Regulation of Bcl-2 proteins during anoikis and amorphosis

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Abstract

Adhesion to extracellular matrix regulates cell survival through both integrin engagement and appropriate cell spreading. Numerous signaling pathways converge to affect the levels and posttranslational modifications of Bcl-2 family proteins. Recent work has defined specific roles for different Bcl-2 proteins in the disruption of mitochondrial function that leads to cell death. Using this understanding of Bcl-2 protein function as a framework, we will consider the molecular mechanisms of apoptosis induced by integrin detachment (anoikis) and cell death stimulated by the loss of cytoskeletal architecture (amorphosis).

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1. Introduction

In multicellular organisms, survival of many cell types depends on adhesion to specific extracellular matrix proteins [1–3]. Detachment of cells from the extracellular matrix often results in apoptotic cell death, known as anoikis [2], which is derived from the Greek word for “homelessness”. Anoikis plays an important role in the physiological induction of apoptosis during development and maintenance of tissue organization and homeostasis in the organism. For example, inner endodermal cells lose adhesion to extracellular matrix and undergo anoikis during vertebrate embryogenesis, which permits cavitation to occur [4]. Widespread proteolysis of the extracellular matrix causes anoikis of mammery epithelial cells in vivo, which contributes to the involution of the mammary gland [5]. In the gut, intestinal epithelial cells lose anchorage as they reach the luminal surface of the crypt, undergo anoikis, and are released into the intestinal lumen [6]. There is evidence that skin keratinocytes also undergo anoikis as they lose attachment to the basement membrane and are eventually shed from the skin [7].

Resistance to anoikis is thought to play a central role in tumor progression and metastatic spread. Most cell lines derived from solid tumors will grow in an anchorage-independent manner in soft agar or suspension culture [8], and experimental evidence demonstrates that suppression of resistance to anoikis in transformed cells strongly inhibits their tumorigenicity in vivo [9]. Conversely, in vitro selection for anoikis-resistant cells increases the in vivo tumorigenicity of intestinal epithelial cells [10] and the metastatic capacity of melanoma cells [11] in mouse model systems. Rescuing human breast tumor cell lines from anoikis through overexpression of either Bcl-2 or Bcl-xL also increases their metastatic potential [12]. Insensitivity to anoikis may allow tumor cells to tolerate the alterations in extracellular matrix that often accompany tumor development and progression [13]. Although tumor cells are often resistant to anoikis, the persistent sensitivity of tumor-associated endothelial cells to anoikis has allowed the development of novel cancer therapies. Disrupting endothelial cell binding to extracellular matrix with either antibody [14] or small molecule [15] antagonists causes tumor regression by stimulating anoikis in the endothelial cells that provide vascularization of the tumor. Anoikis also seems to play a role in other disease states, particularly fibrotic conditions where excessive or inappropriate

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secretion of extracellular matrix suppresses a normal role for anoikis in maintaining tissue architecture [16].

Cellular attachment to the extracellular matrix is mediated by the integrin class of heterodimeric transmembrane receptors. Integrins serve as a direct physical link between the extracellular matrix and the intracellular actin cytoskeleton [17], through multiprotein complexes, known as focal adhesions [18]. Engagement of integrin receptors results in cell spreading which depends on both the integrity and dynamic rearrangement of actin microfilaments [19]. In endothelial cells, survival after attachment to extracellular matrix requires not only integrin engagement, but subsequent cell spreading [20,21]. Direct disruption of actin-mediated cell spreading also stimulates apoptosis in epithelial cells that remain attached to extracellular matrix [22–24]. These findings suggest that adoption of a specific cellular morphology is required for survival. For the purposes of this review, we will refer to apoptotic cell death that is induced specifically through the disruption of cell shape as amorphosis, which is derived from the Greek word *amorphos* for “lack of shape” or “misshapen”.

Disruption of mitochondrial function is one of the hallmarks of apoptosis [25,26]. Loss of mitochondrial membrane potential and release of cytochrome *c* leads to activation of Apaf-1, which activates the cysteine proteases, caspase-9 and caspase-3. Cleavage of cellular substrates by these caspases constitutes the “execution” phase of apoptosis, which is characterized by membrane blebbing, nuclear condensation and DNA fragmentation. Inhibition of caspases or genetic deficiency in Apaf-1, caspase-3 or caspase-9 can prevent many of the cellular changes associated with apoptosis, but does not rescue cell viability [27,28]. The loss of mitochondrial function therefore represents a commitment point of the cell to death.

