Chapter 9

CD13/Aminopeptidase N in Tumor Growth and Angiogenesis

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Abstract: The CD13/aminopeptidase N cell surface peptidase was originally identified as a marker for tumors of the hematopoietic system. Recent evidence however extends the role of CD13/APN to solid tumors as well where it is postulated to regulate angiogenesis and tumor metastasis.

Key words: aminopeptidase, cancer, metastasis, biomarker, angiogenesis, proteolysis

1. CD13/AMINOPEPTIDASE N AS A LINEAGE MARKER IN LEUKEMIA

Since its initial description, the thirteenth 'cluster of differentiation' antigen or CD13, has been associated with cancer. CD13 was originally observed in studies seeking to identify lineage specific antigens to facilitate the classification of human leukemias, where it was found to be a valuable marker in distinguishing subsets of normal and malignant myeloid cells (Hogg and Horton 1987; Griffin *et al.* 1981; Shipp and Look 1993). In acute lymphoid leukemia, distinct clinical and biological subsets can be delineated based on aberrant patterns of CD13 expression. Similarly, in acute non-lymphoblastic leukemia, CD13 expression (along with other antigens such as myeloperoxidase, CD33, and CD117) is a hallmark of a leukemia of myeloid cell origin (Casasnovas *et al.* 2003). Cloning of the gene encoding CD13 revealed that it was identical to the cell surface peptidase, aminopeptidase N (APN, EC 3.4.11.2), thus extending its range of expression beyond the

hematopoietic system to include fibroblasts and epithelial cells in the liver, intestine, brain, and lung (Look *et al.* 1989). The human gene encoding *CD13/APN* is located on the long arm of chromosome 15 at bands q25-26 and spans over 40 kb with 20 exons (Lerche *et al.* 1996; Look *et al.* 1989). Analysis of *CD13/APN* transcriptional regulation revealed that it is regulated by two mutually exclusive promoters, one distal (8kb upstream of the initiation codon) that is active in hematopoietic cells and fibroblasts, and a second proximal promoter (adjacent to the translational start) that controls expression in epithelial and endothelial cells (Shapiro *et al.* 1991).

CD13/APN is a 165 kDa, 967 amino acid integral membrane protein. This type II metallopeptidase spans the outer cell membrane and contains a short intracytoplasmic N-terminal segment of 9 amino acids and a large extracellular COOH-terminal domain. This ectodomain includes the HEXXH pentapeptide motif which is characteristic of members of the M1 zinc-binding metalloprotease superfamily (Dixon *et al.* 1994; Shipp and Look 1993; Look *et al.* 1985; Sakai *et al.* 1987). CD13/APN and other ectopeptidases in this family cleave (thereby activating or inactivating) short bioactive peptides, thus regulating their activity and availability to adjacent cells (Hoffmann *et al.* 1993; Lucius *et al.* 1995; Zini *et al.* 1996). Early biochemical analysis of CD13/APN showed its preference for peptide substrates displaying neutral N-terminal amino acids, while peptides initiating with proline and glutamic acid are poor substrates (Feracci and Maroux 1980; Sjostrom *et al.* 1985). The broad expression pattern of CD13/APN and its location on the cell surface dictates that its function in a specific tissue is directed by the available substrates and receptors in the immediate intercellular space. Therefore, in the renin-angiotensin system CD13/APN participates in the processing of vasoactive peptides (Reaux *et al.* 2001), in the intestine it is responsible for the scavenging of amino acids and catabolism of the peptide products of digestion (Turner *et al.* 1987), while in the brain it contributes to the degradation of neuroactive peptides (Matsas *et al.* 1984; Noble *et al.* 2001). CD13/APN can also function as a cell surface receptor independent of its peptidase activity, where it has been identified as the receptor for certain coronaviruses (Yeager *et al.* 1992) and for tumor homing peptides (Pasqualini *et al.* 2000; Curnis *et al.* 2002). Recently, accumulating evidence suggests that cell-surface peptidases may play an important role in the control of growth and differentiation of many cellular systems (Imamura and Kimura 2000; Bertolini *et al.* 2003; Riemann *et al.* 1999; Lendeckel *et al.* 2003; Kehlen *et al.* 2000; Appasamy *et al.* 1997; Rosenzweig *et al.* 2000), including, as we will present here, endothelial cell functions that are necessary for tumor angiogenesis.

