CHAPTER

10

Altered Hyaluronan Biosynthesis in Cancer Progression

Naoki Itano and Koji Kimata

OUTLINE

INTRODUCTION

Hyaluronan (HA) is a sugar-chain macromolecule in which *N*-acetylglucosamine and glucuronic acid are linked together by alternating β-1,3 and β-1,4 linkages (Fig. 10.1) (Laurent and Fraser, 1992). Despite its apparently simple structure, HA exhibits multiple properties depending on its molecular size and its binding molecules (Fraser and Laurent, 1989). For instance, high molecular weight HA forms part of the extracellular matrix (ECM) by linking HA-binding molecules into macromolecular aggregates and regulating a variety of cell behaviors, such as cell adhesion, motility, growth, and differentiation. HA oligosaccharides also

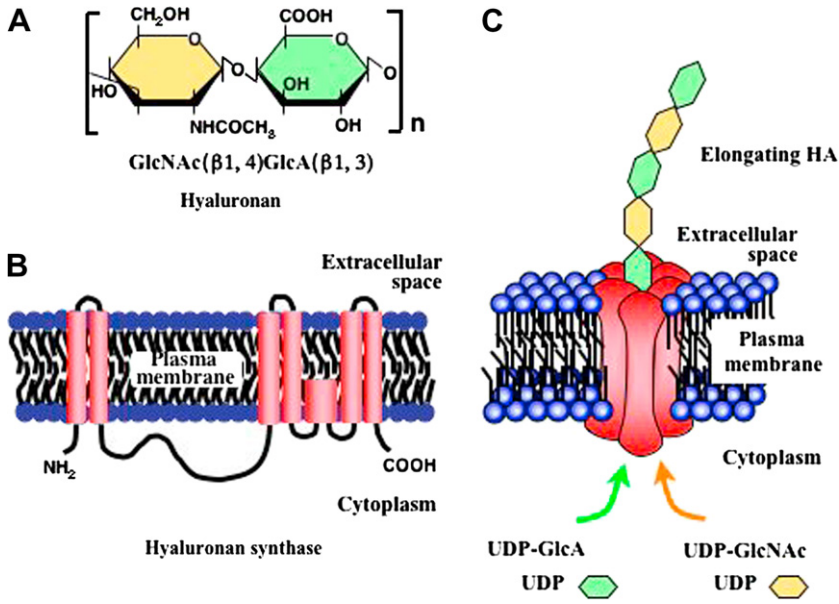

FIGURE 10.1 Scheme illustrating the HA structure (**A**), a predicted structure of mammalian HAS (**B**), and a proposed secretion process of HA (**C**).

regulate such cell behaviors in different ways by acting on intracellular signaling pathways through interaction with cell surface receptors (Toole et al., 1989). Accumulating evidence has demonstrated that the production of HA is excessive in cancer malignancies (Knudson, 1996; Toole, 2004); increased serum levels and deposition in tumor tissue are often associated with malignant progression in many cancers, including breast cancer and colorectal cancer (Ponting et al., 1992; Ropponen et al., 1998).

Although the close association of HA production in the progression of cancer cells is now being established, the entire picture of diverse and complex HA functions in cancer progression remains to be elucidated. Fortunately, animal models with genetically manipulated HA Synthase (HAS) expression provide powerful tools for understanding the *in vivo* function of HA, particularly in connection with cancer cell behavior. Thus the central aim of the present review is to highlight the role of HA in cancer progression from the viewpoint of abnormal HA biosynthesis.

HA BIOSYNTHESIS

The discovery of three members of the HAS gene family (HAS-1, HAS-2, and HAS-3) has enabled great strides in understanding the unique process of HA biosynthesis and mode of chain elongation (Weigel et al., 1997; Itano and Kimata, 2002). Structurally, all HAS proteins are composed of multiple

