredith, Jr. et al., 1993; Schwartz, 1997). Integrins are comprised of a large family of cell surface receptors mediating adhesive interactions and transducing signals across the plasma membrane (Hynes, 1992; Clark & Brugge, 1995; Dedhar & Hannigan, 1996). In addition to providing a physical link by binding other cell-surface receptors or constituents of the ECM extracellularly, integrins bind cytoskeletal elements (such as talin and vinculin) on the cytoplasmic side (Hynes, 1992). Integrin ligand association/dissociation can trigger the reorganization of the actin cytoskeleton and initiate cascades of signaling events (Yamada & Miyamoto, 1995; Clark & Brugge, 1995). For example, both endothelial cells and human melanoma cells are known to require the integrin α5β1 signaling, in its absence undergo apoptosis (Brooks et al., 1994; Montgomery et al., 1994).

This ‘outside-in’ signaling elicits numerous events such as tyrosine phosphorylation (Kapron-Bras et al., 1993) and activation of serine-threonine kinase families such as protein kinase C (PKC) (Vuori & Ruoslahti, 1993), mitogen activated protein kinase (MAPK) (Chen et al., 1994; Zhu & Assoian, 1995) and focal adhesion kinase (FAK) (Schlaepfer et al., 1999; Guan, 1997; Frisch et al., 1996), as well as, Ras (Kapron-Bras et al., 1993) and other small GTPases (Nobes & Hall, 1994; Burbelo et al., 1995). Ultimately, these changes lead to modulation of gene expression, regulation of cell cycle progression and/or apoptosis.
Cellular dependence on adhesion is often lost early in tumour progression, giving rise to one of the best known hallmarks of cancer, 'anchorage independent growth'. Although there are some instances where cell-cell interactions are required for malignant cell survival, e.g. LIM 1863 colon carcinoma cells where the αv integrin subunit facilitates organoid growth in vitro (Bates et al., 1994), these instances are rare (Hague et al., 1997; Kantak & Kramer, 1998). Lack of adhesion may facilitate early events in tumour metastasis, such as detachment of tumour cells and intravesation. However, increased adhesion may actually promote malignant growth at later stages of disease progression, e.g. ectopic growth and survival of nascent microscopic metastases. Understanding the survival signals provided by adhesive interactions and the apoptosis resulting from their absence is of considerable interest for cancer progression and drug resistance (Green et al., 1999), and hence for potential therapeutic implications.

1.5.2 Signaling Cascades Implicated in Anoikis

Apoptosis can be triggered by a wide variety of cellular stresses including DNA damage, ultraviolet (UV) radiation, ionizing radiation, heat shock and oxidative stress, and in the specific case of anoikis, lack of extracellular stimuli acting through the cell-surface receptors (see section 1.5.1). Understanding regulation of each differently induced apoptotic pathway requires (1) the determination of which signaling pathways are involved; and (2) the functional effects of the transcriptional activity initiated by this signaling. The last several years have produced increasing numbers of reports regarding the many forms of cellular stress causing apoptosis by activation of the recently identified cellular signaling cascades of protein kinases, the stress activated protein kinases (SAPKs) and p38/RK kinases (Kyriakis et al., 1994; Zanke et al., 1996). Interestingly, signals that promote apoptosis in one cell model, or resulting from one type
of stress, can suppress death in another (Mcconkey & Orrenius, 1994). These kinds of observations indicate that cellular responses may be determined by intrinsic programming of the cell in question, and forestall generalizations regarding apoptotic signaling across cell types and inducing stress.

1.5.3 Extracellular Stress Induced Signaling: The MAPK/SAPK Family of Proteins

The components of the SAPK pathways, known for their activation in a physiological context by inflammatory cytokines, are distinct from the components of the extracellular signaling related kinase (ERK) (also known as MAPK1/2) which translates primarily proliferative signals (Cosulich & Clarke, 1996). However, all of the above protein kinases belong to the same family of mitogen activated protein (MAP) kinases. These MAPK pathways use sequential phosphorylation reactions to amplify the original signal (Figure 1.2). Although the different MAPK pathways contain structurally similar components, they most likely have different downstream targets and mediate very distinct biological functions. It has been proposed that the balance and/or threshold level of activation of one pathway versus another – MAPK and ERK, versus SAPK and p38/RK - may in fact determine if a cell will ultimately proliferate or die (Cosulich & Clarke, 1996). However, it is not yet clear whether different pathways are concurrently activated within a cell, or if the full commitment to cell death requires survival and proliferation signals be turned off and stress pathways to be activated. As upstream signals are common to both cellular proliferation and death, cross-talk between the different ERK/SAPK/p38/RK pathways is a given. The emerging picture is one of independently regulated parallel cascades of structurally related kinases functioning to integrate extracellular growth promoting and growth inhibitory signals (Woodgett et al., 1995) (see Figure 1.2).
Figure 1.2 Overview of Mitogen and Stress Activated Protein Kinase (MAPK and SAPK) cascades in mammalian cells

SKK1/MKK4 activates SAPK1/JNK, which subsequently binds to the c-jun or ATF2 substrate, in response to a variety of stressful stimuli. Numerous protein kinases, including MEK kinases (MEKKs) activate SKK4/MKK4 (with the possible existence of a positive feedback loop to caspase 7). Mkk1 and Mkk2 activate MAPK1/ERK1 and MAPK2/ERK2 but are inactive toward SAPKs. MAPK1/ERK1 and MAPK2/ERK2 activate the three isoforms of MAPK-activate protein kinase-1 (MAPKAP-1) as well as MAPK-interacting kinases 1 and 2 (Mnk1 and Mnk2). Growth factors initially trigger the activation (GTP-loading) of Ras, which then interacts with Raf. Raf is recruited to the plasma membrane and further mediates the activation of Mkk1 and Mkk2. The proteins mediating activation between extracellular signals and SKK2/SKK3/SKK4/SKK5 are still undetermined. SKK2 activates SAPK2a/p38 efficiently, while SKK3 is capable of activating SAPK2a/p38, SAPK2b/p38β, SAPK3 and SAPK4. SAPK2a and b/SAPK3/SAPK4 activate MAPKAP-K2/MAPKAP-K3 as well as Mnk1/Mnk2. SKK5 interacts specifically with SAPK5 although the precise mechanism is unknown.

Table 1 Nomenclature used for mitogen-Activated and Stress-Activated Kinases *

<table>
<thead>
<tr>
<th>MAPKs and SAPKs</th>
<th>Alternate names</th>
<th>Upstream activators of MAPKs and SAPKs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Class</td>
<td>Proposed names</td>
<td>MAPKs and SAPKs</td>
</tr>
<tr>
<td>MAPK</td>
<td>MAPK1</td>
<td>MAPK1/42MAPK</td>
</tr>
<tr>
<td></td>
<td>MAPK2</td>
<td>ERK2/42MAPK</td>
</tr>
<tr>
<td>SAPK1</td>
<td>SAPK1a</td>
<td>SAPKα/JNK1</td>
</tr>
<tr>
<td></td>
<td>SAPK1b</td>
<td>SAPKβ/JNK3</td>
</tr>
<tr>
<td></td>
<td>SAPK1c</td>
<td>SAPKγ/JNK2</td>
</tr>
<tr>
<td>SAPK2</td>
<td>SAPK2a</td>
<td>p38/CSBP/Mxi2/RK</td>
</tr>
<tr>
<td></td>
<td>SAPK2b</td>
<td>p38β</td>
</tr>
<tr>
<td>SAPK3</td>
<td>SAPK3</td>
<td>ERK5/p38γ</td>
</tr>
<tr>
<td></td>
<td>SAPK4</td>
<td>none</td>
</tr>
<tr>
<td>SAPK5</td>
<td>SAPK5</td>
<td>ERK5/BMK1</td>
</tr>
</tbody>
</table>

