(Figure 2B). Next, to visualize lymphatic function, Evans blue dye was injected intradermally into mouse ear. Lymph leakage was found in the whole ear of UVB-irradiated WT mice at 5 and 10 minutes after dye injection as previously described,[8] whereas lymphatic vessels of UVB-exposed K14-Ang1 mice were still visible at 5 and 10 minutes after dye injection, indicating that the increase of lymphatic capillary permeability was attenuated in K14-Ang1 mice. Moreover, at 1 minute after dye injection, dye spots were found close to the branching points of lymphatic vessels only in UVB-irradiated WT mice, suggesting the presence of abnormal and leaky collecting vessels (Figure 2C). In contrast, in non-UV, there was no significant difference between WT mice and K14-Ang1 in terms of lymph leakage at 5 minutes after dye injection, however, at 10 minutes, K14-Ang1 mouse ears showed inhibited lymph leakage as compared with WT mice.

Double-immunofluorescence analysis using antibodies for blood vessel–specific antigen, panendothelial antigen-1,[21] and a lymphatic specific marker, LYVE-1, was performed. UVB induced marked enlargement of LYVE-1–positive lymphatic vessels in WT mice as compared with ear skin not exposed to UVB (Figure 3, A and C), whereas in K14-Ang1 mice, the enlargement of lymphatic vessels and blood vessels after UVB was attenuated, as compared with WT. In contrast, the density of lymphatic vessels was increased in K14-Ang1 mice compared with WT mice (Figure 3, B and D). Morphometric analysis of lymphatic vessels using IP-LAB software showed an increase in the average size of the inflamed lymphatic vessels of WT mice as compared with the vessels of nonirradiated WT mice, although no significant difference was found in the vessel density. As we had expected, the average size of lymphatic vessels was decreased in K14-Ang1 mice after UVB irradiation. In contrast, the density of lymphatic vessels was increased in K14-Ang1 mice, as compared with WT mice (Figure 3, E and F). To analyze if the increased area of lymphatic vessels in K14-Ang1 mice resulted from the presence of more lymphatic endothelial cells, double-immunofluorescence analysis for podoplanin and a proliferation marker, Ki-67, was performed. The results showed that the number of Ki-67–positive cells was increased in K14-Ang1 mice as compared with WT mice (1.75 ± 0.96 cells/slide in K14-Ang1 mice and 0.25 ± 0.5 cells/slide in WT mice; $P = 0.016$) (Figure 3, G and H). Under physiological conditions, the size and density of blood vessels were increased in K14-Ang1 mice, as previously described (Figure 3, A and B).[14] After UVB irradiation, a relatively small change of blood vessel size was found in K14-Ang1 mice as compared with WT mice. The vessel density was comparable in skin exposed and not exposed to UVB (Figure 3, I and J).

Enhanced Lymphatic Integrity in Inflamed Ears of K14-Ang1 Mice

We previously found that the enlarged lymphatic vessels induced by UVB irradiation are leaky and hyperpermeable, suggesting that lymphatic function is impaired.[8] Therefore, we hypothesized that altered expression pattern of tight junction molecules might be associated with the leakiness of inflamed lymphatic vessels. To test this idea, we performed whole-mount staining for claudin-5 and/or podoplanin/CD31 in ears of animals exposed or not exposed to UVB irradiation. Interestingly, UVB irradiation led to the loss of claudin-5 protein, which was localized exclusively to cell–cell junctions at the tips of lymphatic capillaries without UVB, whereas claudin-5 expression already was redistributed at cell–cell junctions in UVB-irradiated K14-Ang1 mice (Figure 4A). Confocal

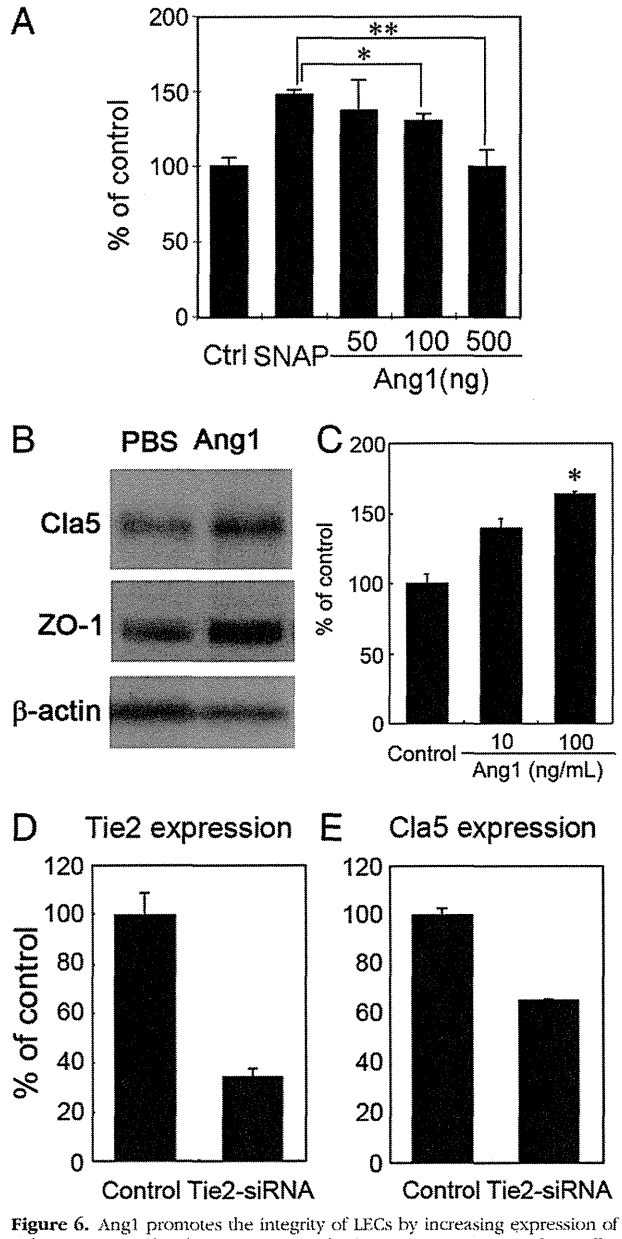

Figure 6. Ang1 promotes the integrity of LECs by increasing expression of tight junction molecules. **A:** Nitric oxide donor S-nitroso-N-acetylpenicillamine (SNAP) increased the lymphatic permeability, whereas co-incubation of LECs with Ang1 and SNAP dose-dependently inhibited the nitric oxide–induced hyperpermeability of LECs. **B:** Treatment with Ang1 for 4 hours markedly increased the levels of tight junction molecules claudin-5 and ZO-1. β-Actin is shown as a loading control. **C:** Claudin-5 expression was dose-dependently increased in the presence of Ang1. (**D** and **E**) LECs transfected with Tie2-siRNA showed decreased expression of Tie2 (**D**) and claudin-5 (**E**). At least three different experiments were performed for each assay to calculate P values. $^{*}P < 0.05$, $^{**}P < 0.01$.

microscopy revealed that claudin-5 protein was present in the cellular membrane of collecting lymphatic vessels in skin, whereas its expression was diminished in inflamed skin (Figure 4B). These data suggested that the lymphatic capillaries and collecting vessels became leaky after UVB irradiation because of loss of claudin-5.

Next, to determine the expression pattern of the lymphatics in K14-Ang1 mice, immunofluorescence analyses using antibodies for the endothelial tight junction molecule claudin-5 and lymphatic vessels were performed. Double-immunofluorescence analysis using antibodies against podoplanin and claudin-5 also confirmed that UVB irradiation resulted in the loss of claudin-5 expression at cellular membranes of lymphatic vessels in WT mice. Moreover, loss of claudin-5 in lymphatics of UVB-irradiated WT mice (Figure 4D), already was redistributed in cell–cell junctions of K14-Ang1 mice (Figure 4E). Another tight junction molecule, ZO-1, also was expressed in podoplanin-positive lymphatic vessels. Double-immunofluorescence staining for podoplanin and ZO-1 showed that ZO-1 expression in lymphatic vessels was lost in the UVB-irradiated skin of WT mice, and the level was markedly less than that in UVB-exposed K14-Ang1 mice. These data indicate that the lymphatic integrity of inflamed skin of UVB-exposed K14-Ang1 mice was enhanced, as compared with WT.

Tie2 Activation by Ang1 Induces Cell Migration and Cord Formation in Vitro

To examine the role of Tie2 signaling in LECs, immunoblot analysis was performed after immunoprecipitation of Tie2. In the presence of Ang1, pronounced Tie2 phosphorylation was found as compared with the control cells, although immunoblot analysis of Tie2 protein confirmed equal loading of protein (Figure 5A). Next, we investigated the effect of Ang1 on LECs. Treatment of LECs with Ang1 dose-dependently induced migration (Figure 5B). The effect of VEGF-A also was assessed as a positive control. Further, Ang1 dose-dependently promoted cord formation of LECs. Our results are consistent with a previous finding of Tie1 phosphorylation of LECs in the presence of Ang1.

To determine which pathway is critical in the mediation of Ang1 signaling of LECs, the Tie2 or Tie1 receptor was specifically knocked down by the transfection of silencer RNAs. We confirmed specific knockdown of Tie1 or Tie2 expression by immunoblotting (Figure 5C). In addition, a cord-formation assay of these cells was performed. Ang1 promoted cord formation of Tie1-knockdown cells as well as control siRNA-transfected cells, whereas in cells transfected with siRNA of Tie2, the effect of Ang1 was reduced, showing the importance of Ang1/Tie2 signaling in LECs (Figure 5, D–G).

Ang1 Enhances Lymphatic Integrity by Increasing Levels of Tight Junction Molecules in Vitro

In vivo, K14-Ang1 mice showed increased levels of tight junction molecules in inflamed skin. To analyze the contribution of Ang1/Tie2 signaling to lymphatic integrity, a Transwell permeability assay was performed in the presence or absence of Ang1 together with a nitric oxide donor, S-nitroso-N-acetylpenicillamine.[20] Ang1 dose-dependently inhibited the lymphatic permeability (Figure 6A). Furthermore, we detected increased expression of claudin-5 as well as ZO-1 after the addition of Ang1, whereas β-actin expression was similar in the two groups (Figure 6B). Moreover, Ang1 treatment of LECs dose-dependently enhanced the expression of claudin-5 (Figure 6C), suggesting that Ang1 induces an increase of lymphatic integrity by promoting expression of tight junction molecules. In contrast, the transfection of LECs with siRNA-Tie2 decreased claudin-5 expression (Figure 6, D and E). These data indicate that Ang1/Tie2 signaling influences lymphatic permeability by modulating expression of tight junction molecules.