membrane-spanning regions and large cytoplasmic loops (Fig. 10.1). Unlike typical glycosyltransferases, the cytoplasmic loop in HAS molecules possesses two active sites which participate in the transfer of UDP-GlcNAc and UDP-GlcA substrates. Characterization of the three HAS isoforms has revealed differences in enzymatic properties, particularly in their ability to form HA matrices and determine product size (Itano et al., 1999a). The expression profiles of HAS genes are temporally and spatially regulated during embryogenesis and pathogenesis (Sugiyama et al., 1998; Kennedy et al., 2000; Recklies et al., 2001; Pienimaki et al., 2001), and divergence in the transcriptional regulation of HAS genes during these processes can be explained to some extent by upstream signaling pathways that are triggered by various growth factors, cytokines, cellular stress, and so on. The dynamic turnover of HA is therefore tightly regulated by altering the expression profiles of HAS isoforms to have different enzymatic properties (Weigel et al., 1997; Itano and Kimata, 2002).

ALTERED HA SYNTHESIS IN CANCER

The malignant transformation of cells frequently impairs regulation of HA synthesis and induces excessive HA production (Hamerman et al., 1965; Hopwood and Dorfman, 1977; Leonard et al., 1978). During this process, multiple transcriptional regulation of HAS genes allows cells to optimize the extracellular environment for tumor growth and malignant progression, and a transcriptional switch in HAS isoforms has been demonstrated in cells undergoing oncogenic transformation (Itano et al., 2004). Of the three HAS isoforms, only HAS-2 gene expression was increased in *v-Ha-ras* transformed cells, which showed lowered malignancy. Conversely, both HAS-1 and HAS-2 expression were elevated in highly malignant cells transformed with *v-src*. This implies that HAS isoforms may be involved in different stages of malignant tumor progression. From this point of view, it is increasingly necessary to confirm the relationship between HAS expression and prognosis by statistical analysis using clinical samples. Thus far, these clinicopathological studies have indicated that elevated HAS-1 expression and/or intronic gene splicing correlate with poor prognosis in human colon cancer, ovarian cancer, and multiple myelomas (Yamada et al., 2004; Yabushita et al., 2004; Adamia et al., 2005).

Emerging evidence is providing new insight into the functional aspect of this polysaccharide, particularly in respect to the involvement of HA in tumor malignancy; forced expression of HAS-2 and HAS-3 genes results in excess HA production and enhanced tumorigenic ability of fibrosarcoma and melanoma cells (Kosaki et al., 1999; Liu et al., 2001; Li and Heldin, 2001). Moreover, induced expression of HAS-1 restores the metastatic potential of mouse mammary carcinoma mutants, previously having low

levels of HA synthesis and metastatic ability (Itano et al., 1999b). Inversely, suppression of HAS-2 or HAS-3 decreases HA production and reduces the tumorigenic potential of various cell lines (Simpson et al., 2002a, b; Nishida et al., 2005; Udabage et al., 2005). Although the above clearly demonstrates the important role of HA in tumorigenesis, the tumor promoting ability of excess HA is still somewhat controversial since HAS-2 overexpression also suppresses the tumorigenesis of glioma cells (Enegd et al., 2002). Furthermore, the *in vitro* growth of human prostate cancer cells decreased dramatically when transfected with an HAS-2-expression plasmid, but co-expression of HAS-2 and hyaluronidase HYAL-1 restores the growth of these cancer cells (Bharadwaj et al., 2007). Here, since HA accumulation is the result of a balance between the activities of HAS and hyaluronidases, the presence of hyaluronidase may have promoted HA turnover in the cancer cells and overcome the tumor suppression by excess amounts of HA. Alternatively, the biphasic effects of HA on tumorigenesis can be explained by considering its dose-dependent properties (Itano et al., 2004). To assess this idea, we generated stable transfectants expressing various levels of HAS genes and examined their tumorigenicity in nude mice. Although significant growth promotion was observed within a narrow range of HAS-2 expression, this growth was inhibited at high expression levels. The dose-dependency of HA may help us consider statements regarding the physiological significance of changes in HA concentration with tumor grade or stage, since HA accumulation in clinical samples varies and occasionally shows little statistical changes with tumor grade.

