Hyaluronan Deficiency in Tumor Stroma Impairs Macrophage Trafficking and Tumor Neovascularization

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Abstract

Despite the importance of stromal cells in tumor progression, our overall understanding of the molecular signals that regulate the complex cellular interactions within tumor stroma is limited. Here, we provide multiple lines of evidence that tumor-associated macrophages (TAM) preferentially traffic to stromal areas formed within tumors in a manner dependent on a hyaluronal (HA)-rich tumor microenvironment. To address the role of stroma-derived HA in macrophage recruitment, we disrupted the HA synthase 2 (Has2) gene in stromal fibroblasts using conditional gene targeting. The Has2 null fibroblasts showed severe impairment in recruiting macrophages when inoculated with tumors into nude mice, which shows the contribution of stroma-derived HA in intratumoral macrophage mobilization. Furthermore, a deficiency in stromal HA attenuated tumor angiogenesis and lymphangiogenesis concomitantly with impaired macrophage recruitment. Taken together, our results suggest that stromal HA serves as a microenvironmental signal for the recruitment of TAMs, which are key regulatory cells involved in tumor neovascularization.

Introduction

Cancers are not autonomous cells that develop independently but rather are cellular masses formed through dynamic interactions with their microenvironment. As carcinomas evolve, the adjacent stroma undergoes profound alterations in composition to provide important support functions in the early stages of cancer establishment and later to facilitate tumor progression and extravasation (1–4). The tumor stroma is composed of various host cells, including endothelial cells, fibroblasts, and infiltrating inflammatory cells. Notably, tumor-infiltrating fibroblasts and macrophages, known as tumor-associated fibroblasts (TAF) and tumor-associated macrophages (TAM), respectively, have been increasingly implicated as active participants in tumor progression (5–7). Several lines of evidence have shown that these cells are exposed to activators within tumor stroma to commence de novo synthesis of a repertoire of growth factors and cytokines. Both TAFs and TAMs have the potential to release both angiogenic and lymphangiogenic growth factors and thereby enhance the formation of tumor vasculatures (8–12). There is therefore no doubt that stromal cells, via complex interactions with tumor cells, play a critical role in the promotion of tumor malignancy, but relatively little is known about the molecular basis regulating the cellular interactions within tumor stroma.

Inside tumor stroma is a dynamic microenvironment where extracellular matrix (ECM) is extensively remodeled during tumor development and progression (13–15). Hyaluronan (HA) plays a central role in tumor angiogenesis and lymphangiogenesis as a component of the ECM (16), and the increased HA deposition in the stromal compartments of tumors is often associated with tumor aggressiveness and adverse clinical outcome (17–19). The biosynthesis of HA, which is critical in establishing its biological function, is regulated by three mammalian HA synthases (Has): Has1, Has2, and Has3 (20). Emerging evidence has shown upregulation of Has gene expression in aggressive and metastatic tumors (21–24). Furthermore, our animal studies using a conditional transgenic mouse model allowing specific expression of Has2 in mammary tumors (25, 26) showed that HA production by tumor cells caused rapid development of...
aggressive breast carcinomas in association with marked stromal induction. Concurrent with stromal HA accumulation, considerable blood and lymphatic vasculatures had been formed within or near the stroma surrounding tumor cell islets. Increased stromal HA may therefore modulate tumor malignancy by controlling the complex cross-talk among host and tumor cells.

To define the roles of stromal HA in tumor malignancy, we developed Has2 conditional knockout mice and showed that this gene disruption in stromal fibroblasts attenuated trafficking of TAMs to tumor stroma. Along with the reduction of TAM recruitment, a deficiency in stromal HA also suppressed tumor angiogenesis and lymphangiogenesis. On the basis of our current findings, we postulate that stromal HA, via TAM recruitment, remodels the local microenvironment to promote the formation of tumor vasculatures.

Materials and Methods

Antibodies

The antibodies used in this study are listed in Supplementary Table S1.

