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Review

Signalling mechanisms of anoikis

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Summary. Apoptosis following loss of cell anchorage ('anoikis') is of relevance for development, tissue homeostasis and disease. Integrins regulate cell viability through their interaction with the extracellular matrix and they can sense mechanical forces arising from the matrix and convert these stimuli to chemical signals capable of modulating intracellular signal transduction. Recently it has been shown that protein kinase signalling pathways and apoptosis-related molecular control anoikis both positively and negatively. Focal adhesion kinase, when activated by integrins, can suppress anoikis. Phosphatidylinositol 3-kinase/Akt and mitogen-activated protein kinase may mediate the anoikis-suppressing effects of cells. Conversely, the stress-activated protein kinase/Jun amino-terminal kinase pathway promotes anoikis. In addition, certain bcl-2 and bcl-2-related proteins may also participate in the regulating of anoikis. In this review, molecular mechanisms of signal pathway inducing and perpetuating detachment-induced apoptosis will be discussed with special emphasis on the role of integrins, focal adhesion kinase, phosphatidylinositol 3-kinase/Akt, mitogen-activated protein kinase and bcl-2 family members.

Key words: Anoikis, Integrin, Extracellular matrix, Focal adhesion kinase, Phosphoinositide-3 kinase, Mitogen-activated protein kinase, bcl-2

Introduction

Normal cell and tissue homeostasis reflects a dynamic balance of cell proliferation, differentiation and apoptosis. Anoikis - the subset of apoptosis triggered by inadequate or inappropriate cell-matrix contacts - maintains the correct cell number of tissues. The loss of integrin-mediated cell-matrix contact induces anoikis in certain cell types.

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Examples of anoikis include apoptosis after detachment and shedding of terminally differentiated colon epithelial cells into the lumen (Strater et al., 1996) and apoptosis of mammary epithelial cells upon regression of the mammary gland, where the cells start expressing various matrix-degrading enzymes, detach from the degraded matrix, and undergo apoptosis (Lund et al., 1996). Anoikis has been described in many types of epithelial cells, including keratinocytes (Tamada et al., 1994), thyroid cells (Bajt et al., 2000), kidney epithelial cells (Frisch and Francis, 1994), as well as in endothelial cells (Meredith et al., 1993; Re et al., 1994), fibroblasts (Folkman and Moscona, 1978), and osteoblasts (Sakai et al., 2000). Anoikis has also been documented *in vivo*. It occurs in normal skin (Polakowska et al., 1994), in colonic epithelial tissues (Hall et al., 1994), and in the involuting mammary gland (Boudreau et al., 1995). Interestingly, it is also important developmentally in the first cavitation step of embryogenesis (Coucouvanis and Martin, 1995).

Survival signals are induced by attachment to extracellular matrix through integrins or by growth factor receptor signalling summarized. Disruption of these signals after cell detachment from the matrix induces anoikis. The signalling mechanisms involved in anoikis are still not completely understood. However, many of the signal transduction pathways activated upon cell detachment from matrix are quite similar to those occurring during apoptosis. Role of protein kinase signalling pathways, such as MAPK, FAK, in regulation of anoikis is important. Further more, the existence of anoikis implies that integrin signalling regulates critical components of the apoptotic machinery, because integrins are the major matrix receptors in vivo. In this review, we summarize the integrin and protein kinase signalling pathways of anoikis and the current understanding of its molecular mechanisms.

Role of the integrin signalling in anoikis

Attachment of MDCK cells to surfaces coated with polylysine, or any other surface on which cell spreading occurred, rescued cells from anoikis. However, attachment of endothelial cells to surfaces coated with

fibronectin or anti-ß1-integrin antibodies, but not to surfaces coated with polylysine, rescued cells(Meredith et al., 1993). This implied a specific requirement for integrin signalling. A *de novo* synthesis of matrix molecules by the MDCK cells followed by integrin-mediated attachment may give a reason to this promiscuity, and does not imply that non-integrin-mediated attachment can rescue these cells.

Only specific ligated integrins suppress anoikis in certain cell types. For example, serum-starved HT29 carcinoma cells readily underwent apoptosis, which was partially suppressed by transfection of the α integrin subunit (O'Brien et al., 1996). The ectopic expression of α5ß1 integrin, but not αvß1 integrin, suppressed anoikis of fibronectin-bound CHO cells under serum-free conditions (Zhang et al., 1995). Similar results were obtained from study of endothelial cells (Wary et al., 1996), MG-63 human osteosarcoma cells (Zhang et al., 1995). They were similarly protected on fibronectin (by αvß1 integrin), but not on vitronectin (the vitronectin receptor is integrin) , demonstrating a privileged role for α5ß1 integrin.