The involvement of HAS in tumor progression has also been evaluated using genetically manipulated animal models. In one study, a transgenic (Tg) mouse model allowing overexpression of murine HAS-2 in mammary glands was generated in order to simulate hyperproduction of HA found in human breast cancer (Koyama et al., 2007). In this model, the expression of exogenous HAS-2 was conditionally controlled by the expression of Cre-recombinase driven by a mammary epithelial cell-specific MMTV promoter. By intercrossing the Tg mice with a mouse mammary tumor model expressing rat c-*neu* protooncogenes in mammary epithelial cells, mammary tumors with aggressive growth rates were developed. Histologically, these tumors were classified as poorly differentiated adenocarcinomas with numerous intratumoral stroma (Fig. 10.2).

TUMOR–STROMAL INTERACTION

Most aggressive tumors are composed not only of cancer cells, but also of many host stromal cells (Kalluri and Ziesberg, 2006), and the importance of interactions between cancer cells and their surrounding stroma in facilitating tumor progression has been demonstrated both by clinical and

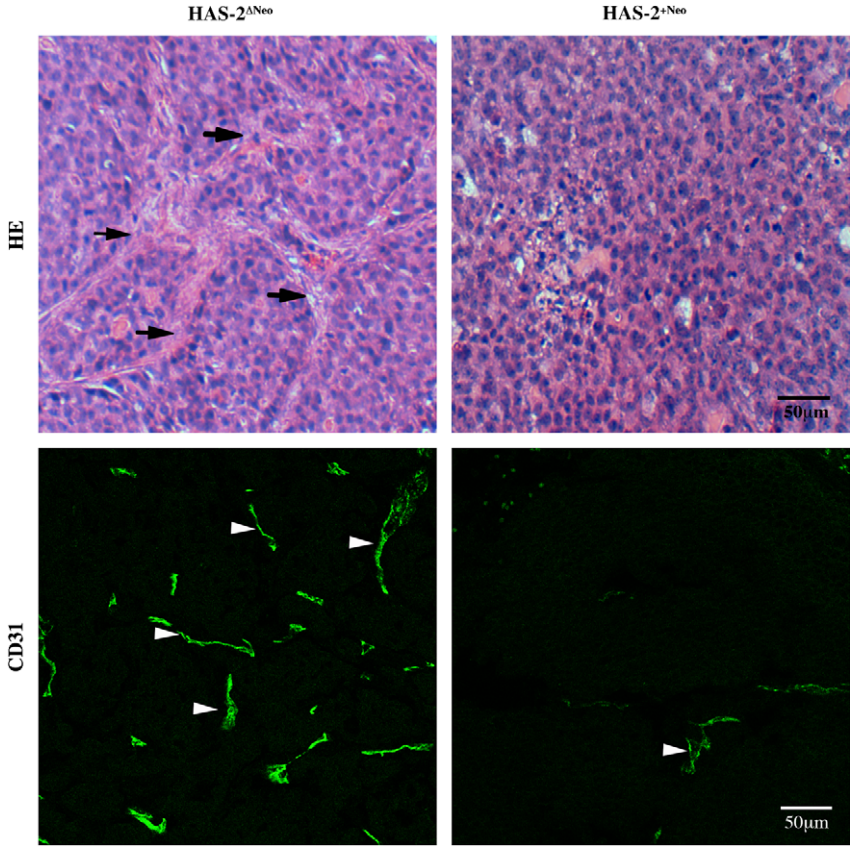

FIGURE 10.2 HA overproduction promotes the formation of intratumoral stroma. Tumor sections from HAS-2-overexpressing transgenic (HAS-$2^{\Delta Neo}$) and control (HAS-2^{+Neo}) mice were stained with hematoxylin and eosin (upper panels). The most prominent histological feature of the HAS-$2^{\Delta Neo}$ tumors was increased formation of intratumoral stroma (arrows). In contrast, control tumors had the characteristics of ductal carcinoma with much less stroma. Tissue sections from HAS-$2^{\Delta Neo}$ and HAS-2^{+Neo} tumors were stained with an antibody against murine CD31 (lower panels). Tumor microvessels (arrowheads) of smaller

experimental evidence (Bhowmick and Moses, 2005). Carcinoma cells actively recruit several distinct stromal cells, such as inflammatory cells, vascular cells, and fibroblasts within the tumor, by secreting chemoattractant factors (Desmouliere et al., 2004; Mantovani et al., 2006). Furthermore, crosstalk between carcinoma cells and adjacent stromal cells influences the composition and arrangement of the tumor microenvironment to support tumor progression by allowing angiogenesis and facilitating the invasion and metastasis of tumor cells (Bhowmick et al., 2004).

