Overcoming anoikis – pathways to anchorage-independent growth in cancer

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Summary

Anoikis (or cell-detachment-induced apoptosis) is a self-defense strategy that organisms use to eliminate 'misplaced' cells, i.e. cells that are in an inappropriate location. Occasionally, detached or misplaced cells can overcome anoikis and survive for a certain period of time in the absence of the correct signals from the extracellular matrix (ECM). If cells are able to adapt to their new environment, then they have probably become anchorage-independent, which is one of the hallmarks of cancer cells. Anoikis resistance and anchorage-independency allow tumor cells to expand and invade adjacent tissues, and to disseminate through the body, giving rise to metastasis. Thus, overcoming anoikis is a crucial step in a series of changes that a tumor cell undergoes during malignant transformation.

Tumor cells have developed a variety of strategies to bypass or overcome anoikis. Some strategies consist of adaptive cellular changes that allow the cells to behave as they would in the correct environment, so that induction of anoikis is aborted. Other strategies aim to counteract the negative effects of anoikis induction by hyperactivating survival and proliferative cascades. The recently discovered processes of autophagy and entosis also highlight the contribution of these mechanisms to rendering the cells in a dormant state until they receive a signal initiated at the ECM, thereby circumventing anoikis. In all situations, the final outcome is the ability of the tumor to grow and metastasize. A better understanding of the mechanisms underlying anoikis resistance could help to counteract tumor progression and prevent metastasis formation.

Key words: Anchorage-independent-growth, Anoikis, Metastasis

Introduction

For most cells, the ability to proliferate depends on two signals. First, cells need to detect that they are appropriately positioned within the tissue. This information is provided by integrins – surface receptors that sense the extracellular matrix (ECM) and connect it to the cytoskeleton – which activate many signaling cascades and influence cell responses to other stimuli (Lee and Juliano, 2004; Shattil et al., 2010; Stupack, 2007). Second, cells require growth factors and cytokines for proliferation. It is the precise modulation of these two elements that ensures the correct development and size of all organs.

Occasionally, cells acquire migratory or proliferative properties that result in their relocation to an inappropriate environment. When cells lose their normal cell–matrix interactions, the cell cycle is arrested and a specific form of caspase-mediated programmed cell death (apoptosis), known as anoikis, is initiated (Frisch and Ruoslahti, 1997). Anoikis ensures that 'misplaced' cells (i.e. those that are in an inappropriate location) are eliminated and thus prevents dysplastic growth. In adherent cells, cell-specific activation of integrins and their downstream signaling mediators, including the non-receptor tyrosine kinase Src, focal adhesion kinase (FAK) and integrin-linked kinase (ILK), provides protection from anoikis (Frisch and Screaton, 2001; Meredith et al., 1993). In addition to eliminating misplaced cells, anoikis also contributes to physiological developmental processes, such as the hollowing of glands and involution processes (Chiarugi and Giannoni, 2008; Gilmore, 2005).

Anoikis can be mediated by both the intrinsic and extrinsic apoptotic pathways (Fig. 1). In the intrinsic pathway, the pro-apoptotic members of the Bcl-2 (B cell lymphoma-2) protein family [including Bad (Bcl-2-associated death promoter), Bax (Bcl-2-associated X protein), Bid (Bcl-2-interacting domain) and Bim (Bcl-2-interacting mediator of cell death)] permeabilize the outer mitochondrial membrane. These proteins cooperate to form pores in the mitochondrial membrane that result in the release of pro-apoptotic factors, such as cytochrome *c* and SMAC/DIABLO (second mitochondria-derived activator of caspases/direct inhibitor of apoptosis binding protein with low pI), into the cytosol, which leads to the activation of caspase enzymes (Chiarugi and Giannoni, 2008; Simpson et al., 2008). The extrinsic pathway is initiated by the stimulation of death receptors, such as Apo1/Fas or TRAIL [tumor necrosis factor (TNF)-related-apoptosis-inducing ligand] receptor, which are both members of the TNF superfamily. This induces the formation of a death-inducing signaling complex (DISC), which in turn activates caspase-8. Caspase-8 activation alone can be sufficient to induce apoptosis, but it can also activate Bid, which triggers the intrinsic pathway (Chiarugi and Giannoni, 2008). Both apoptotic pathways induce the sequential activation of caspases, the final outcome of which is DNA degradation and cell death (Reddig and Juliano, 2005) (Fig. 1).

In some instances, defects in the signaling pathways that lead to anoikis can induce alternative mechanisms of cell death that allow the clearance of the superfluous cells, as described for the ductal hollowing of the mammary gland in the absence of Bim-mediated apoptosis (Mailleux et al., 2007). Unligated integrins can also act as cell-death promoters, through a process named 'integrin-mediated death' (IMD) (Brassard et al., 1999; Stupack et al., 2001). In contrast to 'classical' anoikis, IMD does not necessarily imply

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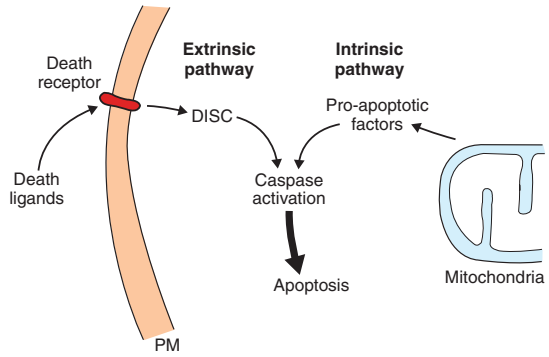