*The amino acid sequences of SAPK1a, SAPK1b and SAPK c are about 90% identical. SAPK2a and SAPK2b are 79% identical. SAPK3 and SAPK4 are 60% identical to each other or to SAPK2a and SAPK2b. SAPK2, SAPK3 and SAPK4 are 45% identical to SAPK1 and 40% identical to MAPKs. Several additional forms are produced in mammalian cells by alternative splicing of the precursor mRNAs for SAPK1a, SAPK1c, SAPK2a and SAPK3.
The biological roles and physiological substrates for members of the MAPK/SAPK families are still being clarified (Cohen, 1997). The lack of standardized nomenclature for the various MAPK/SAPK families, in conjunction with ongoing discoveries of extensive cross-talk between signaling cascades (exhibiting both stimuli and cell specific differences (Carletti et al., 1995; Liu et al., 1996; Hu et al., 1997; Matsuda et al., 1999; Plattner et al., 1999) increase the complexity of these signaling cascades. However, some studies show that many known apoptotic stimuli activate SAPKs specifically, among them UV light, heat stress, interleukin-1, TNFα, and γ-irradiation (Davis, 1994; Derijard et al., 1994; Verheij et al., 1996; Zanke et al., 1996). As previously mentioned (section 1.5.2), extracellular (stress) signals can initiate tyrosine phosphorylation and activation of serine-threonine kinase families, MAPK, as well as, Ras and other small GTPases. The SAPKs are activated by MAPK kinase (MKK) family members, termed here SAPK kinases (SKKs). The major activators of SAPK1a/b/c are SKK1/4, whereas SAPK2a/p38, SAPK2b/p38β, SAPK3 and SAPK4 are instead activated by SKK2/3 (Yan et al., 1994; Lawler et al., 1997). Once activated, the three isoforms of SAPK1 are capable of tightly binding, primarily the transcription factor c-jun, but also ATF-2, thereby modulating gene expression.

In our search for other transducing molecules that could be involved in the regulation of anoikis we became particularly interested in SAPK1 (also known as Jun-N-terminal kinase (JNK)) since previous studies by others had implicated this enzyme was involved in anoikis of Madin Darby Canine Kidney (MDCK) epithelial cells (Frisch et al., 1996). Initial reports indicated that SAPK activity was both necessary and also required caspase activation for the execution of anoikis in this cell system (Frisch et al., 1996). Moreover, the activation of the upstream kinase, MEKK-1 in the SAPK cascade initiated a positive feedback loop to caspases.
(specifically caspase 7) (Cardone et al., 1997). Once activated, either by MEKK-1 or exogenously, caspase 7 cleaves MEKK-1, an event required for its activation, although additional events such as autophosphorylation may be required for full activation.

In direct contrast, subsequent studies by others have reported that although SAPK is indeed activated by MDCK cell detachment, this molecular change fails to correlate with cell death (Khwaja & Downward, 1997). A study of cell lines expressing activated signaling proteins usually controlled by ras determined that activated PI3K and PKB/Akt protect MDCK cells from anoikis without affecting SAPK activation. Conversely, activated Raf and dominant negative SEK1 (upstream of SAPK kinase) attenuate SAPK activity, but fail to prevent anoikis (Khwaja & Downward, 1997). Caspase inhibition was shown to rescue cells, but had no affect on SAPK activity. Furthermore, p38 although stimulated by cell detachment, is unlikely to be functionally involved, as its inhibition does not result in cell survival (Khwaja & Downward, 1997).

The controversy that surrounds the role of SAPK in anoikis (Frisch et al., 1996; Cardone et al., 1997; Khwaja & Downward, 1997), combined with the fact that all the aforementioned observations were obtained using one cell line, illustrate the need for a better definition of the stimuli inducing SAPK activation and of the functional downstream effectors. Moreover, SAPK was also proposed to serve as a 'death pathway' in the context of anticancer therapy (Haq & Zanke, 1998) and hence is of considerable clinical interest.

1.5.4 Cellular Survival Effectors in Normal and Transformed Cells

To better understand the process of cell death one must also examine factors that can override it. In direct opposition to signals initiating and promoting apoptosis there are cellular survival signals. Members of the bcl-2 and IAP family of proteins (as previously described in
section 1.4) can function as either agonists (e.g. Bax, Bak) (Datta et al., 1997; Rosen et al., 1998), or antagonists (bcl-2, bcl-XL, IAP1, 2, 3 survivin) (Chinnaiyan & Dixit, 1996; Cosulich & Clarke, 1996; Reed, 1999) of apoptosis (Figure 1.1). Together, these two families of apoptosis regulators provide a complex and potentially multi-level inhibition of the apoptosis machinery. Ongoing studies revealing the complex nature of these signaling interactions are integral in the overall understanding of the many types of stress induced apoptosis. Thus, the targeting of apoptosis antagonists is of potential therapeutic value for a variety of diseases, either the restoration of normal signaling in various degenerative pathologies or the induction of apoptosis in proliferative pathologies, such as cancer.

Oncogeneic transformation utilizes survival factors described above (section 1.4), among others, as a means of overriding several physiological death processes, including anoikis. In contrast to normal cells, transformed, tumorigenic epithelial cells (i.e., carcinoma cells) appear readily capable of survival under similar detachment conditions (Schwartz, 1997). This relative resistance to anoikis may be an important factor in allowing detached tumour cells to survive as single cells, or small emboli, in the bloodstream, and hence, to establish distant metastases in foreign tissues (Schwartz, 1997). It also may help explain the well-known property of cancer cells to survive and grow in vitro in an anchorage-independent fashion, e.g. as small three-dimensional colonies in soft agarose (Schwartz, 1997).

We recently reported that cells from an immortalized but non-tumorigenic line of rat intestinal epithelium, called IEC-18, undergo a massive apoptotic cell death process when forced to grow as multicellular tumor spheroids in culture (Rak et al., 1995b). In marked contrast, tumorigenic sublines of IEC-18, obtained by transfection with a mutant H-ras oncogene, survived and grew under similar growth conditions (Rak et al., 1995b). The results suggested
that mutant ras oncogenes may have an important anti-apoptotic/pro-survival function which is preferentially expressed under anchorage-independent multicellular growth conditions (Rak et al., 1995b). Similar conclusions have been reported by others (Lebowitz et al., 1997; Khwaja et al., 1997).

These results prompted us to initiate a series of investigations to uncover the molecular basis of activated ras-mediated survival mechanisms in epithelial cells growing anchorage independently. Understanding the ability of mutant ras oncogenes to abrogate anoikis has been somewhat overlooked in the literature compared to other functions such as cell proliferation. Numerous reports have shown ras and ras associated signaling cascades to signal SAPK, both directly and indirectly (Kawasaki et al., 1996), are in stark contrast with the proposal that SAPK is a critical mediator of anoikis. We found that oncogenic ras can induce a significant downregulation of the pro-apoptotic effector protein known as Bak (Rosen et al., 1998) and prevent downregulation of bcl-XL (Rosen et al., 1999 unpublished) a member of the bcl-2 gene family of apoptosis regulators (Reed, 1997; Kroemer, 1997). Moreover, this change appears necessary for ras-dependent resistance to anoikis, at least in the IEC-18 intestinal epithelial cells (Rosen et al., 1998). However, ras transformed IEC cells, transfected with the bak gene failed to fully revert to the parental IEC-18 phenotype either in terms of survival in anchorage-independent conditions in vitro or tumorigenic growth in vivo (Rosen et al., 1998), thus suggesting that additional changes are involved in the ability of ras-transformed IEC-18 cells to resist anoikis. Another important conclusion of this study was that the ras-induced downregulation of Bak requires phosphatidylinositol 3-kinase (PI3K) activity. This conclusion is consistent with results published by several laboratories indicating that PI3K is a critical inhibitor of anoikis (Downward, 1998; Khwaja & Downward, 1997). In our studies, however,
we found that PI3K is not the only mediator of ras-induced resistance to anoikis, since treatment of IEC-ras cells with the PI3K inhibitor LY294002, only partially restored sensitivity to anoikis.

The anoikis resistance of cancer cells provides a therapeutic target for cancer. Differences between normal and transformed cells and their potential exploitation are the basis for the majority of cancer therapies. One such example of oncogene focused anti-cancer therapy is the targeting of ras by farnesyltransferase inhibitors (FTIs) (Gibbs et al., 1994; Gibbs et al., 1996). It is the essential association of ras or a ras-related protein, to the outer cell membrane, mediated by the small 15 carbon isoprenyl (farnesyl) group, that might prove to be a successful therapeutic target to prevent ras-dependent cell transformation (Schafer & Rine, 1992; Newman & Magee, 1993). FTIs, using competitive inhibition, are aimed at the farnesyl transferase enzyme that catalyzes protein prenylation.

Observations, like those outlined above, are a direct result of the considerable interest in uncovering the nature of the genetic changes and the signaling pathways associated with them, which are responsible for the relative resistance of cancer cells to undergo anoikis.