Animals

The details of animals used in this study are described in Supplementary Materials and Methods.

Isolation of primary mammary tumor cells and TAFs

Primary mammary tumor cells and TAFs were established from mouse mammary tumors developed spontaneously in Has2 conditional transgenic mice as described previously (26). These cells were verified morphologically and by immunostaining for mesenchymal-specific vimentin and epithelial cell-specific cytokeratin. Both tumor cells and TAFs were subcultured and used within the nine passages.

Tumor transplantation

Mammary tumor cells derived from Has2ΔNeo, Has2ΔNeo, and nontransgenic mice were transplanted together with stromal fibroblasts into the mammary fat pads of nude mice (8-week-old female BALB/c nu/nu mice; SLC) as described previously (26). Control Has2ΔNeo tumor cells were also transplanted together with 100 μg of native HA or an equivalent amount of HA-versican aggregates into the mammary fat pads of mice (8-week-old female BALB/c nu/nu mice). Animals were sacrificed at an end point when either tumor size reached a mean diameter of 10 mm or on day 15 after the inoculation of tumor cells. Transplanted tumors were dissected out and weighed at that time.

Histologic and morphometric analyses

Excised tumors were immediately fixed in neutralized 10% formalin or in Tris-buffered zinc fixative, dehydrated, and embedded in paraffin wax. Deyparaffinized sections (5-μm thickness) were rehydrated and stained with Azan-Mallory staining. Immunofluorescence staining was carried out using one or more of the following primary antibodies: anti-F4/80 (diluted 1:500) for macrophages, anti-type I collagen (diluted 1:100) for stromal compartments, anti-CD31 (diluted 1:100) for blood vessels, and anti-podoplanin (diluted 1:200) for lymphatic vessels. Some sections were also stained with the biotinylated HA-binding region of aggrecan (b-HABP, Seikagaku Corp.) or with anti-versican antibody (diluted 1:200) to identify HA-rich ECM as described previously (25). Immunolocalization of the antigens was performed using Alexa Fluor-conjugated secondary antibodies under a Leica TCS SP2 AOBS confocal microscope (Leica Microsystems CMS GmbH). Images were exported in TIFF format and analyzed using the Metamorph software (Universal Imaging). The average positive cell density or area per objective field was calculated after immunohistochemical staining.

Preparation of tumor-infiltrating cells and peritoneal exudate macrophages

A single-cell suspension of tumor-infiltrating cells was obtained from tumor transplants as previously described (27). Peritoneal exudate cells (PEC) were obtained from green fluorescent protein (GFP) mice as described previously (28). Fluorescence-activated cell sorting (FACS) analysis revealed the isolated PECs to be 84% of F4/80+CD11b+ monocyte/macrophage lineage.

FACS analysis

Freshly prepared tumor-infiltrating cells were suspended in cold PBS supplemented with 1% fetal bovine serum. To minimize false positive staining, cell surface Fc receptors were blocked by incubation with 10 μg/mL FcBlock at 4°C for 5 minutes before antibody staining. Tumor-infiltrating cells (1 × 10⁶ per sample) were incubated with anti-F4/80 PE-Cy5 and anti-CD206 Alexa Fluor 488 at 4°C for 30 minutes. A total of 100,000 viable cells were analyzed by FACScaliber (BD Bioscience) with CellQuest software version 5.1. Gates were determined with the use of appropriate isotype controls. Results are given as the positive percentage minus background from appropriate isotype controls.

Selective depletion of macrophages using clodronate liposomes

Clodronate liposomes, termed clodrolip, were prepared as described previously (29). Clodrolip was transplanted into the mammary fat pads of nude mice the day before tumor cell injection. Has2ΔNeo or Has2ΔNeo tumor cell suspension was then injected into the mammary fat pads of nude mice as described above. The mice received additional clodrolip every 5 days during the experimental period (30). Control groups received liposomes containing PBS (PBSlip) at the same time points. The mice were sacrificed on day 15 after the inoculation of tumor cells, and excised tumor transplants were fixed and subjected to histologic examination.