The Bcl-2 family of proteins are important regulators of mitochondrial membrane potential and permeability [29]. Recent work has helped define specific signaling roles for the different members of the Bcl-2 family [27,30]. We will consider the current molecular understanding of apoptosis by either detachment (anoikis) or disruption of cell spreading (amorphosis), specifically with regard to the ultimate effect on signaling by Bcl-2 proteins.

2. Regulation of mitochondrial function by Bcl-2 family proteins

The Bcl-2 proteins comprise a protein family of at least 22 members, related through their conservation of α-helical sequences, known as Bcl-2 homology (BH) domains [29]. For simplicity, we will focus on those Bcl-2 proteins which play a known role in cell death induced by disruption of extracellular matrix contact (Fig. 1). The antiapoptotic proteins, Bcl-2 and Bcl-xL, contain all four known BH domains, and promote cell survival by binding proapoptotic Bcl-2 proteins and inhibiting their function. The proapoptotic Bcl-2 proteins can be separated into two classes, the first being the multidomain proteins Bax and Bak, which contain only the first three BH domains. The second class of proapoptotic Bcl-2 proteins are the BH3-only proteins (Bid,
Bim, Bad, Bik and Bmf), which are the most structurally diverse, sharing only the single BH3 domain [31].

The multidomain proapoptotic proteins, Bax and Bak, are the only Bcl-2 proteins capable of directly disrupting mitochondrial function [29]. Either Bax or Bak can homooligomerize into pore-forming complexes that permeabilize the outer mitochondrial membrane [29,32], leading to release of cytochrome c and activation of apoptotic caspases (Fig. 2). Oligomerization of Bak is regulated, but it is constitutively localized to the outer mitochondrial membrane [33]. Bax is often found in the cytosol in viable epithelial cells, and is regulated both through translocation to the mitochondria and subsequent oligomerization [34,35]. Recent evidence suggests that Bax can form oligomers of many subunits, and that these large complexes may be necessary for permeabilization of the outer mitochondrial membrane in epithelial cells [36,37]. Mouse embryo fibroblasts which are deficient in both Bax and Bak are resistant to a wide range of apoptotic stimuli, confirming the critical role of these two proteins in mitochrondrially induced cell death [27].

The BH3-only proteins have recently been separated into two distinct classes, through studies measuring the effects of the BH3 domain peptides on isolated mitochondria [30]. Bid and Bim have been termed “activators”, since they are capable of binding directly to the proapoptotic Bax and Bak proteins and inducing release of cytochrome c. Bad and Bik, on the other hand, bind preferentially to Bcl-2 and Bcl-xL and are incapable of inducing activation of Bax and Bak. Binding of Bad or Bik displaces the activators Bid or Bim from associating with the antiapoptotic proteins Bcl-2 or Bcl-xL. Consequently, this liberates Bid and Bim to activate the apoptotic function of Bax and Bak. The BH3-only proteins Bad and Bik have been termed “sensitizers”, as they block the ability of antiapoptotic Bcl-2 proteins to sequester activators, thereby enhancing the death-promoting effects of Bid and Bim. The specific classification of the BH3-only protein, Bmf, remains undetermined. The protein sequence of the BH3 domain of Bmf bears the most similarity to Bid and Bim, suggesting it may be an activator [30,38]. However, yeast two-hybrid and co-immunoprecipitation experiments show that Bmf binds Bcl-2 and Bcl-xL, but not Bax, suggesting a role as a sensitizer [38].

