ORAL DISEASES

Oral Diseases (2011) 17, 355–361 doi:10.1111/j.1601-0825.2010.01763.x

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REVIEW ARTICLE

Anoikis mediators in oral squamous cell carcinoma

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Anoikis – apoptotic cell death triggered by loss of extracellular matrix (ECM) contacts – is dysregulated in many chronic debilitating and fatal diseases. Mechanisms rendering tumor cells resistant to anoikis, although not completely understood, possess significant therapeutic promise. In death receptor-mediated anoikis mechanisms, focal adhesion kinase (FAK) and receptor-interacting protein (RIP) dissociate, leading to association of RIP with Fas, formation of the death-inducing signaling complex (DISC), activation of caspase-3, and propagation of anoikis. In contrast, anoikis resistance is accomplished through constitutive activation of survival pathways that include integrin-dependent activation of FAK and extracellular-signal-regulated kinase (ERK). In addition, FAK and RIP association confers anoikis resistance by inhibiting the association of RIP with Fas and formation of the death signaling complex, which allows cells to escape anoikis. Up-regulation of CD44 also contributes to survival signals and promotes anoikis resistance. This review will focus on the roles of death receptors, prosurvival pathways, and the molecular players involved in anoikis escalation and resistance in oral squamous cell carcinoma.
Oral Diseases (2011) **17**, 355–361

Keywords: anoikis; oral squamous cell carcinoma; extracellular matrix; focal adhesion kinase; Fas/CD95; receptor interacting protein

Introduction

During development and normal physiologic processes, unwanted cells die by a process known as apoptosis. Tissue homeostasis is achieved when the rate of mitosis is in balance with apoptosis. When unbalanced, cells either divide too rapidly and resist normal apoptosis, leading to excessive cell accumulation as in cancer, or undergo premature apoptosis, leading to a net cell loss, as in the autoimmune diseases of pemphigus and pemphigoid (Gniadecki, 1998).

Apoptosis resulting from loss of cell adhesion to the extracellular matrix (ECM), or inappropriate adhesion is defined as 'anoikis' (Figure 1) (Frisch and Francis, 1994). Malignant tumors are not self-limiting in their growth, and capable of invading adjacent tissues and organs, as well as metastasizing to lymph nodes and other distant sites. Human tumor cell lines derived from metastatic lesions are more resistant to anoikis than tumors derived from primary oral squamous cell carcinomas (OSCC), and those tumor cell lines are more anoikis resistant than primary oral keratinocytes (Swan *et al*, 2003).

Oral squamous cell carcinoma, the most common malignant neoplasm of the oral cavity, is responsible for most deaths related to oral cancer and has a poor 5-year survival rate that has not changed in decades (Jemal *et al*, 2008). Patients with OSCC are commonly treated by radical surgical excision, resulting in significant loss of function and disfigurement, leading to a decrease in overall quality of life. As anoikis resistance leads to a more aggressive phenotype in cancers in general and in OSCC (Swan *et al*, 2003) understanding the processes that regulate anoikis will be paramount to advancing novel therapeutics to improve these stagnant statistics.

Anoikis and OSCC

Although anoikis resistance is implicated in different human malignancies, including ovarian (Tang *et al*, 2010), lung (Balsara and Testa, 2002), breast (Streuli and Gilmore, 1999), colon (Shanmugathasan and Jothy, 2000), and head and neck (Bockmuhl *et al*, 1997) cancers, novel findings are emerging about the role of anoikis in OSCC. When a tumor is confined to an organ or tissue, surgical removal and radiation therapy have proven effective; however, there are limited curative treatment options currently available for patients with metastatic disease (Garrison and Kyprianou, 2004). Tumor cells that acquire malignant potential have developed mechanisms to resist anoikis, allowing survival of cancer cells in systemic circulation, thereby facilitating secondary tumor formation in distant organ sites (Eble and Haier, 2006).