Fig. 1. Intrinsic and extrinsic apoptotic pathways can converge at different levels to induce cell death. In the intrinsic pathway, pro-apoptotic factors are released from the mitochondria, activating caspase signaling. The extrinsic pathway requires the binding of extracellular apoptotic 'death' ligands to their specific receptors at the plasma membrane (PM). Both pathways can flow independently until the last step of DNA degradation, or they might crosstalk at an earlier step and act simultaneously.

loss of ECM attachment because it can be triggered by unligated integrins in an otherwise normal adherent situation. IMD has been described in epithelial cells, for both integrins β1 and β3; it involves the recruitment of caspase-8 to the integrin-β-subunit cytoplasmic domain and the onset of an apoptotic response (Lotti et al., 2010; Stupack et al., 2001; Zhang et al., 2011). A similar direct interaction of integrins and caspase-3 has been described in fibroblasts undergoing anoikis (Rajeswari and Pande, 2006).

Tumor cells use a variety of strategies to circumvent anoikis and thus escape the requirement of integrin–ECM interactions for cell proliferation. The resulting anchorage-independent growth (AIG) is a crucial step in the acquisition of malignancy (Freedman and Shin, 1974). Cells that become anchorage independent have the potential to migrate through the body, colonize other tissues and grow metastatically (Gassmann and Haier, 2008). In addition, tumor cells that have defects in IMD-mediated apoptosis can be more likely to metastasize. In neuroblastoma cells, caspase-8 that is induced by unligated integrins prevents metastasis formation, and deletion of caspase-8 is associated with increased dissemination in vivo (Stupack et al., 2006).

In this Commentary, we describe some of the strategies that allow tumor cells to escape anchorage dependence. To adapt to the new environment, tumor cells can avoid anoikis by acquiring an epithelial–mesenchymal transition (EMT)-like phenotype, in which cells partially de-differentiate to regain the ability to proliferate and migrate, or by altering their repertoire of surface integrins. We also discuss strategies that counteract the absence of integrin signaling by hyperactivating survival and proliferative cascades. Other strategies used by tumor cells to avoid anoikis include two recently described processes, autophagy (or catabolic self-degradation) and entosis (cell-in-cell invasion). These processes, originally described as mechanisms of cell death, can, under certain conditions, become additional strategies for cells to escape apoptosis and promote survival. Finally, we discuss the relevance of anoikis avoidance and AIG for cancer progression.

Overcoming anoikis: adaptive strategies

EMT-like phenotype and metastatic transformation

EMT is characterized by the loss of epithelial features and reversion to a less-differentiated phenotype. During development, EMT is essential for the formation of mesodermal tissue from early embryonic epithelial cells, which allows the morphogenesis of the heart, the neural crest, the muscular system and craniofacial structures (Bolender and Markwald, 1979; Kang and Svoboda, 2005; Savagner, 2001). In the adult, EMT is induced during wound healing, tissue inflammation and organ fibrosis (Boyer et al., 2000; Kalluri and Neilson, 2003). During EMT, epithelial cells undergo a series of morphological changes, including the detachment of cells from one another, due to changes in their cell–cell adhesion structures. Cells then modify their baso-apical polarity and shape by altering their cytoskeleton, which enables them to migrate and overcome anoikis (reviewed in Voulgari and Pintzas, 2009). This correlates with increased ECM deposition and remodeling, as well as with enhanced invasion (Iwatsuki et al., 2009).

These characteristics resemble the metastatic transition undergone by transformed epithelial cells in carcinomas (epithelial-derived cancers): to metastasize, these tumor cells must detach from neighboring cells by downregulating their cell–cell adhesion structures and must be able to survive, at least transiently, under these conditions. As the acquisition of these characteristics is more a de-differentiation process than a complete EMT, some authors prefer to use the term EMT-like process (Klymkowsky and Savagner, 2009). At every step in the acquisition of this EMT-like phenotype, from detachment to invasion and implantation in a new metastatic niche, tumor cells must bypass or overcome anoikis, and cells adopt a variety of strategies to achieve this. The regulation of anoikis involves a potent crosstalk between integrin–ECM and growth factor signaling pathways, which are partially shared with signaling pathways that are related to EMT (reviewed by Chiarugi and Giannoni, 2008; Frisch and Screaton, 2001; Wang, 2004). This type of crosstalk occurs in the phosphoinositide 3-kinase (PI3K)–Akt pathway, which has a pivotal role in regulating the integrin-, growth-factor- and EMT-mediated survival response (reviewed by Chiarugi and Giannoni, 2008; Frisch and Screaton, 2001; Wang, 2004). The activation of this pathway has multiple effects in promoting survival because it inhibits several pro-apoptotic proteins, such as Bad, caspase-9, glycogen synthase kinase 3β (GSK3β) and forkhead transcription factors, and promotes AIG (reviewed by Chiarugi and Giannoni, 2008; Wang, 2004). In addition, several key regulators of EMT are also involved in survival signaling and anoikis resistance, such as nuclear factor κB (NF-κB), Snail and Twist, as discussed below.