Overexpression of either Bcl-2 or Bcl-xL can prevent epithelial cell death upon disruption of extracellular matrix contact, whether during anoikis following detachment [2,39], or amorphosis after cytoskeletal disruption [24]. Protection by Bcl-2 and Bcl-xL is thought to occur through either sequestering of BH3-only activators or association with the pore-forming proteins Bax and Bak. Recent data

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Fig. 2. Bcl-2 protein function at the mitochondrial membrane. Multidomain apoptotic proteins (Bax and Bak) are the only Bcl-2 proteins capable of permeabilizing the outer mitochondrial membrane during cell death. Multimerization of either Bax or Bak results in pore formation, release of cytochrome c and activation of executioner caspases. The pore-forming activity of Bax and Bak is stimulated by the BH3-only “activator” proteins (Bid and Bim). Antiapoptotic Bcl-2 proteins (Bcl-2 and Bcl-xL) can associate with Bax and Bak and prevent their pore-forming ability, or indirectly inhibit pore formation by binding the “activators”, Bid and Bim. The BH3-only “sensitizer” proteins (Bad and Bik) cannot stimulate pore-formation directly, but displace “activator” proteins from binding to Bcl-2 or Bcl-xL, enhancing “activator” function toward Bax and Bak.
show that a mutant form of Bcl-xL that could not bind BH3-only proteins [27] was incapable of preventing anoikis in mammary epithelial cells, despite binding to Bax [40]. The protective effect of the multidomain antiapoptotic Bcl-2 proteins may therefore be primarily through their effect on the signaling of BH3-only proteins. Adhesion and spreading of cells on extracellular matrix alters both the levels and activities of Bcl-2 family proteins, which can affect the
balance between antiapoptotic and proapoptotic signals, and determine the life or death of the cell. There are multiple mechanisms of Bcl-2 protein regulation by integrins, which could be a reflection of cell-type specificity in the signaling pathways. We will first examine what is known about transcriptional regulation of the Bcl-2 proteins by attachment to extracellular matrix and then consider the role of appropriate cell spreading (Fig. 3).

3. Transcriptional regulation of Bcl-2, Bax and Bim by integrin engagement

Integrin-mediated attachment to extracellular matrix increases the level of Bcl-2 protein, and this survival effect is mediated largely through enhanced transcription of the Bcl-2 gene [41,42]. Conversely, detachment of endothelial cells from extracellular matrix enhances transcription of both cell cycle arrest and apoptosis genes through activation of p53 [43]. Transcription of the proapoptotic protein, Bax, is directly regulated by p53 in murine leukemia cells and the resulting increase in Bax protein can tip the balance of the Bcl-2/Bax ratio toward cell death [44,45]. Fibroblasts which are deficient in p53 are resistant to anoikis, and are more metastatic when injected into the tail vein of mice [46]. The resistance to anoikis in p53-deficient fibroblasts is presumably mediated through the resulting loss of Bax induction, since this effect can be mimicked by overexpression of Bcl-2 [46]. Residual p53-dependent cell cycle arrest can reduce the outgrowth of metastatic lesions from Bcl-2 overexpressing fibroblasts, but the resistance to anoikis induced by Bcl-2 overexpression does enhance tumor cell survival in the blood circulation [47]. Epithelial cells may have additional p53-independent pathways to induce apoptosis, because acute suppression of p53 actually enhances apoptosis when primary mammary epithelial cells are growing in a polarized manner on extracellular matrix [48].

Recent data demonstrate that transcription of the BH3-only protein, Bim, is increased following detachment of immortalized mammary epithelial cells from extracellular matrix, but the levels of other BH3-only proteins are not altered [40]. Transcriptional regulation of Bcl-2 family members by extracellular matrix therefore appears to occur primarily with Bcl-2, Bax and Bim, but numerous posttranscriptional and posttranslational effects on Bcl-2 proteins are also observed during anoikis.

4. Movement of Bax and Bid during anoikis

Fusion of Bax to green fluorescent protein demonstrated that induction of apoptosis leads rapidly to the movement of Bax from the cytosol to mitochondria [35]. Detachment of mammary epithelial cells from extracellular matrix also causes translocation of Bax to mitochondria, concurrent with a conformational change that exposes the BH3 domain [34]. These changes in Bax precede activation of caspsases, including initiator caspsases, such as caspsase-8 [49]. Since oligomers of Bax are capable of directly permeabilizing the outer mitochondrial membrane, this event may seem to be sufficient to induce cytochrome c release and anoikis [29]. However, in apoptotically resistant human neuroblastoma cells, cytotoxins also led to a conformational change in Bax and its translocation to mitochondria, but cytochrome c release was not observed [50]. Mitochondrially localized Bcl-2 or Bcl-xL can antagonize the pore-forming ability of Bax [29], so signaling by BH3-only protein sensitizers and activators may still be required to neutralize the survival effect of Bcl-2/Bcl-xL and promote efficient Bax-dependent pore formation.