2. THE ROLE OF ANGIOGENESIS IN TUMOR PROGRESSION

During embryonic development new blood vessels are created *de novo* from endothelial-cell precursors (angioblasts) in a process called vasculogenesis. After initial formation, the circulatory system begins to differentiate and new blood vessels sprout and branch from pre-existing capillaries by a distinct process called angiogenesis (Carmeliet 2000). In the adult, growth of the vasculature is usually quite limited and apart from growing tumors, only a few adult tissues require ongoing angiogenesis including the female reproductive organs, organs that are undergoing physiological growth, or injured tissue (Hanahan and Folkman 1996). While vasculogenesis and angiogenesis have been historically described as different processes, they may be more similar than originally believed. Recent evidence suggests that endothelial progenitors may contribute to vessel growth both in the embryo and in ischemic, malignant or inflamed tissues in the adult (Lyden *et al.* 2001; Rafii *et al.* 2002). However, the percentage of endothelial-precursor-cell incorporation is generally low and depends on the nature of the tumor, supporting the concept that the majority of tumor neo-vascularization seems to occur via angiogenesis (Lyden *et al.* 2001).

The formation of a tumor in mammalian tissues is a multistep process that generally progresses through the acquisition of numerous genetic changes that lead to the uncontrolled growth of a transformed cell. The most critical of these genetic alterations have been determined to be oncogenic activation, loss of telomerase activity, and induction of aneuploidy (Duesberg *et al.* 1999; Hahn and Weinberg 2002; Pelengaris *et al.* 2002). Following cellular transformation, tumorigenesis is controlled by common physiological mechanisms; the newly formed tumor requires a constant supply of nutrients and oxygen for its growth and maintaining an adequate supply of these factors rapidly becomes a growth-limiting concern. At early stages (tumors less than 1–2 mm in diameter), tumor cells are oxygenated by simple diffusion in a phase defined as avascular. Tumors of this size can remain dormant for many months after reaching a steady state between proliferation and apoptosis (Black and Welch 1993).

The demand for oxygen and nutrients varies widely among tumors and is often dependent upon tumor type, their stage of growth, and their location. These requirements can change over the course of tumor progression (Hlatky *et al.* 2002). Tumor cells characteristically show a reduced oxygen dependency as compared to normal cells, and are thought to sustain their metabolism under relatively anaerobic conditions by increasing glycolysis to maintain their ATP production (Papetti and Herman 2002). However, despite

their increased tolerance of hypoxia, tumors remain strictly dependent on an adequate oxygen supply for their exponential growth. During initial chronic oxygen deprivation, and as growth progresses and the lack of oxygen becomes acute, induction of the expression of a battery of genes allows tumors to overcome hypoxic conditions by increasing their blood supply by angiogenesis. This neovascularization signals the change from a relatively dormant state to a tumor capable of rapid growth and is characterized by a set of physiological events termed the “angiogenic switch” that is provoked by a change in the balance of pro- and antiangiogenic molecules (Hanahan and Folkman 1996).

In addition to physiological stimuli, factors influencing the angiogenic switch include genetic mutation, metabolic and mechanical stress, inflammation, activation of oncogenes, or the inactivation of tumor-suppressor genes (Kerbel and Folkman 2002; Carmeliet and Jain 2000). Factors affecting the onset of the angiogenic switch may be tumor- and tissue-specific and may also change during tumor progression (Folkman 2002; Dor *et al.* 2001). The switch can occur at different stages of tumor growth, again depending on the type of the tumor and the surrounding tissue. The process of angiogenesis begins with perivascular detachment and vessel dilation, followed by angiogenic sprouting, new vessel formation and maturation, and the recruitment of perivascular cells. New blood vessel formation will continue as long as the tumor grows in order to feed the hypoxic and necrotic areas of the tumor. Finally, the induction of angiogenesis appears to be a crucial process that impacts not only malignant tumor growth, but also tumor invasiveness and metastasis (Hanahan 2000).