1.6 Thesis Objective

Anoikis, namely the apoptosis caused by lack of proper adhesion, has increasingly been recognized as playing a critical role in the maintenance of epithelial and endothelial tissue homeostasis. Failure of cells to undergo anoikis, also termed "anoikis resistance", is now regarded as one of the crucial steps in the tumorigenesis of epithelial systems (Khwaja & Downward, 1997; Frisch et al., 1996; Schwartz, 1997; Rak et al., 1999)(Rosen et al. 1999 unpublished). Elucidation of the mechanisms governing anoikis will not only increase understanding of the safeguards that ensure normal cellular architecture, but may potentially
provide a therapeutic target in cancer research. In light of this, there are two important questions which should be addressed: (1) what are the cellular and molecular mechanisms of physiological induction of anoikis? (2) how are these pathways subverted by malignant transformation? Recent reports have implicated SAPK signaling as a candidate mechanism for anoikis in MDCK cells. My studies were directed primarily towards the following:

1. To investigate the role of SAPK in relation to anoikis in both normal epithelial and endothelial models.

2. To analyze the role of SAPK in relation to the anoikis abrogation in a ras transformed epithelial cell line.

3. To determine the potential involvement of direct acting survival effectors, e.g. the IAP family of genes, in the anoikis abrogation in transformed cells.
Chapter 2: Results

2.1 Survival Properties of Epithelial and Endothelial Cell Lines Under Three-dimensional Growth Conditions

IEC-18 cells were originally established from embryonic rat intestinal (crypt) epithelium. Shown to be highly sensitive to anoikis (Figure 2.2), these cells undergo massive apoptosis when detached from the substratum (Rak et al., 1995b) and are also unable to form colonies in soft agar (Buick et al., 1987). Oncogenic transformation has long been observed to impart enhanced growth and survival to cells, with ras transformation of IEC-18 cells serving as a paradigm to study the subsequent changes and their resulting functionality (Figure 2.1 data from Rak et al., 1999). Additionally, anoikis resistant variant cell lines were derived from the parental population by serially selecting cells in spheroid culture (see section 2.2 for a detailed description of cell selection). Together these cell lines comprise an admirable model system to study the phenomenon of epithelial cell anoikis.

Since it has been reported that SAPK is involved in the ras-induced resistance to anoikis in MDCK cells, we decided to use those cells as our positive controls for our studies of IEC-18 cells. In addition, we have also included in our studies PK-3E and HUVECs, as numerous epithelial and endothelial cells (Meredith. Jr. et al., 1993) are also known to be highly sensitive to anoikis. Previous studies have reported that cell-cell contact sensitizes MDCK cells to anoikis, with maximal apoptosis being detectable when cells were confluent prior to suspension (Frisch et al., 1996). Accordingly, all cells were grown until confluent prior to plating for the anoikis assay; cells were then placed in either monolayer or three-dimensional culture conditions for 24 hours before being assayed for apoptosis. Apoptosis, although virtually undetectable in
Figure 2.1 Cellular viability of IEC-18, IEC-18 Ras4, IEC-18 AR1.10 and IEC-18 AR2.10 cells in three-dimensional culture

Cumulative survival curves (MTS assay) of anoikis resistant variants of IEC-18 cells derived by spheroid selection protocol (AR1.10, AR2.10) or transformation with mutant H-ras oncogene (Ras4).
Figure 2.2 Apoptosis of IEC-18, IEC-18 ras3, PK-3E, PK-Kras, MDCK, and HUVEC in suspension culture.

Following detachment from the plates matrix and 24 hours in either monolayer or three-dimensional culture conditions the apoptosis of IEC-18, IEC-18 ras3, PK-3E, PK-Kras, MDCK, and HUVEC was quantitated by ELISA. The enrichment of mono- and oligonucleosomes in the cytoplasm (apoptotic enrichment factor) was detected by biotin-labeled anti-histone and peroxide conjugated anti-DNA antibodies (see Methods). The data represents the average of two independent experiments, each one was done in duplicate, error bars are representative of the range between experimental data.
cells placed in monolayer conditions, was detected in all the non-transformed cell lines or populations tested, except the ras transformed IEC-18 or PK variants, after incubation in three-dimensional culture (Figure 2.2). Interestingly, although all four anoikis sensitive cell lines exhibited marked apoptosis after substratum detachment, differences were found between cell types; HUVECs being the most sensitive, whereas immortalized MDCK, IEC-18 and PK-3E cells were found to be somewhat less sensitive.

2.1.1 SAPK is Differentially Activated after Detachment of MDCK, IEC-18, PK-3E, HUVECs

After trypsinization and suspension, SAPK was assayed at various times points in the anoikis sensitive cell lines (MDCK, IEC-18, PK-3E and HUVEC). All cells were confluently plated prior to assay in an effort to ensure maximal cell-cell interactions. MDCK cells showed a strong and rapid induction of SAPK activity upon detachment (Figure 2.3 A), which was sustained for up to 4 hours (data not shown). This temporal order was previously interpreted as an indicator of a linkage between SAPK activation and commitment to undergo cell death in three-dimensional culture (Frisch et al., 1996). In contrast, IEC-18 cells exhibit a markedly different pattern of SAPK induction. SAPK activity was weak and transient after the detachment of IEC-18 cells. Maximal activity was attained approximately 15 minutes after detachment and decreased rapidly to below the basal level after 45 minutes (Figure 2.3 B). This was not due to inability of IEC-18 cells to activate SAPK. A known inducer of the stress pathway, anisomycin was capable of a strong and sustained activation of SAPK in these cells (Figure 2.3 B) (Hazzalin et al., 1998). These results prompted us to further examine the kinetics of SAPK activation in PK-3E and HUVECs, two additional anoikis-sensitive cell lines, in order to determine if the
Figure 2.3 Differential activation of Stress Activated Protein Kinase in four anoikis sensitive cell lines.

SAPK immunoprecipitates from MDCK (A), IEC-18 (B), HUVEC (C) and PK-3E (D) cell line lysates were assayed for associated c-jun activity. Radioactivity in c-jun bands is shown in the insert and quantititated (in arbitrary units) in the graphs shown below. For positive assay controls IEC-18 cells treated with 250 μg/mL of anisomycin were used while negative controls are IEC-18 cell lysates with no c-jun substrate added. Data is representative of a minimum of 4 independent assays.
kinetics of SAPK activation is similar to either IEC-18 cells or MDCK cells. Results show that HUVECs exhibit a strikingly similar pattern of SAPK activation to that of IEC-18 cells; maximal activity is, again, reached within 15 minutes of detachment and activity declines to almost the basal level after 1 hour in suspension (Figures 2.3 C). Furthermore, PK-3E cells demonstrate yet another pattern of SAPK activation when detached. SAPK fails completely to become activated upon cell detachment, instead activity declines below the basal level over time spent in three-dimensional conditions (Figure 2.3 D). This observation may be reflective of the decreased sensitivity to undergoing anoikis demonstrated by these cells in relation to the other cell lines examined (Figure 2.2).

2.1.2 Activated ras Alters the Kinetics of SAPK Activation in IEC-18 cells

A number of reports have indicated that Ras can signal through a variety of pathways to activate SAPK (Kawasaki et al., 1996; Plattner et al., 1999; Hunter, 1997; Cohen, 1997; Clark et al., 1997). The involvement of SAPK activation in triggering apoptosis seems somewhat paradoxical in view of the survival abilities imparted by Ras expression in anchorage-independent growth conditions. We decided then to examine the degree and kinetics of SAPK induction in cells overexpressing oncogenic ras when placed in three-dimensional culture conditions. Not surprisingly, a different pattern of SAPK activation upon cell detachment was detected. Similar to previous reports, in MDCK cells (Frisch et al., 1996), ras does not abrogate SAPK activity in IEC-18 cells placed in suspension. The maximal activity of SAPK occurs between 45 and 60 minutes after detachment of IEC-18 ras3 cells (Figure 2.4 A) contrasting sharply with the transient SAPK activation expressed after 15 minutes by IEC-18 parental cells upon detachment. Although PK-3E cells fail to activate SAPK

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Figure 2.4 Delayed activation of Stress Activated Protein Kinase in anoikis resistant ras transformed cells.