Detachment can stimulate caspsase-8 in epithelial cells [51,52], which is known to cleave the activator, Bid, to a truncated form, tBid. Resulting exposure of a glycine motif at the cleavage site leads to myristoylation of tBid and enhances its localization to mitochondria [53]. tBid can then induce the release of cytochrome c, but fails to do so in Bax/Bak-deficient fibroblasts, indicating that tBid functions by activating the pore-forming ability of Bax/Bak [32,54,55]. The BH3 domain of tBid is not required for its localization to mitochondria, but is required to induce release of cytochrome c by Bax/Bak [32]. Since Bcl-2 or Bcl-xL can bind and sequester the BH3 domain of tBid [27], additional control over anoikis exists even following mitochondrial translocation of both Bax and tBid. Phosphorylation can regulate the activity of the BH3-only proteins, Bad, Bim and Bik, as well as Bcl-2, and the kinases responsible are often regulated by the binding of integrins to extracellular matrix.
5. Integrin-dependent kinase activation and Bcl-2 protein phosphorylation

Ligation of integrins results in activation of numerous intracellular signaling pathways, and the ligand binding status of integrins also regulates growth factor signaling. Thus, signal transduction by growth factor receptors is attenuated in cells detached from extracellular matrix [56,57]. A number of studies have demonstrated mechanisms by which integrin-dependent adhesion serves to recruit and activate proteins involved in transmitting growth factor signals (reviewed in Refs. [58,59]). While integrins affect cell cycle progression, gene expression and motility at many levels, we will primarily consider how integrin-dependent activation of MEK, JNK or PI3K/Akt/PAK modulates Bcl-2 protein function by direct phosphorylation.

5.1. MEK

Binding of integrins to extracellular matrix leads to the assembly of multiprotein complexes, known as focal adhesions, which connect integrin cytoplasmic domains to the actin cytoskeleton [18]. In addition to this physical role, focal adhesions play an important role in integrin signaling that protects cells against anoikis. Constitutive activation of the focal adhesion kinase (FAK) [60] can render epithelial cells resistant to anoikis and increase their tumorigenic capacity [61]. Loss of FAK signaling induces apoptosis in a p53-dependent manner [62]. During fibroblast attachment, FAK autophosphorylates and activates c-Src to respond by phosphorylating tyrosine 925 of FAK [63,64]. This site has been reported to serve as a binding site for the SH2 domain of Grb2-Sos, leading to GTP exchange on p21-Ras and activation of MEK phosphorylating tyrosine 925 of FAK [63,64]. This site has been reported to serve as a binding site for the SH2 domain of Grb2-Sos, leading to GTP exchange on p21-Ras and recruitment of Raf, which phosphorylates and activates the kinase, MEK [65,66]. Tyrosine phosphorylation of Shc also promotes recruitment of the Grb2/Sos complex to the plasma membrane, thereby activating Ras, Raf, MEK and the MAPK cascade in response to integrin-mediated cell attachment [67].

Increased MEK activity impacts Bcl-2 family members in several ways. First, MEK mediates phosphorylation of serine-112 of Bad, leading to its release from Bcl-2 and Bcl-xL [68,69]. This effectively removes the apoptosis-sensitizing function of Bad, since Bcl-2 and Bcl-xL are now free to associate with Bax/Bak and sequester activator Bcl-2 family proteins, Bim and Bid. MEK-dependent activation of MAPK1/2 also leads to phosphorylation of Bcl-2 at multiple sites, preventing ubiquitin-mediated destruction of Bcl-2 [70]. MEK signaling is also important for suppressing transcription of Bim. Loss of MEK signaling through downregulation of the EGF receptor following mammary epithelial cell detachment stimulates anoikis, in part, by increasing Bim expression [40]. Constitutive activation of MEK can reduce anoikis by preventing induction of Bim [40]. Oncogene-mediated stimulation of the Ras–Raf–MEK pathway can promote resistance to anoikis by inactivating Bad and protecting Bcl-2 through phosphorylation, and suppressing Bim transcription. In untransformed cells, full activation of MEK in response to integrin engagement requires reinforcement by PI3K, Akt and PAK [71–74], and these proteins can also affect Bcl-2 protein signaling directly at several levels.