Each cell type can potentially communicate with other cells, or among themselves, through the release of auto-/paracrine factors and formation of a complex ECM network (Orimo et al., 2005; Shekhar et al., 2003). A strong correlation has been drawn between cancer progression and the degree of HA accumulation within it, especially in the invading edges of carcinomas (Setala et al., 1999; Auvinen et al., 2000). As such, HA-rich tumor microenvironments, which may be favorable for cancer invasion, are likely generated from complex interactions between tumor cells and stromal cells infiltrating from adjacent host connective tissue. In fact, *in vitro* HA synthesis was synergistically increased in co-cultures of human lung tumor cells with fibroblasts (Knudson et al., 1984; 1989), and similar synergistic effects have been demonstrated with the combination of fibroblasts and other tumor cell types (Merrilees and Finlay, 1985).

Although the main roles of tumor-associated stromal cells in modulating tumor cell behavior is well-established, critical questions still remain as to the molecular mechanisms underlying communication carcinoma and stromal cells and regulating stromal cell recruitment within tumor tissues.

NOVEL FUNCTION OF HA IN STROMAL CELL RECRUITMENT

Genetic evidence supporting the role of tumor-derived HA in stromal cell recruitment has recently come from our experiments using HAS-2 Tg mice; ectopic expression of HAS-2 in mammary tumor cells leads to a marked recruitment of stromal cells inside tumors followed by formation of intratumoral stroma (Koyama et al., 2007). To date, considerable efforts have been made to purify the stromal cell chemotactic factors produced by tumor cells, but only a few factors, such as PDGF, have been identified (Dong et al., 2004). The above notion therefore strongly suggests the function of an HA-rich ECM as a stromal cell chemotactic factor.

Several complex and multifaceted mechanisms can be considered for understanding how tumor-derived HA intratumorally recruits stromal cells during tumor formation. Extracellular accumulation of highly hydrated HA provides microenvironments amenable to easy fibroblast-penetration by increasing turgidity (Laurent and Fraser, 1992), and the fact that forced expression of HAS-2 impairs the intercellular adhesion machinery of tumor cells. This also explains how HA-rich matrices provide an environment favorable for fibroblast-infiltration (Itano et al., 2002; Zoltan-Jones et al., 2003).

HA-induced signaling pathways govern the migratory phenotypes of stromal cells via interaction with HA receptors (Turley et al., 2002). For instance, CD44 and RHAMM, both typical HA receptors, have been implicated in the HA-dependent cell migration and invasion of stromal

fibroblasts. Additionally, the interaction of HA and CD44 can activate receptor tyrosine kinases, which in turn induce the activation of downstream Ras/MAPK and PI3K/Akt signaling pathways (Turley et al., 2002). HA binding to CD44 also activates Rac1 signals, which regulate actin assembly associated with membrane ruffling and cell motility (Bourguignon et al., 2000). Thus, HA appears to promote cell motility by acting on intracellular signaling pathways and controlling the assembly of the actin cytoskeleton. Alternatively, HA–CD44 interactions may recruit mesenchymal stem cells (MSCs) (Zhu et al., 2006); in a mouse model of acute renal failure, MSCs injected into mice migrated to the injured kidney, where HA is abundant (Herrera et al., 2007). Renal localization of MSCs is blocked by preincubation with CD44 blocking antibodies or soluble HA. Likewise, MSCs derived from CD44 knockout mice do not localize to the injured kidney, but are rescued by transfection with cDNA encoding CD44. This same mechanism may participate in the recruitment of MSCs to HA-rich tumors.