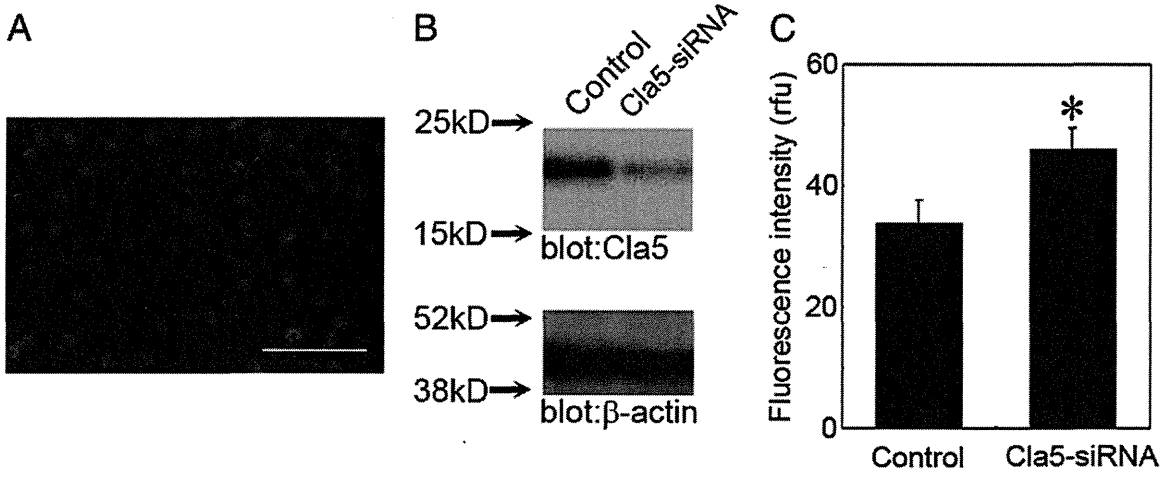

Figure 7. Claudin-5 regulates lymphatic integrity *in vitro*. **A:** Immunofluorescence of LECs for claudin-5 (red) confirmed the expression of claudin-5 on the cellular membrane of LECs. **B:** Western blot analyses revealed a significant decrease of claudin-5 protein in the presence of claudin-5–specific siRNA, whereas β-actin protein levels were comparable in LECs transfected with scramble-siRNA and with claudin-5 siRNA. **C:** Claudin-5 siRNA transfection resulted in increased permeability of LECs *in vitro*. Three different experiments were performed for each assay. $^*P < 0.001$. Scale bar = 100 μm.

Claudin-5 Influences Lymphatic Permeability

Claudin-5 expression was increased in the cellular membrane of lymphatic endothelial cells *in vitro* (Figure 7A). To elucidate claudin-5 function in LECs, claudin-5 expression was knocked down with claudin-5 siRNA; β-actin expression remained similar in the control and claudin-5 siRNA transfectants (Figure 7B). Claudin-5 knockdown resulted in increased permeability of LECs *in vitro* (Figure 7C).

Discussion

Complex phenomena occur during skin inflammation, including epidermal hyperplasia, erythema, edema formation, vessel dilation, and infiltration of inflammatory cells.[1] It also has been shown that lymphatic function actively participates in the resolution of inflammation by modulating lymphangiogenic factors, VEGF-A and VEGF-C/D, secreted from macrophages.[4] In our skin inflammation model, the subcutaneous delivery of VEGF-C attenuated skin inflammation by promoting lymphangiogenesis.[6] In contrast, VEGF-A up-regulation in keratinocytes triggered lymphatic impairment, whereas systemic blockade of VEGF-A attenuated skin inflammation by inhibiting the enlargement of lymphatic vessels.[8] These data indicate that lymphangiogenic factors could play distinct roles in inflammation resolution.

Our results showed that activation of Ang1/Tie2 signaling attenuated inflammation by promoting lymphatic integrity, as well as inhibiting blood vascular hyperpermeability in inflamed tissue. Blood vessels of K14-Ang1 mice were shown to be resistant to leakage induced by an inflammatory stimulus.[22] Therefore, the effect of Ang1 on blood vessels may contribute to the attenuation of inflammation in K14-Ang1 mice. Recently, Ang1 was shown to promote lymphatic formation and hyperplasia.[9,10] We also confirmed increased lymphatic density in the skin of K14-Ang1 mice as compared with WT mice under physiological conditions. Interestingly, the enlargement of lymphatic vessels in inflamed skin of UVB-exposed K14–Ang1 mice was reduced, compared with UVB-exposed WT mice. Furthermore, intravital lymphangiography by injection of Evans blue dye into the ear of UVB-exposed K14-Ang1 mice indicated that the increase of lymphatic permeability also is reduced in these mice. Unlike VEGF-C, Ang1 had no effect on proliferation of lymphatic endothelial cells *in vitro* (data not shown), although Ang1 dose-dependently promoted cell migration and cord formation. Taken together, these results indicate that, in addition to the well-known effect of Ang1 on blood vessels in inflammation,[23] Ang1 has a distinctive functional role in lymphatic vessels of inflamed tissue, serving to modulate lymphatic integrity.

How is lymphatic integrity regulated in inflammation? Lymphatic hyperplasia, together with lymphatic enlargement, is found in several models of inflammation.[3,12] Surprisingly, loss of the tight junction molecule claudin-5 was found in inflamed lymphatic capillaries, as well as collecting vessels. In inflamed skin, lymphatic vessels could be pulled open by anchoring filaments that connect lymphatic endothelial cells with elastic fibers in the extracellular matrix, presumably to wash out increased interstitial tissue fluid resulting from the increased vascular permeability.[24] However, overextension of lymphatic endothelial cells also can cause edema formation.[25] Our results showed that overextension of lymphatic endothelial cells in capillaries as well as collecting vessels was caused by loss of tight junction molecules, claudin-5 and ZO-1 at cell–cell junctions, resulting in lymphatic impairment and prolonged edema and inflammation.

Recent results have indicated that VEGF-A induces disruption of claudin-5 in the blood-brain barrier.[26] Further, up-regulation of VEGF-A was detected in keratinocytes after UVB exposure *in vitro* as well as *in vivo*, and VEGF-A was identified as a mediator of skin inflammation after UVB exposure.[15,27] It would be of interest to see if VEGF-A up-regulation after UVB also mediates decreased expression of tight junction proteins in lymphatic vessels, followed by lymphatic impairment in inflamed skin. Furthermore, we found that activation of Ang1/Tie2 signaling increased the TJPs *in vivo* and *in vitro*. Adrenomedullin has been shown to control murine lymphatic development by stabilizing the lymphatic endothelial barrier,[28,29] and its receptor-modulator, receptor activity modifying protein–2 (RAMP2), modulated vascular integrity in mice.[30] We also confirmed that an endothelial-specific TJP, claudin-5, is a key modulator of the lymphatic endothelial barrier *in vitro*. However, in contrast to adrenomedullin, no obvious change of TJPs was found in K14-Ang1 mice under physiological conditions *in vivo*, whereas inflamed lymphatic vessels showed TJPs at cell–cell junctions *in vivo*, suggesting a distinct role of Ang1/Tie2 signaling in maintaining the lymphatic integrity of inflamed tissue.

In conclusion, we have identified a regulatory pathway that serves to maintain lymphatic integrity during inflammation by controlling TJP components. Ang1, or small molecules that directly activate Tie2, may have potential for the treatment of lymphatic dysfunction during inflammation.

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Guest editorial: mutual relationship between vascular biology and hematology

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Vascular biology has rapidly progressed in a molecular level since the identification of growth factors regulating blood vessel formation in the mid-1990s. In contrast to hematology, in which cytokines involved in the development and proliferation of hematopoietic cells (HCs) were first identified and analyzed in the 1980s, vascular biology has a short history, as analysis of the mechanisms underlying blood vessel formation were started at the molecular level. Inhibitors of vascular endothelial growth factors (VEGFs) or their cognate receptors have, however, already entered clinical use in the treatment of cancer and retinopathy. Moreover, therapeutic angiogenesis by such methods as gene transfer, bone marrow cell injection, and cytokine administration has also entered clinical use. It is no exaggeration to say that translation from bench to bed side has proceeded extremely rapidly in vascular biology.

Recognition of the intimate interaction between hematopoiesis and blood vessel formation emerged from histological analyses showing that hematopoietic cells and vascular endothelial cells (ECs) originate from a common ancestor, known as the hemangioblast. However, several lines of evidence suggest that hematopoietic cells are derived from cells which have already committed to ECs, so-called hemogenic angioblasts, during embryogenesis. In the adult, however, bone marrow hematopoietic cells can differentiate into vascular cells, such as ECs and vascular smooth muscle-like cells. Clearly, these two populations follow complex developmental routes. Moreover, functionally, hematopoietic cells support angiogenesis as an accessory cell component, and, conversely, blood vessels provide a niche for the maintenance of the stemness of hematopoietic stem cells.

Clinically, bone marrow hematopoietic cell infusion therapy to induce angiogenesis for ischemic diseases, such as chronic lower extremity occlusive disease or ischemic heart disease, is one example of the utilization of the intimate interaction between hematopoiesis and vascular development. Usage of the hematopoiesis-related cytokine, G-CSF, in the mobilization of bone marrow hematopoietic stem/progenitor cells into peripheral blood to facilitate the recruitment of such cells to ischemic regions is one example of a strategy that brings together the fields of hematology and vascular biology.

In this PIH review series, a number of research approaches linking hematopoiesis and vascular biology are introduced. Dr. Beate Heissig overviews the mechanism of fibrinolysis for bone marrow cell mobilization associated with induction of angiogenesis, while Dr. Hideto Matsui discusses a strategy for the treatment of congenital coagulation defects using gene transfer into bone marrow endothelial progenitors. It is widely accepted that suppression of angiogenesis is a promising method for inhibiting tumor growth. By contrast, Dr. Yusuke Mizukami argues that induction of angiogenesis in tumor may also represent an effective alternative for tumor growth inhibition, as a means of providing routes of drug delivery. He introduces new blood vessel formation in tumor using bone marrow cells. Finally, the function of hematopoietic stem cells in the promotion of angiogenesis is reviewed, along with recent topics pointing to angiogenesis-related functions in cancer stem cells. The function of stem cells in promoting blood vessel formation may be closely associated with the formation of the vascular niche for stem cell maintenance, and, therefore, stem cells themselves may construct the foci needed to maintain their own stemness.

