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Functions of the extracellular matrix and matrix degrading proteases during tumor progression

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Abstract

Cell interactions with extracellular matrices are important to pathological changes that occur during cell transformation and tumorigenesis. Several extracellular matrix proteins including fibronectin, thrombospondin-1, laminin, SPARC, and osteopontin have been suggested to modulate tumor phenotype by affecting cell migration, survival, or angiogenesis. Likewise, proteases including the matrix metalloproteinases (MMPs) are understood to not only facilitate migration of cells by degradation of matrices, but also to affect tumor formation and growth. We have recently demonstrated an *in vivo* role for the RGD-containing protein, osteopontin, during tumor progression, and found evidence for distinct functions in the host versus the tumor cells. Because of the compartmentalization and temporal regulation of MMP expression, it is likely that MMPs may also function dually in host stroma and the tumor cell. In addition, an important function of proteases appears to be not only degradation, but also cleavage of matrix proteins to generate functionally distinct fragments based on receptor binding, biological activity, or regulation of growth factors.

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Introduction

The ultimate fate of a tumor is determined by its ability to productively interact with its host. The alterations in gene expression that occur in a tumor due to cumulative genetic mutations would be inconsequential if they did not provide a means to exploit the supportive host responses while escaping the destructive ones. The host responds to the presence of the tumor initially through changes in gene expression found in the connective tissue immediately surrounding the tumor cells, a response whose function is largely unknown. A clue that this stromal response is not straightforward may lie in the variety of obvious, but contrary, host responses such as infiltrating cytotoxic cells, which the tumor must evade, and infiltrating blood vessels, which the tumor must attract. Two sets of proteins that have an intuitive role in host/tumor interactions are extracellular matrix (ECM) components, which provide both a substrate for tumor growth and

migration as well as a barrier to tumor invasion and metastasis, and the matrix metalloproteinases (MMPs) which, by degrading the ECM, can theoretically nullify its effects, whether supportive or obstructive.

It has long been recognized that following cell transformation and the initiation of a tumor, the environment of the stroma surrounding the tumor changes. A parallel between the tumor stroma and a wound environment has been proposed (1) due to increases in fibrinogen, increased permeability of vessels, and an inflammatory response in both cases (2,3). Indeed, the extracellular milieu is further altered in various tumors with changes in ECM proteins including fibronectin, thrombospondin, osteopontin (OPN), laminin, SPARC, and hyaluronan proteoglycans, as well as MMPs such as collagenase, stromelysins and gelatinases. The ability of the tumor cell to survive, migrate, invade, and eventually colonize a secondary site is dependent on its interactions with ECM proteins and the ability to modify its extracellular environment either by the expression of ECM proteins or matrix-degrading proteases. Much of the interaction with the surrounding environment can be understood by the interaction of cell surface receptors such as integrins with extracellular proteins. The modulation of integrins during tumor progression has been the subject of several recent reviews (4) and will not be discussed comprehensively here.

ECM proteins during tumor formation and growth

The altered nature of the tumor stroma has suggested that proteins that are either suppressed or induced may function during tumor growth or metastasis. Molecules including thrombospondin-1, laminin, fibronectin, proteoglycans, SPARC, and OPN have been implicated. Analyses of the functions of these proteins have been complicated, and in some instances the activities of a particular protein are dependent on the cell lines studied. Evidence suggests a positive role in tumor progression for laminin and proteoglycans and their receptors (5,6). Antisense reduction of thrombospondin reduced the growth rate of a carcinoma line both *in vitro* and *in vivo* (7). In contrast, subcutaneous growth of tumor cells expressing high levels of thrombospondin-1, or injection of purified thrombospondin-1, has been shown to inhibit growth of experimental lung metastases in the same animal (8). SPARC overexpression in carcinoma cells was shown to suppress tumorigenesis (9), although in melanoma lines, antisense inhibition of SPARC had a similar effect of abolishing tumorigenicity (10). Additionally, a positive role for SPARC in the process of angiogenesis has been indicated (11). Some of the difficulties in interpreting these data may lie in the fact that, as mentioned, these matrix proteins are often host stromal cell products as well as tumor products, and thus may affect primary tumor cell growth and migration, as well as host-derived properties such as angiogenesis and inflammation.