The main component of adherent junctions in epithelial cells is the transmembrane glycoprotein E-cadherin. Through homophilic interactions with other cells, E-cadherins create a bridge that connects the actin cytoskeleton of neighboring cells. This allows the establishment of adhesion points between cells and the regulation of the characteristic epithelium cell cohesion and shape (reviewed by Voulgari and Pintzas, 2009). An early event in EMT is the 'cadherin switch', which consists of the downregulation of E-cadherin and a concomitant increase in the expression of N-cadherin (which is characteristic of mesenchymal cells). N-cadherins tend to destabilize cell adhesion complexes, disrupting the intercellular cohesion and leading to a mesenchymal phenotype (Nieman et al., 1999). In addition to morphological changes, this cadherin switch is also associated with resistance to anoikis; the depletion of E-cadherin promotes mammary cell survival following loss of cell adhesion to the ECM (Derksen et al., 2006; Onder et al., 2008), and N-cadherin expression protects melanoma cells from anoikis (Grossmann, 2002; Li et al., 2001). The relationship between EMT and resistance to anoikis is also reflected in the

existence of common regulators; these are able to modulate both the increased expression of cell survival genes and the coordinated balance of expression of epithelial and mesenchymal genes, which underlies EMT. Some examples are the transcriptional co-repressor CTBP1 (C-terminal-binding protein 1) in fibroblasts (Grooteclaes et al., 2003), which coordinately mediates the downregulation of epithelial genes, such as desmoglein-2, plakoglobin, keratin-8 and E-cadherin, and the expression of the pro-apoptotic proteins PERP (p53-effector related to pmp22), p21, Bax and Noxa; the guanine-nucleotide-exchange factor Tiam1 in colon tumor cells (Minard et al., 2006), which leads to resistance to anoikis through the coordinate downregulation of E-cadherin and the upregulation of N-cadherin; and tuberous sclerosis complex 2 (TSC2), also known as tuberin in epithelial cells (Barnes et al., 2010), whose absence leads to E-cadherin downregulation and mislocation and resistance to anoikis. Furthermore, the expression of some master transcriptional regulators of EMT, as mentioned above, is linked to the acquisition of anoikis resistance. The transcription factor NF-κB confers apoptosis resistance in a PI3K–Akt-pathway-dependent manner (Madrid et al., 2001; Ozes et al., 1999). Snail, which induces EMT by repressing E-cadherin transcription, suppresses cell death by inhibiting caspase-3 and by activating the pro-survival PI3K–Akt pathway (Barrallo-Gimeno and Nieto, 2005; Vega et al., 2004). Twist, which regulates EMT by inducing expression of N-cadherin and fibronectin, promotes survival by modulating the levels of anti-apoptotic Bcl-2 and pro-apoptotic Bax proteins (Kwok et al., 2005), which coordinately modulate the release of death promoting proteins from mitochondria to cytoplasm (reviewed by Simpson et al., 2008). Moreover, the Twist–Snail signaling axis is involved in EMT, anoikis resistance and metastasis, which are triggered by the neurotrophic tyrosine receptor kinase (TrkB) (Smit et al., 2009), which is itself associated with tumor malignancy and metastasis. Finally, the coordinated regulation of EMT and anoikis is highlighted by the fact that the polarity regulator Scribble disrupts normal three-dimensional acinar lumen by both inhibiting the establishment of apical–basal polarity, which leads to EMT-like de-differentiation, and promoting anoikis resistance through a pathway involving Rac and JNK (Zhan et al., 2008).

Changes in integrin repertoire

Another strategy to avoid anoikis that is used by cells undergoing EMT-like or metastatic transformation is to alter their integrin repertoire (Guo and Giancotti, 2004) in response to either direct oncogenic signaling (Plantefaber and Hynes, 1989) or selective microenvironmental pressures. By expressing integrins that are appropriate to the local matrix, cells can transduce ECM stimuli and therefore suppress anoikis (Frisch and Screaton, 2001; Meredith et al., 1993). By contrast, expression of unligated integrins in misplaced cells leads to cell death through IMD (Stupack et al., 2001). The ability to modify integrin expression is thus a survival advantage for cells that are migrating to a new cell–matrix environment. Examples of this strategy include the switch from expressing αvβ5 integrins to αvβ6 integrins in squamous cell carcinoma given that αvβ5 integrins induce the intrinsic apoptotic pathway when unligated, whereas αvβ6 integrins activate the pro-survival PI3K–Akt pathway (Janes and Watt, 2004). Another example is the expression of αvβ3 integrins in melanoma cells, which is needed for the cells to adhere to the dermal collagen and suppresses anoikis by modifying the proportion of anti-apoptotic Bcl-2 and pro-apoptotic Bax proteins (Montgomery et al., 1994). The expression of αvβ3 integrins is also important in the survival of carcinoma cells; unligated integrin β3 tail can recruit Src, leading to its activation, which triggers tumor cell survival (Desgrosellier et al., 2009).

Overcoming anoikis: compensatory strategies

Bypassing integrin signaling: hyperactivation of receptor tyrosine kinases

In some situations, despite inappropriate integrin expression, cells can survive loss of adhesion by 'ignoring' signals that would normally lead to cell cycle arrest and anoikis. One way for detached tumor cells to bypass the requirement of integrin signaling for cell survival is to constitutively activate downstream pro-survival signals, such as PI3K, Ras–Erk, NF-κB and Rho GTPase (Tsuji et al., 2009). This can be achieved through autocrine secretion of growth factors, such as basic fibroblast growth factor (bFGF), hepatocyte growth factor (HGF), interleukin (IL)-8 and platelet-derived growth factor AA (PDGF-AA) in melanoma cells (Li et al., 2003), which results in activation loops that trigger proliferation, survival and migration pathways. Alternatively, the overexpression of receptor tyrosine kinases (RTKs), such as EGF receptor (EGFR) in the MCF-10A mammary epithelial cell line (Reginato et al., 2003), and TrkB receptors and the HGF receptor MET in ovarian cancer cells (Douma et al., 2004; Tang et al., 2007; Yu et al., 2008), also leads to the suppression of anoikis. In addition, overexpression of the RTK ErbB2 induces anoikis resistance in mammary epithelial cells by inhibiting the expression of the pro-apoptotic protein Bim (Reginato et al., 2003); this process requires expression of the α5 integrin subunit and subsequent Src activation (Haenssen et al., 2010). Cells overexpressing RTKs are thus able to uncouple survival from adhesion and, furthermore, to use integrins as adaptors to amplify survival signals. Overexpression of ErbB2 can also rescue integrin β1 expression by maintaining EGFR expression and stability in detached cells, through a mechanism, dependent on ERK and Sprouty2, which inhibits EGFR degradation in the lysosome (Grassian et al., 2010).