Macrophage adhesion assay

Quantification of macrophage adhesion to fibroblasts was done using PECs isolated from GFP mice as described
previously (31). Fluorescence intensity was measured using a CytoFluor Multiwell Plate Reader Series 4000 (Applied Biosystems) with an excitation of 485 nm and an emission of 530 nm. The number of adherent cells was calculated using a standard curve, and adhesion index was expressed as a percentage of total inoculated cells. All measurements were carried out in triplicate.

**Statistical analysis**

Statistical analysis was performed using the two-tailed Student’s t test. All results were expressed as mean ± SD. A P value of <0.05 was considered to be statistically significant.

**Results**

**TAMs preferentially traffic to stromal compartments formed within HA-producing tumors**

To define the molecular basis of the complex cellular interactions within tumor stroma, we first focused our investigation on the effect of a HA-rich tumor microenvironment on the recruitment and function of TAMs. For this purpose, we used a conditional transgenic mouse model allowing specific expression of Has2 in mammary tumors, where Has2ΔNeo transgenic mice actively produce HA in spontaneous mammary tumors by specifically expressing exogenous Has2, whereas control Has2ΔNeo mice, with a silent transgene cassette, do not (25, 26). HA overproduction resulted in marked stromal induction and HA accumulation within the stromal compartments of the aggressive tumors (Fig. 1A, top). Concurrent with stromal HA accumulation, considerable blood and lymphatic vasculature had formed within or near the stroma surrounding tumor cell islets (Fig. 1A, top).

Mammary tumor tissues from Has2ΔNeo and control Has2ΔNeo transgenic mice were initially evaluated by immunostaining with the pan-macrophage marker F4/80. F4/80-positive (F4/80+) macrophages were far more numerous in the stroma of Has2ΔNeo tumors compared with control Has2ΔNeo tumors (Fig. 1A, top), and quantitative analysis showed that the average density of infiltrating F4/80+ macrophages was 7-fold greater in HA-producing tumors than in controls (Fig. 1B, left). Notably, macrophages were seen to preferentially traffic to HA-enriched and type I collagen–enriched stromal structures in spontaneous Has2ΔNeo mammary tumors (Fig. 1A, top), which is strongly suggestive of the stromal involvement in macrophage recruitment. To specify the participants responsible for macrophage recruitment, we established primary tumor cells from Has2ΔNeo and control Has2ΔNeo mice and transplanted them with or without TAFs into the mammary fat pads of nude mice. When Has2ΔNeo tumor cells were inoculated with TAFs, F4/80+ macrophages trafficked to tumor transplants as they did in the spontaneous mammary tumors (Fig. 1A, bottom and B, right). In spite of single inoculation, the macrophages trafficked to a similar degree to stromal structures in Has2ΔNeo tumors, which may have been due to their high capability of stromal induction. On the other hand, Has2ΔNeo tumors induced much less macrophage mobilization (Fig. 1A, bottom and B, right). With TAFs, however, the number of infiltrating macrophages was markedly increased, suggesting a critical contribution of TAFs to intratumoral macrophage mobilization.

TAMs, which have little cytotoxicity against tumor cells and can actually promote tumor aggressiveness, share a phenotype similar to that of immunosuppressive M2 macrophages (32–34). To evaluate TAM infiltration into tumor stroma, we examined the M2 macrophage population in both Has2ΔNeo and Has2ΔNeo tumors. FACS analysis revealed that F4/80+CD206+ M2 macrophages were more abundant in Has2ΔNeo tumors than in Has2ΔNeo tumors (Fig. 1C and D). We further examined the mobilization of F4/80+ total and F4/80+CD206+ M2 macrophages using tumor cells from non-transgenic mice and found that the control experiment gave similar results to those of Has2ΔNeo tumor cells (Fig. 1D).