Angiogenic endothelial cells are particularly dependent on the αvß3 integrin *in vivo* and can be induced to undergo apoptosis by treatment with antagonists of this integrin (Brooks et al., 1994). The same antagonists can also induce apoptosis in melanoma cells (Montgomery et al., 1994).

The differing abilities of specific integrins to rescue cells from anoikis suggests that they utilize distinct signalling pathways. For example, αvß3 integrin uniquely associates with insulin receptor substrate-1 (IRS-1), linking it to the growth-regulated pathways that are mediated by insulin, insulin-like growth factor (IGF) and platelet-derived growth factor (PDGF) (Vuori and Ruoslahti, 1994). The α5ß1, αvß3, α5ß1ß1 integrins, but not some other integrins, activate tyrosine phosphorylation of the adaptor protein Shc and through this pathway possibly activate mitogen-activated protein (MAP) kinases (Wary et al., 1996).

The role of integrin ß1 in transmitting survival signals to epithelial cells attached to a solid substrate has been demonstrated for several cell types. Normal mammary epithelial cells differentiate and form acinar structures when embedded into a reconstituted basement membrane. However, function-blocking integrin ß1 antibodies cause inhibition of cell growth and induce apoptosis (Howlett et al., 1995).

Human colon intestinal crypts undergo apoptosis within 4 h after isolation, but embedding them in collagen I gels prevents anoikis (Strater et al., 1996). Preincubation of the crypts with anti-integrinß1 inhibitory antibodies induces much higher rate of apoptosis after cell re-adhesion (Strater et al., 1996). Unlike growth factor receptors, integrins are activated by their ligand only when it is substrate immobilized, so that the same ligand presented in soluble form may act as a receptor antagonist. The clustering of integrins at the focal adhesion site is essential for integrin signalling, and soluble integrin ligands may block the integrins, but may not be sufficient to induce this necessary clustering. This inhibition of integrin signalling by soluble matrix molecules is relevant for epithelial cells only, because anoikis in fibroblasts may be prevented by addition of soluble collagen VI (Ruhl et al., 1999).

Moreover, not only cell detachment, but also changes in cell shape, such as cell rounding, induced by actin microfilaments and microtubules disruption is a signal for anoikis (Flusberg et al., 2001). Anoikis in this case results from the disruption of focal adhesions. Another case in which anoikis can be triggered in the absence of cell detachment from the substrate has been recently described (Stupack et al., 2001).

Anoikis occurs in tumor cell lines that have free, unoccupied integrins. These integrins directly bind and activate caspase-8 through their cytoplasmic tails and cause apoptosis in extracellular matrix (ECM)-attached cells (Stupack et al., 2001).

Integrins can act through the integrin-linked kinase (ILK) that is activated by integrin binding to extracellular matrix (e.g., by adhesion of cells to fibronectin), and ILK activity is inhibited in cells in suspension. Activated ILK can directly phosphorylate and activate the survival signal PKB/Akt (Persad et al., 2001). Moreover, ILK induces a tumorigenic, anchorage-independent phenotype when overexpressed in intestinal epithelial and mammary epithelial cells (White et al., 2001).

The anoikis-negative (survived in suspension) human intestinal carcinoma Caco-2 cells demonstrated markedly decreased levels of αvß3 on the cell surface and of transcription of the αv subunit gene in comparison to the original Caco-2 cell population. Activation of the signalling function of αvß3 in the original Caco-2 cells led to substantial stimulation of anoikis, while the inhibition of expression of this receptor resulted in better resistance of the cells to anoikis (Morozevich et al., 2003). Treatment of non-adherent human osteosarcoma cell line SAOS with an anti-α4 mAb increased anoikis while anti-ß1 integrin mAbs did not alter anoikis, thus indicating a novel function for the α4 subunit in the control of cell anoikis. The data provide the evidences that certain integrin can generate apoptosis-stimulating signals (Marco et al., 2003).

All of the above results suggest the possibility that the same integrin might connect to different pathways in different cell types and that a given integrin could, therefore, provide a survival signal in one cell type and an anoikis signal in another. It also seems like that integrins differ in their ability to suppress anoikis in a single cell type and much more work needs to be done to understand integrin-specific signalling and its role in anoikis.

Role of focal adhesion kinase in anoikis

Focal adhesion kinase (FAK), which has received

much attention as a transducer of integrin-mediated signals, is a good candidate for a mediator of protection from anoikis. Upon activation by integrins, FAK undergoes autophosphorylation as well as associations with several other intracellular signalling molecules.