Another possible way to maintain pro-survival signaling in the absence of anchorage is to prevent the internalization of signaling platforms from the plasma membrane. These platforms are distinct regions of the plasma membrane that are sites of assembly and initiation of signaling pathways. In attached cells, activation of ECM-coupled integrins triggers the relocation of effector molecules, such as Rac1, to plasma membrane cholesterol-enriched membrane microdomains (CEMMs), which act as platforms to regulate the localization of these effector molecules, as well as their coupling to specific adaptors (del Pozo et al., 2004). Following detachment, integrin inactivation leads to the internalization of CEMMs and the shut-down of associated signaling pathways (Fig. 2) (del Pozo and Schwartz, 2007). The inwards trafficking of CEMMs after the loss of integrin signaling requires caveolin-1 (Cav1), and hence the absence of Cav1 impairs detachment-induced CEMM internalization, which correlates with increased signaling through Ras–MAPK, PI3K–Akt and Rac–p21-activated-kinase (PAK) pathways (del Pozo et al., 2005). Antisense-mediated Cav1 downregulation has been linked to AIG (Galbiati et al., 1998). Consistent with this, AIG by Cav1-deficient cells is independent of ERK and dependent on Rac–PI3K signaling (Cerezo et al., 2009), and the activation of the Rac–PI3K signaling pathway probably prevents anoikis.

These findings support a role for Cav1 as a tumor suppressor; however, this issue remains controversial because several other

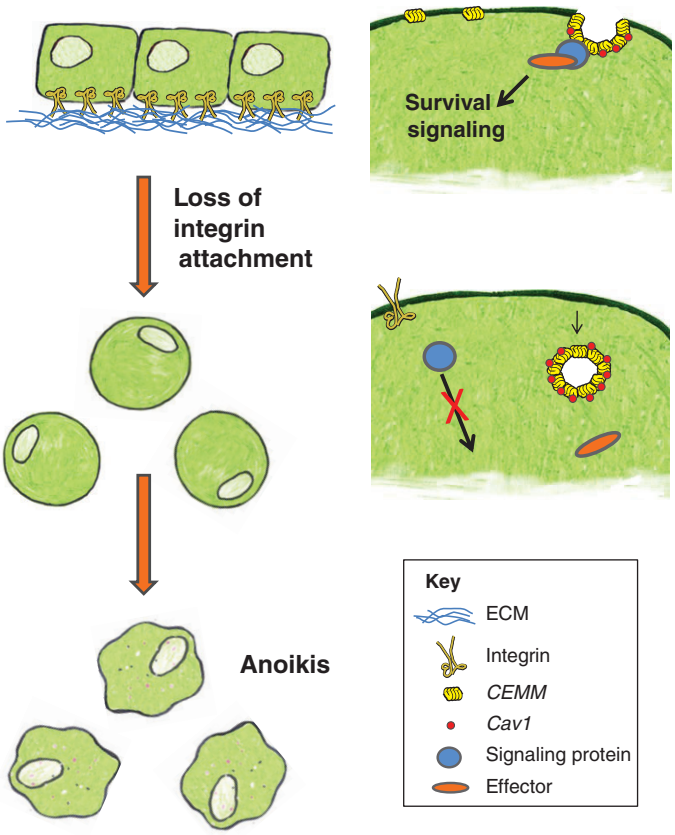

Fig. 2. Cav1-dependent inwards trafficking of CEMMs following loss of adhesion leads to the shutdown of signaling pathways. In adhered cells, CEMMs at the plasma membrane allow correct activation and coupling of signaling molecules to their effectors (such as Rac–PAK and PI3K–Akt), which results in cell survival and growth. After cell detachment from the ECM, integrin signaling is shutdown and CEMMs are internalized in a Cav1-dependent manner, resulting in cell cycle arrest and anoikis.

studies support a tumor promoter role for Cav1. For example, in lung carcinoma cells, Cav1 confers resistance to anoikis (Chanvorachote et al., 2009; Rungtabnapa et al., 2010), and Cav1 also prevents anoikis in MCF-7 cells by suppressing p53 activation and upregulating expression of insulin growth factor-1 (IGF-1) (Ravid et al., 2005). These opposing roles of Cav1 support the hypothesis that Cav1 levels have to be modified during tumor development and progression; lower levels of Cav1 at early stages of tumor development are needed to confer the capacity for AIG and resistance to anoikis, whereas increased levels at later stages could contribute to the development of metastatic potential (Shatz and Liscovitch, 2008). Thus, the role of Cav1 appears to be highly dependent on the origin and stage of the tumor. Indeed, Cav1 expression is decreased in most primary tumors, whereas some of the most aggressive cancers, particularly those refractory to drug or hormonal therapy and those with more invasive and metastasizing abilities, such as basal breast, certain prostate cancers, and certain pancreatic and small cell lung carcinomas, tend to show increased Cav1 levels (Shatz and Liscovitch, 2008).