SAPK immunoprecipitates from IEC-18 ras3 (A) and PK-Kras (B) cells cell lysates were assayed for associated c-jun activity. Radioactivity in c-jun bands is shown in the insert and quantitiated in the graph shown below. Positive control is IEC-18 ras3 cells treated with 250 μg/mL of anisomycin. Data is representative of a minimum of 4 independent assays.
upon detachment, ras transformation of these cells does alter the kinetics of SAPK activity. PK-Kras cells, which similar to IEC-18 ras3 cells are resistant to anoikis (Figure 2.2), activate SAPK within 15 minutes after detachment with activity remaining elevated for 60 minutes (Figure 2.3 B). This observation further supports the arguments for both ras dependent signaling of SAPK (Kawasaki et al., 1996), and the lack of correlation between SAPK signaling and cell death.

2.1.3 Maximal Activation of SAPK in Spheroid Culture does not Commit Cells to Undergo Anoikis

To investigate whether SAPK activity is sufficient to induce anoikis, we designed a rescue experiment to test cell viability after the maximal SAPK activation in suspension culture has been reached (Figure 2.5 A). The various cell lines, in which we had previously established the pattern of SAPK induction, were plated and allowed to become confluent. After 24 hours, cells were trypsinized and then either replated in monolayer or in three-dimensional culture conditions for 2 hours. This time point was selected to ensure that all cell lines would attain the maximal activation of SAPK induced by substratum detachment. After such 2 hour incubation in suspension culture the cells were replated in monolayer culture for a further 22 hours. Apoptosis was determined 24 hours after the initial cell plating by a cell death ELISA (Roche). Interestingly, none of the cells pre-incubated in suspension showed detectable levels of apoptosis (Figure 2.5 B) thus, confirming the hypothesis that SAPK activity induced by cell detachment is not sufficient to commit cells to apoptosis.
Figure 2.5 Activation of SAPK in suspension culture is insufficient to cause anoikis. A. Schematic of anoikis rescue experiment. B. Apoptosis of IEC-18, IEC-18 Ras3, HUVEC, PK-3E cells and PK-Kras was assessed after the cells remained in three-dimensional culture for the time exceeding the requirement for maximal activation of SAPK (2 hours). The enrichment of mono- and oligonucleosomes in the cytoplasm was detected by cell death ELISA assay. The data represents the average of two independent experiments, each one was done in duplicate, error bars are representative of the range between experimental data.
2.1.4 Inhibition of p38 by SB203580 fails to affect anchorage independent IEC-18 cell survival

The p38 MAPK, together with its upstream and downstream targets comprise another MAPK family. Like the SAPK pathway, p38 is known to be activated by a variety of cellular stresses, including osmotic shock, inflammatory cytokines, UV light and growth factors (Han et al., 1994; Lee et al., 1994; Khwaja & Downward, 1997). The compound SB203580, which effects a specific and potent inhibition of p38, was used at a variety of doses to determine if p38 played a role in effecting the anoikis of IEC-18 cells. A recent study by Khajwa in 1997 using dominant negatively transfected cells reported that although activated when MDCK cells were detached, p38 activity was immaterial to the subsequent anoikis of these cells (Khwaja & Downward, 1997). Similar to these observations, p38 does not appear to be involved in IEC-18 cell anoikis. None of the concentrations (1, 5 or 10 μM) of SB203580 we examined had any significant effect on the anoikis of IEC-18 cells (Figure 2.6).

2.2 Anoikis resistant phenotype confers tumorigenicity in vivo.

As previously stated (section 2.1) IEC-18 cells are highly susceptible to anoikis. During this death process, a proportion of the cells initially form a tight aggregate while remaining cells disintegrate outside the spheroid, forming a halo of debris (Rak et al., 1999). Due to the heterogeneity observed between different IEC-18 derived clones with respect to their sensitivity to cell death induced by three-dimensional conditions, it was hypothesized that cryptic subpopulations resistant to anoikis might exist. Furthermore, such cells may be selected (and/or further induced) by long-term growth in three-dimensional conditions. Subsequently, IEC-18 cells were subjected to repeated cycles of selection for survival and growth in spheroid culture
Figure 2.6 No effect of p38 inhibition on IEC-18 cell death in spheroid culture

Apoptosis of IEC-18 was assessed after the cells were incubated in three-dimensional culture for 24 hours with the indicated concentration of SB203580. Inhibition of p38 with SB203580 fails to demonstrate any significant effect on the anoikis of IEC-18 cells. The enrichment of mono- and oligonucleosomes in the cytoplasm (apoptotic enrichment factor) was detected by cell death ELISA assay. Error bars are the standard deviation between experimental data (n=4 for each sample).
After each 4- to 7-day incubation period on agarose-coated dishes, the remnants of IEC-18 cell spheroids were collected and replated into monolayer culture where they were left to recover for several weeks. The few surviving clones were expanded and plated for another cycle of three-dimensional culture. After 10 such selection cycles the cellular variants were able to form larger spheroids and were comprised of cells that showed a reduced rate of cell death, albeit to a lesser extent that transformed cells, such as, IEC-18 cells expressing mutant H-ras oncogene (Rak et al., 1995b).

Independent series of selections resulted in the derivation of two anoikis-resistant variants, designated IEC-18 AR1.10 and IEC-18 AR2.10. These cells were found to be stable in their ability to retain their survival properties in three-dimensional culture even after several weeks of prior growth in monolayer cultures (Figure 2.1). These cells have further been shown to harbour a number of molecular alterations, such as upregulation of cyclin D1, bcl-XL and VEGF, which in combination may explain some of the observed survival and growth advantages displayed by these cells.

Previous work with parental IEC-18 cells have shown these cells to be absolutely nontumorigenic after subcutaneous injection into nude mice, regardless of cell number at the inoculum (1-5 x 10^6) or the duration of the follow up time (up to 12 months). In contrast, expression of the H-ras oncogene had a pleiotrophic transforming effect on IEC-18 cells manifested by tumorigenic properties attributable to the combination of effects of the oncogene on cellular survival, proliferation, and induction of an angiogenic response in vivo (Filmus et al., 1994; Rak et al., 1995b; Rak et al., 1995a).

Surprisingly, selection for anoikis resistance alone resulted in acquisition of a tumorigenic phenotype by both AR1.10 and AR2.10 cell lines (Figure 2.7 B).
Figure 2.7 Tumour forming capacity of IEC-18 variant cell lines

A. Experimental protocol for derivation of apoptosis resistant variants (see text). B. Tumorigenicity of IEC-18 AR1.10 and IEC-18 AR2.10 cells in nude mice (2 X 10^6 cell per subcutaneous inoculum) (Inset) C. Induction of anoikis resistance in vivo. Cumulative cell survival after 3 days in spheroid culture (MTS assay). (Inset) Rate of nucleosome release (apoptotic enrichment factor) as measured after 48 hours in spheroid culture by Cell Death Elisa) D. SAPK immunoprecipitates from IEC-18 AR1.10 and IEC-18 AR2.10 cell lysates were assayed for associated c-jun activity. Radioactivity in c-jun bands is shown in the insert and quantitated in the graph shown below. Positive control is IEC-18 AR1.10 cells treated with 250 µg/mL of anisomycin. Data is representative of three independent assays.
Injection of $2 \times 10^6$ cells of each variant has been shown to result in the formation of slow-growing tumours in 9 out of 10 and 5 out of 8 nude mice, respectively (Rak et al., 1999). Despite their slow growth, tumours eventually reached considerable sizes, e.g. 500 to 2000 mm$^3$, within 4 to 5 months. Cell lines reisolated from some of these tumours, designated AR2T1 and AR2T2 were even more resistant to anoikis in three-dimensional culture and morphologically indistinguishable from their parental AR2.10 cell line (Figure 2.7 C and data not shown).

Interestingly, the tumour derived variant cell lines, although clearly exhibiting enhanced survival characteristics compared to their parental (in vitro) passed cell lines, failed to demonstrate any changes in the kinetics of SAPK activity upon cell detachment in vitro (Figure 2.7 D). This provides further evidence for the lack of direct involvement of SAPK signaling in the transformation and anoikis resistance of IEC-18 cells. Thus the SAPK pathway is not involved in triggering anoikis of IEC-18 cells or anoikis resistance of their transformed tumorigenic counterparts.