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Received 3 June 2010; revised 13 August 2010; accepted 10 September 2010

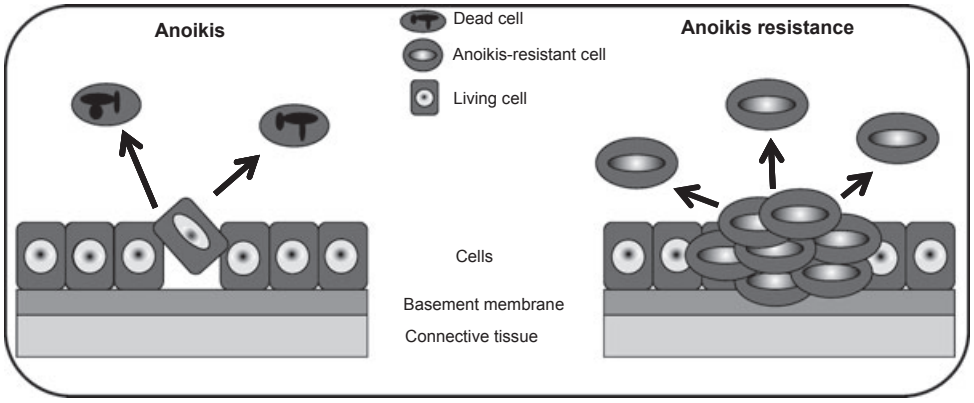

Figure 1 Schematic representation of anoikis *vs* anoikis resistance

Emerging data indicate that multiple pathways regulate anoikis, whereby involvement of integrin receptors, death receptors, adhesion molecules, and complex signaling cascades can lead to anoikis resistance and spread of metastatic cancer cells (Ishida *et al*, 2007; Kamarajan and Kapila, 2007; Kupferman *et al*, 2007; Chiarugi, 2008; Neiva *et al*, 2009). Anoikis-resistant OSCC cell lines exhibit a unique karyotypic and genotypic fingerprint that differs from that of anoikis-sensitive OSCC cells (Kupferman *et al*, 2007). Knowledge of these specific anoikis-resistance processes may help in developing therapies that explicitly target metastatic spread, new opportunities for less invasive and more successful treatments, and potential prognostic markers for aggressive OSCC.

Anoikis mediators

Anoikis mediators, and thus potential anoikis targets for therapy of aggressive OSCC, include ECM molecules, receptors that regulate cell survival pathways, such as integrins and proteoglycans, and cell death pathways, such as Fas/CD95, TNFR1, TNFR2, DR4, and DR5, and their cognate signaling molecules, plus molecules that engage in crosstalk between these two pathways.

ECM

The ECM is comprised of collagenous and non-collagenous proteins, proteoglycans and glycoproteins, including laminin and fibronectin, and growth factors, and alteration of their composition may promote cancer development and progression (Stetler-Stevenson, 1996). The ECM supports tissue homeostasis, is a physical scaffold for support and anchorage of cells, and it regulates the behavior of cells, influencing their survival, apoptosis, anoikis, differentiation, development, migration, proliferation, shape, and function.

Of the ECM proteins, type I collagen is more effective than fibronectin in delaying anoikis in some cell types (Bozzo *et al*, 2006). Furthermore, collagens are disrupted in invasive SCC, yet normal in carcinoma *in situ* or oral premalignant lesions (Giannelli *et al*, 2001). Matrix metalloproteinases (MMPs) can regulate OSCC tumor cell migration, invasion, survival, and apoptosis or anoikis presumably by degrading ECM proteins including collagens (Kurihara *et al*, 2009). Thus, altered collagen metabolism may play an important role in anoikis regulation in OSCC.

The other major adhesive ECM proteins, laminin, fibronectin, and tenascin have been closely linked to anoikis regulation (Ziober *et al*, 2001; Zhang *et al*, 2004b; Dai *et al*, 2005; Boisvert-Adamo and Aplin, 2006; Kamarajan and Kapila, 2007; Lange *et al*, 2007; Ordonez *et al*, 2007). Laminin-5, which is present in normal epithelial tissues and the primary component of the basement membrane of epithelium, is increased in SCC (Ziober *et al*, 2001). Overexpression of laminin-5 correlates with increased tumor invasiveness and motility of OSCC, and this increased motility is thought to be controlled and enhanced by $\alpha 2\beta 1$ and $\alpha 3\beta 1$ integrins (Decline and Rousselle, 2001). Fibronectin is a multi-adhesive matrix protein with domains that bind collagen/gelatin, fibrin, and heparin and regions that undergo alternatively splicing, which bind integrin and proteoglycan receptors. Fragments of fibronectin containing an alternatively spliced V region and function-perturbing point mutations in the high-affinity heparin-binding domain disrupt cell adhesion to the ECM and mediate invasion, motility and anoikis of OSCC cells (Kapila *et al*, 1997; Kamarajan and Kapila, 2007). Laminin and fibronectin are key ECM adhesion glycoprotein components involved in OSCC anoikis regulation.