In the case of fibronectin, tumor growth has been associated with reduced levels of the protein or its receptor. Both transformed cells and tumors have been shown to have reduced levels of fibronectin (12), and the restoration of fibronectin, or its receptor, the $\alpha 5\beta 1$ integrin, can reverse the transformed phenotype (13). Recently the roles of the $\alpha 5$ integrin and fibronectin during tumorigenesis have been tested by analyzing animals heterozygous for a null mutation in either gene alone, or in the p53-null background (14), in which tumor incidence is increased. In this case, the decrease in the levels of fibronectin or its receptor did not reflect a difference in the incidence of tumors or metastasis of those tumors. These findings do not eliminate the possibility that fibronectin can function to suppress tumor growth, since the levels in the heterozygous animal may be sufficient to complete this function.

We have focused our studies on OPN, a multifunctional secreted protein whose overexpression is associated with cell transformation. Recent analyses of a variety of human tumor specimens demonstrated that OPN expression is present in tumor cells and/or stromal cells in human carcinomas of the colon, duodenum, stomach, breast, lung, prostate, melanoma, bladder, ovary, thyroid, and pancreas (15). Evidence for the functional consequences of OPN in tumors has been obtained using antisense OPN constructs designed to eliminate secretion of OPN in transformed cells. Gardner et al. (16) have expressed antisense OPN in transformed malignant Rat 1 fibroblasts and shown that the reduction in OPN protein secretion correlates with a decrease in tumor growth in the lung as well as growth in soft agar. Su et al. (17) reported that antisense OPN constructs in epidermal cells could inhibit the induction of OPN following tetradecanoylphorbol acetate treatment, and clones stably expressing antisense OPN failed to grow in an anchorage-independent manner in soft agar. Consistent results were also obtained by Feng et al. (18) who found that OPN-targeted ribozymes in H-*ras*-transformed 3T3 cells had reduced tumorigenicity, perhaps due to a greater sensitivity to the cytotoxic activity of macrophage-like cells. Finally, overexpression of OPN in a benign mammary epithelial cell line was sufficient to cause significant metastases of the injected transfectants (19). These results support a causal role for OPN in the ability of tumor cells to survive and metastasize to secondary sites, and suggest that initial OPN induction at stages as early as cell transformation may be critical to the tumor cell phenotype.

One hypothesis as to why OPN-producing tumors are more successful is that the protein provides an adhesive matrix suitable for tumor cell survival and invasion. As mentioned, in comparison to normal tissues, the tumor stroma is of unique composition. As a parallel to this, many transformed cells alter their complements of receptors for extracellular matrix, including modulating cell surface integrins (20). Expression of any of a number of OPN receptors (see below) may facilitate interaction of the tumor cell with the tumor stroma. Denhardt and Chambers (21) have also demonstrated that production of OPN by tumor cells promotes survival by inhibiting cytotoxic attack from host cells via regulation of genes such as nitric oxide synthase, which decreases the ability of the host cell to target the tumor cell.

We have addressed the roles of OPN *in vivo* in a murine model of squamous cell carcinoma using OPN null mutant mice (22). In this system, the carcinogen causes development of benign papillomas, which progress to invasive carcinomas, metastatic tumors, and frequently form secondary tumors in the lungs (23). We have shown that in papillomas, OPN expression is limited to the stroma surrounding the tumor, and it is not until the tumor becomes invasive that the tumor cells produce OPN (24). The extent of expression also correlates with progression state in that tumors graded as metastatic spindle cell carcinomas express high levels of OPN.

Our studies demonstrated that on an OPN null background, chemically induced squamous cell carcinomas grow faster, apparently progress faster, and have more, albeit smaller, lung metastases compared to wild type animals. Tumor lines were derived from carcinomas of wild type and OPN null animals, and characterized *in vivo* and *in vitro* based on OPN production. When injected into nude mice, tumor lines producing OPN grew more slowly than OPN null lines, and this correlated with a higher number of infiltrating macrophages within the OPN-producing tumor. However, further analysis demonstrated that although more macrophages were present in the OPN-producing tumor, most displayed characteristics of differentiated but non-activated cells. One feature was a high level of the mannose receptor, which is downregulated in macrophages

with an activated phenotype. Levels of macrophage mannose receptor are decreased following activation with interferons, lipopolysaccharide, and antigen challenge, and inversely correlate with the generation of superoxide radicals and production of plasminogen activator (25,26). Finally, OPN-producing versus OPN null tumor lines also behaved differently *in vitro*, where survival of cells at low density was compromised in the absence of OPN.