2.3 Deregulation of members of the IAP family of survival genes in transformed cells, resistant to anoikis

2.3.1 IAP upregulation in ras transformed IEC-18 cells

We sought to determine what changes expressed in survival genes are associated with and possibly responsible for resistance to anoikis in ras transformed cells. In that regard, we chose members of the IAP family, as they directly antagonize the activity of cell death inducing 'distal' caspases. A growing number of reports are implicating the IAP gene family in the enhanced survival of tumour cells (Clem & Duckett, 1997; LaCasse et al., 1998). We decided to examine different members of the IAP family to determine if they played a role in the
circumvention of anoikis by transformed cells. Initial investigations were undertaken in the IEC-18 cell system. Using rat specific antibodies, IAP protein expression, was determined by western blot analysis, for monolayer and spheroid cultures after 24 hours. No significant change in protein level was found between monolayer and spheroid culture for IEC-18 cells. However, this observation does not rule out the possibility that constitutive ras-dependent changes in IAP levels may regulate susceptibility to anoikis as has been shown for members of the bcl-2 family e.g. Bak. Indeed, changes were observed in expression of only one of the three IAP members, namely RIAP1, in that a significant upregulation of RIAP1 was consistently observed in ras transformed IEC-18 cells (Figure 2.8).

Similar to the parental cells no change was found between the monolayer and spheroid cultures of IEC-18 ras3 cells. However, the functional consequences of the increased expression of RIAP1 in IEC-18 ras3 compared to the parental cells may be sufficient to impart some resistance to anoikis. Additional investigation was undertaken to examine mRNA levels of RIAP3 using a RIAP3 probe. There was no significant change in RIAP3 mRNA expression between monolayer and spheroid of both IEC-18 and IEC-18 ras3 cells (data not shown). Furthermore, there was no upregulation of RIAP3 mRNA expression in the ras transformed cells. This is not surprising as IAP expression is reported to be regulated at the post-transcriptional level.

2.3.2 IAP levels exhibit specificity based on cell type and oncogene

The observation that RIAP1 protein expression was upregulated in ras transformed IEC-18 cells prompted us to further investigate the impact of other oncogenes. In contrast to ras, src expression does not appear to upregulate RIAP1 in the IEC-18 cell system as determined by
Figure 2.8 Expression of RIAP1 protein in IEC-18 cells and IEC-18 ras3 cells

Western blot analysis of RIAP1 expression in IEC-18 and IEC-18 ras3 cells in monolayer and spheroid culture after 24 hours. Protein expression was normalized based on expression of cdk4.
Figure 2.9 Expression of RIAP1 protein in IEC-18 ras3 and IEC-18 src2 cells

Western blot analysis of RIAP1 expression in IEC-18, and IEC-18 src2 cells in monolayer culture. Protein expression was normalized based on expression of cdk4.
western analysis (Figure 2.9). A panel of rodent and human cell lines, harbouring various oncogenic changes, including activated \textit{neu} expression, was examined for IAP expression. Again, the only consistent trend observed was the \textit{ras} upregulation of RIAP1. This was observed in both IEC-18 epithelial cells and also in NIH 3T3, a mouse fibroblast system. No changes were observed for either RIAP2 or RIAP3 protein levels in IEC-18 cells compared to IEC-18 \textit{ras3} and IEC-18 \textit{src2} cells, or NIH 3T3 cells compared to NIH 3T3 \textit{ras} (3T3 ras) and NIH 3T3 \textit{neu} cells (B104.1.1). The above results indicate that IAP expression exhibit specificity based on the oncogene.
Chapter 3: Discussion

3.1 Role of Adhesion in Tumorigenesis

In the progression of cancer, disruptions of normal mitogenic and survival mechanisms are frequently the main driving forces initially behind, early cellular transformation, and in later stages invasion and metastasis. Investigations are ongoing to unravel the specific events, such as signaling and transcriptional activity, which ultimately culminate in abnormal cellular behaviour. One important survival mechanism is cellular adhesion, which is a requirement, albeit to varying degrees, for the majority of normal cell types.

Adhesive interactions regulate numerous cellular processes such as growth, gene expression and apoptosis. In fact, Raff's proposal of 'death by default', where signals are continuously required by cells for proliferation/survival, and in their absence initiate apoptosis, is substantiated by one particularly striking manifestation of apoptosis involving cellular adhesion (Raff, 1992). Anoikis, or the apoptosis resulting from loss of adhesion, has been reported in numerous cell types (Meredith, Jr. et al., 1993; Frisch & Francis, 1994; Boudreau et al., 1995; Ishizaki et al., 1995; Rak et al., 1995b; Vachon et al., 1996). Anoikis sensitivity, which presumably acts as a critical safeguard in the maintenance of normal tissue architecture, is often lost in transformed cells. Moreover, among the numerous alterations exhibited by transformed cells conferring formidable growth and survival advantages over normal cells, it is the ability of cells to grow in an anchorage independent manner that has received prominence and become one of the most widely accepted in vitro hallmarks of cancer.

Although increasing at a rapid rate, the number of available reports investigating the death effectors of anoikis have to date been limited to a handful of papers. Prompted by our
discovery of the strikingly massive apoptosis response of IEC-18 cells when grown as spheroids, and the marked reversal of this effect in ras transformed IEC-18 cells (Rak et al., 1995b), we undertook a study to explore possible regulators of this system, which seemed particularly suitable for such an analysis given the magnitude of the differences. When I began my investigations of potential signals controlling IEC-18 cell anoikis there were no available reports implicating any one particular signaling cascade. Based on the knowledge that anoikis is thought to occur mainly by the disruption of integrin mediated survival signals (Meredith, Jr. et al., 1993; Schwartz, 1997) we reviewed the literature to determine a suitable target and thus narrow the focus of our investigation.

3.2 Biological Role of the SAPK Family

Apoptosis is now one of the ‘hottest’ areas of cancer research and this trend has resulted in an explosion of papers on the topic. At the onset of this study one cellular signaling cascade, aptly named SAPKs, was being reported in the apoptosis observed resulting from a wide variety of cellular stresses, including UV irradiation, heat shock, oxidative stress, and ischemic reperfusion (Kyriakis et al., 1994; Zanke et al., 1996). Our hypothesis was that the disruption of integrin interactions occurring from cell detachment may signal through the SAPK signaling cascade thereby effecting, at least in part, anoikis and thus add loss of adhesion to the growing list of cellular stresses that signal SAPK. It was upon this foundation that we began our examination of SAPK in the anoikis of IEC-18 cell system. In this study I have attempted to elucidate and more clearly define the role of SAPK in the relation to anoikis in both normal epithelial and endothelial cell models.
Primary experiments in the IEC-18 cell system, although initially slowed by several technical problems (involving available antibodies for immunoprecipitation assays), were unable to detect significant SAPK signaling after 30 and 60 minutes of IEC-18 cell detachment from the substratum. It was at this point that the first published paper appeared implicating SAPK in the anoikis of MDCK cells (Frisch et al., 1996). This was closely followed by another report from the same group indicating a positive feedback loop between SAPK signaling and caspase activation (Cardone et al., 1997). However, the casual role of SAPK was examined only in the MDCK cell system and even this result was found to be controversial, and has not yet been confirmed. Taken together these results prompted us to undertake a more rigorous and in-depth analysis of the role of SAPK in anoikis. We decided to examine not only the IEC-18 cell system, but also additional epithelial and endothelial anoikis-sensitive cell systems and to investigate further the anoikis abrogation resulting from mutant ras expression in two variants of the chosen cell lines.

The initial paper regarding SAPK and anoikis reported that not only was SAPK activation strongly induced by cell detachment from the matrix, but that this activity was required for anoikis (Frisch et al., 1996). These investigations involved only the MDCK cell system, and it was further reported that caspase 3 activation was another early response to cell detachment. Upon closer examination the resulting conclusions were twofold. First, it was reported that both crmA, an inhibitor of caspase 3, and bcl-2 suppressed both caspase 3 activation and anoikis. Surprisingly, bcl-2 and crmA attenuated the activity of SAPKs suggesting that signaling may serve in a regulatory manner, either indirectly or directly. Interestingly, it was also demonstrated that the transformation of MDCK cells by H-ras abrogated anoikis and suppressed the activation of SAPK. The final observation involved the
creation of a dominant negative cell line in which a mutated form of SEK (JNKK) was shown to block signaling to SAPK and thus result in anoikis resistance. The hypothesis put forth on the basis of these experiments was the following sequence of information flow in anoikis: integrins→bcl-2/bax ⇔ (caspase 3/ SAPK) → apoptosis (Frisch et al., 1996) (see also Figure 1.2).