Lastly for consideration of HA-mediated stromal cell recruitment is the action of HA-binding molecules. Versican (also called PG-M), an HA-binding proteoglycan, is highly expressed in tissue compartments undergoing active cell proliferation and migration (Wight, 2002) and participates in the formation of an HA-rich ECM (Fig. 10.3). In the peripheral invasive areas of infiltrating ductal carcinomas, the most intense staining by a versican-specific antibody is visualized in the mesenchymal

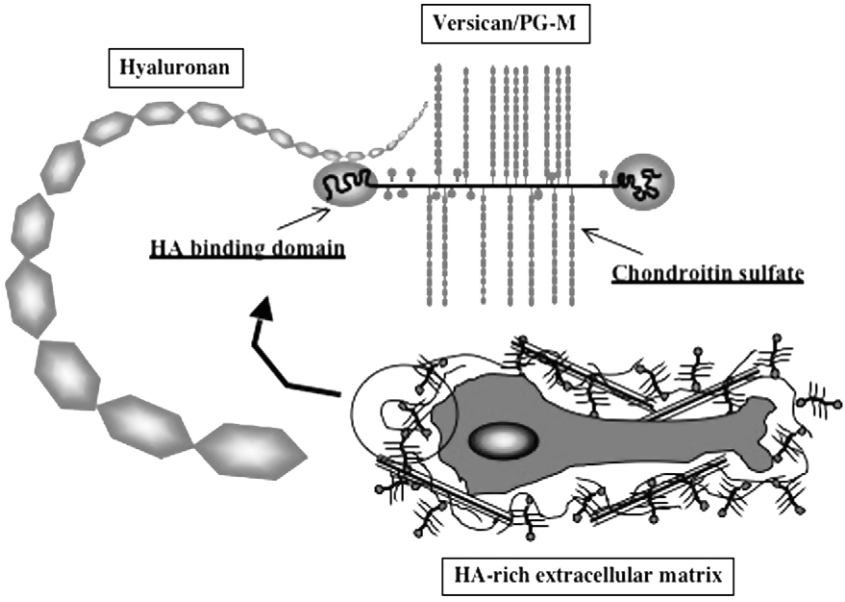

FIGURE 10.3 Scheme of HA-rich extracellular matrix and its constituents.

tissues between carcinoma cell clumps and surrounding tissues, where HA can be demonstrated histochemically (Nara et al., 1997). This cooperative action of HA and versican in mobilizing stromal cells has been demonstrated by the Matrigel plug assay (Koyama et al., 2007). When Matrigel plugs are subcutaneously implanted into nude mice, only trace numbers of stromal cells migrate into the Matrigel plugs containing high molecular weight (HMW) HA alone. However, the infiltration of stromal cells is markedly increased in the presence of HA/versican aggregates. In tumor xenograft models, exogenously added HA/versican aggregates also significantly promote the infiltration of stromal cells within tumors (Koyama et al., personal communication). In concert with versican, HA may therefore allow cells to prepare for migration by enhancing cell detachment from the ECM (Fig. 10.3). The anti-adhesive and motility-promoting effects of versican is evident by combining an earlier observation that versican can inhibit cancer cell attachment to fibronectin, together with the recent finding that formation of an HA/versican pericellular matrix promotes prostate cancer cell motility (Yamagata and Kimata, 1994; Ricciardelli et al., 2007). Current studies have also implied a role of versican in enhancement of cell motility in the assembly of cytoskeletal machinery and transmitting signals.

EPITHELIAL–MESENCHYMAL TRANSITION CAUSED BY HA OVERPRODUCTION

Epithelial–mesenchymal transition (EMT) is the process whereby epithelial cells convert into mesenchymal cells (Thiery and Sleeman, 2006). Following a series of events, epithelial cells lose their epithelial polarity and characteristics while simultaneously acquiring the mesenchymal phenotype. Typically, EMT switches gene expression characteristics from epithelial E-cadherin and cytokeratin to mesenchymal vimentin and α-smooth muscle actin (α-SMA).