In fibroblasts, activated FAK leads to the activation of the MAP kinases extracellular signal regulated kinase (ERK)-I and ERK-2 (Schlaepfer et al., 1994). In the absence of adhesion, SW620 human colon cancer cells exhibit increased p38 but decreased FAK activation, signals that may promote cell death. Inside-out signals, from p38 to FAK, may regulate both adhesion and anoikis in SW620 cells (Walsh et al., 2003). Tissue inhibitor of metalloproteinase (TIMP-1) is a natural protease inhibitor of matrix metalloproteinases (MMPs). Recent studies revealed that TIMP-1 is also a potent inhibitor of apoptosis in mammalian cells. It activates cell survival signalling pathways involving FAK in human breast epithelial cells and down-regulates caspase-mediated apoptotic pathways induced by anoikis (Liu et al., 2003).

Transformed and neoplastic cells are anchorage independent and usually have developed anoikis resistance. Various oncogenes expressed in these cells cause constitutive activation of the integrin signalling pathway. FAK can also signal downstream of src oncogenes. For example, c-src causes constitutive phosphorylation of FAK and may lead to anoikis resistance in transformed cells (Calalb et al., 1995). In another study, anchorage-dependent mock- or ras-transfected HAG-1 human epithelial cells underwent anoikis. In contrast, anchorage-independent v-Src-transformed cells did not exhibit such apoptotic features. FAK was only activated in v-Src-transformed cells. However, both protein kinase C (PKC) and phophatidylinositol-3 (PI-3) kinase inhibitors failed to induce anoikis. This data suggests that the ability of activated Src to prevent anoikis may be mediated by Src to a downstream signalling pathway involving FAK, but not Ras, PI-3 kinase, or PKC (Hisano et al., 2003).

FAK-related non-kinase (FRNK) is an autonomously expressed carboxyl-terminal region of FAK, which acts as an inhibitor of FAK function. A constructed replication-defective adenovirus encoding a GFP-FRNK fusion protein (Adv-GFP-FRNK) inhibition of FAK and PYK2 (the other member of the FAK family) phosphorylation, resulted in the loss of detectable FAK and paxillin in focal adhesions. It was accompanied by reduced levels of total paxillin and, ultimately, resulted in cell detachment and apoptosis (Heidkamp et al., 2002).

Development of a constitutively activated form of FAK made it possible to test the role of FAK in anoikis. Fusion of FAK to the ectodomain and transmembrane domain of the CD2 antigen targeted it to the plasma membrane, resuiting in constitutive activation of FAK (Chan et al., 1994). Specifically, FAK's auto-phosphorylation and src-interaction functions, which are normally active in only matrix-attached cells, were found to remain active in the CD2-FAK chimeric proteins expressed in suspended cells (Chan et al., 1994; Frisch et al., 1996a,b). The CD2-FAK chimeras substantially protected MDCK cells from anoikis (Frisch et al., 1996b), demonstrating the importance of FAK in regulating adhesion-dependent cell survival; this conclusion is supported by peptide microinjection experiments where the inactivation of FAK caused apoptosis (Hungerford et al., 1996).

Role of hosphoinositide-3 kinase-related signalling in anoikis

Numerous kinase/phosphatase signalling molecules have been implicated in anoikis as central regulators. The kinases PI3K (phosphoinositide-3 kinase) and Akt is involved in diverse survival signalling scenarios, including anoikis. For example, Activated ras oncogene activates PKB/Akt and confers resistance to detachment-induced apoptosis (Khwaja et al., 1997). Events downstream of FAK that lead to rescue from anoikis were less clear until a recent study implicated the involvement of the FAK- and ras-interaction partner PI3K (Khwaja et al., 1997). Activation of ras or adhesion of MDCK cells was shown to activate this enzyme, and the resulting lipid products activated the kinase Akt. Interestingly, introduction of activated forms of the PI3K p110 subunit, or of Akt, rescued the MDCK cells from anoikis. This was consistent with the ability of non-raf-interacting ras mutants, but not non-PI3K-interacting mutants, to rescue cells. Activated ras was also shown to promote the interaction of FAK with PI3K but not the phosphorylation of FAK (Khwaja et al., 1997). Taken together, this data strongly suggests that ras or FAK controls anoikis through PI3K, which in turn regulates kinases such as Akt.

Drug resistance of bone-marrow minimal residual disease (MRD) is induced by the attachment of very late antigen (VLA)-4 on leukemic cells to fibronectin on bone-marrow stromal cells. Interaction of VLA-4 and fibronectin leads VLA-4-positive cells resistance to anoikis through the PI-3K/Akt/bcl-2 signalling pathway (Matsunaga et al., 2003).

Overexpression of bad, a proapoptotic protein, preferentially augments anoikis when MDCK cells were cultured in suspension. Cotransfection of bad with constitutively active Akt cDNA strongly inhibited this change. Similar results were observed in COS-7 cells (Idogawa et al., 2003).