Integrin receptors

Integrins are alpha and beta heterodimeric receptor complexes that connect ECM components to intracellular signaling pathways. Integrins regulate cell adhesion, migration, survival, and anoikis resistance by activating complex signaling networks (Mitra *et al*, 2005). Integrin suppression leads to anoikis and apoptosis in human mammary epithelial cells (Haenssen *et al*, 2010). Overexpression of integrin $\alpha 4$ increases phosphorylation of its downstream mediator, focal adhesion kinase (FAK), and suppresses anoikis in primary fibroblasts (Joo *et al*, 2008). In addition, inhibiting the $\beta 1$ integrin induces anoikis in epithelial cells (Bouchard *et al*, 2007). Several integrins recognize

fibronectin and are present on the surface of OSCC cells, including $\alpha4\beta1$, $\alpha5\beta1$, and $\alpha v\beta1$ (Zhang *et al*, 2004a). Suppression of integrin αv and its downstream phosphorylation of FAK and ERK induces anoikis in human OSCC cells (Zhang *et al*, 2004b; Kamarajan and Kapila, 2007). Integrin switching from $\alpha v\beta5$ to $\alpha v\beta6$ protects OSCC cells from anoikis (Janes and Watt, 2004). Furthermore, integrin $\alpha v\beta6$ and the enzyme cyclooxygenase-2 are implicated in OSCC progression and have been suggested as possible therapeutic targets (Nystrom *et al*, 2006).

Focal adhesion kinase, a key regulator of integrin signaling, interacts with p130cas, paxillin, talin, Src, phosphoinositide 3-kinase, Shc, ERK, and p53 to regulate cell survival and anoikis resistance (Kapila *et al*, 1999; Janes and Watt, 2004; Shiraki *et al*, 2005; Kamarajan and Kapila, 2007; Koschny *et al*, 2007; Sakamoto *et al*, 2010). Disrupting FAK signaling and genetic deletion of FAK induce anoikis and apoptosis in cells (Taylor *et al*, 2008; Schwock *et al*, 2009). Furthermore, anoikis is associated with reduced levels of FAK phosphorylation in multiple cell types including OSCC (Frisch *et al*, 1996; Valentinis *et al*, 1998; Zhang *et al*, 2004b; Masuda *et al*, 2005; Kamarajan and Kapila, 2007; Johnson *et al*, 2008; Wendt *et al*, 2008; Noda *et al*, 2009) and some cell types seem to undergo FAK-independent anoikis (Wei *et al*, 2004; Diaz-Montero *et al*, 2006). Conversely, FAK overexpression is present in numerous human malignancies, including SCC, with the degree of overexpression correlating with the magnitude of aggressiveness; demonstrating a protective role for FAK in apoptosis (Aronsohn *et al*, 2003; Jiang *et al*, 2010). FAK activates multiple signaling pathways that may fine tune cell-type-specific phenotypes and cell survival (Zouq *et al*, 2009). For example, treatment of OSCC cells with safingol, a protein kinase C (PKC) inhibitor, leads to a rapid decrease in FAK phosphorylation, a subsequent decrease in FAK protein levels, and anoikis; suggesting that FAK suppression via PKC inhibition contributes to safingol-induced anoikis (Noda *et al*, 2009). Emerging data from early-phase cancer clinical trials with orally available small-molecule inhibitors of FAK are promising (Schultze and Fiedler, 2010). Integrin receptors and their downstream signaling pathways, including FAK, show promise as anoikis regulatory targets.