These findings were supported shortly afterward by the publication that anoikis was also regulated by MEKK-1 and caspase activation. Again in the MDCK cell system, it was demonstrated that cell detachment activates caspase activity, specifically caspases 3 and 7, that are in turn responsible for the cleavage of MEKK-1 (Cardone et al., 1997). This MEKK-1 cleavage product is not only required for subsequent kinase activity supposed to be necessary for apoptosis, but can also stimulate additional caspase activity, once more indicating a positive feedback loop. Dominant negative constructs were also employed to illustrate the stimulation of apoptosis in cells overexpressing the cleavage product of MEKK, the sensitization of cells to anoikis expressing wild type MEKK and finally, the partial protection of cells from anoikis by cells expressing a cleavage resistant mutant MEKK (Cardone et al., 1997).

Controversy became apparent when the most recent publication linking SAPK and anoikis was unable to reconfirm the earlier studies, even though it utilized the same MDCK cell line, (Khwaja & Downward, 1997). Khwaja et al. reported that although SAPK was indeed activated upon cell detachment, a study of cell lines expressing activated signaling proteins usually controlled by ras showed the stimulation of SAPK failed to correlate with the induction of anoikis (Khwaja & Downward, 1997). It was further reported that PI3-kinase and PKB/Akt protect MDCK cells from anoikis without any suppression of SAPK activity. Conversely, activated Raf and dominant negative SEK (the same kinase targeted in the previous studies)
attenuate detachment-induced SAPK activation without protecting the cells from anoikis. Interestingly, here zVAD-fmk, a peptide inhibitor of caspases, was reported to prevent apoptosis without affecting SAPK activity (Khwaja & Downward, 1997). p38 was also shown to be activated early in response to loss of cell adhesion, but inhibition by SB203580 failed to protect the cells from undergoing anoikis. It was concluded from these studies that SAPK and p38 are both unlikely to play a role in the anoikis response of MDCK cells (Khwaja & Downward, 1997).

It is the aforementioned published data that provides the context for the publication of my own findings (Krestow et al., 1999). Two main findings are presented in this thesis. First, the magnitude of SAPK activation upon cell detachment from the extracellular matrix appears to be a cell-type specific phenomenon, and, second, SAPK activation is dissociable not only from the onset of anoikis in four anoikis-sensitive cell lines, but also, from the lack of anoikis in the ras transformed variants of two of these lines.

Our results shed some light on the role of SAPK in the broader phenomenon of anoikis as a result of investigating a number of different cell lines. Furthermore, this work is consistent with the growing number of reports indicating that SAPK activation is highly dependent on both the nature of the inducing stimulus and the cellular context (Carletti et al., 1995; Liu et al., 1996; Hu et al., 1997; Plattner et al., 1999; Matsuda et al., 1999).

The second finding, namely the complete lack of correlation between SAPK activity and the induction of anoikis in any of the cell lines that I examined, is not surprising in light of my first finding because two out of three cell lines exhibited only a weak and transient activation of SAPK upon substratum detachment, while the third failed altogether to activate SAPK, although all cells displayed comparable levels of apoptosis in three-dimensional culture. p38 was also
examined briefly in the IEC-18 cell system. However, no survival advantage was detected when p38 activity was inhibited by SB203580. In addition, ras transformed IEC-18 and PK-k ras cells are completely resistant to anoikis but, nevertheless, show SAPK activity when detached, albeit with different kinetics from the parental cells. These results support the findings of Khwaja et al. who, as outlined above, reported that although SAPK is strongly activated under the conditions that initiate anoikis in MDCK cells, it is neither necessary nor sufficient for the induction of anoikis in this particular cell line (Khwaja & Downward, 1997). My results confirm this conclusion in other cell lines as well, such as rat intestinal and kidney epithelial cells, and human endothelial cells.

3.3 Interference with Death Pathways by Oncogenes and Survival Effectors

What factors, then, are responsible for induction of anoikis, and development of resistance to this form of apoptosis in normal epithelial and transformed epithelial cells, respectively? The answer, to some extent, may depend upon the nature and origin of the cell population, and perhaps other contextual factors as well.

For example, we have previously reported that pro-apoptotic effector molecule known as Bak plays an important role in the induction of anoikis of IEC-18 intestinal epithelial cells (Rosen et al., 1998). Oncogenic ras can induce a significant downregulation of the pro-apoptotic effector protein known as Bak (Rosen et al., 1998), a member of the bcl-2 gene family of apoptosis regulators (Kroemer, 1997; Reed, 1999). In this regard it is interesting to note that Bak has been previously implicated as a critical endogenous promoter of apoptosis in the intestinal epithelium (Moss et al., 1996). For example, Arber et al. (Arber et al., 1997) found that a K-ras
oncogene can increase resistance to sulindac-induced apoptosis in IEC-18 cells and that this may be the result of downregulation of Bak. Similarly, Houghton et al. found that apoptosis was inhibited after 'thymineless stress' by oncogenic K-ras in thymidylate synthase deficient human colon cancer cells, and that this may be due to altered levels of Bak and Bcl-X\textsubscript{L}, an anti-apoptotic effector, (i.e., upregulation of Bcl-X\textsubscript{L} and downregulation of Bak) by oncogenic K-ras (Houghton \textit{et al.}, 1998).

The findings by Houghton et al. have recently been further supported by the discovery that activated \textit{ras} not only induces the downregulation of Bak, but also prevents the downregulation of Bcl-X\textsubscript{L} upon cell detachment in IEC-18 cells (Rosen, 1999 unpublished). Since exogenous Bak was insufficient to fully restore the susceptibility of \textit{ras}-transformed cells to anoikis, this indicated the necessary involvement of additional components of apoptotic regulation in mediating the effect(s) of \textit{ras} on anoikis. It was demonstrated that non-malignant rat and human intestinal epithelial cells both exhibit a significant downregulation of Bcl-X\textsubscript{L} upon cell detachment, and that this downregulation was completely abrogated by expression of either H- or K-\textit{ras} oncogenes. Moreover, enforced downregulation of Bcl-X\textsubscript{L} in \textit{ras}-transformed epithelial cells not only promotes anoikis, but also significantly inhibits tumorigenicity (Rosen \textit{et al.}, 1999 unpublished).

Although the \textit{ras} oncogene-Bak inter-relationship appears to be mediated, at least in part by PI3K (Kroemer, 1997; Khwaja & Downward, 1997), the effect of \textit{ras} on Bcl-X\textsubscript{L} was PI3K independent. Simultaneous downregulation of Bcl-X\textsubscript{L} and inhibition of PI3K had a co-operative effect in overcoming anoikis resistance (Rosen \textit{et al.}, 1999 unpublished). Together these findings suggest that there exists both cooperation and redundancy in the signaling that regulates anoikis and hence a variety of means exist to abrogate apoptosis in transformed cells. Moreover,
mutant ras may function as an important survival factor in colon cancer through its ability to modulate (downregulate) Bak and (stabilize) Bcl-X\textsubscript{L} expression.

However, as discussed in the Introduction, despite the pre-eminence of the bcl-2 family in apoptosis studies there are numerous families of genes involved in the regulation of this process (see Figure 1.1). In fact, additional factors appear to be involved in ras-mediated resistance to anoikis, the nature of which are currently under investigation. Initial work was undertaken to investigate the potential role of the IAPs, anti-apoptotic effectors functioning through caspase inhibition (LaCasse et al., 1998), in the phenomenon of anoikis and its subsequent abrogation in transformed cells. Little is presently known about IAP expression and functionality in cancer, although the family member called "survivin" is reported to be overexpressed in fetal tissues and numerous human malignancies, but not in adult tissues (Ambrosini et al., 1997).

Preliminary investigations have determined that, although not involved (by downregulation) in the anoikis of non-transformed IEC-18 cells, one family member appears to be involved in the anoikis abrogation of ras-transformed IEC-18 cells. RIAP1 is the only family member that demonstrates consistent changes in expression, becoming upregulated, by activated ras. The following studies, which were extended to include both additional ras transformed cell types, and also cell lines transformed by other oncogenes, e.g. neu, and src, found no other marked patterns in IAP upregulation and oncogenic transformation. This finding may suggest that IAP involvement in anoikis regulation, and perhaps by extension apoptosis resulting from other stimuli, exhibits specificity based on oncogene expressed.
3.4 Collateral Regulation of Anoikis Resistant Phenotype and Tumorigenecity

To date the majority of studies investigating anoikis have been carried out primarily in vitro. Although providing valuable preliminary observations, the relevance to the in vivo situation remains uncertain. As stated previously, anchorage-independent growth is widely used as a marker of cell transformation; however, the biological implications of this property are poorly understood. Direct evidence for a causal relationship between anoikis resistance and tumorigenecity has not been firmly established. This lack of evidence can be attributed to the multitude of functional abnormalities usually expressed by cancer cells, such as resistance to various apoptosis-inducing stimuli, deregulated mitogenic activity, reduced growth factor requirements, and expression of pro-angiogenic properties, all of which are difficult to separate functionally from one another.