As insulin and IGF receptors are major activators of Akt activity and their ligands are critical survival factors for numerous cell types, several laboratories have addressed the role of these factors in anoikis. One study demonstrated that IGFs protected serum-starved mouse embryo fibroblasts (Valentinis et al., 1999) and LNCap prostate carcinoma cells (Yu et al., 2001) from anoikis. In the LNCap system, IGF levels are reported to dissipate slowly over the course of 16 hours of cell detachment, which is immediately followed by anoikis

(Yu et al., 2001). The authors attribute the observed anoikis in LNCap cells to this decrease in IGF. Moreover, the absence of IGFs was proposed to initiate a decline in Akt activity and the following cascade of events: activation of Gsk-3, phosphorylation-driven degradation of cyclin D, Rb hyperphosphorylation and consequent repression of the IGF promoter. With the IGF promoter repressed, the cells are committed stably to anoikis. Another study demonstrated that insulin and IGF receptors did not inhibit anoikis in primary mammary epithelial cells - even though the mammary cells normally require insulin for survival when attached to the extracellular matrix (Farrelly et al., 1999). When mammary cells attached to collagen (a non-permissive matrix required for their survival) were stimulated with insulin, the insulin receptor was still capable of autophosphorylation. Further signalling, however, was blocked: the receptor was not able to phosphorylate and recruit the adapter IRS-1 or activate downstream PI3K/Akt survival signalling (Farrelly et al., 1999). Thus, in this cell system, integrin ligation is a prerequisite for insulin signalling.

Integrins also regulate cell survival via the PI3K/Akt pathway directly or indirectly. ligation of ß1 integrin with anti-ß1 integrin antibodies protected fibroblasts from apoptosis during contraction of collagen matrices by activation of PI3K through Akt (Tian et al., 2002). Although FAK (focal adhesion kinase), Shc and ILK (integrin-linked kinase) can signal by distinct mechanisms, these three primary integrin signalling molecules that linked to cell survival may also impinge upon the PI3K/Akt pathway (Aplin et al., 1999; Giancotti and Ruoslahti, 1999; Dedhar, 2000).

When overexpressed in cell lines, ILK activates Akt activity either directly or indirectly. Inactivation of the phospholipid phosphatase PTEN, which occurs frequently in prostate cancer cell lines, constitutively activates both ILK and Akt (both kinases possess lipid-binding pleckstrin homology (PH) domains, which probably explains this activation). Interestingly, transfection of a dominant-negative ILK into PTEN-deficient prostate carcinoma cells or treatment of these cells with a specific chemical inhibitor of ILK decreases serine-473 phosphorylation of Akt and, in turn, its kinase activity (Persad et al., 2000). This implicates ILK as an in vivo activator of Akt, which was recently confirmed biochemically (Persad et al., 2001). Overexpression of ILK can also suppress aniokis in certain epithelial cell lines (Attwell et al., 2000).

FAK has been demonstrated to regulate the expression of caspase inhibitors of the IAP (inhibitor of apoptosis) family (Sonoda et al., 2000), by a proposed mechanism involving PI3K/Akt activation of the NF-κB pathway. FAK may also activate Akt through direct PI3K activation as well as indirectly through a p130cas-crkII-DOCK180-rac pathway (Kiyokawa et al., 1998).

Shc is also a potent PI3K/Akt activator (Gu et al., 2000). In hematopoietic cells, activated Shc recruits gab2 through grb2. Gab2 in turns recruits the regulatory subunit of PI3K, p85, leading to PI3K activation. In colon tumor cells, increasing Src expression and activity led to increased phosphorylation of Akt. With high Src activity, the PI3 kinase inhibitor LY 294002 sensitized cells to anoikis. It suggests that Src activation may contribute to colon tumor progression and metastasis in part by activating Akt-mediated survival pathways that decrease sensitivity of detached cells to anoikis (Windham et al., 2002).

A precise determination of the role of Akt activity in anoikis is complicated by the fact that Akt itself is a target for activated caspases (Bachelder et al., 1999). Thus the loss of Akt activity may be a consequence and not a cause of anoikis.