Recent advances have fostered a more detailed understanding of the molecular machinery and networks governing EMT. TGF-β and related growth factors mainly influence the process of EMT and receptor tyrosine kinases, which induce the activation of downstream Ras/MAPK and PI3K/Akt signaling pathways, govern EMT in cooperation with growth factors. The nuclear translocation of β-catenin is a key downstream signal that triggers EMT, and all of these pathways crosstalk with each other and transmit signals towards a common endpoint to promote EMT. ECM molecules and degrading enzymes can also convert epithelial cells into mesenchymal cells by triggering EMT, and recent studies using gene-targeted mice have revealed that HA plays a central role in EMT as well (Camenisch et al., 2000); in one report, HAS-2 null mice showed severe

cardiac and vascular abnormalities, and died during midgestation due to a lack of transformation of cardiac endothelial cells to mesenchyme.

EMT was originally defined as a morphological conversion during normal development, but has recently gained attention as a central mechanism for carcinoma progression and metastasis (Huber et al., 2005). The progression of carcinoma cells to metastatic cells frequently involves an EMT-like epithelial change towards a migratory fibroblastic phenotype, particularly at the invasive front of tumors. During tumor progression, downregulation of E-cadherin, a hallmark of EMT, aids tumor cells in spreading. In one study, infection of recombinant HAS-2 adenoviral vectors converted normal Madin-Darby canine kidney and MCF-10A human mammary epithelial cells to mesenchyme as assessed by upregulation of vimentin, dispersion of cytokeratin, and loss of E-cadherin at intercellular boundaries (Zoltan-Jones et al., 2003). All of this suggests that increased HA production appears to be sufficient to induce EMT. Our recent observation using HAS-2 conditional Tg mice has also revealed that overproduction of HA in mammary carcinoma cells results in the suppression of E-cadherin expression and nuclear translocation of β-catenin, further providing evidence for HA-mediated promotion of EMT (Koyama et al., 2007). Carcinoma cells having undergone EMT may then participate in the formation of intratumoral stroma observed in HA overproducing tumors, such as those seen in human breast cancers (Petersen et al., 2003).

ROLES OF HA-RICH ECM IN TUMOR ANGIOGENESIS

Angiogenesis, the formation of new capillaries from preexisting vessels, is an absolute requirement for tumor growth and metastasis (Carmeliet, 2003) and is controlled by the aberrant production of angiogenic factors expressed by malignant tumor cells, host cells, or both. Among such factors, vascular endothelial growth factor (VEGF) has emerged as a central regulator. In addition, the local composition of the ECM surrounding the vasculature can affect angiogenesis either positively or negatively (Sottile, 2004), and HA oligosaccharides have been implicated in the promotion of angiogenesis (West et al., 1985). Studies in chick chorioallantoic membranes and rat skin have demonstrated that HA degradation products of specific size (3–10 disaccharide units) have the potential to induce neovascularization (Sattar et al., 1994; Slevin et al., 1998). Furthermore, HA oligosaccharides, together with angiogenic factors such as VEGF and basic fibroblast growth factor (bFGF), synergistically stimulate endothelial cell proliferation, migration, and capillary formation *in vitro*. The angiogenic activity of HA depends on its molecular mass; HMW native HA is antiangiogenic by inhibiting endothelial cell proliferation and migration and capillary formation in a three-dimensional matrix (Feinberg and Beebe,

1983). Because angiogenesis is the result of complex interactions between positive and negative regulators of angiogenesis (Slevin et al., 1998), the balance of regulatory HMW HA and effecter HA oligosaccharides may be important for controlling the angiogenic response.

HAS gene manipulation provides an opportunity to assess the role of HA during angiogenesis *in vivo*. The significance of HA has been highlighted in HAS-2 deficient mice having vascular defects, implicating a critical function of HA in embryonic vasculogenesis (Camenisch et al., 2000). HAS-2 Tg mouse models of breast cancer have also shown that overproduction of HA in tumor cells accelerates formation of intratumoral neovasculature (Koyama et al., 2007). This altered formation in genetically manipulated mice may be explained by the well-known fact that HA degradation products induce an angiogenic response. Indeed, HAS-2-overexpressing tumors contained significant amounts of small HA oligosaccharides, as assessed by gel filtration chromatography of tumor homogenates, whereas control tumors contained mostly HMW HA (Koyama et al., 2007). This supports the conventional notion that HA oligosaccharides influence tumor-induced angiogenesis. Although the physiological significance of HA oligosaccharides in the promotion of angiogenesis is well-established, it is still open to debate whether the ECM consisting of HMW HA and HA-binding molecules has any role in angiogenesis. Interestingly, in HAS-2-overexpressing mammary tumors, most neovasculature is predominantly found penetrating into the intratumoral stroma where HA is abundant as a constituent of ECM (Koyama et al., 2007). The constituents of stromal ECM therefore likely provide a supporting framework for easy penetration of endothelial cells and subsequent neovascularization.