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It will be important to gain a better understanding of the precise molecular mechanisms behind blood vessel formation by stem cells, and to determine the vascular niche component if we are to develop effective strategies in both regeneration and cancer therapy.

Compared with research in hematology, in which extensive molecular analyses of lineage commitment from hematopoietic stem cells to well-differentiated mature hematopoietic cells have been performed, lineage analysis of the differentiation of vascular stem cells to mature ECs is yet to be addressed in vascular biology. While hematopoietic stem cells can be identified using a profile of surface molecules and isolated to analyze their differentiation, there are still no molecular markers of endothelial stem cells, and indeed, the endothelial stem cell itself has not been definitively identified. It is still unclear whether endothelial stem cells are present in the adult; however, as there are three different types of ECs during angiogenesis, they may still await identification. Tip cells are sprouted from pre-existing blood vessel in the initiation of angiogenesis and located in front of new vascular branch; however, these lack proliferative ability. Stalk cells situated behind the tip cells proliferate and induce the elongation of new branches. Finally, phalanx cells emerge, stabilize and mature into newly developed blood vessels. This heterogeneity of ECs suggests that there may be endothelial stem cells that produce different types of ECs. Vascular biology may grow even further once endothelial stem cells have been defined, and therapy for vascular diseases, including the suppression and induction of blood vessel formation, is improved.

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Involvement of non-vascular stem cells in blood vessel formation

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Abstract Blood vessels clearly act as conduits for blood flow, but recently the concept that they are also involved in organ maintenance, especially by providing a niche for organ-specific stem cells, has begun to emerge. Moreover, several lines of evidence suggest that hematopoietic stem cells can differentiate directly into cells composing blood vessels. Recently, cancer stem cells (CSCs) have also been assigned these roles in the cancer microenvironment. Although anti-angiogenic drugs have been developed and are utilized in the clinic for their anti-tumor activity, their suppressive effects on tumor growth have been disappointing. This may be caused by transferring drug resistance from CSCs to endothelial cells. It has been suggested that CSCs localize in the peri-vascular niche. Therefore, it is extremely important to know how the vascular niche maintains CSCs, as such knowledge may enable us to develop promising new approaches to cancer treatment.

Keywords Endothelial cells · Hematopoietic stem cells · Angiogenesis · Vasculogenic mimicry · Cancer stem cell

Introduction

Blood vessel formation takes place by two main processes [1]. "Vasculogenesis" is mainly observed in the embryo, where angioblasts derived from mesodermal cells give rise to endothelial cells (ECs) which form vascular tubes (Fig. 1). Blood vessels generated in this way are immature, but gradually mature via several additional processes collectively termed "remodeling." These include interactions of ECs with mural cells for stabilization of the new blood vessels, fusion of blood vessels resulting in bore enlargement, intussusceptions that increase vascular density, and regression of surplus vessels. The second main process, unlike vasculogenesis, results in the generation of new blood vessels from pre-existing vessels and is termed "angiogenesis" (Fig. 2a). Blood vessel formation is closely associated with the progression of many diseases, including cancer, retinopathy, inflammation, and atherosclerosis, which are classified as vascular diseases. Angiogenesis is the main contributor to new blood vessel formation in vascular diseases. For this reason, the mechanisms responsible for angiogenesis have been extensively analyzed at the molecular and cellular levels with the aim of developing novel strategies to regulate angiogenesis.

For the past two decades, investigations of the molecular mechanisms of blood vessel formation have focused especially on receptor tyrosine kinases, such as vascular endothelial growth factor (VEGF) receptors (VEGFR1, 2, and 3), platelet-derived growth factor (PDGF) receptor beta (PDGFRβ), and the Tie2 receptor for angiopoietin (Ang) [2]. VEGFs regulate development, tube formation, and proliferation of ECs, PDGF-BB induces recruitment of mural cells near ECs, the Tie2 agonist Ang1 mainly induces EC–EC and EC–mural cell adhesion for stabilization of blood vessels, and another Tie2 antagonist, Ang2, inhibits EC–mural cell adhesion, thereby enabling the initiation of sprouting angiogenesis. Prolonged dissociation of mural cells from ECs mediated by Ang2 also induces blood vessel regression. Efforts to target these pathways have resulted in the development of many VEGF/VEGFR inhibitors that are already utilized clinically [3].

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Angiogenesis inhibitors effectively prevent blood vessel formation and tumor growth in mouse models, but it is widely accepted that these drugs are not effective in humans when used individually as single agents. However, they do improve permeability by normalization of abnormally leaky blood vessels with the result that a combination of angiogenesis inhibitor with anti-cancer drugs does have an effect on tumor growth in humans [4]. Clinical application of angiogenesis inhibitors is of course usually aimed at disrupting the blood supply to tumors to starve them of nutrients and oxygen. If blood vessels cannot be destroyed by means of a strategy based on knowledge of physiological angiogenesis, it is clear that research on tumor-specific processes of blood vessel formation is required.

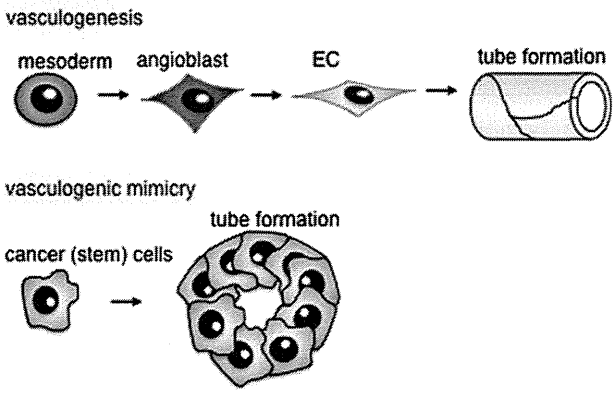

Fig. 1 Schema of vasculogenesis by endothelial cells (ECs) and vasculogenic mimicry by cancer (stem) cells. See text for details

Recently, several mechanisms indicating a direct contribution to blood vessel formation by cancer cells themselves, particularly cancer stem cells (CSCs), have been described [5, 6]. Here, we will review new data on the contribution of CSCs to blood vessel formation, in addition to the classical function of hematopoietic stem cells (HSC) in promoting angiogenesis.

Roles of hematopoietic stem cells in promoting blood vessel formation

Much attention has been focused on the source of ECs derived from bone marrow (BM; endothelial progenitor cells, the so-called "EPC"). These are characterized as non-hematopoietic immature ECs that can differentiate into mature ECs. However, it has also been reported that BM hematopoietic cells can act as a source of ECs as well. We have also determined that CD45-positive BM HSCs can differentiate into ECs [7]. Moreover, we found that HSCs in adult BM, as well as in the embryo, can transdifferentiate into mural cell populations via CD11b-positive myeloid progenitor cells (Fig. 2b). HSCs from mouse embryonic brain can differentiate into mural cells in vitro under the usual culture conditions in serum-containing media, without the addition of specific factors [7]. However, TGFβ stimulation is required for transdifferentiation of adult BM HSCs into mural cells. Recently, it was reported that conversion of ECs into mesenchymal stem cells can be induced by TGFβ or BMP4 [8]. Therefore, it is

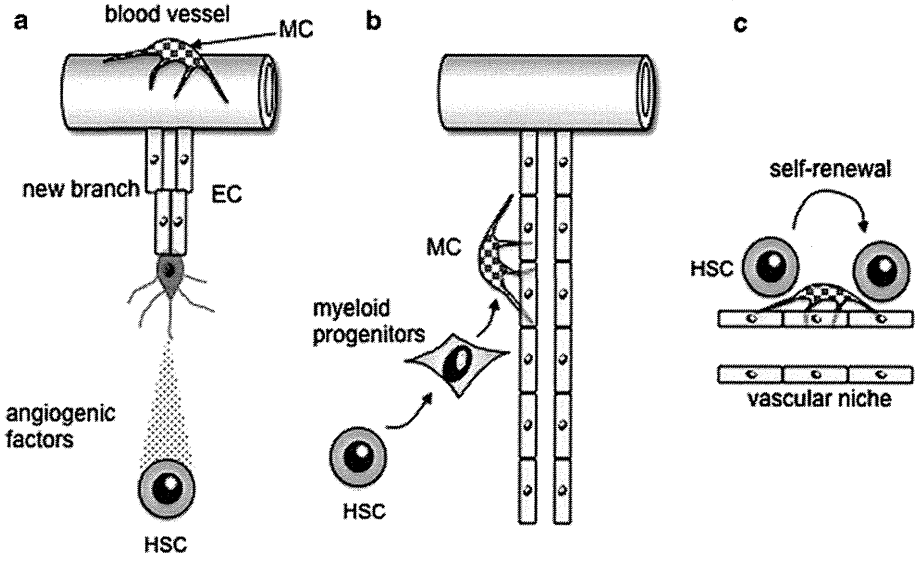

Fig. 2 Intimate interactions of hematopoietic stem cells with vascular cells. **a** In several situations, angiogenesis, new blood vessel formation from preexisting blood vessels, can be induced. During angiogenesis, hematopoietic stem cells (HSCs) produce angiogenic factors to induce angiogenesis by affecting proliferation and migration of endothelial cells (ECs). *MC* mural cells. **b** HSCs have the ability to differentiate into MCs via myeloid progenitors and thus to enhance the stability of newly developed blood vessels. **c** Vascular cells such as ECs and MCs support stemness of HSCs in the vascular niche

possible that the emergence of a mural cell population from HSCs is the result of differentiation of ECs derived from HSCs into mesenchymal stem cells. As previously suggested, Crisan et al. [9] recently showed that pericytes (mural cells observed in capillaries) expressing CD146, NG2, and PDGFRβ are mesenchymal stem cells possessing osteogenic, chondrogenic, and myogenic properties. It is possible that a certain population of pericytes is derived from HSCs.

AML1/Runx1 is a transcription factor required for the development of HSCs; AML1-deficient mice show complete lack of definitive (adult)-type HSCs. Abnormal blood vessel structure, and subsequently massive hemorrhage in the ventricles of the central nervous system, causes lethality in AML1 mutant embryos. Mural cell development in the brain of AML1 mutant embryos is deficient. In the brain of wild-type mice at the same stage, HSCs first appear in the parenchyma, adhere to ECs, and start to expressing smooth muscle actin, a marker of mural cells. Because we found that transdifferentiation of HSCs into mural cells was possible, we concluded that disruption of the blood vessels observed in AML mutant mice was caused by their instability due to the absence of mural cell development from HSCs. In terms of the biological significance of differentiation of HSCs into vascular cells, we failed to unequivocally demonstrate a crucial role for HSC-derived ECs, but we did show that mural cell-lineage cells physiologically derived from HSCs at least promote the maturation/stabilization of blood vessels [7].