Proteoglycan receptors

Proteoglycan receptors also regulate cell survival and apoptosis, and alterations in these receptors are associated with tumorigenesis (Misra *et al*, 2008). CD44 is a multifunctional protein involved in cell adhesion, migration, apoptosis, and drug resistance. CD44 is a primary receptor for hyaluronan (HA), a major component of the ECM, and it plays a critical role in cell signaling and cell–ECM interactions in cancer (Ponta *et al*, 2003; Hauptschein *et al*, 2005; Hao *et al*, 2010). CD44 undergoes alternative splicing and several of its spliced variants are associated with tumorigenesis. In fact, expression of various CD44 protein variants correlates with aggressive human cancers, including head and neck SCC and breast cancer (To *et al*, 2010). Overexpression of CD44 is associated with apoptotic resistance and down regulation of CD44 is associated with anoikis, as is the case in head and neck SCC (Roehlecke *et al*, 2000; Harper *et al*, 2010). CD44 is also believed to be a stem cell marker in head and neck tumors as in other cancers (Ailles and Prince, 2009; Harper *et al*, 2010). Inhibition of the proteoglycan receptor CD44s inhibits breast cancer cell adhesion, motility, and invasion (Afify *et al*, 2009). Downregulation of CD44 is associated with tumor metastasis in OSCC cells and treatment with an anti-CD44 antibody enhances the invasive potential of OSCC cell lines (Sato *et al*, 2004). Furthermore, OSCC patients demonstrating irregular staining and expression of CD44 exhibited more advanced disease and shortened survival (Kosunen *et al*, 2007). Monoclonal antibodies (BIWA 4, bivatuzumab) against specific CD44 isoforms have been tested in patients with HNSCCs for potential imaging or targeting therapy against tumors (Lyons and Jones, 2007). A clinical trial using anti-CD44v6 antibodies to treat head and neck cancer showed promise but the death of a patient suspended the trial (Orian-Rousseau, 2010). CD44 expression plays an important role in the behavior of malignant tumors, and its variants may be future targets for OSCC anoikis regulation and treatment.

Another family of proteoglycan receptors of potential significance in anoikis regulation are the syndecans as they modulate cancer cell apoptosis and survival (Choi *et al*, 2009; Shimada *et al*, 2009). Syndecans interact with a number of ECM ligands including fibroblast growth factor, vascular endothelial growth factor, transforming growth factor-beta, epithelial growth factor, and fibronectin (Tkachenko *et al*, 2005). In many cell models, overexpression of syndecans enhances cell adhesion and migration in normal and neoplastic cells, whereas interference with syndecan function decreases cell migration, consequently enhancing cell spreading (Choi *et al*, 2009; Khotskaya *et al*, 2009). Alterations in syndecan-2 levels can also regulate cancer cell apoptosis (Orosco *et al*, 2007). Like CD44, syndecans interact with integrins and have multiple adhesion and cosignaling functions, but their mechanistic contribution to anoikis remains poorly understood. Proteoglycan receptors regulate key tumorgenic functions in OSCC progression, and are therefore promising therapeutic targets for regulation of cancer cell anoikis regulation.

Death receptors and their ligands

Apoptosis is regulated by both intrinsic and extrinsic cell death pathways. The intrinsic pathway is mediated by intracellular signals induced by DNA damage or cytokine deprivation leading to caspase activation and cell death (Green, 2000). In the extrinsic pathway, cell surface death receptors, such as Fas/CD95, TNFR1, TNFR2, DR4, and DR5, are characterized by the presence of a death domain and are activated by extracellular death ligands. Activation or oligomerization of death receptors leads

to formation of a death-inducing signaling complex (DISC) and subsequent caspase activation that mediates cell death (Sharma *et al*, 2000; Taylor *et al*, 2008).