In an effort to explore the potential relationship between the acquisition of an anoikis-resistant phenotype and tumorigenic capacity we decided to study serially selected anoikis resistant variant cells (IEC-18 AR1.10 and AR2.10) derived from the parental IEC-18 cell population (Rak et al., 1995b; Rak et al., 1999). We designed an in vitro selection protocol, which theoretically would specifically favour the acquisition of resistance of IEC-18 cells to anoikis. From the parental population of IEC-18 cells, which shows some intrinsic heterogeneity as to the kinetics of cell death induced under three-dimensional growth conditions, we found that sequential exposure of IEC-18 cells to such 3D growth conditions spontaneously leads to the derivation of rare cellular variants with a markedly reduced susceptibility to anoikis. Cells were subjected to repeated cycles of selection for survival and growth in 3D culture. After each 4- to 7-day incubation on agarose coated dishes, the remnants of IEC-18 cells spheroids were
collected, replated in monolayer culture and left to recover for several weeks. The few surviving clones were expanded and plated for another cycle of spheroid culture. After 10 cycles of selection the variant cells were capable of forming larger spheroids comprised of cells that exhibited a reduced sensitivity to apoptotic cell death, albeit to a lesser extent than IEC-18 sublines overexpressing a mutant H-ras oncogene (Rak et al., 1999). Two cell lines were established from independent serial selections, which were shown to be stable in that even after several weeks in standard monolayer culture they retained their survival properties in spheroid culture.

This sequential selection for survival in three-dimensional culture was sufficient for acquisition of tumorigenic competence of these cells in vivo. Unlike the parental cells, the anoikis-resistant cells can form relatively large, (although slow growing tumours) in nude mice. Interestingly, the tumour-derived variant cells showed no alterations in SAPK activity on cell detachment in vitro, indicating the irrelevance of SAPK signaling in the anoikis-resistant phenotype, at least in this experimental model. Furthermore, the anoikis-resistant phenotype was associated with the collateral and spontaneous upregulation of VEGF, a potent stimulator of angiogenesis. Both cell lines exhibited upregulated expression of VEGF, which may be similar in some aspects to the angiogenic switch exhibited by some types of cancer during the premalignant phases of tumour development (Smith-McCune & Weidner, 1994; Hanahan & Folkman, 1996; Erhard et al., 1998). However, transfection-mediated overexpression of VEGF demonstrated that VEGF expression alone was insufficient to confer a tumorigenic phenotype with cells remaining highly susceptible to anoikis. Clearly other factors may contribute to this tumorigenic (and perhaps angiogenic) switch as well, e.g. hypoxia (Shweiki et al., 1992) and/or other genetic alterations (Bouck et al., 1996).
These observations prompted us to postulate that both anoikis resistance and angiogenic-competence contribute to tumour formation (Rak et al., 1999). Development of anoikis-resistance can be then viewed as a precondition for expression of the tumorigenic phenotype. Even when angiogenesis is not a rate-limiting factor, e.g. in vitro, the selective pressures of solid tumour-like, three-dimensional growth conditions favoring anoikis resistance result in collateral induction of a proangiogenic phenotype. This study, where cell selection was solely based on survival under anchorage-independent growth conditions, provides a relatively unbiased system, which illustrates the principle that cellular survival is a critical component in the evolution of a transformed phenotype. Although, the mechanisms are as yet unknown, cell survival may act in a manner to facilitate the selection and progression of additional transforming changes. It is exactly this type of functional coupling (anoikis resistance and angiogenic competence) that suggests the selection of cells to grow three-dimensionally favours certain types of pleiotrophic molecular defects in conjunction with cell survival. Therapeutic targeting of such pleiotrophic defects could be highly effective, as it could simultaneously suppress or obliterate multiple essential components of the tumorigenic phenotype, such as tumour angiogenesis and the anoikis resistant phenotype of tumour cells.

3.5 Anoikis as a Potential Target of Anti-cancer Therapy

Regardless of whether cellular survival is sufficient on its own or instead acts as a facilitator, requiring one or more additional genetic changes, to confer a transforming phenotype to a cell, it remains an attractive target for anti-cancer therapy. Surviving noxious conditions more effectively than the cells surrounding it will enable a cell to establishing dominance and is essential for cancer progression. Lack of adhesion correlates quite well with tumorigenicity and
may facilitate early events in tumour metastasis, such as detachment of tumour cells and intravesation. Consequently, understanding the survival signals and the anoikis resulting in their absence is of considerable interest for cancer development and drug resistance (Green et al., 1999).

Although SAPK does not appear to have a regulatory function in anoikis, it has been implicated as a ‘death pathway’ in the context of anticancer therapy (Haq & Zanke, 1998). This has prompted considerable clinical interest and illustrates the need for a better definition of the stimuli inducing SAPK activation and of the functional downstream effectors. It further indicates the necessity of more encompassing investigations to determine the actual functional regulators of anoikis, both pro- and anti-death.

One of the best examples of anoikis resistance on the survival of tumour cells is a number of experiments designed to impair the function of the IGF-1 receptor through either antisense strategies or dominant negative mutants. Studies revealed that antisense oligodeoxynucleotides targeting IGF-1 receptor RNA resulted in massive apoptosis of melanoma cells (Resnicoff et al., 1995). However, the same treatment of cells in standard monolayer in vitro culture had only a very modest apoptotic result, whereas cells grown in three-dimensional in vitro culture (soft agar) showed apoptotic death levels somewhere in between. These results were repeated with additional human and rodent tumour cell lines (Baserga et al., 1997) and further recapitulated in tumour cells stably transfected with dominant negative IGF-1 receptor (IGF1-R) (D'Ambrosio et al., 1996). Evidently, the IGF-1R plays a crucial role for cells in (anchorage-independent) soft agar or in vivo, and only has minimal involvement in monolayer growth. A similar type of difference between monolayer and three-dimensional growth to that of the IGF-1R is that of the massive apoptosis and its subsequent abrogation by the mutant ras protein in the IEC-18 cell
system (Rak et al., 1995b). In conjunction with the IGF-1R experiments, these observations point to the existence of targets that are much more important for anchorage independent growth than for normal monolayer growth (Baserga, 1997). Thus, once anoikis abrogation is understood in more depth it could provide a much more selective target for cancer, and hence, anti-cancer drugs having a better therapeutic index. One such example is already underway, with the targeting of transformed cells by Ras FTIs, which may act directly on ras (Gibbs et al., 1994; Gibbs et al., 1996; Leonard, 1997) or, as more recently reported, ras and/or RhoB (Prendergast et al., 1994; Prendergast et al., 1996).

In the broader context of cancer therapeutics Baserga elegantly outlines the necessity of examining the anoikis phenomenon (Baserga, 1997). The increasing observations of differential effects under diverse conditions lead to three main questions: (1) are potential therapeutics being overlooked due to their testing against tumour cells only in monolayer culture? (2) could the differential response of cells to anoikis and it’s abrogation in transformed cells be used to treat cancer, where cells frequently become anchorage independent (e.g. metastases and local recurrences) during disease progression? and (3) what are the mechanisms regulating anoikis?