Another role of HSCs in blood vessel formation is to guide the direction of migration of blood vessels sprouting from pre-existing vessels (Fig. 2a). Indeed, HSCs are frequent in peripheral blood and organs in adults as well as embryos. AML1-deficient mice also provide a good tool to analyze interactions between HSCs and ECs because the former are totally absent, as described above. We found that sprouting of capillaries generated during angiogenesis is retarded in the brain, pericardium, and other organs. During embryogenesis, HSCs migrate into the parenchyma of the brain first and subsequently ECs migrate toward the HSCs. We found that Angl, which is produced in large amounts by HSCs, induces EC migration in vitro in the manner of a chemoattractant. Therefore, we concluded that Angl produced by HSCs stimulates Tie2 on ECs; this results in recruitment of ECs near HSCs, and thus in promotion of sprouting angiogenesis (Fig. 2a) [10]. The main role of HSCs remains the production of all types of mature hematopoietic cells, but another role for these cells may be in blood vessel formation closely associated with stem cell vascular niche formation, i.e., HSCs themselves generate their own niche for maintenance of stemness (Fig. 2c).

Vasculogenic mimicry

In less evolutionarily derived species, especially small animals, diffusion is sufficient for supplying fluids (carrying oxygen and nutrients) to all parts of the body. However, as body size increases, this becomes inefficient at supplying blood to all tissues and active pumping of some kind becomes a requirement. Moreover, the space between organs/tissues provides an initial space for development of tubes which formed an open circulatory system for blood flow coming from the heart. As far as we have been able to determine, in *Halocynthia roretzi* (the common sea squirt), there are no ECs in the space between organs but fluid from the heart flows through these spaces. Therefore, in this case at least, this space without ECs is indeed utilized in the same manner as blood vessels.

In mammals, blood vessel-like tubes as observed above are induced in the tumor environment. Maniotis et al. [11] reported that melanoma cells themselves generate tube-like structures which might be involved in microcirculation. They designated this process "vasculogenic mimicry" (Fig. 1). When typically highly invasive melanoma cells were cultured in three-dimensional collagen gels or Matrigels, they were found to form tubes. In this culture system, co-culture with ECs and/or fibroblasts is not required. It has been suggested that the very malignant cancer stem cell-like melanoma cells with the ability to form tubes express laminin 5 and matrix metalloproteinases 1, 2, and 9 which might be involved in tube formation.

It has been known for some time that tumor cells can form sac-like structures connecting to blood vessels, as shown by their being filled with red blood cells. However, it had not been demonstrated that the tubes generated by tumor cells themselves were actually involved in tumor microcirculation. Therefore, when this concept was published, several questions arose, such as: Are red blood cells to be found in these tubes? Where is the interface between tumor cells and ECs forming these so-called "blood vessels"? What is the biological significance of this tube formation by tumor cells for tumor microcirculation? [12]. Based on electron microscopy, subsequent reports suggested that other tumors can also generate tube-like structures (e.g., ovary and breast cancer) [13]. Researchers who first documented "vasculogenic mimicry" have further analyzed the features of tumor cells constructing tubes more precisely and found that they express vascular endothelial (VE)-cadherin. It is well established that VE-cadherin is specifically expressed in ECs, and is required for the formation of adherent junctions in ECs. Hence, it seems to function to facilitate tube formation in tumor cells [14].

After publication of this work, it was reported that ECs in the tumor environment frequently exhibit chromosomal

abnormalities [15]. Moreover, these were sometimes common between tumor cells and ECs in the same tumor environment [16]. Therefore, it was tempting to conclude that the tumor cells had directly differentiated into ECs, resulting in the hypothesis that CSCs have the ability to give rise to ECs. Thus, "vasculogenic mimicry" leads to a similar concept: that tumor cells themselves adapt to the absence of blood vessels by differentiating into ECs.

Cancer stem cells as source of ECs in tumors

Differentiation of normal tissue-specific stem cell populations into vascular cells such as ECs and mural cells had already been reported for HSCs, as described above [7], as well as for neuronal stem cells [17]. Recently, cancer stem cell isolation has been greatly facilitated by the establishment of CSC markers. Thus, it has been reported that CSCs isolated from a cultured glioblastoma cell line can differentiate into neuronal cells and glial cells [18]. Neuronal stem cells were previously reported to differentiate into ECs. Moreover, it is known that the density of vascularization in glioblastoma is higher than in other tumors. Taken together, these findings support the notion that glioblastoma CSCs can directly differentiate into ECs.

Wang et al. [5] reported that ECs from glioblastoma showed aberrant overexpression of receptors for epidermal growth factor (EGF) as observed in glioblastoma cells themselves. The expression of VE-cadherin and CD133 (a stem cell marker) by tumor cells has been examined and it was concluded that VE-cadherin-positive cells were already committed to ECs regardless of CD133 expression. When candidate CSCs defined as VE-cadherin-negative and CD133-positive were cultured once with cancer cells in vitro and then transferred to collagen gels, they generated tube-like structures and began to express the EC marker CD31. ECs derived from CSCs in this case arose via CD133 and VE-cadherin double-positive cells, possibly EPC (Fig. 3). Interestingly, without prior co-culture with cancer cells, CSCs could not differentiate into ECs. This suggests two possibilities, i.e., cancer-derived factors directly induce differentiation of CSCs into ECs, or an autocrine loop of factors derived from CSCs affected by co-culture with cancer cells is established which induces transdifferentiation of CSCs to ECs.

CD133-positive CSCs have tumor initiation ability but only those from the tumor that are VE-cadherin-negative had the ability to differentiate into ECs. CSCs can produce not only a multitude of cancer cells, resulting in large masses, but may also participate in blood vessel formation as ECs. In terms of anti-tumor angiogenesis blockade, because CSCs originally show drug resistance, those ECs derived from them may also show resistance against

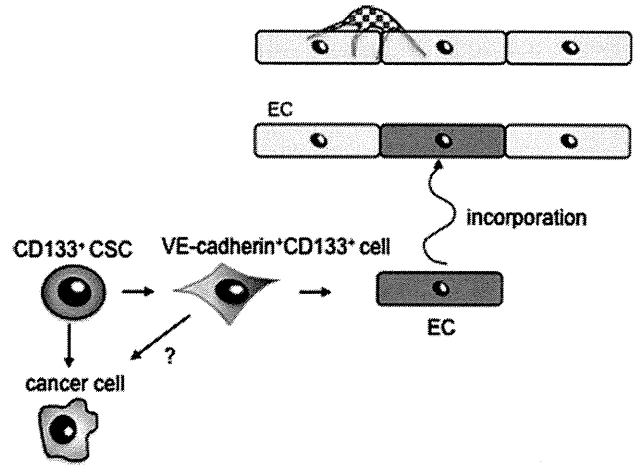

Fig. 3 Differentiation of $CD133^+$ glioblastoma cancer stem cells (CSCs) to ECs through VE-cadherin$^+$CD133$^+$ endothelial progenitor cells. It is not fully understood whether VE-cadherin$^+$CD133$^+$ cells are cancer cells or differentiate into cancer cells

angiogenesis inhibitors. Therefore, it is important to design a strategy for effectively inhibiting the growth of ECs from CSCs. When notch signaling is suppressed in CSCs by γ-secretase inhibitors, differentiation of CD133 and VE-cadherin double-positive endothelial progenitors from CSCs is blocked. Moreover, neutralization of VEGF or suppression of VEGF receptor expression inhibits differentiation of CD133 and VE-cadherin double-positive ECs. Such strategies may contribute to the development of anti-angiogenic therapy targeting the CSC–EC transition.

Along the same lines, Ricci-Vitiani et al. have shown that a comparable proportion of a cell population expressing EC markers and a population of neighboring tumor cells shared a mutated version of the oncogene p53. This also strongly suggested that ECs in the tumor had originated from the cancer cells [6]. Moreover, they found that 30% of ECs express both the EC marker vWF and the glial cell marker GFAP. As also observed by Wang et al., they reported that CD133-positive CSCs could differentiate into ECs both in vitro and in vivo. This work also revealed the biological significance of CSC-derived ECs for tumor growth. A suicide gene, herpes simplex virus thymidine kinase (tk), was transfected into neurosphere-forming CSCs retrovirally and expressed under the transcriptional control of an endothelial-specific gene (Tie2) promoter. In this model, ECs derived from CSCs would be killed by treatment with ganciclovir if it was the CSCs which had differentiated into ECs. It was indeed found that tumor growth was greatly suppressed by the death of CSC-derived ECs. Therefore, it was concluded that ECs derived from CSCs directly contribute to blood vessel formation at least in their model of glioblastoma.

Conclusion

Direct and indirect effects of stem cell populations on blood vessel formation have been reviewed here. In terms of stem cell function in promoting angiogenesis as accessory components indirectly, probably both normal stem cells and CSCs have such an ability in the context of blood vessel formation. However, direct differentiation of CSCs into ECs needs to be verified in cancer histotypes other than glioblastoma, although the possibility of direct differentiation of CD44-positive CSCs into ECs has already been suggested [19].

In the tumor microenvironment, ECs originally play a role in the formation of blood vessels. However, as the stemness of HSCs is supported in the vascular region, the so-called "vascular niche", ECs in the tumor may also act as a niche cell component to support maintenance of stemness of CSCs as well. In terms of stromal cells, it has been suggested that myofibroblasts, cancer-associated fibroblasts, and vascular mural cells all support tumor growth. Recently, it was even reported that ECs can differentiate into mesenchymal stem cells by an endothelial–mesenchymal transition, designated End-MT, when stimulated with TGFβ or BMP2 [8]. Therefore, it is conceivable that ECs derived from CSCs may also differentiate into mesenchymal cells, as above, to support growth of tumor cells. In summary, it is important to understand the molecular mechanisms of EC development from CSCs to develop optimal strategies to inhibit tumor growth.

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Conflict of interest The authors declare no competing financial interests.