5.2. PI3K/Akt/PAK

Adhesion to extracellular matrix activates the integrin-linked kinase (ILK) in epithelial cells, which can prevent anoikis when activated constitutively [75,76]. Signaling by ILK, FAK and growth factor receptors likely all contribute to integrin-dependent activation of PI3K, and the resulting 3,4,5-PIP3 lipid product can then serve to recruit and activate Vav [77–79], which catalyzes GTP exchange on p21-Rac [80,81]. FAK also regulates adhesion-dependent Rac activation by promoting the p130-Cas/Crk complex, which stimulates the novel guanine nucleotide exchange factor DOCK180 [82–84]. In addition to inducing actin rearrangement and cell spreading, p21-Rac stimulates the kinase PAK [85], which feeds back directly on the Ras–MAPK pathway by phosphorylating and activating both Raf and MEK [71,74]. An additional integrin-dependent feedback from PAK to the MAPK pathway may occur through FAK. Phosphorylation of p130-Cas by FAK induces its association with the adapter protein Nck in fibroblasts [86]. Association of Nck and PAK in fibroblasts is integrin-dependent [87], and together with PI3K-dependent activation of Rac, likely allows reinforcing feedback to Raf and MEK. In support of this model, activation of Raf following fibroblast adhesion to fibronectin requires both PI3K and PAK signaling [88]. Association of the focal adhesion protein, paxillin, with PAK [89] and the Rac exchange factor, PIX [90], provides yet another mechanism for integrin signaling to enhance MAPK signaling.

In addition to reinforcing the Ras–MAPK pathway, ILK and PI3K cooperate to activate Akt. In prostate tumor cells, ILK has been reported to phosphorylate Akt at serine-473, while PI3K-dependent activation of PDK-1 leads to phosphorylation of Akt at serine-308 [91]. Phosphorylation of both serine-308 and serine-473 is required for full activation of Akt [91]. Overexpression of Akt can suppress anoikis in epithelial cells [92], as well as apoptosis induced by expression of the Bcl-2 proteins, Bax, Bak, Bik or Bad in rat fibroblasts [93]. In intestinal epithelial cells, part of the protective effect of Akt can be traced to its ability to stimulate NFkB-dependent transcription of the antiapoptotic proteins, Bcl-2 and Bcl-xL [94,95]. More importantly, Akt protects from apoptosis by phosphorylating serine-136 of Bad in neurons [96]. Mutation of this site in Bad prevents the ability of Akt to enhance survival in rat fibroblasts [93]. Bad associates with 14–3–3 proteins after MEK-dependent phosphorylation at serine-112 and Akt-dependent phosphorylation of serine-136 [97]. Binding to 14–3–3 proteins both sequesters Bad in the cytoplasm, and enhances phosphory-
lation of serine-155, which disrupts the ability of Bad to bind Bcl-2 [97]. PAK can also phosphorylate both serine-112 and -136 of Bad in fibroblasts, providing a parallel input to suppress anoikis [98].

Signaling by ILK, FAK, PI3K, Akt, PAK and MEK therefore combine to enhance survival, through both transcriptional responses and phosphorylation of Bad. In this regard, it is interesting to note that p53-dependent transcription of the lipid phosphatase PTEN can block this survival pathway at several points, and promote apoptosis. PTEN can inhibit PI3K signaling by dephosphorylating 3,4,5-,PIP_3, but can also directly dephosphorylate FAK [99,100]. Since PI3K and FAK are essential for integrin-mediated activation of Akt and MEK, it is understandable that expression of PTEN can suppress integrin survival pathways. Conversely, loss of PTEN removes an important negative control on this survival pathway and contributes to anchorage-independence in tumor cells [99,101].

