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Changes in blood vessel maturation in the fibrous cap of the tumor rim

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It is widely accepted that blood vessels in the tumor microenvironment are immature because mural cell (MC) adhesion to endothelial cells (ECs) is broadly lacking. Hyperpermeability of the tumor vasculature then results in interstitial hypertension that mitigates against penetration of anticancer drugs into the depths of the tumor. It has been suggested that treatment with angiogenesis inhibitors normalizes blood vessels, resulting in restoration of normal permeability and improved drug delivery. However, recent reports suggest that cancer cell invasion is induced from the edge of the tumor into peripheral areas after treatment with angiogenesis inhibitors. Therefore, it is important to assess the status of blood vessels in the fibrous cap at the tumor rim after antiangiogenesis therapy. In the present study, we found that mature blood vessels in which ECs are covered with MCs are present in the fibrous cap. After treatment with angiogenesis inhibitors, immature blood vessels were destroyed and vascular function was significantly improved, but maturing blood vessels in which ECs were covered with MCs remained visible. These maturing blood vessels showed a less dilated character after treatment with the angiogenesis inhibitors. It is widely accepted that well-matured blood vessels are sheathed in extracellular matrix (ECM) and that cancer cells migrate along tracks made of ECM collagen fibers. Therefore, our data indicate the importance of destroying maturing blood vessels outside the tumor parenchyma to prevent cancer cell invasion. (*Cancer Sci* 2012; 103: 433–438)

Tumor growth commonly depends on newly developed blood vessels that supply oxygen and nutrients to the tumor microenvironment; indeed, antiangiogenic therapy has been clinically available for some time.[1] Vascular endothelial growth factor (VEGF) plays a fundamental role in this process by inducing the proliferation, migration, and tube formation of endothelial cells (ECs) in physiological as well as pathological angiogenesis. Accordingly, many agents have been developed targeting VEGF itself or VEGF receptors.[2] Although effective antiangiogenic activity is mediated by these agents in mouse tumor models, suppression of tumor growth by angiogenesis inhibitors alone in clinical studies has been disappointing. However, enhanced efficacy of combination therapy using anticancer drugs together with angiogenesis inhibitors for tumor suppression has been reported.[3] Therefore, a new concept has emerged that the normalization of tumor vasculature by angiogenesis inhibitors may act to attenuate tumor growth.[4]

In the tumor microenvironment, proangiogenic factors are abundantly produced by the tumor cells themselves, as well as by stromal cell components such as fibroblasts, myofibroblasts, hematopoietic cells, ECs, and other cell types, in amounts sufficient to overcome antiangiogenic factors in normal homeostasis. This can result in the disorganized growth of immature blood vessels lacking mural cell (MC) coverage. Moreover, the EC–EC junctions are loose; therefore, blood vessels in the tumor exhibit hyperpermeability and both spatially and temporally non-uniform blood flow, which may result in interstitial hypertension. This contributes to an inability of anticancer drugs to penetrate deeply into the tumor mass. Angiogenesis inhibitors seem to cause maturation and normalization of the tumor vasculature by rebalancing the dysregulated production of pro- and antiangiogenic factors. This results in normalized vascular permeability and enhanced ability of anticancer drugs and oxygen to exit from the intravascular lumen and penetrate into the parenchyma of the tumor mass. Therefore, normalization of blood vessels may increase the efficacy of standard drug therapy and radiation therapy.

Although this benefit of angiogenesis inhibitors has been advocated, it has also been reported that cancer cells tend to acquire invasive potential when treatment with angiogenesis inhibitors is terminated.[5] Moreover, one line of evidence suggests that cancer recurrence is induced from the edge of the tumor after treatment with vascular disrupting agents.[6] It is hypothesized that responsiveness to angiogenesis inhibitors can differ depending on the structure or localization (i.e. center, periphery, or fibrous cap) of the tumor. The sequential changes to the vasculature inside the tumor after antiangiogenic therapy have been studied extensively;[7] however, changes outside the tumor mass have not. Therefore, in the present study we investigated how tumor blood vessels respond to angiogenesis inhibitors depending on their characteristics, with a particular focus on the MC coverage of the tumor vasculature in the fibrous cap of the tumor rim because cancer cell invasion or recurrence occurs from these peripheral areas.