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Review Article

Formation and regulation of the cancer stem cell niche

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It is widely accepted that tumors contain cancer stem cells (CSC) possessing self-renewal potential as well as the ability to generate numerous cancer cells. Cancer stem cells are resistant to conventional cancer therapy and have greater invasive and metastatic behavior. It has been suggested that blood vessels provide a niche that maintains stemness in normal organs. This role also extends to the field of cancer biology. Cancer stem cells have been isolated from leukemias and solid cancers. Identification of these cells and their niche is critical for identifying molecular targets in order to inhibit their growth and to destroy their niche. For this purpose, sorting of living CSC is required to monitor their presence in the presumptive niche to establish whether a CSC candidate actually shows malignant features. Based on and referring to analyses in normal tissues, molecules including nitric oxide, Wnt, neuropilin-1, hepatocyte growth factor and others involved in the maintenance of CSC have been isolated. Stem cells might affect niche cells and niche cells produce stemness factors on such stimulation. Therefore, the niche might be flexible to support self-renewal or differentiation of stem cells even in the same niche cells. (*Cancer Sci* 2012; 103: 1177–1181)

As elucidation of stem cell dynamism is directly related to the development of therapies for cancer as well as tissue regeneration, the mechanisms responsible for self-renewal and maintenance of immature status in stem cells have been extensively analyzed. It has been asked whether stem cells maintain stemness autonomously or whether the interaction of stem cells with other cell types is required for the maintenance of stemness. It seems that stemness might be sustained by interactions with other cells because most stem cells isolated from tissues cannot be maintained independently *in vitro*.

Thus far, the location of tissue-specific stem cells has been analyzed to identify niche cells supporting stemness.[1] Among several types of stem cell systems in mammals, research on the stem cell niche in hematopoiesis is better developed because surface phenotypes of hematopoietic stem cells (HSC) have been well defined compared with other stem cell systems. In mice, HSC localize and adhere to endothelial cells (EC) in intraluminal parts of the omphalomesenteric artery at embryonic day (E) 9.5 and form clusters suggesting self-renewing activity.[2] We found that the receptor tyrosine kinase, Tie2, expressed on both HSC and EC, is required for the formation of this HSC vascular niche via activation of integrin.[2] Localization of HSC in the vascular area is also observed in the dorsal aorta and placenta at midgestation.[3] Moreover, formation clusters of HSC around EC are observed in fetal liver where hematopoiesis is ongoing during embryogenesis. Around the time of birth, HSC home to and migrate into the bone marrow (BM). It has been established that there are two different types of niche for HSC in BM. Osteoblasts in the endosteum constitute the niche for maintenance of dormancy in HSC.[4,5] Here, firm cell–cell adhesion between HSC and osteoblasts via N-cadherin expressed on both cell types is induced on activation of Tie2 expressed on HSC with angiopoietin-1. Deficiency of the bone morphogenic protein (BMP) type 1A receptor results in osteoblast proliferation in mouse BM, resulting in increased numbers of HSC.[4] Therefore, it seems that osteoblasts control the number of HSC by spatially limiting the pool size. The other niche for HSC in BM is the vascular area termed the sinusoid. Here, in a vascular niche, reticular cells located next to EC release CXCL12/SDF1, a chemokine ligand for CXCR4, and induce recruitment of HSC.[6] In the vascular niche, it is not clear whether direct interactions between HSC and EC are required but proliferation (self-renewal) of HSC in this area has been suggested.[6]

In this way, when the niche region has been identified, analysis of niche formation and self-renewal in stem cells can be performed at the molecular level. In the cancer field, the cancer stem cell (CSC) niche is being intensively investigated. In this review, different types of niches for several cancers are overviewed, including the function of the niche.

Perivascular niche for glioblastoma CSC

In the adult brain, neuronal stem cells localize in close proximity to blood vessels in the hippocampus and subventricular zone. During embryonic brain development, the neural ectoderm produces vascular endothelial growth factor (VEGF) for mobilization of neovascularization. Thus, EC and neuronal stem cells localize together in the embryo and this interaction continues after birth. It has been shown that stemness characteristics of neuronal stem cells, such as self-renewal activity and maintenance of immature status, are induced by the notch signaling pathway.[7]

Among the cancer cells in glioblastoma tumors, it has been reported that there are CSC having elevated DNA repair capacity and tumor initiating ability. These are nestin$^+$ CSC that localize near CD34$^+$ EC.[8] On inoculation of CSC into immunodeficient mice, tumor incidence was higher when EC from the original tumor were injected together with them. In this case, nitric oxide produced from EC seemed to be acting as a factor for self-renewal of CSC via activation of the notch pathway. In contrast, it has been suggested that CSC prevent EC from undergoing apoptosis by tissue hypoxia, a function that may relate to their resistance to vascular disrupting agents (Fig. 1).

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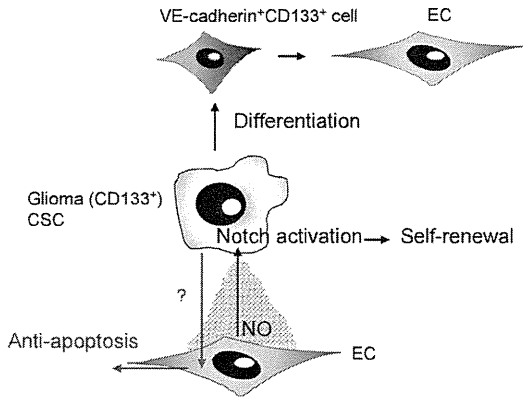

Fig. 1. Perivascular niche for glioma stem cells. It has been suggested that cancer stem cells (CSC) in glioma localize near endothelial cells (EC). Nitric oxide (NO) produced from EC seems to induce self-renewal of CSC mediating activation of the notch pathway. Conversely, CSC prevent EC from undergoing apoptosis when exposed to tissue hypoxia. Consistent with evidence of a higher vascular density in glioblastoma compared with other cancers, it has been reported that $CD133^+$ glioblastoma CSC can differentiate into EC via $CD133^+$VE-cadherin$^+$ endothelial progenitors.

It is well known that glioblastoma is a tumor with a high vascular density. Recently, several lines of evidence have suggested that glioblastoma CSC can differentiate into EC and participate in blood vessel formation. Wang *et al.*[9] showed that $CD133^+$VE-cadherin$^-$ CSC differentiate into $CD133^-$VE-cadherin$^+$ EC via $CD133^+$VE-cadherin$^+$ endothelial progenitors. Ricci-Vitiani *et al.*[10] also showed differentiation of $CD133^+$ CSC into EC both *in vitro* and *in vivo* (Fig. 1). When EC derived from CSC were killed, tumor growth was greatly suppressed, indicating that such EC directly contribute to blood vessel formation, at least in glioblastoma. Whether EC derived from CSC can function as CSC-supporting cells in the vascular niche needs to be addressed, so that methods can be developed to disrupt the CSC niche.

Niche for colorectal cancer stem cells

Niches for normal stem cells in the intestinal tract are also being gradually uncovered subsequent to the acquisition of data on HSC and neuronal stem cells. The crypt is the functional unit in the small and large intestine. Intestinal stem cells localize at the bottom of the crypt and self-renew beside epithelium or mesenchymal cells such as myofibroblasts. In this area, the Wnt signaling pathway is involved in maintaining stemness.[11]

In the case of colon cancer CSC, the location of the niche has not yet been identified. However, myofibroblasts or mesenchymal stem cells have been suggested to be niche cell components. As observed in the normal intestinal stem cell system, activation of the Wnt signaling pathway is induced in CSC in colon cancer. Such colon cancer cells expressing β catenin in their nuclei form clusters and localize to the invasive front of the tumor together with myofibroblasts.[12] Those cancer cells co-locating with myofibroblasts have not yet been proven to be CSC and therefore it is not clear whether myofibroblasts are niche cells for colon cancer CSC. However, it has been suggested that type I collagen stimulates conversion of cancer cells into CSC in colon cancer cell lines; the main producer of type I collagen is the myofibroblast (Fig. 2). Moreover, killing of myofibroblasts by $CD8^+$ T lymphocytes suppresses the growth and metastasis of cancer.[13] Therefore, myofibroblasts have been suggested to be niche cells for induction and maintenance of CSC.

Recently, a system for isolation of CSC utilizing the degree of Wnt signaling activation has been reported.[14] Using this method it has been demonstrated that hepatocyte growth factor (HGF) produced by myofibroblasts enhances the Wnt signaling cascade of CSC in colon cancer (Fig. 2). Myofibroblasts have been suggested to come from BM and act as stromal cell components in a mouse tumor xenograft model.[15] Myofibroblasts might play roles in the maintenance of CSC as niche cell components not only in colon cancer but various other cancers.

Niche for cancer stem cells in a skin tumor model

Keratinocyte stem cells are located in a line in the basal layer of the epidermis and regularly self-renew in response to keratinocyte turnover. It has been recently reported that CSC in skin papillomas and EC in a perivascular niche interact in a chemically induced skin carcinoma model.[16] In this model, CSC express neuropilin-1 (Nrp1) that acts as a co-receptor with the VEGF receptor (VEGFR); VEGF produced by CSC activates Nrp1 by an autocrine and paracrine loop, resulting in their proliferation. In contrast, VEGF from CSC also promotes blood vessel formation via its primary function on EC and increases the area of the vascular niche, providing a site for maintenance of stemness in CSC (Fig. 3). Indeed, a crucial role for CRC-derived VEGF for tumor growth was proven by knocking down the VEGF gene specifically in CSC.

Nrp1c expressed on EC forms a heterodimer with VEGFR2 and enhances downstream signaling of the latter compared with the activity of the homodimer of VEGFR2, resulting in excess proliferation and migration of EC for angiogenesis.[17] Nrp1 is a cell membrane protein but does not have a kinase domain and therefore another binding partner, that is, VEGFR2 in EC, is required for intracellular signaling. It has been reported that deletion of VEGFR1 (flt1) in epidermal cells delays the appearance of skin papilloma in a model using K5-Sos transgenic mice.[18] This suggests that VEGF signaling in keratinocytes can directly regulate the initiation of skin cancer;

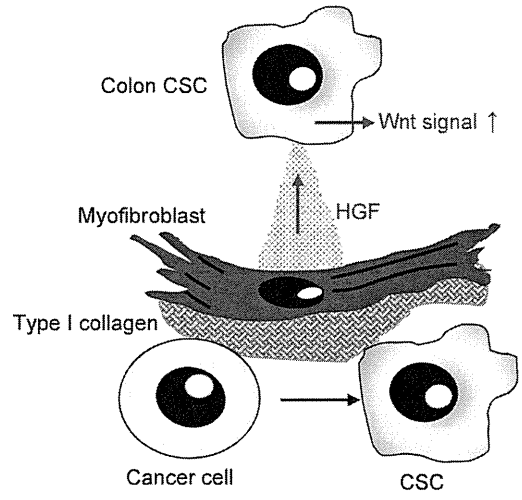

Fig. 2. Myofibroblasts are the cell source for the cancer stem cell (CSC) niche in colon cancer. It has been suggested that myofibroblasts promote phenotypic changes from cancer cells to CSC in colon cancer. Type I collagen produced by myofibroblasts is probably involved in this dedifferentiation. Moreover, hepatocyte growth factor (HGF) produced by myofibroblasts enhances the Wnt signaling cascade of CSC in colon cancer. It has been suggested that a bone marrow stem cell population enters the tumor and differentiates into myofibroblasts.