Akt phosphorylates regulators of apoptosis such as (human) caspase-9 and bad (Datta et al., 1999), as well as transcription factors whose target genes program the survival phenotype (e.g. Forkhead factors). However, Akt-mediated survival is likely to involve as yet unidentified novel Akt substrates as well as kinases possessing Akt-like activity. The fact that Akt is required for the activation of p21-activated kinases (PAK) by ras (Tang et al., 2000), lead us catalog PAK itself or some PAK activator as a critical new Akt substrate. Several anti-apoptotic effects of PAK which, like Akt, can phosphorylate bad (Schurmann et al., 2000) have been reported (Gnesutta et al., 2001). Forkhead transcriptional regulators are also substrates for a set of Akt-related kinases known collectively as serum- and glucorticoid-inducible kinases (SGKs). Both Akt and SGK1 can inhibit Forkhead function by promoting cytoplasmic accumulation of the phosphorylated Forkhead product (Brunet et al., 2001). Whether the other Akt substrates are also SGK substrates, and the relevance of SGKs to anoikis, remains to be determined.

Raf–ERK-related signalling in anoikis

Although cell adhesion to extracellular matrix (ECM) has been shown to influence many signalling pathways (Aplin et al., 1998; Giancotti and Ruoslahti, 1999), the extracellular signal regulated kinase/mitogen-activated protein kinase (ERK/MAPK) cascade remains the most intensely studied pathway in which growth factors and integrin-mediated adhesion converge, and with good reason. ERK activity has been implicated as a regulatory component in nearly every fundamental cellular activity and therefore regulation of this activity by cell adhesion is a matter of far-reaching importance.

There is much evidence to implicate Raf–ERK related signalling activation in cell anoikis (Tran et al., 2001). For example, expression of active Raf in estrogen-inducible CCL 39 fibroblast and MDCK cell lines leads to protection from anoikis (Le. Gall et al., 2000). Raf-mediated protective effects are blocked by a ERK kinase/MEK inhibitor and similar pro-survival effects are imparted by expression of an activated MEK, indicating that the Raf -MEK-ERK pathway conveys the anti-apoptotic signal downstream of Raf. U0126 and

PD98059, MEK inhibitors, also selectively repressed anchorage-independent growth of human breast cancer cell line MDA-MB231 (Fukazawa et al., 2002).

Tumor cells display a remarkable resistance to anoikis in concert with deregulated cell proliferation (Evan and Vousden, 2001). More recent reports, have pointed that Raf-ERK activation providing one route that bypasses adhesion signals and elicits resistance to anoikis. Enhanced ERK activity, via activation of the EGF receptor, in suspended keratinocytes provides protection against anoikis (Jost et al., 2001). As raf-1 can in turn be activated by EGFRs, this result implicates a positive feedback loop that promotes cell survival involving raf and autocrine growth stimulation. It has been found recently that hepatocyte growth factor (HGF) inhibited anoikis of human head and neck squamous cell carcinoma (HNSCC) cells. The inhibition of anoikis mediated by HGF was dependent on activator protein-1 activity by activating the ERK-signalling pathway (Zeng et al., 2002).

It is well established that raf-1 function is critical for ERK activation; a possible alternative function and novel activation mechanism for raf-1 are considered here. The bcl-2-interacting protein BAG-1 activates raf-1 kinase activity and targets it to mitochondria, where its survival effect is apparently enhanced (Tran et al., 2001). Interestingly, BAG-1 also interacts with the p53-inducible inhibitor of the ras-raf-MAP-kinase pathway, Siah-1, and prevents Siah-1 from inducing growth arrest (Matsuzawa et al., 1998). Whether this phenotypic effect of Siah-1 is dependent on association with raf-1 through BAG-1 is not yet clear. In any event, the existence of critical mitochondrial raf-1 substrates is strongly implied by these results.

PAK is an important regulator of anchorage-dependent ERK activation. Growth factor activation of PAK, itself, is profoundly dependent upon anchorage (del Pozo et al., 2000; Howe and Juliano, 2000). Importantly, expression of constitutive active PAK in non-adherent cells permits anchorage-independent growth factor activation of ERK (Howe and Juliano, 2000), similar to the effects seen with FAK (Howe and Juliano, 2000). The ability of PAK to modulate anchorage dependent ERK signalling may be attributable to its ability to regulate, via direct phosphorylation, the upstream kinases Raf and MEK (Frost et al., 1997; King et al., 1998), both of which have been identified as points of convergence between cell adhesion and growth factors (Assoian and Schwartz, 2001).

Although the precise mechanism of raf-1 kinase activation remains unclear, it is known that the PAKs can activate raf-1 by direct phosphorylation of serine 338 (Chaudhary et al., 2000). PAK activity is controlled both by phosphoinositides and PI3K, as the former interact with the PH domains of the rac/cdc42 GEFs, and the latter may directly activate PAK through the adaptor protein PIX (Yoshii et al., 1999). Integrin ligation to fibronectin can stimulate raf phosphorylation at serine 338 by this pathway, which provides an interesting new mechanism for the control of raf-1 activity by integrin stimulation of PI3K activity. It may also explain the critical involvement of PAKs in the stimulation of ERK activity by integrins. However, PAK may also contribute to anchorage dependent signalling by virtue of its ability to regulate the cortical actin cytoskeleton (Sells et al., 1997; Bagrodia and Cerione 1999), the integrity of which is required for efficient growth-factor activation of ERK (Aplin and Juliano, 1999).