3. MOLECULAR MECHANISMS REGULATING TUMOR ANGIOGENESIS

The hypoxic effects resulting from tumor growth that trigger angiogenesis are mediated predominantly by the hypoxia-inducible transcription factors (HIFs; reviewed in Pugh and Ratcliffe 2003; Semenza 2002). HIFs induce the expression of many pro-angiogenic genes such as growth factors, cytokines, proteases, and cell surface proteins, all of which contribute to angiogenesis by distinct mechanisms. Angiogenic growth factors, such as VEGF, bFGF, PlGF, HGF, the angiopoietins, and cytokines such as TGF β initiate signaling cascades by binding to their endothelial-expressed receptors and inducing further expression of a wide array of genes that regulate angiogenesis by distinct mechanisms (Klagsbrun and D'Amore 1991). Signals initiated by specific growth factors generate diverse effects on angiogenesis such as stimulation of endothelial cell proliferation and

migration, leukocyte adhesion, promotion of endothelial cell survival, and attraction and activation of inflammatory cells to the angiogenic site. Growth factor signals also modulate the affinity and avidity of the integrin extracellular-matrix-binding integrin receptors from inside the cell in a process known as 'inside-out' signaling and so influence adhesion, migration and growth (Eliceiri *et al.* 1998).

Degradation of the extracellular matrix (ECM) is an essential prerequisite for the migration of endothelial cells and growth of new vessels. This degradation is facilitated by proteases which not only degrade the basement membrane and other ECM components, but also release ECM-bound growth factors [e.g. bFGF; (Falcone *et al.* 1993)] and activate latent growth factors and other proteases (reviewed in Noel *et al.* 1997). A growing number of studies indicate that the invasive behavior and the vascularization of a tumor is related to a coordinated expression of proteolytic enzymes including serine-, cysteine-, aspartic-proteinases and metalloproteinases such as the matrix metalloproteinases and plasminogen activators (Twining 1994; Andreasen *et al.* 1997; Chapman *et al.* 1997; Murphy and Knauper 1997; Kute *et al.* 1998). Matrix metalloproteinases degrade the ECM to allow cell movement, affect signaling, and release bioactive peptides. Expression of matrix metalloproteinases is induced by various pro-angiogenic factors, e.g. VEGF (Wang and Keiser 1998), bFGF (Andersen *et al.* 1998), TGF- β (Santibanez *et al.* 2002), EGF (Kheradmand *et al.* 2002), IGF (Long *et al.* 1998) and IL-8 (Inoue *et al.* 2000). Plasminogen activators belong to the class of serine proteases that play critical roles in membrane degradation, the proteolytic activation of growth factors (Saksela and Rifkin 1990), and regulation of cell adhesion and motility by interaction with the actin cytoskeleton via various integrins (Kjoller 2002; Wei *et al.* 1996; Planus *et al.* 1997).

In addition to the secreted proteins, many membrane bound proteins play prominent roles in angiogenesis. Integrins and cadherins are membrane proteins that mediate many functions involved in blood vessel assembly. Integrins are the primary receptors for cell-matrix interactions, whereas cell-cell contacts are mediated by cadherins. Integrins promote endothelial cell proliferation and motility by directly controlling adhesion to the matrix and regulating intracellular signaling pathways controlling cytoskeletal organization, force generation, and survival (Eliceiri *et al.* 1998; Leavesley *et al.* 1993; Hood and Cheresch 2002). *In vivo*, angiogenesis in response to different growth factors is mediated via distinct integrins (Friedlander *et al.* 1995) and numerous studies have emphasized the important role of these proteins in tumor growth, vascularization and metastasis (Bello *et al.* 2000; Sato *et al.* 2001; Vonlaufen *et al.* 2001; Cooper *et al.* 2002). Cadherins are critical for the maintenance of Ca⁺⁺-dependent cell-cell adhesion. Vascular