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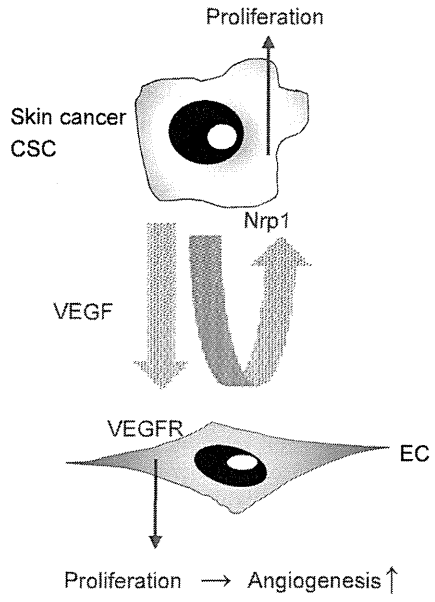

Fig. 3. Cancer stem cell (CSC) niche of a skin cancer model. Vascular endothelial growth factor (VEGF) produced by CSC in skin cancer stimulate endothelial cells (EC) and CSC themselves by paracrine and autocrine loops. Vascular endothelial growth factor induces angiogenesis, resulting in expansion of the vascular niche region and also induction of self-renewal of CSC at the same time. It has been suggested that Nrp1 on CSC is a receptor for VEGF; however, a co-receptor of Nrp1 has not been identified. VEGFR, VEGF receptor.

however, it has not been shown whether Nrp1 binds to VEGFR1 for enhancement of VEGF signaling in CSC or whether Nrp1 can transduce signals via binding of VEGF to receptors other than VEGFR1. Identification of the VEGF receptor with Nrp1 on CSC might help to clarify whether the direct effect of VEGF produced by CSC can be generalized to other cancer models.

Vascular niche for CSCs in a cancer cell xenograft model

PSF1 is a marker for detection of stem cells in normal tissues. Thus far, the importance of the vascular niche for the maintenance of CSC in various cancer models has been suggested. Xenograft models using mouse cancer cells provide tools to identify niches for CSC and to analyze the precise function of the niche for CSC. To identify the niche, good markers of CSC are required in mice. Several markers to identify CSC in humans have been reported, that is, CD44 for breast cancer and CD133 for lung, brain, liver and other.[19] However, in mice, there are no good markers to identify CSC.

It is widely accepted that tissue-specific stem cells actively self-renew during embryogenesis for acute expansion of tissue in organs; however, most of these stem cells become dormant in adulthood because the tissue/organ is already established and because of pool (niche) size limitation. In contrast, in tumor tissue, tumor growth is not restricted and there is no limitation of tumor size, so that CSC actively proliferate but do not become dormant. Therefore, molecules expressed in stem cells specifically in the embryo but not the adult might be useful to identify CSC. Following this hypothesis, we tried to identify such molecules by comparing gene expression in HSC cDNA libraries from embryo and adult BM. Among molecules isolated by this method, PSF1 (partner of SLD five 1) was found to be specifically upregulated in HSC in the embryo.[20] Thus far, the function of PSF1 has not been identified; however, in yeast, it has been reported that PSF1 forms a complex with PSF2, PSF3 and SLD5 termed GINS (this name is derived from the Japanese words for numbers, i.e., 5: Go; 1: Ichi; 2: Ni; 3: San), which plays a critical role in the generation of the DNA replication fork associated with CDC45.[21] To determine whether PSF1 is functional in mice, we generated PSF1-deficient animals and found that they die at around E 6.5. Lethality was caused by deficient proliferation of epiblasts, a type of totipotent stem cell.[20] Moreover, we found that PSF1 is essential for acute proliferation of HSC in experiments following their recovery after BM ablation in PSF1 heterozygous mice.[22] Therefore, it was suggested that PSF1 plays an important role in promoting stem cell proliferation.

Visualization of CSC by PSF1 promoter activity. We found that cells strongly positive for PSF1 in human lung and esophageal cancers are localized to the perivascular region of the tumor edge.[23] Because we observed that PSF1 is specifically expressed in stem cells such as HSC, spermatogonia, intestinal stem cells, etc. in the normal organ, this suggested that PSF1-positive cells in cancer are CSC. However, it was difficult to prove whether PSF1-positive cells in the perivascular region are indeed CSC, because PSF1 is an intracellular protein and therefore we could not sort PSF1-positive viable cells to evaluate their biological activities *in vitro* and *in vivo*. To overcome this problem, we transduced a reporter gene expressing EGFP under the transcriptional control of the PFS1 promoter into several cancer cell lines. Compared with $EGFP^{low}$ cells, we found that sorted $EGFP^{high}$ cells have greater tumor-initiating abilities with small numbers of cells (100 cells), show a highly invasive ability by digesting extracellular matrices and generate many more metastatic foci on intravenous injection. These findings suggest that promoter activity of PSF1 correlates with tumor cell malignancy. Moreover, gene expression profiles of the $EGFP^{high}$ cells were similar to ES cells. Taken together, these data suggest that $EGFP^{high}$ cells are in the CSC population. By histology, these $EGFP^{high}$ cells are found to be abundant in the perivascular region of the tumor edge, as observed in human cancer.[23]

Based on our model and together with other models showing localization of CSC,[8,16,23] it is possible that highly malignant cancer cells like CSC generally utilize vascular regions as their stem cell niche. Here, in our model, it is interesting that $EGFP^{high}$ cells cultured *in vitro* did not show malignant CSC activities on inoculation into mice. This suggests that some molecular cues derived from cells composing the tumor microenvironment might change cancer cells into CSC and that cells in perivascular niches such as EC, mural cells (pericytes and vascular smooth muscle cells), myofibroblasts, fibroblasts and mesenchymal stem cells are possible sources producing such educational factors.

Molecules affecting niche cells alter the function of the niche for stem cells

Niche cells support stemness by definition; however, it is difficult to imagine that niche cells autonomously support stemness independently of stem cells localizing near the niche cells. Most likely, molecular cues derived from stem cells affect niche cells and on receiving these cues, niche cells produce stemness factors or differentiation factors for stem cells (Fig. 4). Here, two lessons from the HSC system showing such abilities can be learned.

As described above, it has been suggested that osteoblasts in the BM osteoblast niche support dormancy of HSC and EC in the vascular niche support self-renewal.[5,6] However, it is possible that any niche cells, such as EC, osteoblasts or other mesenchymal cells, can support both dormancy, self-renewal

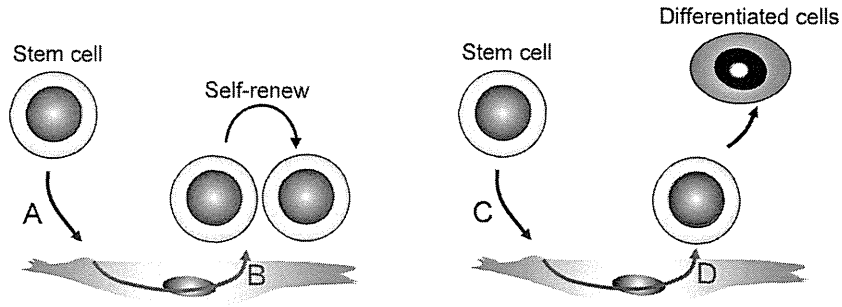

Fig. 4. Altered function of niche cells for self-renewal or differentiation of stem cells. It is hypothesized that niche cells do not autonomously determine the fate of stem cells locating near them, but stem cells might affect the niche and thus influence their behavior. In brief, when stem cells producing factor A come near the niche and factor A then stimulates the niche cells, factor B produced from the niche cells induces self-renewal of the stem cell. In contrast, when stem cells producing factor C come near the niche and factor C stimulates the niche cells, factor D produced from the niche cells induces differentiation of the stem cell.

and differentiation of stem cells depending on factors derived from the stem cells. We developed a co-culture system of HSC with OP9 stromal cells to analyze how the self-renewal or differentiation of HSC is affected by the stromal cells.[24] OP9 cells were derived from calvaria of *op/op*, M-CSF-deficient mice and are defined as osteoblastic cells with adipocyte differentiation ability. When OP9 cells were stimulated with epidermal growth factor (EGF) and co-cultured with HSC, immature hematopoietic progenitor cells (HPC) including HSC proliferated and the undifferentiated state of such HPC or HSC was maintained long term. In contrast, when fibroblast growth factor-2 (FGF2) was used instead of EGF, differentiation of HSC into mature hematopoietic cells was induced. When the EGF receptor on OP9 was transactivated by the transfection of a constitutively active erbB2 gene into OP9 to eliminate the direct effect of EGF on HSC, differentiation of HSC was suppressed and proliferation of immature hematopoietic cells was maintained long term.[24] Therefore, this suggests that the fate of HSC, that is, maintenance of immature state or differentiation, is altered by factors derived from niche cells (in this case, OP9 cells) and whether niche cells produce stemness or differentiation factors is dependent on exogenous factors stimulating the niche cells.

Recently, Raffi's group also developed a similar culture system using primary EC and drew similar conclusions.[25] In brief, they constitutively activated Akt signaling in EC by transduction of the adenoviral region 4 gene.[25] When HSC were co-cultured with these EC, self-renewal of HSC was induced. In contrast, when MAPK was activated in EC by the transfection of a constitutively active form of c-raf, differentiation of HSC was induced on co-culture with these EC. They proposed that production of BMP4, angiopoietin-1 and other stemness factors for HSC was elevated in the former condition and angiopoietin-2 and interleukin-6, which are differentiation factors for HSC, were upregulated in the latter.