**Selective macrophage depletion reduces tumor angiogenesis and lymphangiogenesis in HA-producing tumors**

Recent clinical and experimental studies have indicated that TAMs promote malignant tumor progression through their proangiogenic activity (10, 11), so we explored whether TAMs are responsible for angiogenic promotion in HA-producing tumors. Because liposome-encapsulated clodrolip (clodrolip) is commonly used for selective macrophage depletion (29, 30), we conducted experiments in which local TAMs were depleted by peritumoral administration of clodrolip. HA-producing Has2ΔNeo tumor cells were inoculated into the mammary fat pads of nude mice and then evaluated for tumor growth after macrophage depletion. Clodrolip treatment significantly suppressed tumor growth and caused a significant reduction in tumor dry weight compared with PBSlip treatment (Fig. 2A), suggesting a pivotal role of macrophages in growth acceleration of HA-producing tumors. The degree of macrophage depletion was verified by F4/80 staining of transplanted tumors (Fig. 2B and C), showing successful elimination of local TAMs in clodrolip-treated tumors.

To investigate whether macrophage depletion had an effect on neovascularization, tumor-associated vasculatures were visualized by immunostaining with an antibody to endothelial cell–specific CD31. In agreement with a previous study (25), the blood vessels in HA-producing Has2ΔNeo tumors were mostly small and fragmented (Fig. 2B). CD31-positive blood vessels were significantly decreased by clodrolip treatment (Fig. 2C). Macrophages in tumor stroma have been suggested to be the major cellular sources of lymphangiogenic growth factors and participate in the promotion of tumor lymphangiogenesis (8), and our previous study also suggested that tumor lymphangiogenesis was closely associated with the formation of tumor stroma in HA-producing tumors (26). Quantitative evaluation of podoplanin-positive areas showed a significant reduction in lymphatic vessels in clodrolip-treated tumors (Fig. 2B and C).

To further investigate the link between HA and macrophage mobilization and neovascularization, a similar experiment was performed using Has2ΔNeo tumor cells. Clodrolip treatment suppressed macrophage mobilization and vascular...
formation in the transplanted tumors but to a lesser extent than those of HA-overproducing Has2ΔNeo tumors (Fig. 2C), suggesting that HA works synergistically with macrophage recruitment to promote vascular formation. It was also considered that clodrolip treatment may suppress macrophage mobilization and subsequent tumor neovascularization by decreasing HA production. To clarify this possibility, we measured the HA content in Has2ΔNeo tumors and found a significant reduction after clodrolip treatment (Fig. 2A). HA distribution was also examined with biotinylated HABP (b-HABP), a specific probe for HA. HA staining was detected at the intercellular boundaries of tumor cells and was prominent in the tumor stroma of Has2ΔNeo tumors (Fig. 2B). Interestingly, HA accumulation had disappeared from the stromal compartments after clodrolip treatment, suggesting that macrophages are crucial for the maintenance of HA-rich stromal structures. On the other hand, macrophages preferentially trafficked to HA-rich stromal compartments (Fig. 1A), which may indicate that a paracrine loop between macrophage mobilization and accumulation of stromal HA is required for sustained expansion of tumor stroma and vasculatures.