The ERK pathway is also affected by the state of the actin cytoskeleton. In 3T3 cells, both detachment and treatment of attached cells with cytochalasin D is sufficient to prevent ERK translocation into the nucleus, and the consequent phosphorylation of its target, elk-1, in response to mitogenic stimulation (Aplin et al., 2001). It is difficult to reconcile the adhesion requirement for ERK translocation with the ability of EGF to rescue cells in suspension from anoikis, although cell-type differences between these studies may contribute. Nevertheless, this highlights the important challenge of elucidating how integrins control ERK nuclear import or export through cytoskeletal regulation.

One study contrasts with the above findings in which expression of a membrane-targeted form of Raf fails to protect MDCK cells from anoikis (Khwaja et al., 1997). Other studies have also indicated a partial role for the ERK pathway in providing protection from anoikis. In epithelial cells, macrophage-stimulating protein elicits an antiapoptotic effect via both the ERK and PI3K/Akt pathways (Danilkovitch et al., 2000). Interestingly, ERK and Akt act independently of each other. Thus, macrophage-stimulating protein protects epithelial cells from anoikis even in the presence of MEK inhibitors through activation of the Akt pathway. In contrast, Akt does not confer such resistance in suspended rat intestinal epithelial cells, although pharmacological inhibition of MEK partially blocks protection inferred by active Ras (McFall et al., 2001). Studies in human umbilical vein endothelial cells also indicates that Raf-ERK provides only limited protection from anoikis (Aoudjit and Vuori, 2001).

Clearly, a myriad of pathways contribute to protection from anoikis and the relative importance of the ERK pathway must be considered in the light of cell context. Nonetheless, there is a growing consensus that lack of signalling through the ERK pathway, through loss of integrin engagement, contributes to anoikis, a role that is consistent with ERK promoting survival against a variety of apoptotic stimuli. A future research focus will be to determine the adhesion dependent ERK targets that are important in eliciting these pro-survival signals through proteomic techniques.

Jun N-terminal kinases in anoikis

The Jun amino-terminal kinases (JNKs) are a family of MAP kinase related serine/threonine kinases that respond to a variety of 'stress-inducing' stimuli, such as UV or y irradiation, ceramide, tumor necrosis factor-α

and interleukin-1 (Waskiewicz and Cooper, 1995; Su and Karin, 1996). Recently, the dissociation of epithelial cells from the extracellular matrix was found to rapidly and dramatically induce the activity of the JNKs as well (Frisch et al., 1996a). Furthermore, the blockage of the JNK pathway by a dominant-negative form of JNK kinase partially inhibited anoikis.

The 5-lipoxygenase inhibitor nordihydroguaiaretic acid potently inhibits anchorage-independent growth of human pancreatic and cervical cancer cells and induces anoikis of these cancer cells in vitro and in vivo. Treatment with nordihydroguaiaretic acid led to a disruption of the filamentous actin cytoskeleton in cells, which was accompanied by the activation of JNK and p38MAPK. It suggests that nordihydroguaiaretic acid induces anoikis as a result of disruption of the actin cytoskeleton in association with the activation of stress activated protein kinases (Seufferlein et al., 2002).

Other evidence supporting a pro-apoptotic role for JNKs comes from the observation that mouse embryo fibroblasts lacking both JNKs 1 and 2 (due to a compound knockout) were resistant to UV-, anisomycinand DNA damage-induced apoptosis (Tournier et al., 2000). Thus, the JNK pathway plays a critical pro-apoptotic role in anoikis. The apoptosis-inducing activity of JNK is counteracted by ERK, and the balance between the activation of these pathways has been proposed to control cell survival versus apoptosis (Xia et al., 1995).

Caspase activity is required for the activation of the JNK pathway (Frisch et al., 1996a), this may result from the requirement of the upstream kinase MAP kinase/ERK kinase kinase-1 (MEKK-1) to be activated by caspase-mediated cleavage (Cardone et al., 1997). This suggests that bcl-2 may suppress anoikis in part by suppressing JNK activation. Thus, the signals regulating anoikis appear to flow from the integrins through bcl-2-related proteins to caspases, then to MEKK-1 and JNK. The MEKK/JNK pathway and caspases also communicate in a positive feedback loop, because blockage of MEKK/JNK inhibits caspases and blockage of caspases inhibits MEKK/JNK (Cardone et al., 1997). The MEKK substrates or JNK substrates that mediate anoikis remain to be identified, but c-jun itself should be considered, because it can induce apoptosis when overexpressed in at least one system (Bossy-Wetzel et al., 1997). Activation of the IκBkinase complex by MEKK-1 (Lee et al., 1997) might also be important. Clearly, JNK regulation is significantly different in certain cell types. This might reflect the different circumstances under which different types of cells have evolved to undergo anoikis *in vivo*.