Taken together, these data strongly suggest that any niche cells, such as osteoblasts or EC, can alter self-renewal or differentiation of HSC by extrinsic factors stimulating the niche cells. The CSC must produce several factors and stimulate niche cells such as EC, myofibroblasts and others in the vascular niche of cancer. In this vascular niche, differentiation factors as well as stemness factors might be produced from niche cells; both of these would be useful drug targets to suppress self-renewal or enhance differentiation of CSC. Therefore, molecular mechanisms regulating stemness or differentiation of CSC in the vascular niche must be identified to develop better strategies to combat cancer.

Conclusions

Based on the knowledge acquired from stem cell research in normal organs, technology to identify CSC niches has been developed. It is suggested that CSC localize to the perivascular region. Blood vessels are generally composed of luminal EC, mural cells such as pericytes and smooth muscle cells, with EC adhering on the basal side, and extracellular matrices such as collagen and fibronectin covering the mural cells. In addition to such components, myofibroblasts expressing α-smooth muscle actin are abundant near mural cells in tumor blood vessels. To elucidate molecular cues to induce stemness of CSC, it is important to determine which cells are critically involved in the maintenance of CSC. Although it has been reported in colon cancer that myofibroblasts are the candidate niche cells supporting CSC,[14] other reports suggest that EC are important to support *in vivo* tumor initiation from small numbers of CSC.[8] Therefore, further analysis is required to determine the roles of cells composing the vascular niche in terms of their effects on survival, proliferation and differentiation of CSC.

Regarding the origin of myofibroblasts in the tumor, it has been reported that a mesenchymal stem cell population recruited from BM differentiates and is involved in supporting tumor growth, as described above.[15] Moreover, CSC themselves have the ability to differentiate into EC[9,10] and these can transdifferentiate into mesenchymal stem cells via the endothelial–mesenchymal transition (End-MT) on stimulation with BMP2 and/or TGFβ.[26] This suggests that CSC can differentiate into myofibroblasts and act as niche cells in the tumor.

Molecules associating with stemness of HSC have been identified due to the success of the co-culture system of HSC with different stromal cells. Therefore, establishment of stromal cell lines that can support stemness of CSC might help to identify the molecular cues inducing self-renewal and differentiation of CSC.

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Disclosure Statement

There are no conflicting financial interests in this work.

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Monoclonal Antibody Selectively Recognizing Murine But Not Human CD44

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Here we report on the generation of a monoclonal antibody (MAb) specific for murine, but not human CD44 obtained by immunization with cancer-associated fibroblasts. The monoclonal antibody MS44 recognizes CD44, as evidenced by immunoblotting of cell lysates. Additionally, MAb MS44 reacts with mouse but not human CD44 in flow cytometry. Thus, this antibody provides an effective tool to analyze CD44 from host (mouse) cells in human cancer cell xenograft models in mice.

Introduction

THE TUMOR ENVIRONMENT CONSISTS of stromal cell components, including endothelial cells, activated fibroblasts, inflammatory cells, and others, together with the extracellular matrix. In the tumor stroma, cancer-associated fibroblasts (CAFs) are a major component, contributing to tumor initiation, growth, and progression. Recent studies show that there are several different subpopulations of CAFs expressing partially overlapping markers, including α-smooth muscle actin, platelet-derived growth factor (PDGF) receptors, fibroblast specific protein-1, and others.[1,2]

CD44 is a class I transmembrane glycoprotein expressed ubiquitously in a number of isoforms generated by alternative splicing of 10 exons.[3] It has been reported that some variant isoforms of CD44 (CD44v) induced a metastatic phenotype.[4] Therefore, CD44v is implicated as a cancer-initiating cell marker in several cancers.[5,6] In contrast, the shortest, standard or hematopoietic, isoform (CD44s) is expressed on the membrane of most vertebrate cells.[3] CD44s is an adhesion molecule that is upregulated following tissue injury and is implicated in many chronic inflammatory diseases such as rheumatoid arthritis.[7–10]

Here we report the production of a monoclonal antibody (MAb) raised against cloned tumor stromal cells, CAFs, and show by immunoblotting and flow cytometry that it specifically recognizes murine, but not human CD44s.

Materials and Methods

Cell culture and animals

Mouse melanoma cell line (B16) and African green monkey kidney fibroblast-like cell line (COS7) were maintained in DMEM (Sigma, St. Louis, MO) with 10% fetal bovine serum (FBS, Sigma) and penicillin/streptomycin (Invitrogen, Carlsbad, CA). Human umbilical vein endothelial cells (HUVEC) were maintained in Humedia EG2 (Kurabo, Osaka, Japan). The animals were housed in environmentally controlled rooms of the animal experimentation facility at Osaka University. All experiments were done in compliance with the laws and institutional guidelines of Osaka University.

Immunohistochemical staining

Tissue fixation, preparation of tissue sections, and staining of sections with antibodies were performed as described previously.[11] For immunohistochemistry, rat anti-PDGFRβ antibody (APB5, eBioscience, San Diego, CA) and phycoerythrin (PE)-conjugated anti-rat IgG antibody (BD Biosciences, San Jose, CA) were used. Stained sections were assessed using a CTR 5500 (Leica, Wetzlar, Germany).

Establishment of tumor stromal cell lines

Tumor stromal cells defined as CAFs were isolated from B16 melanoma xenografts in CAG-EGFP/C57BL/6 mice (Jackson ImmunoResearch Laboratory, West Grove, PA). Single cell suspensions of tumor tissues were prepared using a standard protocol. The antibodies used for flow cytometry were biotin-conjugated APB5 (eBioscience), PE-conjugated anti-lineage (a mixture of ter119, Gr-1, Mac-1, B220, CD4, and CD8), and CD31 antibodies (all purchased from BD Biosciences). Biotinylated antibodies were visualized with APC-conjugated streptavidin (BD Biosciences). Cell sorting was done by JSAN (Bay Bioscience, Kobe, Japan). GFP^+ Lin^- $CD31^-$ $PDGFR\beta^+$ cells were isolated as CAFs and grown in DMEM with 10% FBS and penicillin/streptomycin. CAFs were immortalized with the simian virus 40 large T antigen (SV40LT, a gift of Dr. M. Yutsudo) and cloned.

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Preparation of cell extracts for use as antigen

The collected cells were washed with ice-cold phosphate-buffered saline (PBS) and lysed in an ice-cold lysis buffer I (1 mM EDTA, 50 mM Tris-HCl [pH 7.6], 150 mM NaCl, supplemented with Complete Protease Inhibitor Cocktail [Roche, Indianapolis, IN]). The cell lysates were homogenized using a PT 3100 polytron homogenizer (Ishii Laboratory Works, Osaka, Japan) and centrifuged at 20,400 *g* for 15 min. Pellets were suspended in ice-cold lysis buffer II (1% NP-40, 1 mM EDTA, 50 mM Tris-HCl [pH 7.6], 150 mM NaCl, Complete Protease Inhibitor Cocktail), sonicated, and centrifuged at 20,400 *g* for 10 min. The supernatants were collected as cell extracts.

Production of monoclonal antibody

CAF cell extracts were used as antigen to immunize rats, and rat/mouse hybridomas were established by standard procedures.[12] The mouse myeloma cell line X63Ag8653 was used for cell fusion. A stable hybridoma cell line, MS44, was obtained.

ELISA

The cell extracts in 1% bovine serum albumin (Sigma) in PBS (1% BSA/PBS) were absorbed to the surface of 96-well plates (Becton Dickinson, Franklin Lakes, NJ) overnight at 4°C. To avoid non-specific binding, the plates were blocked with 5% BSA/PBS. The hybridoma supernatants were incubated for 2 h at room temperature, then washed three times with 0.05% Tween-20 in PBS (T-PBS). The plates were incubated for 1 h at room temperature with horseradish peroxidase (HRP)-conjugated anti-rat IgG antibody (BioSource, Camarillo, CA). After washing with T-PBS five times, immunoreactivity was visualized with substrate 3,3′,5,5′-Tetramethylbenzidine (Wako Pure Chemical Industries, Osaka, Japan) and absorbance measured by Powerscan HT (DS Pharma Biomedical, Osaka, Japan).

Immunoprecipitation and immunoblotting

CAF cell extracts were incubated with MAb MS44 or rat IgG2b, κ isotype control (eBioscience) for 2 h at 4°C with end-over-end rotation. Protein G-Sepharose beads (GE Healthcare, Buckinghamshire, United Kingdom) were added to lysate/antibody mixture and incubated for 2 h at 4°C. The mixture was centrifuged and the Protein G-Sepharose beads were washed three times with lysis buffer. Bound proteins were analyzed by immunoblotting using MAb MS44 as the primary antibody. HRP-conjugated anti-rat IgG antibody (Invitrogen) was used as the secondary antibody. The immunoreactive proteins were visualized using the ECL plus Western Blotting Detection system (GE Healthcare). The blots were scanned with a imaging densitometer (LAS-3000 Mini, Fujifilm, Tokyo, Japan).

Identification of antigen recognized by MAb MS44 using LC-MS/MS

The purified protein complexes were separated by SDS-PAGE on a 5–20% gradient gel (DRC, Tokyo, Japan). After visualization of the proteins by silver staining (Daiichi Pure Chemicals, Tokyo, Jpaan), the specific band was excised from the gel and digested *in situ* with TrypsinGold (Promega, Madison, WI). The digested samples were analyzed using a QTOF Ultima (Waters, Milford, MA), as described previously.[13]

Expression vector and transient expression

For transient expression in COS7 cells, the full-length mouse CD44s and full-length human CD44s were cloned into the BglII and XhoI sites of modified pME18S vector (pME18S-V5).[14] Transfection was carried out using Lipofectamine 2000 (Invitrogen). COS7 cell extracts, or extracts of cells expressing V5-tagged mouse CD44s (mCD44-V5) or V5-tagged human CD44s (hCD44-V5), were analyzed by immunoblotting with MAb MS44, anti-V5 mouse monoclonal antibody (Invitrogen), and anti-mouse/human CD44 rat monoclonal antibody (IM7, eBioscience) by the methods described above.

FACS analysis

FACS analysis was performed as described previously[11] using DyLight 649 (Thermo, Rockford, IL)-conjugated MAb MS44 and APC-conjugated IM7 (eBioscience).