Versican is abundant in both the perivascular elastic tissues of blood vessels and stromal ECM (Nara et al., 1997). In our recent study, administration of HA-versican aggregates, but not native HMW HA alone, promote the infiltration of endothelial cells within Matrigel plugs containing angiogenic bFGF (Koyama et al., 2007), suggesting the potency of HMW HA to accelerate angiogenesis in the presence of versican. Currently, one can only speculate as to the function of HA/versican aggregates in the context of angiogenesis (Fig. 10.4). Since HA/versican complexes can stimulate cell migration, their possible role would be one that enhances migration and invasion of endothelial cells. As an alternative explanation, the HA-rich matrix can be proposed to serve as a reservoir for various growth factors involved in vessel development; degradation of the matrix results in the release of various growth factors sequestered within, which in turn promotes an angiogenic response (Fig. 10.4). Further investigation is being conducted to clarify both the functional aspect of HA/versican aggregates in angiogenesis, as well as the relationship between such aggregates and HA oligosaccharides.

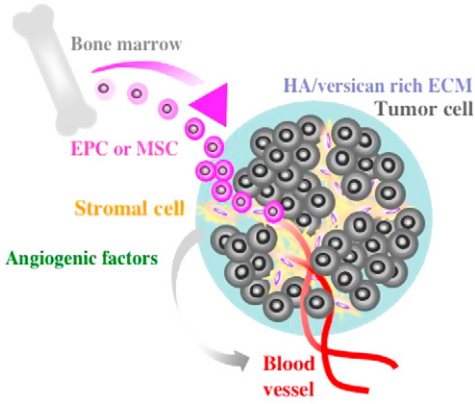

FIGURE 10.4 A model of stroma-induced angiogenesis.

Until recently, new blood vessels in adults were thought to grow exclusively through the sprouting of preexisting vasculature (Hillen and Griffioen, 2007). However, emerging evidence suggests that bone marrow-derived endothelial progenitor cells (EPCs) contribute to tissue vascularization during both embryonic and pathogenetic conditions; circulating bone marrow-derived EPCs are mobilized from the bone marrow and recruited to the foci of neovascularization where they form new *in situ* blood vessels through vasculogenesis. However, the homing process of EPCs remains unclear. Similarly to the recruitment of MSCs, HA-rich matrices may provide a stem cell niche for recruitment and retention of circulating EPCs (Fig. 10.4). In the future, a greater understanding of the mechanisms regulating selective cell movement and recruitment will lead to the development of novel anticancer therapeutic agents targeting reparative progenitor cells.

CONCLUSION AND PERSPECTIVES

This review focused on the role of HA in cancer progression with respect to its biosynthesis and function. A wealth of data has been accumulated on HA function in the promotion of malignancies, showing that enhanced cancer invasion and dissemination may be partly dependent on the mesenchymal conversion of cancer cells by HA overexpression. Furthermore, recent studies have enabled postulation of reliable mechanisms by which HA influences tumor growth and invasion by modulating the tumor microenvironment to recruit stromal cells and vasculature. Although the angiogenic function of HA oligosaccharides has been

well-established, the anti-angiogenicity of HMW HA being modulated by HA-binding molecules needs further clarification and study.

The roles of HA in cancer progression may differ according to the HAS isoforms expressed, meaning cancer cells at different stages may differentially utilize the three HAS isoforms to maximize their survival. Studies are now in progress to identify exactly which HAS proteins are associated with cancer progression. This will provide an opportunity to develop new strategies for cancer therapy targeting specific cancer-associated HAS species.

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