It is not well established how increasing levels of Cav1 could contribute to metastasis; we herein speculate with two possible explanations. First, that Cav1 induces an elongated and polarized cellular morphology and, most importantly, also provides the cell with the ability to migrate directionally in two dimensions (Grande-Garcia et al., 2007), which actually correlates with increased invasion in three-dimensions and metastatic ability in several animal models (Goetz et al., 2011). Second, the increased metastatic ability could stem from increased Cav1 expression in the stroma, and not in the tumor cell itself. In fact, tumor-associated fibroblasts (TAFs) show increased Cav1 expression in breast, colon and kidney human carcinomas, as well as in melanoma metastases (Goetz et al., 2011). Most studies have focused on Cav1 expression in the tumor cells, not in the stroma, so the reported increase in Cav1 expression in metastatic lesions could stem from stromal cells rather than the metastatic tumor cells (Ghajar et al., 2009; Witkiewicz et al., 2011; Shatz and Liscovitch, 2008). It is worth noting here that the dependence of cell growth on anchorage is a checkpoint that guards against metastasis: tumors shed vast numbers of detached cells into the circulation, but only the few that do not require anchorage for growth can take root at new locations and metastasize. Consistent with this role for Cav1, *Cav1*$^{-/-}$ cells are anchorage independent (Cerezo et al., 2009; Galbiati et al., 1998), and absence of Cav1 leads to a very rapid progression of some tumors to metastasis, such as lung metastasis observed in a *Cav1*$^{-/-}$ and MMTV-PyMT background (Trimmer et al., 2010; Williams et al., 2004) and the ability of B16F10 melanoma cells lacking Cav1 to form lung metastases (Trimmer et al., 2010).

Inside-out control of anoikis

In addition to the ‘outside-in’ role of integrins, in which they transduce information from the ECM to control anoikis, this process can also be regulated in an ‘inside-out’ manner, by the profound cytoskeletal rearrangements that accompany cell detachment. Multiple apoptotic factors and other signaling molecules associate with the cytoskeleton, and can therefore act as sensors of cytoskeletal changes (Chiarugi and Giannoni, 2008; Frisch and Screaton, 2001). For example, the pro-apoptotic Bcl-2-family proteins Bim and Bcl-2-modifying factor (Bmf) are sequestered by myosin motor complexes when cells are attached to the ECM, yet are released following loss of cell adhesion, thereby triggering anoikis (Puthalakath et al., 2001; Strasser et al., 2000). In addtion, the Src family member p66Shc (focal-adhesion-associated 66kDa isoform of the Src homology and collagen) senses attachment through a RhoA-dependent tension created between focal-adhesion-like sites (Ma et al., 2007). Accordingly, in lung tumors that do not express p66Shc, this mechanosensitive test fails, which suppresses anoikis through Ras hyperactivation and RhoA inactivation (Ma et al., 2010). Anoikis is therefore also regulated through ‘inside-out’ mechanisms when cells lose their attachment to the ECM.

Oxidative stress in anoikis

Loss of cell adhesion triggers metabolic and oxidative stress. Oxidative stress can have opposing effects on cell survival (Martindale and Holbrook, 2002). On the one hand, production of reactive oxygen species (ROS) following cell detachment correlates with anoikis (Li et al., 1999). This metabolic stress can be bypassed by the activation of oncoproteins, such as ErbB2. Overexpression of ErbB2 rescues the ATP deficiency by restoring glucose uptake in an EGFR- and PI3K-pathway-dependent manner (Schafer et al., 2009). On the other hand, increased ROS production has been detected during metastasis, which is probably related to hypoxia, and in this situation could have a protective effect that is associated with the ability of ROS to elicit pro-survival signals (Giannoni et al., 2008). The role of ROS in anoikis protection is likely to be

dependent on several factors and also to be influenced by the potent crosstalk between integrins and growth factor signaling.

Overcoming anoikis: alternative strategies

The above description illustrates many strategies that allow tumor cells to escape the integrin control of anchorage dependence. However, the picture is made more complex by the recent discovery of two cellular processes, autophagy and entosis, that provide further potential mechanisms for promoting cell survival after detachment.

Autophagy

Autophagy is a lysosomal self-digestion process through which superfluous organelles, proteins and cytosol are catabolically degraded. Autophagy is generally induced in response to stress and enables cells to obtain energy in starvation conditions and to eliminate damaged organelles or protein aggregates that are generated by oxidative damage (Mizushima and Levine, 2010). Autophagy would appear to be a survival mechanism in the short term, but in certain situations it causes cell death (Debnath et al., 2005), which points to a delicately balanced regulation.

In mammals, the physiological role of autophagy begins at the oocyte-to-embryo transition after fertilization, when maternal mRNA and proteins need to be degraded to allow the expression of zygotic genes. Autophagy also has a crucial role in the early neonatal period because it ensures cell survival during the starvation period, which is associated with the transition from placental to alimentary nourishment (Kuma et al., 2004). In addition, autophagy is thought to account for the clearance of intracellular organelles, at least of mitochondria, during the differentiation of certain cell types, such as erythrocytes, lymphocytes and adipocytes (Mizushima and Levine, 2010). Several studies have also analyzed the role of autophagy in the formation of the mammary gland lumen, but with different conclusions. Although it was originally described that TRAIL-driven autophagy cooperates with apoptosis in the formation of the mammary gland lumen in vitro (Mills et al., 2004), a later report described the induction of cytoprotective autophagy in untransformed epithelial cells through a mechanism involving TGFβ-activated kinase 1 (TAK1) and AMP-activated protein kinase (AMPK). This TRAIL-induced autophagy appears to counteract mammary gland lumen formation, contrary to the observations made by Mills and colleagues (Herrero-Martin et al., 2009) because the inhibition of this process enhanced luminal apoptosis (Fung et al., 2008).