**Generation of Has1 and Has2 double-knockout mice and establishment of Has-deficient stromal fibroblasts**

To define the relative contribution of stromal HA, we explored whether the absence of HA production in stromal fibroblasts, achieved by deleting the Has genes, would abrogate macrophage mobilization and subsequent tumor neovascularization. Our previous report has shown that, of the three Has genes, Has1 and Has2, but not Has3, are endogenously expressed in stromal fibroblasts (26). To ablate the HA synthesis of stromal fibroblasts, Has1 and Has2 double-knockout mice were obtained by intercrossing Has1-null and Has2 conditional knockout mice, and stromal fibroblast cells were established from founder lineages having a different combination of the two knockout alleles (Supplementary Figs. S1 and S2). Genomic PCR verified that a part of exon 5 of the Has1 locus was replaced by a neomycin-resistant gene in Has1-deficient fibroblasts (Supplementary Fig. S2A). To selectively suppress HA synthesis in stromal fibroblasts, the Has2 floxed allele was excised in vitro by infecting Has2ΔNeo fibroblasts with a recombinant adenovirus carrying the Cre recombinase gene. As a result, exon 2 of the Has2 locus was effectively deleted by Cre-mediated recombination in Has2ΔNeo/ΔNeo stromal fibroblasts (Supplementary Fig. S2A). In contrast, an intact floxed allele remained in Has2ΔNeo/ΔNeo fibroblasts after infection of a control virus carrying the LacZ gene. Real-time quantitative reverse transcription-PCR confirmed that the expression of intact Has1 transcripts was lost in Has1-deficient fibroblasts and that Has2 expression was greatly diminished by Cre-mediated recombination in Has2ΔNeo/ΔNeo fibroblasts (Supplementary Fig. S2B). Deletion of a single allele, however, did not change Has2 expression, suggesting the possibility that Has2 gene expression is compensated for by an unknown mechanism to maintain a constant level. We then compared HA content in the conditioned medium of stromal fibroblasts and found that deletion of Has1 gene did not affect HA synthesis in stromal fibroblasts (Supplementary Fig. S2B). In contrast, Cre-mediated recombination of Has2 locus induced an almost complete failure in HA synthesis. The current results, together with those of our previous study showing that Has2 is more catalytically stable than Has1 (35), suggest that Has2 accounts for most of the HA production from stromal fibroblasts. HA staining revealed that no cable-like ECM structures of HA could be detected in Has2-deficient (Has2Δ/Δ) fibroblasts, but such structures could be seen in wild-type and Has2Δ/Δ fibroblasts (Supplementary Fig. S2C).

**A deficiency in stromal HA impairs macrophage trafficking**

To test whether a deficiency in stromal HA influences macrophage trafficking, Has2ΔNeo tumor cells were inoculated together with Has-deficient stromal fibroblasts into the mammary fat pads of nude mice. The generated transplants were then processed for FACS analysis, which showed that F4/80+ macrophages trafficked as expected to the tumor transplants generated by coinoculation of Has2ΔNeo tumor cells and Has2Δ/Δ fibroblasts (termed Has2Δ/Δ tumors; Fig. 3A and B). Heterozygous mutation of the Has2 allele (Has21/2) had a similar effect on macrophage trafficking. However, a significantly lower number of macrophages was recorded in tumor transplants composed of Has2ΔNeo tumor cells and Has2Δ/Δ fibroblasts (termed Has2Δ/Δ tumors). A decrease in the M2 population was also seen in Has2Δ/Δ tumors.
tumors (Fig. 3A and B), which was supportive of the pivotal role of Has2-derived stromal HA in intratumoral TAM mobilization. As the influence of a Has1 deficiency was marginal on macrophage trafficking (data not shown), Has1 null fibroblasts having different combinations of Has2 knockout alleles were used for further experiments.

We next sought to address exactly how the absence of HA synthesis in stromal fibroblasts attenuated macrophage recruitment.
trafficking. Because recent studies have suggested that cable-like HA structures are primarily responsible for the binding of mononuclear leukocytes (31, 36), stromal HA might mobilize macrophages into tumors through direct interaction. We thus examined macrophage adhesion to Has2-deficient fibroblasts to verify the role of stromal HA in macrophage trafficking. When PECs were seeded onto monolayers of stromal fibroblasts having different combinations of the Has2 knockout alleles, the adhesion rate of PECs was almost 2-fold less to Has2Δ/Δ fibroblast monolayers compared with that of Has2flox/flox fibroblasts (Fig. 3C), which was identical to the results obtained from the in vivo transplant study.