5.3. JNK

The specific role of kinase signaling by another MAP kinase, JNK, in anoikis is a little less clear. JNK is activated during anoikis in epithelial cells, and blockade of JNK signaling can protect from cell death following detachment [102]. JNK-mediated phosphorylation of Bim and Bmf releases these BH3-only proteins from their cytoskeletal associations, and promotes cell death via Bax/Bak [103]. Similarly, phosphorylation of Bim at serine-65 by JNK enhances activation of Bax, and JNK can stimulate increased transcription of Bim in neuronal cells [104]. However, epithelial anoikis can be prevented with a broad spectrum caspase inhibitor or by expressing activated PI3K or Akt, without affecting JNK activation [105]. Additionally, integrin engagement has been shown to activate JNK [106], and FAK-dependent activation of JNK is actually linked with protection from cell death and cell cycle progression [107,108].

Differences in the cell types and serum conditions used in these studies, as well as in the kinetics and duration of JNK activation, may explain the differences in the observed results. It is also interesting to note that the ability of JNK to induce epithelial anoikis apparently depends on caspase activation [102]. Very recent data show that apoptotic induction leads to caspase-dependent cleavage of the carboxy-terminal 14 amino acids from JNK in human leukemia cells [109]. While this does not apparent alter the kinase activity of JNK, it is proposed to potentially change its substrate specificity [109]. Since this cleavage only leads to a minor shift in the mobility of the protein from 54 to 52 kDa, it may have been overlooked in previous studies. Full-length JNK could promote survival following integrin engagement, while the caspase-truncated form may select different substrates during anoikis. This could potentially reconcile the disparate data on the role of JNK activation in anoikis, but further testing of this model is clearly required.

6. Death receptor signaling

The potential role of death receptor signaling in anoikis varies somewhat by cell type. Detachment of endothelial cells from extracellular matrix results in transcriptional upregulation of Fas receptor and Fas–Fas ligand interaction [110]. In addition, execution of apoptosis can be blocked by antibodies that inhibit Fas receptor signaling [110]. Binding of Fas ligand to the Fas receptor results in receptor clustering and the recruitment of the adapter protein FADD and caspase-8 to the cytoplasmic domain of the receptor. Anoikis in endothelial cells, MCF10A mammary epithelial cells or MDCK kidney epithelial cells leads to the activation of caspase-8 [39,52,110]. Furthermore, epithelial anoikis can be blocked by dominant negative forms of either FADD or caspase-8 [39]. As noted above, activated caspase-8 cleaves the Bcl-2 protein, Bid, and enhances its localization to mitochondria where it activates apoptosis through Bax/Bak [53]. Bid cleavage is observed during anoikis in MDCK cells [51]. As a counterbalance to this effect, attachment-mediated activation of MEK upregulates transcription of cFLIP, at least in endothelial cells, which directly inhibits caspase-8 [110]. Overexpression of either Bcl-2 or Bcl-xL can block activation of caspase-8 during anoikis [39], demonstrating that mitochondrial events still control death receptor signaling after detachment.

However, there are certainly death receptor-independent models of anoikis. In numerous cell types, unligated integrin receptors directly activate caspase-8, without the involvement of death receptors [111]. In FSK-7 mouse mammary epithelial cells, many of the early events of anoikis occur independently of caspase-8 activation [49]. This includes Bax conformational change, translocation to mitochondria and subsequent release of cytochrome c [49]. In addition, no cleavage of Bid is observed in this system. It is worth noting that many of the measurements of apoptosis in these studies rely on the quantification of apoptotic nuclear morphology. Overexpression of Bcl-2 prevents cell death induced by actin cytoskeletal disruption in mammary epithelial cells, but does not prevent nuclear condensation [24]. It is possible that changes in nuclear morphology are not always indicative of apoptotic execution, especially in systems such as anoikis and amorphosis, where cell shape change already occurs as part of the apoptotic stimulus. However, the caspase-8-independent induction of cytochrome c release in these studies is compelling, since this is thought to be a commitment step to cell death. Chemical inhibition of caspases [28,112,113] or genetic deficiency in caspase activation downstream of mitochondria [27] can prevent many of the morphological and biochemical indications of apoptosis, without rescuing clonogenic cell survival. This is presumably due to permanent damage to the mitochondria that follows release of cytochrome c. With this in mind, it will also remain important to measure clonogenic cell survival in studies addressing the role of caspase-8 in anoikis, since earlier studies have relied on measurement...
of DNA fragmentation. Broad spectrum inhibition of caspases can prevent many hallmarks of apoptosis, including DNA fragmentation, but does not necessarily rescue cell viability [28,112,113]. It is possible that caspase-8 activation plays a role in apoptotic execution, even though early release of cytochrome c by Bax has already committed these cells to death. So, although cell type differences may be responsible for some of the variable findings on the involvement of death receptors in anoikis, a direct comparison of the experimental systems at the level of clonogenic cell survival may still resolve some of the differences. Measuring the endpoint of cell survival remains important in any apoptosis study to determine whether observed changes in signaling events have consequences for the cellular phenotype.