endothelial cells express N-cadherin and vascular endothelial (VE-) cadherin, both of which have been found to play important roles in physiological and pathological angiogenesis (Radice *et al.* 1997; Carmeliet *et al.* 1999; Corada *et al.* 2002; Nakashima *et al.* 2003). A number of additional cell surface molecules are involved in cellular migration and invasion and many of these are regulated in the course of tumor growth and vascularization [e.g. cell adhesion molecules (CAM) and selectins (Fox *et al.* 1995; Friess *et al.* 1996; Verkarre *et al.* 1999)]. Pertinent to this review, cell surface peptidases such as CD13/APN are emerging as important regulators of angiogenesis as well.

4. CD13/APN IN ANGIOGENESIS

We have recently identified a novel role for the CD13/APN cell surface peptidase as a potent angiogenic regulator, where functional CD13/APN is required for tumor growth *in vivo* (Pasqualini *et al.* 2000) and endothelial migration and capillary network formation *in vitro* (Bhagwat *et al.* 2001). Initial immunohistochemical studies of various tissues revealed that CD13/APN is present on the tumor vasculature of both mice and humans and on the vessels of angiogenic corpus luteum, but is essentially absent on the vessels of normal tissues examined (Pasqualini *et al.* 2000; Curnis *et al.* 2002). This expression pattern suggested that CD13/APN expression in endothelial cells may be linked to angiogenesis and predicted a functional role for CD13/APN enzymatic activity in new blood vessel formation. Indeed, treatment of animals with CD13/APN antagonists impaired their ability to mount an angiogenic response, and injection of the functional inhibitor bestatin or anti-CD13/APN monoclonal antibodies significantly inhibited growth of breast carcinoma xenografts (Pasqualini *et al.* 2000). Similarly, these inhibitors were also extremely effective at abrogating the ability of endothelial cells to organize a capillary network or migrate through the extracellular matrix Figure 1a and 2 (from Bhagwat *et al.* 2003). In contrast, treatment of endothelial cells with CD13/APN antagonists had no effect on proliferation rates, implying that CD13/APN does not play a role in endothelial cell proliferation during angiogenesis. These data suggested that CD13/APN may be a functional regulator of angiogenesis and could play a role in later stages of the angiogenic process.

Investigation of the molecular basis for the expression of CD13/APN on activated vs. resting endothelial cells indicated that it is precisely controlled by angiogenic signals present in the tumor microenvironment (Bhagwat *et al.* 2001), predominantly at the transcriptional level. In primary endothelial cells, CD13/APN cell surface expression and mRNA levels were

significantly induced upon serum stimulation and after culture with individual angiogenic factors (bFGF>VEGF>TNF α >IGF-1) as well as hypoxia and other signals in the extracellular matrix, thus explaining its expression in angiogenic endothelium.