3.6 Summary

In summary, the data presented here does not dispute that SAPK is activated by the loss of cell-ECM interactions (Frisch et al., 1996; Khwaja & Downward, 1997), but serves to provide further support for the lack of causative effects this activation has in the overall phenomenon of anoikis. Although implicated in a variety of cellular functions, such as cell cycle regulation (Zanke et al., 1996) or the apoptosis induced by such divergent stimuli as heat stress or chemotherapeutic drugs (Zanke et al., 1996; Hu et al., 1997) SAPK activation appears to be
functionally unimportant in the direct signaling that commits cells to anoikis. Further studies investigating other possible regulators of anoikis have pointed to several members of different gene families, which act to either effect or abrogate anoikis. Foremost among them are the bcl-2 family members Bak and Bcl-X$_L$ (Rosen et al., 1998; Rosen et al., 1999 unpublished) although newly discovered apoptotic regulators such as the IAP family should not be disregarded. It would appear that anoikis regulation is a complex process regulated by numerous and possibly redundant signaling pathways, which may further exhibit cell specific variations.
Chapter 4: Materials and Methods

4.1 Cells Lines and Culture Conditions

IEC-18 and IEC-18 ras3 cells were obtained and cultured as described previously (Rak et al., 1995b). Briefly, all cell lines were maintained in monolayer culture in growth medium composed of α-minimum essential medium (α-MEM) base supplemented with 5% fetal bovine serum (Gibco BRL, Gaithersburg, MD), 4 mmol/L L-glutamine, 20 mmol/L glucose, 10 μg/mL insulin, (Sigma Chemical Co., St. Louis, MO). MDCK cells were obtained from the ATCC and maintained as monolayer cultures in medium composed of Dulbecco’s modified Eagle’s medium (DMEM) base supplemented with 10% fetal bovine serum (FBS, Gibco Canadian Life Technologies) grown on dishes precoated with 1% gelatin (Sigma) in medium composed of a MCDB131 base supplemented with 15% FBS (Gibco), 10 ng/mL epidermal growth factor (Upstate Technologies, Lake Placid, NY), 5 ng/mL basic fibroblast growth factor (R&D Systems Inc., Minneapolis, MN) and 10 units/mL heparin (Gibco). PK-3E and PK-kras cells were maintained in monolayer culture in growth medium composed of DMEM base supplemented with 10% FBS. For spheroid culture over 2 hours in length cells were plated on 100-mm (Nunc) tissue culture dishes precoated with a thin layer of 1% Seaplaque agarose (FMC Corp., Rockland, ME). For spheroid culture under 2 hours in length cells were left in 14 mL Falcon polystyrene tubes (Becton Dickinson, Mississauga, Ont.) at 37°C and agitated every 30 minutes.
4.2 **MTS Staining**

Cell viability was assessed essentially as described previously (Cosimi et al., 1991). This assay is composed of two solutions, a tetrazolium compound (3(4,5-dimethylthizol-2-yl)-5-(3-carboxymethoxphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt; MTS) (Promega, Fischer Scientific) and an electron coupling reagent (phenazine methosulfate:PMS) (Sigma-Aldrich). Briefly, 10⁴ cells/per well were cultured in 100 µL of media in 96-well plates precoated with 2% poly(2-hydroxethyl methacrylate) (Sigma). At the time point indicated, 100 µL of a mixture containing the solutions described above was added and the absorbance at 490 nm recorded after 1 and 3.5 hours. The average absorbance from multiple wells containing spheroids was normalized to absorbance of corresponding monolayer cultures or to day zero in time course experiments.

4.3 **Anoikis Assays**

The characteristics of apoptotic death of IEC-18 cells in spheroid culture, and its abrogation in oncogenic ras transfectants IEC-18 ras-3 have been described in detail (Rak et al., 1995b). For the purpose of the present study we have used the cell death detection Elisa Plus kit (Roche Molecular Biochemicals, Laval, Quebec) to estimate the rate of apoptotic cell death by quantitation of nucleosomes released into the cytoplasm by dying cells. Briefly, cells were allowed to become confluent prior to plating at 5 x10⁴ cells per 100-mm tissue culture dish (Nunc) in both monolayer culture and spheroid culture where the tissue culture dish (Nunc) was precoated with a thin layer of 1% Seaplaque agarose (FMC Corp.). At the time points indicated duplicate samples were collected from the dishes, lysed and incubated with a mixture of
biotinylated antihistone antibody and peroxidase-conjugated anti-DNA antibody, both of which bind to histone DNA complexes and initiate colour reaction in the presence of the ABST (2,2'-Azino-di[3-ethylbenthiazolin-sulfonat] substrate. The OD reading at 405 nm was corrected to negative control (of lysis buffer supplied in the kit) and expressed as an enrichment factor, according to manufacturer's instructions.

4.4 Induction of Tumours in Mice and Establishment of Cell Lines

Single-cell suspensions of all cell lines indicated were prepared from monolayer cultures by trypsinization with 1% trypsin and neutralized with one wash with media containing FBS. Cells were further washed with PBS and 2-5 X 10^6 cells were injected subcutaneously into BALB/c nu/nu mice (Taconic, Germantown, NY). Tumours were measured weekly and their volume calculated according to the standard formula (a^2 X b)/2, where a is the width and b is the length of the horizontal tumour perimeter. The animals were sacrificed before any visible signs of discomfort were present according to the guidelines of the Animal Care Committee at Sunnybrook and Women's Health Sciences Center.

4.5 Immunoprecipitation Kinase Assays

The effect of cell detachment on SAPK activation was measured in IEC-18, IEC-18 ras3, PK-3E, PK-Kras, HUVEC and MDCK cells. Cells were plated at 3.8 x 10^6 cell/100mm plate and detached 24 hours later after a brief incubation with 1% trypsin. Trypsin was neutralized with one wash with media containing FBS (Gibco BRL); the cells were then resuspended in media with no FBS in 14 mL Falcon polystyrene tubes (Becton Dickinson) and incubated at 37°C with agitation every 30 minutes for the period of time indicated. Positive controls were
provided by cells treated with 250 μg/ml anisomycin for 30 minutes at 37°C. Cells were then rapidly pelleted and lysed in lysis buffer containing 50 mM Hepes (pH 7.4), 150 mM sodium chloride (NaCl), 1 % IEGPAL, 1 mM EDTA, 20 μg/ml aprotinin/leupeptin, 1mM phenylmethylsulfonyl fluoride (PMSF) and 1 mM sodium vanadate (Na₃VO₄) for 30 minutes on ice. Lysates were centrifuged at 12 000 g for 10 minutes at 4°C, equalized for protein (determined by Bio-Rad protein assays), and the supernatant immunoprecipitated with rabbit polyclonal JNK antibody sc-474 (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 hour at 4°C. Samples were further incubated in the presence of protein A sepharose beads (Sigma Chem Co., St. Louis, MO) for 1 hour at 4°C. Pellets were washed twice in wash buffer (50 mM Hepes (pH 7.4), 150 mM NaCl, 1 % IEGPAL, 1 mM EDTA) and once in kinase buffer wash (10 mM MgCl₂, 50 mM Tris-Cl, pH 7.5 and 1 mM EDTA, pH 7.5). The pellet was resuspended in kinase buffer and 100 μM [γ-³²P] ATP (Amersham Intl., Arlington Heights, IL); and c-Jun sc-4113 (Santa Cruz). The reaction was stopped with sample buffer after 30 minutes of incubation at 37°C. After boiling for 5 minutes and SDS-PAGE, the phosphorylation of c-Jun protein was quantified by using a phosphoimager (Molecular Dynamics, Sunnyvale, CA).

4.6 Western Blot Analysis

Cells grown in either monolayer or spheroid culture, were lysed for 45 minutes on ice in a buffer containing 50 mM Hepes (pH 7.4), 150 mM NaCl, 1 % IEGPAL, 1 mM EDTA, 20 μg/ml aprotinin/leupeptin, 1mM PMSF and 1 mM Na₃VO₄. Lysates were centrifuged at 12 000 g for 10 minutes at 4°C, equalized for protein (determined by Bio-Rad protein assays). Aliquots of supernatant containing 15-50 μg of protein were mixed with 5X SDS-PAGE sample buffer,
boiled for 5 minutes, and run through a 12% polyacrylamide gel under reducing conditions. The separated proteins were then electrophoretically transferred to Immobilon-P membrane (Millepore, Bedford MA). Following blocking by 5% nonfat dried milk in Tris-buffered saline with 0.125% Tween-20 (TSBS-T) for one hour at room temperature, the membranes were blotted with an anti-cyclin D1 antibody (Santa Cruz) at the concentration of 0.1 μg/ml, an anti-RIAP1/2/3 antibody (Apoptogen Inc., Ottawa, ON) an anti-XIAP antibody (Apoptogen Inc.), and anti-XAF antibody (Apoptogen Inc.) and an anti-survivin antibody (Alpha Diagnostics Inc., San Antonio, TX) at a 1:2000 dilution. After washing in TBS-T, the membrane was incubated with antirabbit immunoglobulin-horseradish peroxidase (1:1000 dilution) for one hour. Protein bands were detected by enhanced chemiluminescence ECL Detection System (Amersham Pharmacia Biotech).
Reference List


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