A deficiency in stromal HA attenuated tumor angiogenesis and lymphangiogenesis

We next investigated whether an HA deficiency in stroma had an effect on tumor neovascularization. In accordance with the massive formation of HA-rich stroma, Has2flox/flox fibroblasts gave rise to highly vascularized tumors when coinoculated with Has2ΔNeo tumor cells (Fig. 4A). In morphometric analyses of CD31-positive blood vessels, the mean vessel density was over 20 vessels per field in Has2flox/flox tumors (Fig. 4B). Conversely, CD31-positive microvessels were significantly fewer in Has2Δ/Δ tumors than in control Has2flox/flox tumors. Immunofluorescence staining of podoplanin was

Figure 2. Macrophage depletion affects tumor growth and neovascularization. A, macroscopic appearance and growth curve of Has2ΔNeo tumors treated with clodrolip and control PBSlip. Clodrolip and PBSlip were given the day before the injection of Has2ΔNeo tumor cells and every 5 d thereafter. Animals were sacrificed at the end point, and the transplanted tumors were excised and evaluated for tumor growth, tumor weight, and HA content. Scale bar, 10 mm. Graphs represent the mean ± SD (PBSlip, n = 6; clodrolip, n = 8). *, P < 0.05; **, P < 0.01. B, photomicrographs of immunofluorescence images. Top, sections from tumors treated with clodrolip were immunostained with anti-F4/80 antibody (green) to identify infiltrating macrophages. Middle, blood (arrowheads) and lymphatic vessels were visualized by immunostaining with anti-CD31 antibody (green) and anti-podoplanin antibody (red), respectively. Bottom, the distribution of HA was evaluated by staining with b-HABP (red). Cell nuclei were counterstained with DAPI (blue). Scale bars, 40 μm. C, quantitative analysis of macrophage density and morphometric analyses of blood and lymphatic vessels. Has2ΔNeo or Has2Δ/Δ tumor cells were transplanted into nude mice, and the animals were treated with clodrolip (white columns) or control PBSlip (black columns). Graphs represent the mean ± SD from more than six tumors per experimental group (at least three sections and five fields were counted for each tumor). **, P < 0.01.
also carried out on tumor transplants, where attenuated expression of podoplanin in Has2Δ/Δ tumors confirmed that a Has2 deficiency resulted in impaired tumor lymphangiogenesis (Fig. 4A and B).

The cooperative effect of HA and versican on macrophage trafficking

Because a wide variety of HA-binding molecules contribute to the assembly of pericellular HA-ECM and tightly regulate HA functions, we hypothesized that such molecules, when produced by stromal fibroblasts, can modulate stromal HA to enhance macrophage recruitment and subsequent neovascularization. Versican, a member of the HA-binding chondroitin sulfate proteoglycan family, is important for the stabilization of pericellular HA coats. Consistent with the previous observation that versican is enriched in stromal compartments (25), we found that this molecule was produced mainly by TAFs and not by tumor cells (Fig. 5A). As shown in Fig. 5B, a deficiency in HA synthesis resulted in the destruction of HA-rich and versican-rich pericellular ECM, and only small pieces of versican cables were detected on the surface of Has2-deficient stromal fibroblasts despite versican levels being unaffected by an HA deficiency (data not shown). The deformed cable-like structures were partially restored by the addition of exogenous HMW-HA (Fig. 5B).

In the final set of experiments, we investigated whether versican exerted cooperative effects with HMW-HA in terms of potentiation of macrophage trafficking. Has2ΔNeo tumor cells were implanted together with exogenous HMW-HA, versican, or their aggregates into the mammary fat pads of nude mice. Administration of HA or versican had little effect on tumor growth or the promotion of macrophage recruitment in the transplanted model (Fig. 5C and D). In contrast, HA-versican aggregates accelerated the mobilization of both F4/80+ total and F4/80+CD206+ M2 macrophages as well as tumor growth (Fig. 5C and D). Histologic staining of HA and versican showed that these were prominent in the tumor stroma and were accompanied with macrophages.
Together with our previous notion that HA-versican aggregates accelerate angiogenesis and lymphangiogenesis (25, 26), all of the above data provide evidence that a HA-rich stromal microenvironment is crucial for TAM trafficking and subsequent tumor neovascularization.