Taken together, our findings support a model where OPN produced by the host and OPN produced by the tumor cells have different functions during tumorigenesis. During the early papilloma stage, we propose that the OPN produced by the stroma surrounding the tumor functions as a chemoattractant for macrophages as a host response. The presence of macrophages at the tumor site can function to inhibit tumor growth. Therefore, on the OPN null background, this host response would be abolished, and the tumors would be able to grow at a faster rate. However, once the invasive/metastatic tumor begins to produce OPN, this tumor-derived protein inhibits the activation of cells including macrophages, allowing greater tumor survival. This concept is consistent with previous findings by Denhardt and Chambers (21) suggesting that tumor-derived OPN provides a survival advantage by inhibiting cells that would cytotoxically attack tumor cells.

Table 1 - Osteopontin (OPN) receptors.

OPN receptor	Possible functions during tumorigenesis
αvß3 integrin (27)	Involved in angiogenesis (28) and endothelial cell survival (29)
αvß5 integrin (30)	Involved in angiogenesis (31)
αvß1 integrin (30)	?
α8ß1 integrin (32)	?
α9ß1 integrin (33)	?
α4ß1 integrin (34)	Leukocyte adhesion (34)
CD44 (35)	Variant forms associated with tumor aggressiveness can confer metastatic potential to tumors (36-38)

Evasion of macrophages may account for the growth differences of the primary tumor, but cannot explain the increased survival *in vitro*. For a metastasis to be successful, the tumor cell must not only reach the secondary site, but should also exhibit growth from clonal density. *In vitro*, this property was reflected in the fact that OPN-producing cells were able to form colonies at clonal densities at which OPN null cells did not survive. The observation that the lung metastases in the OPN mutant mice were significantly smaller than in wild type animals supports the notion that OPN provides a growth advantage under these conditions *in vivo* as well. These results are consistent with the previously discussed antisense experiments in cell lines, where the predominant result of OPN inhibition was reduced clonal growth in soft agar, and reduced experimental lung metastasis.

Our studies of OPN during tumor progression point out that cell compartmentalization (host versus tumor) is very important in determining the overall effects of this protein *in vivo*, and in fact, the effects may be antagonistic. We postulate that this diversity may be explained in part by the presence of multiple cellular receptors, or different activities of multiple forms of the protein. Table 1 indicates the identified OPN receptors, many which have been described recently (27-38). Several of these receptors have been implicated in some stage of tumor growth or progression, including the integrins αvß3, αvß5, and αvß1, and the glycoprotein CD44. Cell adhesion to the matrix is critical for the ability of a tumor cell to migrate and invade. On the other hand, expression of matrix receptors by stromal components such as angiogenic endothelium also is vital to tumor survival. Increasing evidence suggests that both αvß3 and αvß5 are critically involved in the angiogenesis process (28,31). CD44 is a recently identified OPN receptor corresponding to a family of proteins generated by alternate splicing of a single gene. CD44 has been of interest in tumor progres-

sion since variant forms of the protein correlate with progression and metastatic spread of malignant cells. One explanation for this could be CD44 interaction with hyaluronan, a glycosaminoglycan that is enriched in the stroma of carcinomas of the esophagus, stomach, and colon (39), and another possibility is an interaction with OPN, also produced in both tumor and stromal cells during malignant progression. A possible mechanism for the diverse effects of OPN on various cells is that the receptors utilized are different and have distinct signaling cascades. In the case of the CD44 receptor, OPN has been shown to stimulate cell migration, whereas another ligand, hyaluronan, induced cell aggregation (35). We have also shown that OPN is a chemotactic stimulus for αvß3-bearing cells, and did not induce migration even if the adhesive receptors αvß5 and αvß1 were present (40).

Secondly, modified forms of OPN may account for different activities. Biochemical studies of the protein show extensive post-translational modification including phosphorylation, glycosylation, sialylation, and transglutaminase-mediated crosslinking. Several lines of evidence indicate that these post-translational modifications can alter the ability of OPN to bind to other proteins (41,42) or bind to cellular receptors (43). OPN is also a substrate for proteolytic cleavage, and fragments of the protein have different adhesive properties, effects on migration, and receptor-binding capabilities (44, 45). Importantly, proteolytic fragments of OPN occur naturally *in vivo* (45), and thrombin is one known protease that cleaves intact OPN. As discussed further below, proteolytic cleavage of ECM proteins may be one important step regulating their activities.