Materials and Methods

Mice, cell lines, and tumors. All experiments were performed in accordance with the guidelines of the Osaka University Committee for Animal and Recombinant DNA Experiments. Mice were handled and maintained according to the Osaka University guidelines for animal experimentation. KSN nude mice (7–10 weeks of age) were purchased from Japan SLC (Shizuoka, Japan). The HT29 (human colorectal adenocarcinoma) and Colo320DM (human sigmoid colon cancer) cell lines were cultured in RPMI-1640 (Sigma, St Louis, MO, USA) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. Subcutaneous xenografts were established by injecting 1.0 or 2.0×10^6 cells into the flanks of mice and tumor size was measured with calipers. Tumor volume (V) was calculated according to the formula $V = ½ \times \text{length} \times \text{width} \times \text{height}$.[8] Bevacizumab (Avastin; Genentec, San Francisco, CA, USA) and AG-013736 (Axitinib; Selleck Chemicals, Houston, TX, USA) were used as angiogenesis inhibitors. Bevacizumab, a VEGF neutralizing antibody, was dissolved in saline and injected twice a week at a dose of 5 mg/kg, i.p. Axitinib, which inhibits

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multiple kinases, including the VEGF receptor (VEGFR), was dissolved in a solution of PEG-400 (Sigma)–acidified (pH 2–3) water (3:7) and injected at a dose of 25 mg/kg, i.p., for 7 days.[9]

Immunohistological analysis. The procedure for tissue preparation and staining was as reported previously.[10] Briefly, fixed specimens were embedded in OCT compound (Sakura Finetek, Tokyo, Japan) and sectioned at 8 or 12 μm. For immunofluorescence analyses, anti-CD31 mAb (clone MEC 13.3; Pharmingen, BD Biosciences, San Diego, CA, USA) was used to stain ECs and Cy3-conjugated mouse anti-α-smooth muscle actin (α-SMA) mAb (Sigma) was used to stain MCs. Anti-rat IgG Alexa Fluor 488 (Invitrogen, Carlsbad, CA, USA) was used as the secondary Ab. To measure hypoxia in tumor tissues, HypoxyProbe-1 (60 mg/kg, i.v.; Hypoxyprobe, Burlington, MA, USA) was injected 2 h before tissues were harvested. Tumor sections were stained using an anti-HypoxyProbe antibody.[11] To evaluate macromolecule infiltration, mice were injected intravenously with 0.5 mg of FITC-conjugated dextran (MW 40 000; 0.5 mg/body; MP Biomedicals, Solon, OH, USA) and the dextran was allowed to circulate for 2 h. Samples were visualized using a Leica DM5500B or Leica TCS/SP5 confocal microscope (Leica Microsystems, Nussloch, Germany) and processed with the Leica application suite (Leica Microsystems) and Adobe Photoshop CS3 software (Adobe Systems, San Jose, CA, USA). All images shown are representative of more than five independent experiments. The ratio of matured vessels ($CD31^+$ SMA^+ vessels/$CD31^+$ vessels) and the number of $CD31^+$ vessels were counted in more than seven different fields at a magnification of ×100.

Statistical analysis. All data are presented as the mean ± SEM. Statistical analyses were performed using statcel 2 (OMS, Tokorozawa, Japan). Data were analyzed by analysis of variance (ANOVA), followed by Tukey–Kramer multiple comparison tests. When only two groups were compared, two-sided Student's *t*-test was used. $P < 0.01$ was considered significant.

Results

Histological evaluation of sequential changes to the vasculature in the fibrous cap after tumor inoculation. We first evaluated vascularity in the fibrous cap during tumor growth. Five days after inoculation of HT29 colon tumor cells, areas with abnormally high numbers of blood vessels appeared outside the tumor mass (Fig. 1a,b). On Day 7, the vasculature in the fibrous cap did not differ from that observed on Day 5, but the density of the vasculature in the parenchyma of the tumor mass was greater (Fig. 1c,d). On Day 10, blood vessels in which ECs were covered with α-SMA-positive MCs gradually appeared in the fibrous cap (Fig. 1e,f). On Day 14, mature blood vessels covered with α-SMA-positive MCs had increased in number; these were irregular in size, with both small and large diameter vessels seen (Fig. 1g,h). These changes continued after Day 14. Compared with the maturation of blood vessels in the fibrous cap, in the tumor parenchyma the ECs were rarely covered with MCs, despite the presence of α-SMA-positive cells.