Interestingly, in contrast to the above reports, numerous reports have also suggested anti-apoptotic role for JNKs, creating some degree of complexity in the field. For example, Almeida and co-workers (Almeida et al., 2000) observed that serum-starved fibroblasts activated JNKs in response to attachment to, rather than detachment from, the matrix (fibronectin). In these experiments, as reported previously, JNK activation was stimulated by cotransfection of FAK, accompanied by enhanced survival. Thus, a survival pathway from integrins- hrough FAK - to JNKs was proposed.

There is evidence to suggest that JNKs are in fact involved in normal cell cycle progression in fibroblasts: adherent 3T3 cells show a spike of JNK activity in the G1 phase of the cell cycle, and transfection of a dominant negative version of MKK4 - a positive upstream regulator of JNK activity - causes 3T3 cells to growth-arrest in G1 (Oktay et al., 1999). In this study, JNK activity was stimulated both by attachment to the matrix (in HUVEC, 3T3 and 293 cells) and by FAK (in 293 cells), further implicating JNKs in adhesion-dependent cell cycle progression and survival. JNK was assayed immediately after detachment (time zero) versus various time points after reattachment. However, because JNK activity is stimulated rapidly by detachment (Frisch et al., 1996a) and cells reattach and spread slowly, it is possible that the stimulation of JNK observed in these (Oktay et al., 1999) and other studies (Dolfi et al., 1998) was due to detachment - indicating a proapoptotic effect of JNKs - rather than reattachment.

Role of bcl-2 family proteins in anoikis

The expression levels of pro-apoptotic and anti-apoptotic bcl-2 family proteins and related proteins may determine the sensitivity of cells to anoikis (Strasser et al., 2000). Consistent with this, bcl-2 levels were found to decrease to undetectable levels when MDCK became confluent (i.e. sensitive to anoikis (Frisch et al., 1996a), suggesting that the bcl-2 gene may be regulated by cell-cell interactions.

Bcl-2 overexpression has been shown to prevent anoikis in suspended epithelial cell lines (Frisch et al., 1996a). The overexpression of bcl-2, which had previously been shown to suppress anoikis, suppresses the activation of caspases involved in anoikis (Frisch et al., 1996a) and other apoptotic systems (Chinnaiyan et al., 1996). An alternative way for bcl-2 to block anoikis has been proposed (Day et al., 1997). In the prostate carcinoma cell line LNCaP, bcl-2 overexpression suppressed the accumulation of the cyclin dependent kinase inhibitors p21 and p27. Presumably because of this, the retinoblastoma protein (Rb) remained in the inactive, hyperphosphorylated state even in suspended cells. In the absence of active Rb, LNCaP cells were resistant to anoikis. Thus, bcl-2 might directly prevent the Rb-mediated cell cycle arrest that is required for the induction of anoikis in this system.

Studies have shown that activated ras, which renders cells anoikis resistant, prevents bcl-xL downregulation (Rosen et al., 2000). In intestinal epithelial cells high Src activity confers resistance to anoikis, at least in part, by inducing bcl-xL overexpression, and that this induction is mediated by the MEK/MAPK pathway (Coll et al., 2002). One study has found that overexpression of bcl-2 and anti-apoptotic bcl-xL, as well as expression of a

dominant negative form of the Fas-associated death domain (FADD) protein, abolishes anoikis (Rytomaa et al., 1999).

Gene transfer-mediated bad overexpression alone did not induce apoptosis in attached MDCK cells but strongly augmented anoikis when cells were cultured in suspension. In contrast, overexpression of another BH3-only protein bid, displayed much lower augmentation of anoikis, suggesting a preferential contribution of bad to anoikis in this cell model. Similar results were observed in COS-7 cells, suggesting that bad overexpression can increase sensitivity of anchorage-dependent cancer cells to anoikis (Idogawa et al., 2003).

The role of mitochondrial mechanisms in anoikis is being studied. Release of mitochondrial cytochrome c to the cytoplasm is a major mechanism for enhancing apoptosis. Caspase-8 activation induces cleavage of the proapoptotic bcl-2 family protein bid, which then, either by itself or in combination with the pro-apoptotic bax, translocates to the mitochondria, inducing changes in mitochondrial permeability potential and cytochrome c release. Cytochrome c associates with apoptosis protease activator protein-1 (Apaf-1)/ATP to form an apoptosome, which leads to further activation of caspases (Yin, 2000). Interestingly, anoikis also involves mitochondrial cytochrome c release (Rytomaa et al., 2000).