We have also investigated growth factor-mediated signaling mechanisms controlling the expression of *CD13/APN* in endothelial cells. Our study showed that activated Ras is critical for the induction of *CD13/APN* transcription in endothelial cells treated with bFGF, since abrogation of Ras activity with inhibitors or dominant negative expression constructs hinders *CD13/APN* expression (Bhagwat *et al.* 2003). The central role of Ras in signal transduction cascades in tumorigenesis and angiogenesis is well established (D'Angelo *et al.* 1997; D'Angelo *et al.* 1999; Ilan *et al.* 1998; Eliceiri *et al.* 1998; Larrivee and Karsan 2000; Mechtcheriakova *et al.* 2001; Arbiser 1997; Elenbaas *et al.* 2001; Hanahan and Weinberg 2000). These studies have shown these processes to be so critical that Ras growth signaling pathways are postulated to be corrupted in the majority of human tumors (Hanahan and Weinberg 2000). In addition to this important role in cell transformation, aberrant Ras signaling in tumor cells elicits expression of angiogenic growth factors that in turn initiate the angiogenic response, thus amplifying tumor growth. Further investigation of the mediators acting downstream of Ras that participate in *CD13/APN* induction showed that the MAP kinase and PI-3 kinase pathways act in concert to relay signals to the nucleus. Similarly, studies from other laboratories have shown that both PMA- (Ilan *et al.* 1998) and TGF β 1- (Vinals and Pouyssegur 2001) induced capillary network formation are controlled by parallel pathways involving PI-3K and MEK. In agreement with these studies, we found that inhibition of either PI-3K or MEK activity abrogates cellular network formation. Significantly, expression of exogenous CD13/APN or the addition of soluble membrane proteins from cells overexpressing CD13/APN can compensate for the loss of either pathway and rescue network formation *in vitro* (Figure 1b and c) and angiogenesis *in vivo*, thus defining CD13/APN as an important target for these pathways in angiogenesis (Bhagwat *et al.* 2003). Finally, our data also indicate that *CD13/APN* is not the sole target of Ras activation in angiogenesis, since addition of CD13/APN is not sufficient to overcome the inhibition of capillary network formation in the presence of the Ras inhibitor manumycin A. These data may reflect the fact that while Ras is required for processes active early in angiogenesis (Hanahan and Folkman 1996), CD13/APN is operative in later stages after angiogenesis has already been initiated. Therefore, CD13/APN is sufficient to rescue angiogenesis when key signaling pathways are interrupted, and is likely an essential target of Ras signaling pathways during angiogenesis (Hanahan *et al.* 1996; Folkman and D'Amore 1996).

Figure 1. CD13/APN rescues capillary morphogenesis despite MEK or PI-3K inhibition. **a)** CD13/APN is required for capillary morphogenesis. Primary endothelial cells (HUVEC) were plated on Matrigel containing the indicated CD13/APN functional antagonists or isotype-matched control antibody (control) and photographed after 18-24 h. **b)** Increased cell surface CD13/APN can rescue inhibited morphogenesis. Hemangioendothelioma cells (EOMA) engineered to express high levels of CD13/APN (20-fold increase, EOMA-CD13, bottom row) or vector control cells (EOMA-pcDNA, top row) were plated on Matrigel in the presence of the indicated Ras, MEK, or PI-3 kinase inhibitors. **c)** Transmembrane expression of CD13/APN is not required for rescue. HUVEC cells were plated on Matrigel containing membrane preparations of CD13-high cells (+CD13, right column) or vector control cells (left column) in the absence (top row) or presence of the MEK inhibitor PD98059 (middle row) or both PD98059 and the aminopeptidase inhibitor bestatin (bottom row).

Figure 2. CD13/APN rescues endothelial invasion in the presence of Ras-MAPK or PI-3K inhibition. CD13/APN is required for migration/invasion. Primary endothelial cells (HUVEC) in 1% serum in the presence or absence of the indicated CD13/APN functional antagonists or vehicle or isotype-matched control antibodies were plated in the top chamber of Matrigel coated transwell plates and bFGF angiogenic stimulus was placed in the bottom chamber. The number of cells invading and migrating through the Matrigel barrier in response to bFGF was counted after 24 hours.

While these experiments strongly suggest that CD13/APN plays a role in angiogenesis, its precise mechanism and substrate remains under investigation. Accumulating data from our laboratory and others suggests that it acts in its capacity as a cell surface peptidase (Shim *et al.* 2003). Because proteolytic processing is a critical aspect of angiogenesis, it is intriguing to postulate that the sequential breakdown of extracellular matrix proteins or other angiogenic proteins may generate bioactive peptides which can positively or negatively regulate angiogenesis. Indeed, proteolytic fragments of extracellular matrix proteins such as angiostatin (O'Reilly *et al.* 1994), endostatin (O'Reilly *et al.* 1997), and tumstatin (Hamano *et al.* 2003; Sudhakar *et al.* 2003) have been shown to be potent mediators of angiogenesis. While these proteolytic fragments are fairly large to be efficiently cleaved by CD13/APN, it is likely that similar but smaller peptide regulators are produced by endopeptidase cleavage during angiogenesis. In this situation, CD13/APN could convert a pro-peptide activator to its active form, or inactivate a small molecule inhibitor, thereby supporting angiogenesis. Given our observation that CD13/APN can sustain angiogenesis in the absence of MEK or PI-3K signaling, it would appear that its putative peptide substrate is a significant regulator of angiogenesis and could be an attractive target for anti-angiogenic therapy.