Discussion

The present work on the effect of stromal HA on tumor progression has helped to identify the molecular basis of TAM mobilization toward stromal compartments within malignant tumors. To our knowledge, this is the first report showing the HA-dependent interactions between TAMs and stromal fibroblasts to enhance tumor neovascularization by modulation of the local stromal microenvironment.

HA accumulation varies considerably depending on its origin and the histologic type of the cancer. Clinically, increased deposition of HA in stromal compartments within tumors often correlates with tumor aggressiveness and adverse clinical outcome (17–19), and HA has been frequently observed at elevated levels around actively migrating and proliferating tumor cells (37, 38). For these reasons, clarifying the functional aspects of HA on tumor cell surfaces and in the stroma constitutes an indispensable step toward our overall understanding of the roles of a HA-rich tumor microenvironment in tumor progression. Our knockout experiments clearly showed that HA species released by stromal fibroblasts participate in TAM trafficking into tumor masses, thereby boosting tumor neovascularization. Because a wide variety of HA binding molecules contribute to the assembly of pericellular HA-ECM and tightly regulate HA functions (39), the diverging properties of tumoral and stromal HA species may reflect the wide spectrum of molecules constituting HA-ECM. In the present study, we showed that HA and versican derived from stromal fibroblasts promoted macrophage mobilization in a cooperative fashion. Although our work did not fully define the functional diversity of tumoral and stromal HA species, the presence of versican may account for the specific action of stromal HA.

HA is an important ligand for leukocyte recruitment and retention (40, 41), and recent studies suggest that cable-like HA structures are primarily responsible for the binding of mononuclear leukocytes (31, 36). Accordingly, the HA-rich tumor microenvironment may serve as a suitable scaffold for...
macrophage trafficking. The current study suggests at least a partial involvement of HA-enriched and versican-enriched peri-cellular ECM in macrophage recruitment. Consistent with our observations, Potter-Perigo and colleagues have also recently reported an important role of versican in the HA-dependent binding of monocytes to the ECM of lung fibroblasts stimulated with poly IC (42). Additionally, Kim and colleagues showed that versican activates macrophages through the activation of the Toll-like receptor (TLR) family member TLR2 (43). Thus, stromal HA may indirectly activate and recruit TAMs by
versican-1TLR2 interactions, as well as through direct interaction with HA receptors.

In conclusion, our results defined the molecular basis for TAM recruitment and provided evidence that stromal HA, via macrophage recruitment, remodels the local microenvironment to promote the formation of tumor vasculatures. A more detailed understanding of the mechanisms regulating cellular interactions within the HA-rich tumor microenvironment will further contribute to the development of novel therapeutic strategies for the prevention of tumor neovascularization.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank David Gordon for his generous gift of the p-loxp-P2RT-PGKneo clone, Izumu Saito (Tokyo University) for providing the AsCANCRe adenovirus, Jun-ichi Miyazaki (Osaka University) for the CAG promoter, Andrew P. Spicer for his help with the Has1 knockout study, and our laboratory members for their technical support and many helpful discussions.

Grant Support

KAKENHI (grants-in-aid for scientific research) from the Ministry of Education, Culture, Sports, Science and Technology of Japan and a grant from Mitsuoka Foundation for Glycoscience (N. Itano).

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Received 12/24/2009; revised 07/02/2010; accepted 07/14/2010; published OnlineFirst 09/07/2010.

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Cancer Res 2010;70:7073-7083. Published OnlineFirst September 7, 2010.