There is more evidence that supports a counteracting role of autophagy and apoptosis. For instance, tumor cells with apoptotic defects develop cytoprotective autophagy in response to TRAIL treatment, and this situation is reverted to apoptosis by deleting autophagic signaling (Han et al., 2008).

Finally, autophagy appears to have an essential role in ensuring the health of terminally differentiated cells by eliminating dysfunctional mitochondria (Zhang et al., 2007). Autophagy in this scenario might be triggered by the cytosolic accumulation of ROS, which occurs as a consequence of mitochondrial failure (Luce et al., 2010).

The most recent studies indicate that autophagy has a prominent role in promoting cancer progression. Its role here is not simple, however, because autophagy involves both tumor-promoting and -suppressing functions. Autophagy can be induced in response to starvation and hypoxia in the less-vascularized areas of the tumor, which therefore limits primary tumor growth. In addition, necrotic cell death in tumors and the subsequent inflammatory response can induce angiogenesis, promoting tumor growth and metastasis. This situation is prevented if cells undergo autophagy instead of necrosis, which allows elimination of cells without triggering inflammation (Degenhardt et al., 2006; Mathew et al., 2007). By contrast, autophagy can also drive tumor cells into a dormant state, which allows them to survive unfavorable conditions and then reactivate metabolism and the cell cycle when conditions improve (Fig. 3). Indeed, inhibition of autophagy when nutrients are lacking can trigger apoptosis (Boya et al., 2005). In addition, excessive cell consumption, when autophagy is unable to provide enough energy for the cell to survive, can promote tumor cell necrosis, and the ensuing chronic necrosis and inflammation, which is similar to a wound healing process, can stimulate angiogenesis and thus become an additional contribution of autophagy to tumor growth (Mathew et al., 2007).

Autophagy also provides a mechanism for migrating pre-metastatic tumor cells to avoid anoikis. The reduced integrin signaling that occurs following detachment from the ECM can induce autophagy in epithelial cells and fibroblasts. In this situation, autophagy might delay the onset of apoptosis, thus giving cells the chance to survive and reactivate once they reattach to the ECM (Fung et al., 2008). The effects of oxidative stress are intimately linked to growth factor signaling. On detachment from the ECM, normal epithelial cells show a substantial downregulation in EGFR expression, which results in the inhibition of the pro-survival PI3K–Akt pathway (Reginato et al., 2003). Both PI3K and Akt have been found to be crucial for glucose transport and metabolism (Czech and Corvera, 1999; Elstrom et al., 2004) and, accordingly, detached epithelial cells also show a marked reduction in ATP levels, which is a result of the loss of glucose transport. In addition, these cells show an increase in ROS production (Schafer et al., 2009). Oxidative stress can have multiple effects that range from promoting cell survival to the induction of cell death (Martindale

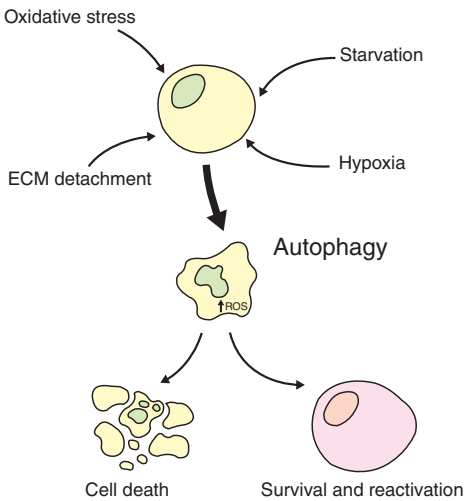

Fig. 3. Autophagy is usually induced under stress conditions, such as lack of nutrients or oxidative stress. In the context of tumor progression, ROS accumulation, which leads to autophagy, can be triggered by hypoxia in the less-vascularized areas of primary tumors or by ECM detachment or mitochondrial failure. Autophagy can result in the apoptotic death of the cell or survival of the cell through reactivation of the cell cycle. The outcome for a specific cell largely depends on additional factors, such as the tissue of origin, availability of nutrients and the presence of oncogenes.

and Holbrook, 2002) or autophagy (Chen et al., 2008). Thus, the increased levels of ROS might be the crucial signal that triggers antophagy in the cells that are detaching from the ECM. However, certain oncogenes can counteract EGFR downregulation, enhance antioxidant capacity and provide cells with ATP by restoring glucose uptake into the cell and enhancing fatty acid oxidation, which thus promotes cell survival (Schafer et al., 2009). In particular, ErbB2 overexpression is enough to restore EGFR expression in detached mammary epithelial cells (MCF10A) by increasing EGFR stability. In this situation, EGFR lysosomal degradation is prevented by ErbB2-dependent ERK activity and Sprouty2 expression (Grassian et al., 2010). Increased glycolysis is one determining feature that allows cancer cells to support their transformation and high proliferation rate (Vander Heiden et al., 2009). In cells that express oncogenic Ras, detachment-induced autophagy contributes to glycolysis, thereby promoting cell proliferation (Lock et al., 2011). Considering all of these factors, the particular conditions of each tumor cell appear to determine whether autophagy will act to limit tumor growth or favor resistance to anoikis and metastasis.