5. CD13/APN AND SOLID TUMORS

Clinical trials testing the efficacy of potential therapies repeatedly illustrate the necessity for the accurate identification, characterization, and validation of biomarkers to determine optimal dosing regimens, risk stratification, and assessment of treatment efficacy (Fabian and Kimler 2001). As previously indicated, CD13/APN is a classic marker for establishing the stage and lineage of various leukemias and thus is a valuable tool for designing therapy that is specific for particular subsets of leukemia. Accumulating evidence regarding the expression of CD13/APN in angiogenic vessels and solid tumors suggest that it may be an effective marker and regulator of solid tumor growth as well. The fact that CD13/APN is expressed on newly formed tumor vessels indicates that the extent of angiogenesis in solid tumors (and the efficacy of anti-angiogenic regimens) may correlate with CD13/APN levels. Similarly, CD13/APN expression on a number of tumors appears to associate with phenotypic characteristics such as invasiveness and metastasis, again implying its potential as a therapeutic marker of solid tumor subsets. Finally, understanding the molecular basis of the regulatory mechanisms controlling biomarker expression will undoubtedly uncover new therapeutic targets, allowing more effective treatment of the disease.

5.1 CD13/APN in Solid Tumor Development

We have shown that CD13/APN is a potent regulator of angiogenesis and treatment of tumor bearing mice with CD13/APN inhibitors blocks xenograft growth, consistent with its inhibition of tumor-induced angiogenesis (Pasqualini *et al.* 2000). Clinical studies indicate that all solid tumors must acquire the angiogenic phenotype for invasiveness, progression, and metastasis (Folkman *et al.* 1989). Therefore, CD13/APN contributes to the growth and development of solid tumors by regulating their supply of nutrients. In addition, however, numerous studies indicate that CD13/APN expression may affect the phenotype of solid tumors as well.

Expression of CD13/APN and the extent of tumor vascularization has recently been investigated in patients with pancreatic carcinoma (Ikeda *et al.* 2003). In this study, fifty percent of human tumors were found to express CD13/APN on the tumor cells themselves. Correlation of expression with disease outcome showed that the median survival time of patients with CD13/APN-positive tumors was significantly shorter than patients with CD13/APN-negative tumors. Multivariate analysis indicated that the CD13/APN status was a significant independent factor in disease outcome. In an independent study of colon carcinoma, a significant association was

found between CD13/APN expression and prognosis as well (Hashida *et al.* 2002). Patients with tumors that were positive for CD13/APN expression again had a lower survival rate than those with CD13/APN-negative tumors. Importantly, a significant association was found between CD13/APN expression and a poor prognosis among the patients with node-positive colon cancers, suggesting a role for CD13/APN in tumor metastasis (see section 5.2). Furthermore, antibodies directed against CD13/APN inhibited tumor cell motility *in vitro*.