Anoikis can be mediated by activation of the death receptor pathway and caspase activation (Frisch and Screaton, 2001). The CD95/Fas pathway has been implicated in chemotherapy-induced tumor cell death in a number of studies. Treatment with anticancer drugs trigger an increase in CD95/Fas ligand (CD95/FasL) expression, which stimulates the Fas receptor pathway in an autocrine or paracrine manner, whereby FasL binds its receptor (Fulda and Debatin, 2004). Fas receptors are expressed in both normal and tumor cells, including OSCC cells (Iwase *et al*, 2003; Ozoren and El-Deiry, 2003). Up-regulation of FasL and down-regulation of Fas expression are early and frequent events associated with the evolution of esophageal SCC (Gratas *et al*, 1998). Furthermore, Fas is expressed in low quantities in OSCC and FasL expression correlates negatively with the degree of differentiation and apoptosis in OSCC (Loro *et al*, 1999). OSCC cells undergo apoptosis in response to treatment with FasL or Fas antibody (Moers *et al*, 1999). Anoikis triggered by activation of Fas pathways in OSCC could be an important defense mechanism against tumorigenesis (Kamarajan *et al*, 2010). However, these approaches have not been applied in OSCC clinical trails (Papenfuss *et al*, 2008).

Tumor necrosis factor alpha (TNF-alpha) is a multifunctional cytokine involved in apoptosis, anoikis, cell survival, inflammation, immunity, and cell signaling through two different death receptor subtypes, TNFR1 and TNFR2 (Simonitsch and Krupitza, 1998; Locksley *et al*, 2001; Engbers-Buijtenhuijs *et al*, 2005). TNFR1 forms a DISC similar to Fas, involving TRADD, FADD, and procaspase-8. Mice lacking TNFR1 and TNFR2 are resistant to death and liver injury induced by an anti-Fas antibody (Costelli *et al*, 2003). Furthermore, the cell surface receptor TNFR1 has been detected only in small numbers in OSCC (Gupta *et al*, 2008).

TNF-related apoptosis-inducing ligand (TRAIL) is a cytokine that interacts with death receptors DR4 and DR5 to facilitate the selective elimination of malignant cells through the induction of apoptosis (LeBlanc and Ashkenazi, 2003; Mahmood and Shukla, 2010). DR5, and to a lesser extent DR4, mediates anoikis in human colorectal carcinoma cell lines (Shiraki *et al*, 2005; Laguinge *et al*, 2008). Cancer cells are more susceptible than normal cells to apoptosis induction by TRAIL, and combinations of TRAIL and chemotherapeutics can act synergistically to kill tumor cells (Koschny *et al*, 2007). Primary cells from OSCC are sensitive to TRAIL, but metastatic cell lines of OSCC are resistant to TRAIL exposure (Noutomi *et al*, 2009). Therapeutic agents targeting TRAIL and its receptors have been developed for clinical application. TRAIL receptor agonists, including recombinant human TRAIL and an agonistic monoclonal antibody against DR4 and DR5, are currently being tested in phase I and II clinical trails (Bellail *et al*, 2009). These results suggest TRAIL resistance imparts metastatic capacity to primary OSCC tumors, and therapeutic agents targeting TRAIL hold promise for OSCC.

Death ligands are critical for control of tissue homeostasis and spread of primary OSCC, and apoptosis by death receptors is considered an important defense mechanism against metastasis that may also be important for anoikis resistance.

Cell survival and cell death receptor crosstalk

Receptor interacting protein (RIP), a kinase with homology to both serine/threonine and tyrosine kinases, interacts with both death receptors and FAK survival pathways and is found in many tissues (Meylan and Tschopp, 2005). RIP is activated and cleaved upon treatment with TNF, Fas, or TRAIL in cell death pathways (Kim *et al*, 2000). Once activated, RIP interacts with both the death receptors TNFR1, TRAF2, FADD, and TRADD, and epithelial growth factor receptors, subsequently playing a key role in inhibiting the activation of NF-kB, thus potentiating apoptosis (Habib *et al*, 2001; Meylan and Tschopp, 2005). RIP also contributes to TNF-induced JNK, ERK, and p38 MAPK kinase activation (Lee *et al*, 2003). Furthermore, RIP seems to play a dual role, as it can bind to FAK in human tumor cells providing a prosurvival signal, and it can interact with Fas in cell death pathways to initiate anoikis (Kurenova *et al*, 2004; Meylan and Tschopp, 2005). Therefore, RIP appears to participate in both prosurvival and cell death pathways, and thus, could constitute a possible target for modulating OSCC anoikis resistance. Indeed, RIP was recently found to mediate crosstalk between the Fas/CD95 death receptor and integrin/FAK signaling pathways in regulating anoikis. Under anoikis conditions, RIP forms a complex with Fas while dissociating from FAK, whereas under survival conditions, it dissociates from Fas and complexes with FAK. RIP is a key shuttling protein that communicates with both integrin/FAK survival signals and Fas/death signals in anoikis (Figure 2) (Kamarajan *et al*, 2010), and may thus serve as a potential target to modulate anoikis.