Entosis

Entosis (from the Greek 'entos', meaning 'inside') is a non-apoptotic cell death process that has recently been described in matrix-detached cells and is initiated by a cell actively invading a homotypic cell. After invasion, the invading cell enters an intermediate state, in which it remains alive within the host cell, until it is either degraded by a lysosomal mechanism or released (Overholtzer et al., 2007).

The cell-in-cell phenomenon was first described in the 1920s (Lewis, 1925) as a non-phagocytic process in which immune cells 'eat' other cells. Later on, similar 'cannibalism' situations were observed in metastatic tumors and are considered to be a survival strategy by which tumor cells kill the immune cells that should eliminate them (Lugini et al., 2006). Cell cannibalism is also a way of ensuring that there is a nutrient supply in response to metabolic stress and starvation (Fais, 2007).

But, although cell cannibalism is non-selective (cells are cannibalized regardless of whether they are dead or alive), entosis is an active invasion of one living homotypic cell by another. The initiation of entosis following detachment appears to require the formation of adherens junctions (which involves cadherins) because the unbalanced myosin II contractile forces that are associated with adherens junctions push one cell into another. Adhesion to the ECM counterbalances these forces, which inhibits cell internalization in a β1-integrin-dependent manner. The internalizing cell participates in an active manner that is dependent on the Rho–ROCK pathway, yet activation of this pathway in host cells is not required for the invading cell to be internalized (Overholtzer et al., 2007). The mechanism, however, by which the invading and recipient detached cells coordinately regulate these processes remains unclear.

Entosis usually results in non-apoptotic, lysosomal cell death, but occasionally the internalized cell can be released. Moreover, a small proportion of internalized cells undergo cell division (Overholtzer et al., 2007), which clearly demonstrates that entotic cells can remain viable within their hosts (Fig. 4). Entosis has been described in tumors in which deposition of ECM was undetectable, regardless of whether the tumor was a primary one or a metastatic exudate, which suggests that this process might be triggered by the loss of ECM attachment (Overholtzer et al., 2007). However, the biological significance of entosis remains unclear. On the one hand, entosis could be an alternative mechanism to anoikis that contributes to the clearance of detached and misplaced cells. Accordingly, inhibition of entosis enhances AIG in soft agar, thereby suggesting a role for entosis in tumor suppression (Overholtzer et al., 2007). On the other hand, the survival and later release of entotic cells points to a pro-survival role. What determines which fate an internalized cell undergoes is still unknown.

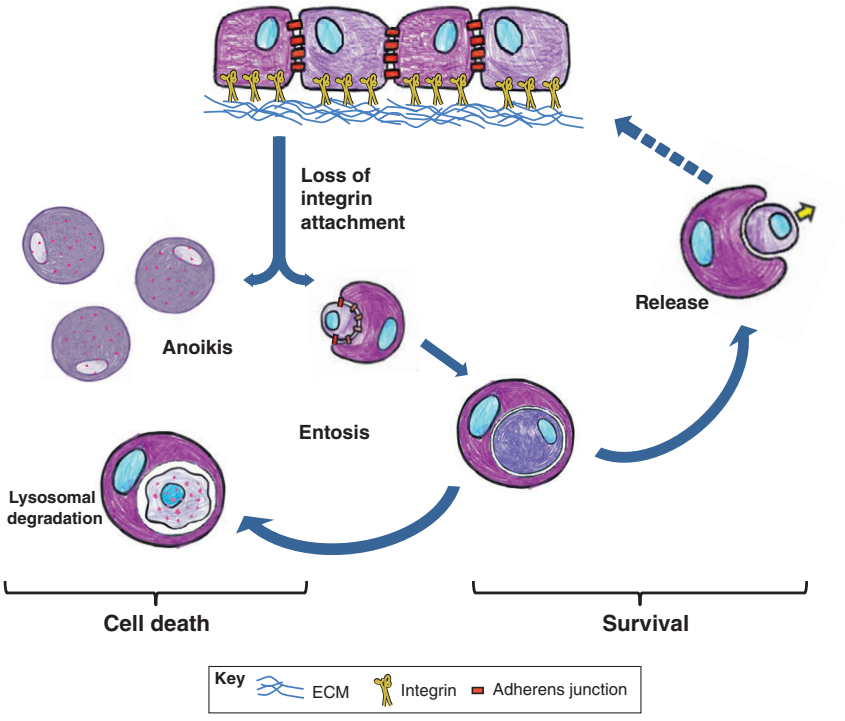

Fig. 4. The potential dual role of entosis. When epithelial cells lose their attachment to the ECM, cells can undergo, instead of anoikis, the process of entosis, which is favored by forces that are associated with adherens junctions. In this intermediate state, where one cell is inside another, an important decision can be made: the internalized cell can either die or be released from the host cell, and potentially reattach to ECM, pointing to a protective role of entosis.