In light of these results, the reported expression of CD13/APN in tumors of numerous other tissues, such as prostate (Ishii *et al.* 2001; Bogenrieder *et al.* 1997), renal (Saiki *et al.* 1993), and lung carcinomas (Tokuhara *et al.* 2001; Prochazka *et al.* 1991), fibrosarcomas (Fujii *et al.* 1996; Saiki *et al.* 1993), and malignant melanomas (Menrad *et al.* 1993; Saiki *et al.* 1993; Fujii *et al.* 1995) may be of prognostic significance. In one broad overview of tissues and tumors of mesenchymal origin, CD13/APN expression was reported to be detected immunohistochemically in some, but not all, cases of a variety of tumors including those of smooth muscle, fibrous, fibrohistiocytic, synovial, osteogenic, and peripheral nerve sheath, and adipose tissue origin, but not in tumors of striated muscle, autonomic ganglia, or cartilage-forming tissues (Mechtersheimer and Moller 1990). In an independent study of tumors of epithelial and lymphoid origin, CD13/APN expression was found in 20% of breast carcinomas, but absent in neoplastic colonic and bronchial tissues and lymphomas (Dixon *et al.* 1994). This is in agreement with our recent studies of CD13/APN expression in normal breast epithelium and murine models of breast carcinoma where we find that normal breast epithelium is CD13/APN negative but tumor epithelial cells derived from three different transgenic models express varying levels of CD13/APN (Figure 3). Immunohistochemical analysis of the vasculature and ductal epithelium of normal breast tissue shows an absence of CD13/APN expression (Figure 3 E), while the blood vessels from all three types of tumors are clearly CD13/APN positive (Figure 3 F, G, and H). Analysis of tumors from the MMTV-Cox-2 transgenic strain (Liu *et al.* 2001) indicates that some but not all cells of hyperplastic ductal nodules from preneoplastic lesions are strongly CD13/APN positive (Fig 3G). Similar analysis of two additional murine transgenic breast cancer models show that tumor epithelial cells from the MMTV-neu model (Guy *et al.* 1996) (Figure 3F) are CD13/APN negative, while their vasculature is positive. In contrast, breast tumors derived from the C3(1)SV40-Tag model (Shibata *et al.* 1998) are nearly all highly CD13/APN positive (Figure 3H). What effects these variations of CD13/APN expression in tumor tissue have on tumor angiogenesis, growth, and invasion is currently under investigation.

A possible consequence of the expression of CD13/APN in tumors may be reflected in studies investigating various peptidase activities in the serum of cancer patients. Although CD13/APN is synthesized as a membrane-bound protein, human plasma contains significant amounts of an active soluble form of CD13/APN suggesting that CD13/APN may be secreted or released from the plasma membrane by shedding or by specific cleavage (Favaloro *et al.* 1993). In two independent reports, CD13/APN enzymatic activity was found to be increased not only in tumor tissue and tumor effusions but also in the serum of breast cancer patients, leading the authors to propose CD13/APN expression as a prognostic marker of tumor progression (Gupta *et al.* 1989; Severini *et al.* 1991). Finally in a recent study, soluble CD13/APN activity was two-fold higher in plasma from cancer patients as compared to healthy subjects (van Hensbergen *et al.* 2002). A significant correlation was found between soluble CD13/APN activity in the plasma and the patient's tumor load. Similarly in tumor exudates, sCD13/APN activity and VEGF levels correlated significantly, while sCD13/APN and other angiogenic protease activities did not (van Hensbergen *et al.* 2002). It is still unclear whether the CD13/APN levels in the serum of cancer patients reflect its expression in the tumors themselves, the degree of angiogenesis in the tumors, or if sCD13/APN is released at higher rates in more aggressive tumors. However, the possibility that sCD13/APN may predict disease outcome and thus may be a useful diagnostic and prognostic marker for solid tumors is intriguing and warrants further investigation.

5.2 CD13/APN in Tumor Metastasis

In addition to its role as an angiogenic regulator and a potential diagnostic marker, CD13/APN has also been reported to contribute to tumor cell invasion and metastasis. A number of studies of malignant melanoma have demonstrated that CD13/APN activity is higher in the more invasive tumors and that specific inhibition of CD13/APN activity decreases invasion and metastasis *in vitro* (Fujii *et al.* 1995; Ishii *et al.* 2001; Menrad *et al.* 1993; Saiki *et al.* 1993). In the colon carcinoma study mentioned earlier, elevated CD13/APN expression significantly correlated with tumor status as well as disease-free and overall survival rate (Hashida *et al.* 2002). In particular, in patients with tumors that had metastasized to the lymph nodes a significant correlation was found between CD13/APN expression and a poor prognosis, again suggesting that CD13/APN might contribute to metastasis. Metastasis is one of the most serious and poorly understood problems encountered in cancer therapy and the prognosis for patients with distant metastasis is extremely poor. It depends on several important processes including cell adhesion, migration, and invasion of the tumor cells (Miyake *et al.* 1991).