Matrix metalloproteinases in tumors

MMPs have had a long history associated with tumor progression. The consistent expression of MMPs in invasive metastatic tumor cells (46) has pigeonholed this large family of proteases into the generic role of clearing ECM components from the path of a migrating tumor cell. However, just as the expression in invasive tumors led to this model, closer examination of MMP expression *in vivo* has forced us to consider more complex functions for these enzymes in tumor progression. In the majority of epithelial tumors, expression of most MMPs is found initially in the surrounding tumor stroma. It is not until the latest stages of tumor progression that these MMPs become widely expressed by the tumor cells. Representative exceptions to this expression pattern range from stromelysin-3, which is virtually never expressed by the tumor cells at any stage of progression, but is highly expressed in the tumor stroma (47), to matrilysin, which is highly expressed in benign epithelial tumors, but not in the tumor stroma (48). Overall, the expression patterns of MMPs are more complicated than simply being associated with metastatic tumors and thus suggest a multifunctional role for MMPs beyond simple invasion and metastasis.

By examining animals with targeted inactivating mutations in MMP genes, the complexity of MMP functions in tumors is just beginning to be unraveled. In gelatinase A (MMP-2) null mice, tumor angiogenesis and progression of injected tumor cell lines is inhibited (49). Chemically induced skin tumorigenesis is inhibited in the stromelysin-3 (MMP-11) null mouse, and stromelysin-3 null fibroblasts fail to support the growth of injected breast tumor cells (50). Multiple intestinal neoplasia (*Min*) mice on a matrilysin (MMP-7) null background have a 60% reduction in the formation of benign intestinal tumors (51). Each of these mutants indicates that MMPs support tumor formation and growth, and do not simply enhance tumor invasion and metastasis. Further support for this hypothesis is found in MMP transgenic mice. For instance, overexpression of either stromelysin or matrilysin in the

Table 2 - Consequences of protein cleavage by matrix metalloproteinases (MMPs).

Protein	Protease	Functional consequence of proteolysis
Laminin-5	MMP-2	Induce cell migration (56)
Decorin	MMP-2, -3, -7	Release of TGFß1 (57)
Entactin	Str-1 (MMP-3)	Cell apoptosis (58)
Fibronectin	MMP-2?	Modulate cell proliferation and migration (59)
Beta 4 integrin	Matrilysin	Regulate cell surface beta 4 levels? (60)
Collagen XVIII	?	Generation of endostatin
Plasminogen	Str-1 (61), MMP-7, -9 (62)	Generation of angiostatin

mammary gland enhances tumorigenesis (52,53). Similarly, collagenase overexpression in the skin of mice increases tumorigenesis of chemically induced tumors (54).

Though there have been no reports of MMPs whose activities inhibit tumor growth, preliminary studies with the stromelysin-1 null mouse (55) indicate that, in the very earliest stages of skin tumor growth, such a function appears to exist. When skin tumors are chemically induced in the stromelysin-1 null mouse, we see a higher rate of initial tumor growth as determined by tumor size. This accelerated growth is completely limited to the first 7 weeks after the appearance of the tumor, a time consistent with the stromal expression of stromelysin-1. However, once the tumors progress beyond 7 weeks, there are no apparent differences in tumor growth, invasion or metastasis. The explanation for this phenotype may lie in the observation that stromelysin-1 is one of the very few MMPs that appears to have a role in normal connective tissue as exemplified by its high fibroblast expression during the cutaneous wound healing process. In fact, the stromelysin null mouse is deficient in wound contraction, a process that we are exploring as having an effect on tumor growth.

Perspectives

Though it is possible to hypothesize that the effects of ECM proteins on tumor behavior are due merely to their altered expression, it is unlikely that this is the case for MMPs. A more likely possibility is that MMPs exert their effects by proteolyzing available substrates, whether matrix components or other effector molecules (Table 2; 56-62). For instance, the tumor growth-enhancing effect of stromelysin-3-producing fibroblasts requires the presence of growth factors bound to the matrix, implying that stromelysin-3 processes matrix components in such a way that growth factors become newly bioavailable to the tumor (50). MMP processing of ECM components has also been shown to create fragments of matrix proteins that were not present in the intact molecule, such as in the case of gelatinase A cleavage of laminin 5 inducing cell migration (56). Conversely, proteolytic processing may also inactivate matrix protein function. MMPs have also been shown to be capable of processing integrin receptors for ECM components (60), another mechanism by which the cellular response to matrix can be modified.

The seemingly diverse and even contradictory activities of particular matrix proteins during tumor progression will likely be reconciled by a more extensive consideration of the specific extracellular environment. Expression and localization of cell surface receptors, expression of activating and inactivating proteases and their inhibitors, and alterations in expression of the matrix components themselves will all integrate to determine the behavioral responses of the tumor cells and the selective pressures that determine tumor progression.

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