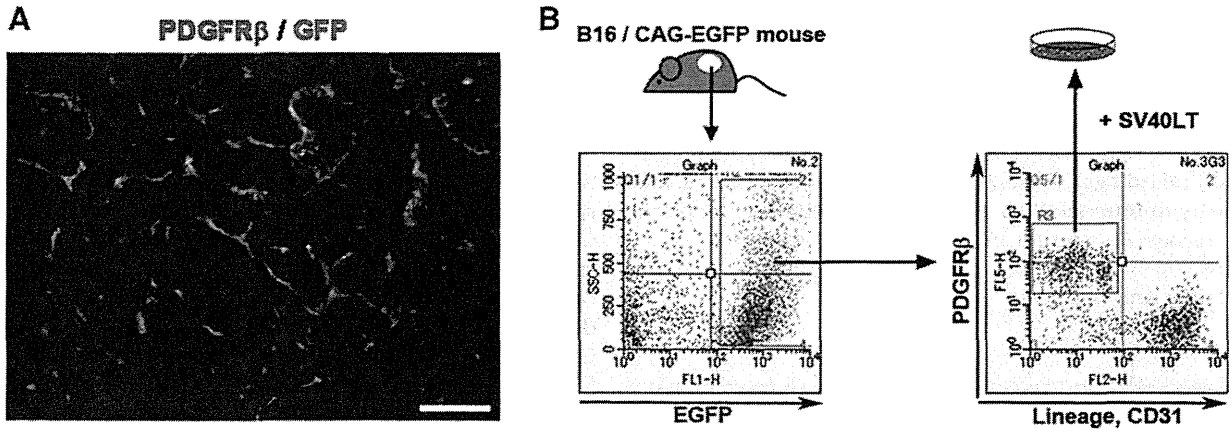

FIG. 1. Expansion of cancer-associated fibroblasts. (**A**) Expression of PDGFRβ (red) and endogenous GFP (green) in tumor tissue generated by inoculation of B16 melanoma cells into a CAG-EGFP mouse. Bar indicates 100 μm. (**B**) Schematic of strategy for establishment of CAF cell lines isolated from B16 melanoma-bearing CAG-EGFP mice. CAFs (right) defined as Lin$^-$ CD31$^-$ PDGFRβ$^+$ cells were isolated from GFP$^+$ host mouse-derived cells (left), immortalized with the simian virus 40 large T antigen (SV40LT), and cloned and expanded for the preparation of cell lysate.

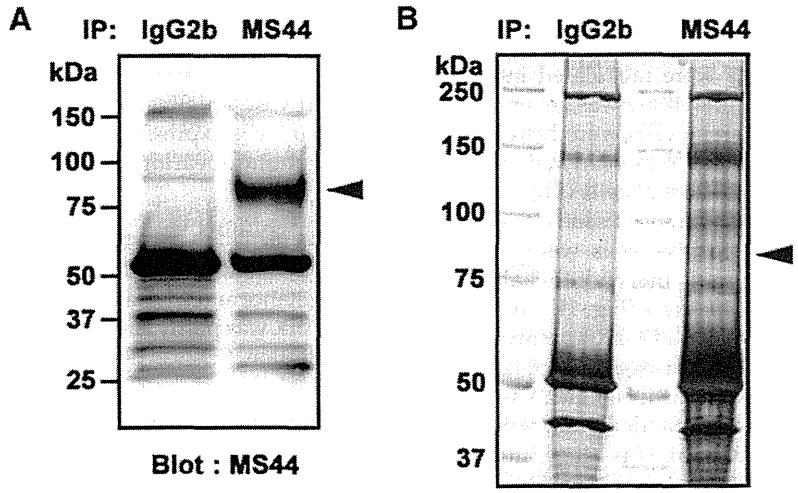

FIG. 2. Identification of MAb MS44 antigen. Cell lysates were prepared from cloned CAFs and immunoprecipitated with MAb MS44. The samples pulled down were immunobloted with MAb MS44 (A) stained with silver (B). IgG2b was the isotype-matched control Ig. The specific band (arrowhead) was analyzed by LC-MS/MS.

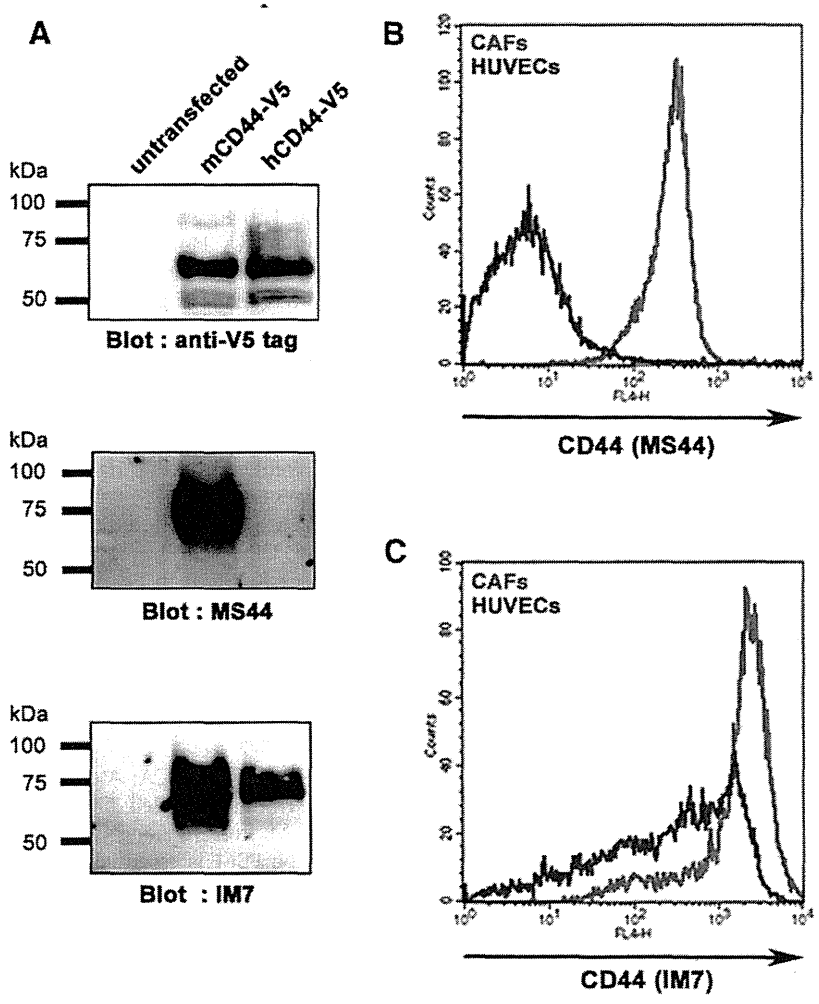

FIG. 3. Specificity of MAb MS44 for mouse CD44. (A) Extracts of COS7 cells transiently expressing V5-tagged mouse CD44s (mCD44-V5) or human CD44s (hCD44-V5) were analyzed by Western blot analysis using anti-V5 antibody (upper panel), MAb MS44 (middle panel), and anti-mouse/human CD44 antibody IM7 (lower panel). CAFs (red) or HUVECs (blue) were stained with MAb MS44 (B) or IM7 (C) and analyzed by flow cytometry.

Results and Discussion

B16 mouse melanoma cells were inoculated into CAG-EGFP/C57BL/6 mice and a tumor mass was generated. The host mouse-derived GFP-positive cells were histologically of two types: PDGFRβ-positive mesenchymal cells and PDGFRβ−negative non-mesenchymal cells (Fig. 1A). Analysis of single cell suspensions of tumor tissue revealed that approximately 50% of GFP-positive cells were PDGFRβ-positive and did not co-express the endothelial marker CD31 or other hematopoietic markers (Fig. 1B). To identify CAF-specific molecules, we sorted PDGFRβ-positive, CD31-negative, or hematopoietic marker-negative cells. To establish immortalized CAFs, we transduced the SV40LT gene into sorted cells and cloned and expanded them. An 8-week-old female F344/N Slc (SLC) rat was then immunized with cell extracts of these immortalized CAFs. Hybridomas obtained after fusing B lymphocytes from spleen of the immunized rat with mouse myeloma cells were tested by ELISA for the production of MAbs that reacted with the CAF cell extracts. Twenty-three supernatants, positive by ELISA, were further examined by immunoprecipitation and Western blot analysis to determine whether the MAb recognized multiple non-specific proteins (data not shown). One of these MAbs, designated MS44, was selected for further study.

To identify the antigen recognized by MAb MS44, CAF cell extracts were immunoprecipitated and the bound proteins separated on an SDS-PAGE gel (Fig. 2). LC-MS/MS analysis of the visible bands (Fig. 2B) resulted in the identification of CD44.

We transduced mouse (m) and human (h) CD44 fused with a V5 tag epitope into COS7 cells and tested for cross-reactivity of MS44 on human CD44. We confirmed a similar level of mCD44 and hCD44 expression in transfected COS7 cells by detection of V5 (Fig. 3A). IM7, a commercially available anti-CD44 (IM7) MAb, recognized both mCD44 and hCD44; however, our MS44 recognized mCD44 but not hCD44 (Fig. 3A). MS44 no longer recognized CD44 deglycosylated by N-Glycanase treatment (data not shown). Therefore, we conclude that MS44 recognizes sugar moieties of the extracellular domain of CD44.

Next we applied flow cytometry to analyze human umbilical vein endothelial cells (HUVECs), which express CD44[15] and CAFs. We found that MAb MS44 stained mouse CD44 on CAFs, but not human CD44 on HUVECs (Fig. 3B). On the other hand, IM7 recognized both mCD44 and hCD44 on these cells (Fig. 3C). These results suggest that MAb MS44 is specific for murine but not human CD44.

As reported here, CD44 is expressed abundantly in CAFs and is probably involved in the formation of the tumor microenvironment. Functional analysis of CD44 in non-cancer cells may shed light on the development of new strategies for managing cancer patients. To this end, it is important to analyze the localization of CD44-positive CAFs in tumor tissue precisely. Because cancer cells may also express CD44, this may perturb visualization of CD44-positive CAFs in the tumor microenvironment when a syngeneic mouse cancer model or chemically and/or genetically induced cancer models in mice are used. However, in a model where human cancer cells are inoculated into nude mice, MS44 will detect host mouse-derived CAFs but not human cancer cells. We have confirmed that MS44 is suitable for use in immunohistochemistry. Therefore the monoclonal anti-mouse CD44-specific antibody MAb MS44 will provide an effective tool for investigating the localization of cells expressing CD44 in tumor stromal tissues of human tumor-bearing mice.

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Author Disclosure Statement

The authors have no financial interests to disclose.

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