Figure 3. Immunohistochemical analysis of breast tissue from control mice and tumor-bearing transgenic animals. Immunohistochemical analysis of normal breast tissue failed to detect significant CD13 levels (E) above background levels (A). Tumor cells from transgenic mice expressing neu are CD13/APN negative while the vasculature is positive (brown color, indicated by arrows in F). In contrast, in MMTV-Cox-2 transgenic breast tissue, the hyperplastic ductal epithelium (arrows in C and G) in the lesion is markedly CD13 positive (brown color, G), as are tumor cells from the C3(1)SV40Tag expressing animals (H). Control sections were stained with the secondary antibody only (A-D).

During migration and invasion, malignant cells degrade basement membranes and extracellular matrix proteins using proteolytic enzymes (as highlighted above) through the surrounding tissue to access the circulation. In this regard, it is possible that CD13/APN could participate in this degradation by hydrolyzing fragments of extracellular matrix components that are the cleavage products of other endoproteases.

CD13/APN has also been postulated to be involved in regulating cell migration, a component of metastasis, because it is expressed by macrophages and fibroblasts that exhibit high degrees of motility in solid tissues (Dixon *et al.* 1994). In this scenario, CD13/APN would participate in invasion through the extracellular matrix by these normal cells as well. Finally, the “priming” of cells for migration/invasion apparently includes expression of CD13/APN, based on the observation that while normal melanocytes do not express CD13/APN, some malignant melanoma cells are CD13/APN-positive (Menrad *et al.* 1993). Melanoma cells engineered to express high levels of CD13/APN degraded type IV collagen and invaded extracellular matrix preparations more actively than did the parental or control vector-transfected cells. These cells also showed an increased ability to colonize the lung in nude mice (Fujii *et al.* 1995). This study also showed that CD13/APN-specific antibodies strongly suppressed invasion. Taken together, these observations suggest that CD13/APN expression by a subset of tumors identifies a more aggressive phenotype perhaps due to a higher capacity for migration or invasion, and that again, CD13/APN expression could be used to predict treatment outcome and monitor response. In addition, one would predict that inhibition of CD13/APN activity would be an effective means of tumor therapy. In this regard, one inhibitor of CD13/APN, Bestatin (ubininex), has been extensively characterized and is the subject of Chapter 13 in this volume.

6. CONCLUSIONS

The expression of CD13/APN in endothelial cells in response to signals in the angiogenic microenvironment and in metastasis-prone cancer cells indicates the importance of this aminopeptidase in tumor progression. Accumulating data suggest that CD13/APN performs dual pivotal functions during cancer growth: i) promoting formation of neovasculature necessary for sustaining tumor progression by its expression on tumor-associated vascular endothelial cells, and ii) enhancing tumor aggressiveness by its expression on a subset of carcinomas. Exactly how CD13/APN might regulate the biological processes involved in angiogenesis and metastasis is still unknown although it is possible that CD13/APN plays a role in cell

migration or invasion, common mechanisms linking both angiogenesis and metastasis. Finally, while the precise role of CD13/APN in neovascularization and metastasis is under active investigation, a comprehensive understanding of the mechanisms controlling the expression of this important regulator of tumor growth is therefore fundamental to the identification of potential therapies targeting not only angiogenesis but metastasis as well.

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Figures 1 and 2 from Bhagwat SV, Petrovic N, Okamoto Y, and Shapiro LH. The angiogenic regulator CD13/APN is a transcriptional target of Ras signaling pathways in endothelial morphogenesis. *Blood* 101:1818-1826, 2003. Copyright American Society of Hematology, used by permission.

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