Reports focused on the relationship of caspases and bcl-2 family proteins activation in anoikis associated with changes within mitochondria also have contrasting results. In mammary epithelial cells, a rapid translocation of apoptotic bax from cytosol to mitochondria occurs shortly after cell detachment from matrix. This translocation is mediated by FAK, and occurs before caspase activation (Beattie et al., 2000).

Bcl-2 oncoprotein overexpression correlates with the progression and metastases of prostate cancer. However, enforced overexpression of bcl-2 did not protect either PC-3P or LNCaP-PRO-5 prostate carcinoma cells from anoikis, even though it rendered them resistant to thapsigargin and inhibited cytochrome c release. Strikingly, cells that survived anoikis expressed much lower levels of the protein. It may be concluded that sensitivity to anoikis is regulated by bcl-2-independent mechanisms in LNCaP and PC-3 prostate cancer cells (Bondar and McConkey, 2002).

Another study found no changes in the bcl-2 levels during anoikis (Park et al., 1999), and bcl-2 is able to block CD95/Fas ligand-induced apoptosis only when caspase-8 concentrations are very low (Kuwana et al., 1998). In epithelial cells bax activation occurs extremely rapidly, within 15 min after loss of integrin-mediated adhesion to extracellular matrix. The conformational changes associated with bax activation are independent of caspases including the initiator caspase-8. Furthermore, cytochrome c release - the downstream events in the apoptosis program - occurs after a delay of at least 1 h, with subsequent activation of the effector caspase-3 (Wang et al., 2003).

Surprisingly, individual integrins can also regulate bcl-2 levels in CHO cells stabling transfected with integrins (Zhang et al., 1995). Keratinocytes enriched for stem cells are protected from anoikis via ß1 integrin, in a bcl-2 dependent manner. Addition of anti-integrin, the anoikis rate was significantly higher in HaCaT cells not expressing bcl-2 than in controls (Tiberio et al., 2002).

Bim, a pro-apoptotic protein, may act as a critical mediator of anoikis in epithelial cells. Bim is strongly induced after cell detachment and downregulation of Bim expression by RNA interference (RNAi) inhibits anoikis. Detachment-induced expression of Bim requires a lack of ß1 integrin engagement, downregulation of EGF receptor (EGFR) expression and inhibition of Erk signalling (Reginato et al., 2003). Cells attached through α5ß1 integrin (and thus capable of survival) expressed bcl-2 at detectable levels, whereas cells that were attached through other integrins and were destined for anoikis did not. Similarly, the blockage of endothelial cell αvß3 integrin with antibodies specifically caused the activation of p53 and the downregulation of bcl-2 (Stromblad et al., 1996). The subsequent decrease in the bcl-2: bax ratio was proposed to induce apoptosis. It will be interesting to identify the integrin signal transduction events that regulate the bcl-2 promoter.

Conclusions

Molecular mechanisms in anoikis have been described in several cell types, and it appears that various cell types activate different pathways leading to anoikis. Relevance of the mechanisms of anoikis is compounded by the fact that most studies have been performed using immortalized cell lines or cancer cell lines, rather than normal epithelial cells. Immortalized cell lines and cancer cell lines are usually not sensitive to anoikis, and many have developed anchorage independence, meaning they do not require adhesion to substrate to proliferate and survive. For example, while normal mammary epithelial cells are anoikis sensitive, mammary carcinoma cell lines are not (Pullan et al., 1996).

It is evident that much work remains to be done to establish mechanistic linkages between cell adhesion and anoikis. Despite significant advances, important questions remain in the regulation of anoikis. What is the exact biochemical and structural nature of the signalling pathways between cell adhesion and anoikis? Currently one of the challenges of anoikis research is to connect cytoskeletal organization with molecular signalling. The architectural state of the cytoskeleton is expected to affect the interactions of signalling molecules in three-dimensional space.

Anoikis in vivo may prevent detached cells from reattaching to new matrices and growing dysplastically; this could be an important safeguard for the organism. In neoplastic cells, alterations in cell-cell adhesion molecules, protein kinases, integrin-associated signalling molecules or apoptosis regulators can lead to anoikis

resistance, facilitating anchorage-independent growth. With the aid of increasingly advanced techniques, such as proteomic and genomic information and resources, molecular mechanics of cell anoikis will be the subjects of future study. Once defined, the elements responsible for collaboration between cell adhesion and signal moleculars may represent a prime target for specific, therapeutic manipulation of anchorage-independent cell growth characteristic of cancerous cells.

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