7. Cytoskeleton-dependent cell spreading and amorphosis

Following engagement of extracellular matrix by integrins, cells spread through rearrangement of the actin cytoskeleton by Rho proteins [114–116]. Many of the signaling pathways we have considered for their role in cell survival also regulate cell spreading. Inhibiting the function of either FAK, PI3K or MEK can inhibit cell spreading [73,117,118]. Interestingly, cell spreading itself seems to feedback on cell survival signaling. Elegant experiments using micropatterned extracellular matrix showed that, even after integrin binding, endothelial cell spreading is actually essential to suppress apoptosis [20,21]. Three-dimensional morphogenesis of mammary epithelial cells in gels of extracellular matrix is also required to prevent cell death [119,120]. Normal epithelial architecture is maintained and malignant growth suppressed by the induction of apoptosis upon loss of appropriate cell morphology, which we term amorphosis.

Stable cellular morphology is promoted through a dynamic balance of counteracting action of actin and microtubule filaments, known as tensegrity [17,121,122]. Contractile force, applied to integrin contacts by the actin cytoskeleton, is sensitive to the rigidity of the extracellular matrix, and loss of rigidity in the matrix results in rapid disassembly of actin filaments, even though integrin receptors remain engaged [123–125]. For this reason, loss of actin organization could serve as a signal for disrupted extracellular matrix contact. Even after attachment and spreading, persistent maintenance of cytoskeletal architecture is required to prevent amorphosis. Reflecting the dynamic nature of cytoskeletal filaments, amorphosis can be induced when filaments are either depolymerized or stabilized artificially. Inhibition of actin polymerization with Cytochalasin D or Latrunculin-A leads to amorphotic cell death [22,24,126]. Overexpression of either Bcl-2 or Hsp-27 can prevent cell death in these cases, as can loss of p53, confirming that amorphosis represents a programmed process rather than nonspecific cytotoxicity. Stabilization of actin filaments by jasplakinolide also induces amorphosis in lung epithelial cells [127]. Similarly, depolymerization of microtubules with vincristine or stabilization with paclitaxel leads to amorphosis in mammary carcinoma cells, which can be inhibited by overexpression of Bcl-2 [128]. The molecular mechanisms by which amorphosis affects Bcl-2 proteins are not completely understood, but could involve either disruption of integrin-mediated signaling pathways or specific cytoskeletal sensors.

Actin depolymerization or inhibition of Rho-dependent actin organization prevents integrin-stimulated FAK phosphorylation in platelets [129]. Raf, even when targeted to the plasma membrane, is not activated when actin is depolymerized in fibroblasts [130]. Both of these effects would impact integrin-mediated stimulation of the MEK signaling pathway. There is also evidence that actin-mediated signaling provides a constant input to the MEK pathway during adhesion to extracellular matrix. Activation of PKA upon cell detachment downregulates PAK signaling, and activation of Raf and MEK [72]. Inhibiting PKA leads to sustained MEK signaling in suspension, although depolymerization of actin blocks the adhesion-independent stimulation of MEK, suggesting an active role for actin polymerization in maintaining MEK activity [72]. As discussed in the previous sections, FAK and MEK signaling impact Bcl-2 protein signaling at multiple points. Interestingly, while integrin-mediated MEK activation is inhibited by Cytochalasin D, integrin-dependent activation of p70-S6 kinase is not affected, indicating that some pathways are actin-dependent and others are not [131]. Tyrosine phosphorylation of p130-Cas in fibroblasts is also dependent on a polymerized actin cytoskeleton, although this requirement is overcome by oncogenic v-Src [132]. This provides one possible mechanism by which an oncogene could promote survival despite cytoskeletal disorganization, as is seen with the amorphotic resistance of the metastatic mammary cell line, MDA-MB-453 [24].