Summary and future directions

Oral squamous cell carcinoma treatments have shown limited success in the clinical setting in recent decades. Currently, there are many new promising potential targets for cancer therapy and OSCC prognostic markers. Regulation of anoikis and apoptosis pathways in tumor cells, including OSCC involves multiple ECM proteins, cell surface receptors and intracellular signaling targets, and these may be cell-type specific and may serve as therapeutic targets for tumorigenesis. The ability to target both death receptor pathways and prosurvival pathways concurrently seems a promising approach in anticancer therapy. Existing potential targets need to be examined to develop more successful therapeutic tools to provide vital and necessary improvements in cancer treatment.

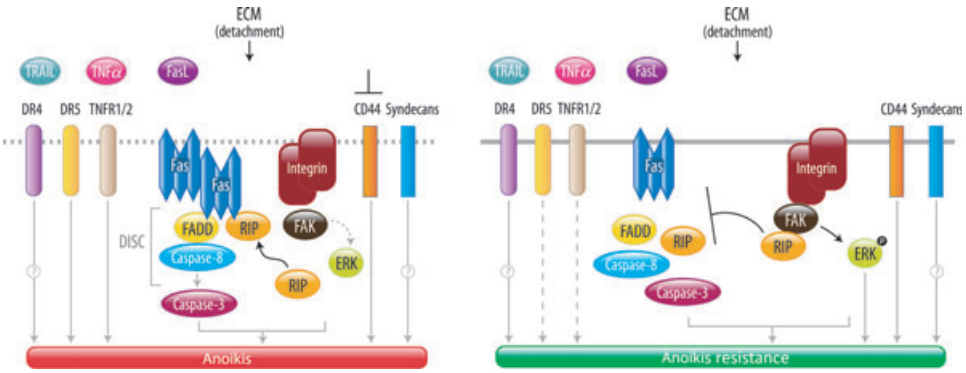

Figure 2 Signaling pathways that mediate anoikis and anoikis resistance. Loss of cell–extracellular matrix (ECM) contacts (detachment) and dissociation of integrin and focal adhesion kinase (FAK) fails to activate survival signals via molecules that include extracellular-signal-regulated kinase (ERK). In death receptor-mediated anoikis mechanisms, FAK and receptor-interacting protein (RIP) dissociate, leading to association of RIP with Fas, formation of the death-inducing signaling complex (DISC), activation of caspase-3, and propagation of anoikis. The DISC is comprised of Fas, FADD, and caspase-8. Anoikis is further activated by an increase in the cell surface expression of Fas and DR5 receptors. The role of the death receptor ligands, TRAIL, TNF-α, and FasL in death receptor activation during anoikis is still unclear. Inhibition of proteoglycan receptors, like CD44 promotes anoikis processes. The role of other proteoglycans, like Syndecans in anoikis regulation is still unclear. Upon detachment from the ECM, cells are capable of evading anoikis and acquire the ability to survive thereby becoming anoikis resistant. This mechanism is achieved through the involvement of constitutive activity of survival pathways that include integrin-dependent activation of FAK and subsequent ERK phosphorylation. In addition, FAK and RIP associate, thereby inhibiting the association of RIP with Fas and formation of the DISC, which favors cell survival. Up-regulation of CD44 also contributes to survival signals and promotes anoikis resistance. Cancer cells, including oral squamous cell carcinoma cells exhibit the unique property of anoikis resistance when detached from their ECM

Acknowledgements

We truly regret that we could not cite the seminal work of many of our colleagues owing to space limitations. This work was supported by Grant R01 DE014429 from the National Institutes of Health to YLK.

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