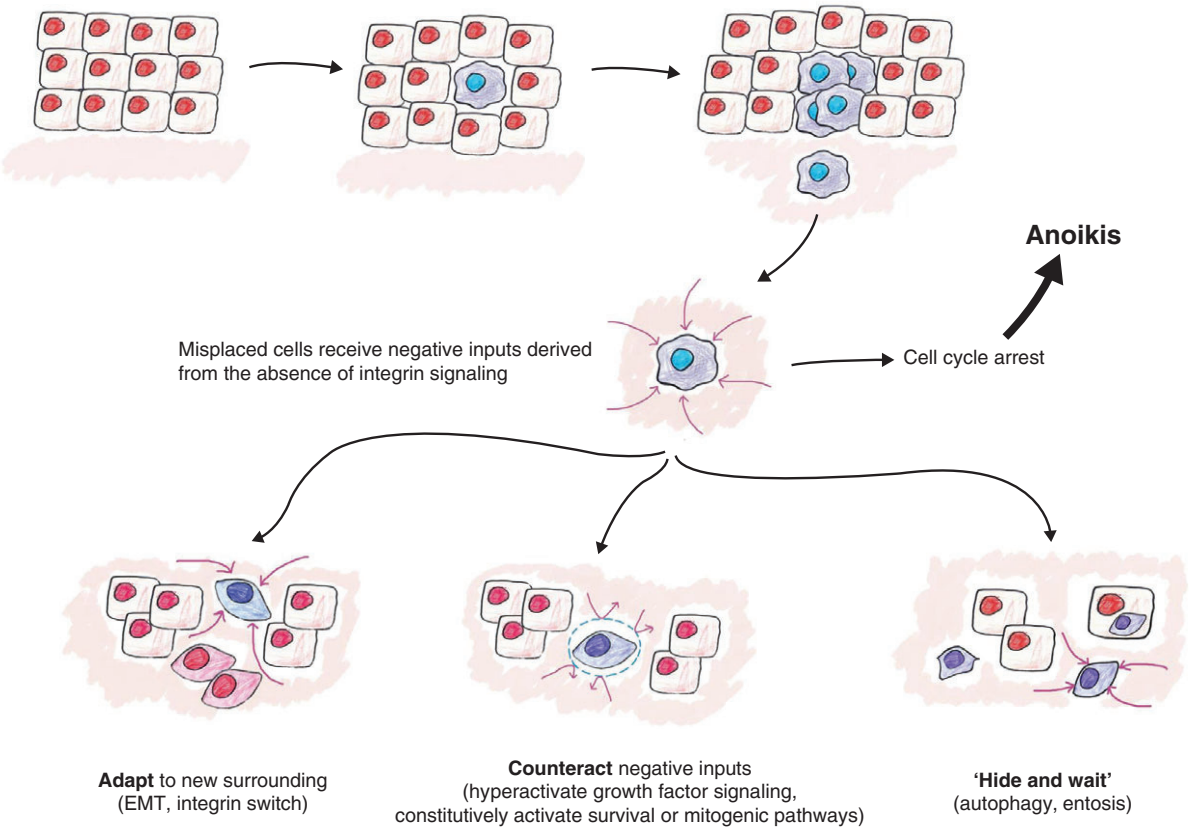

Fig. 5. Transformed cells employ different strategies to compensate for, or circumvent, the anoikis signals and thus become anchorage independent. In general, these strategies follow three different patterns: adapting to the new surroundings, either by EMT or by integrin switching, to avoid anoikis; counteracting negative signaling by hyperactivation of survival or mitogenic pathways; or 'hiding and waiting' by entering into a dormant state through either autophagy or entosis, and then reactivating the cell cycle when conditions are favorable.

The initiation of entosis, instead of anoikis, following loss of adhesion might give the cell additional time in which to decide whether the deleterious conditions are transient or not. Entosis might thus be a strategy for clearing detached cells without killing them, until it is certain they must die. However, this can have deleterious consequences for the organism: a recent study describes how entosis can perturb cytokinesis and induce the formation of aneuploid cells (Krajcovic et al., 2011), thus promoting cell transformation and tumor formation. Like autophagy, entosis is a cellular mechanism with potential antitumor effects, but one that tumor cells are able to subvert under certain conditions and exploit as a sophisticated way of escaping anoikis and becoming anchorage independent. By any interpretation, entosis therefore appears to be much more than just a cell death mechanism.

AIG in cancer progression and metastasis: concluding remarks and perspectives

To ensure the correct organization, size and function of each organ and tissue in the body, the proliferation of adherent cells is strictly dependent on growth factors and attachment to the appropriate ECM. Detachment-induced cell death (anoikis) is a self-defense mechanism that prevents cells from leaving their natural niche and growing dysplastically and has a determining role in preventing tumor cell dissemination and metastatic growth. Tumor cells employ a variety of strategies to escape this process, and the main consequence of these strategies is the loss of anchorage dependency. In the worst-case scenario for a patient, cells partially revert to their undifferentiated phenotype, switching their pattern of expression of integrins and other adhesion molecules, or they develop strategies that overcome detachment-induced cell-cycle arrest or cell death. Tumors release vast amounts of cells into vessels, but only AIG-competent cells can grow and metastasize. After becoming anchorage independent, tumor cells are free to disseminate and colonize foreign tissues, where the plasticity derived from their poorly differentiated state allows them to readapt to the new surroundings and grow dysplastically (Fig. 5).

The ten major causes of death in high-income countries include different types of cancer, meaning that a huge effort to understand the mechanisms underlying cell transformation, tumor development and cancer progression is necessary. Some aspects of this malignization process have been extensively studied and have provided a clear picture of how EMT or anoikis function. Other processes, such as autophagy or entosis, have only been linked to anoikis resistance and tumor progression recently, and the mechanisms that determine whether cells die or survive remain to be explored. Today, cancer treatment is mainly directed towards eliminating the tumor cells. Complementary approaches that impair anoikis resistance or AIG could limit metastasis formation, and this would have a determinant impact in the prognosis of the patients, given that dissemination of the disease could be better prevented. Thus, elucidating mechanisms that allow AIG by tumor cells is a fascinating area of research with substantial therapeutic potential.

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