Although downregulation of FAK-mediated signaling could partially explain the effect of actin depolymerization to induce amorphosis, microtubule depolymerization actually enhances focal adhesion assembly and FAK phosphorylation [133,134], and must operate by a distinct mechanism to induce amorphosis. A separate pathway is also supported by the finding that p53 deficiency in fibroblasts actually increases apoptosis after microtubule disruption [135], despite protecting from cell death after actin depolymerization [22]. Microtubule damage induces JNK-dependent phosphorylation of Bcl-2, which is not observed following DNA damage [128,136], demonstrating that amorphosis is mechanistically distinct from apoptosis induced by DNA damage.

Signaling by death receptors is also affected by disruption of the cytoskeleton. Stabilization of microtubules with paclitaxel induces amorphosis in a manner that depends on signaling by the Fas receptor [137]. Paclitaxel may mediate this effect through upregulation of Fas-L, which is also observed following treatment with Cytochalasin D [137]. Disruption of actin filaments may also directly activate the
Fas pathway by directly promoting Fas receptor clustering [138].

In addition to the effects of cytoskeletal disruption on receptor signaling pathways, Bcl-2 proteins may play a direct role as sensors of cytoskeletal integrity. The BH3-only protein, Bim, is bound to the dynein light chain (DLC1) and sequestered to the microtubule cytoskeleton [139]. Treatment with paclitaxel releases Bim from its binding to microtubules and it translocates to mitochondria after diverse apoptotic stimuli [38,139]. This response is independent of caspase activation, suggesting that Bim may represent a direct sensor of microtubule integrity [139]. Bim can directly stimulate cytochrome c release by binding Bax/ Bak [30], and should induce cell death unless counteracted by free Bcl-2 survival proteins. Cell detachment increases expression of Bim in mammary epithelial cells [40], potentially enhancing the sensitivity to any microtubule irregularities in suspended cells. Increased susceptibility of suspended mammary carcinoma cells to apoptosis induced by paclitaxel supports this model [140]. Another BH3-only protein, Bmf, binds DLC2 and is localized to the actin cytoskeleton through association with the myosin V motor complex [38]. Depolymerization of actin filaments releases Bmf from its cytoskeletal sequestration, and cell detachment induces movement of Bmf to mitochondria and association with Bcl-2 [38]. As noted above, the specific classification of Bmf as a BH3-only activator or sensitizer is currently undetermined. Both Bim and Bmf can be released from their cytoskeletal localizations by JNK-mediated phosphorylation [103], and this provides a potential route of crossstalk between these cytoskeletal sensors and integrin-stimulated JNK signaling. Bim and Bmf may serve as direct modulators of amorphosis by monitoring cytoskeletal integrity, and regulation of either the level or phosphorylation state of these BH3-only proteins may modulate cellular sensitivity to cytoskeletal abnormalities.

8. Conclusions

Cell adhesion regulates cell survival by altering the signaling balance between antiapoptotic and proapoptotic members of the Bcl-2 protein family. Loss of integrin binding to extracellular matrix results in anoikis, while disruption of a defined shape commits cells to death by amorphosis. Either apoptotic pathway results from a loss of survival signals produced by attachment or spreading, combined with the specific induction of death regulators by detachment or cytoskeletal disruption. Independence from anoikis may enhance tumor metastasis by allowing cells to survive detachment from the extracellular matrix of their organ of origin. Resistance to amorphosis may allow tumor cells to tolerate the gross disruptions of cytoskeletal organization that are commonly observed in solid tumors, and provide protection against apoptosis induced by microtubule-directed chemotherapeutic agents, such as paclitaxel. The specific contributions of anoikis and amorphosis to tumor metastasis remain to be separated from a general resistance to apoptosis, since metastatic enhancement by p53 loss and overexpression of Bcl2/BclxL [46,47] allows tumor cells to escape a fairly broad range of apoptotic stimuli. Identification of the cytoskeletally regulated BH3-only proteins Bim and Bmf [38,139], as well as modifications to Bcl-2 that occur with microtubule disruption but not DNA damage [128,136], may allow more specific questions on the roles of anoikis and amorphosis in tumor progression and metastasis in the near future.

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