Mature blood vessels in the fibrous cap selectively survive treatment with angiogenesis inhibitors. Next we treated HT29 tumor-bearing mice with bevacizumab from Day 14 after tumor cell inoculation. As reported previously,[12] tumor growth was significantly slowed by three injections of bevacizumab (Fig. 2a,b). There was a significant decrease in the number of microvessels inside the tumor parenchyma that were not covered with α-SMA-positive MCs (Fig. 3a,b). To determine the effect of VEGF inhibition on the vasculature in the fibrous cap surrounding the tumor, we used anti-CD31 and anti-α-SMA mAbs. The results of these experiments revealed that there was a decrease in the number of immature vessels in which ECs were not covered with MCs and that the ratio of mature:total blood vessels was increased from 22.2 ± 3.9% to 59.1 ± 3.5% after treatment with bevacizumab (Fig. 2c,d). Moreover, compared with untreated controls, most of the mature blood vessels covered with MCs were shrunken and looked squashed

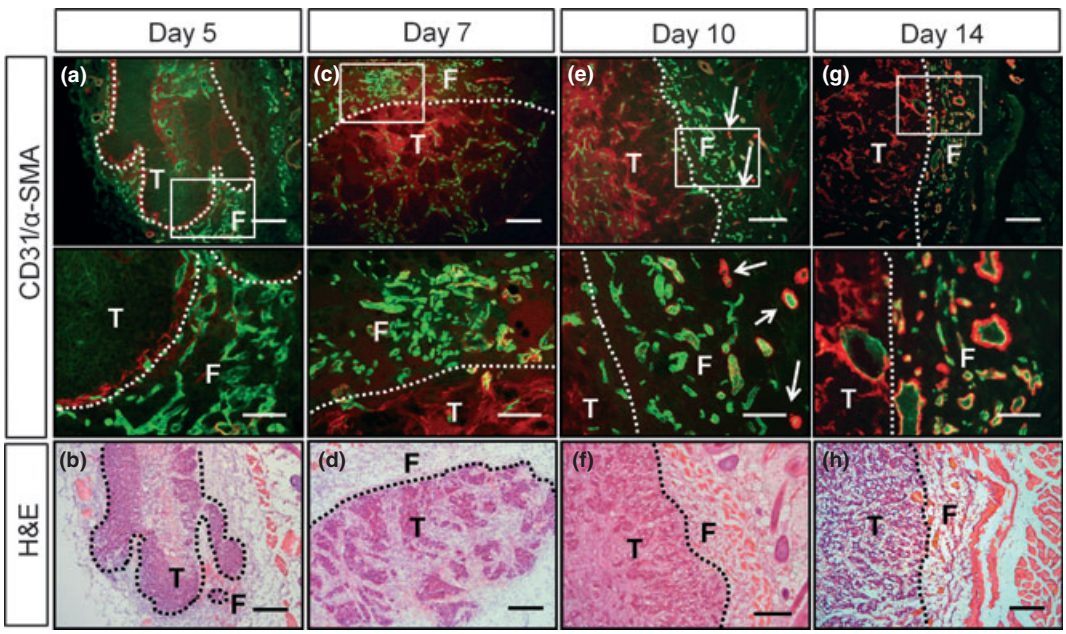

Fig. 1. Sequence of histological changes in the vasculature of the fibrous cap after inoculation of tumor cells. Sections of HT29 tumor tissues 5 (a,b), 7 (c,d), 10 (e,f), and 14 days (g,h) after inoculation of tumor cells were stained with (a,c,e,g) anti-CD31 mAb (green) and anti-α-smooth muscle actin (α-SMA) Ab (red) or (b,d,f,h) H&E. (a,c,e,g) High-power views of the areas indicated by the white boxes in the top panels are shown in the bottom panels. Dotted lines show the border between the fibrous cap surrounding the tumor mass (F) and the tumor mass (T). Arrows in (e) show mature vessels covered with α-SMA^+ mural cells. Scale lines: 200 μm.

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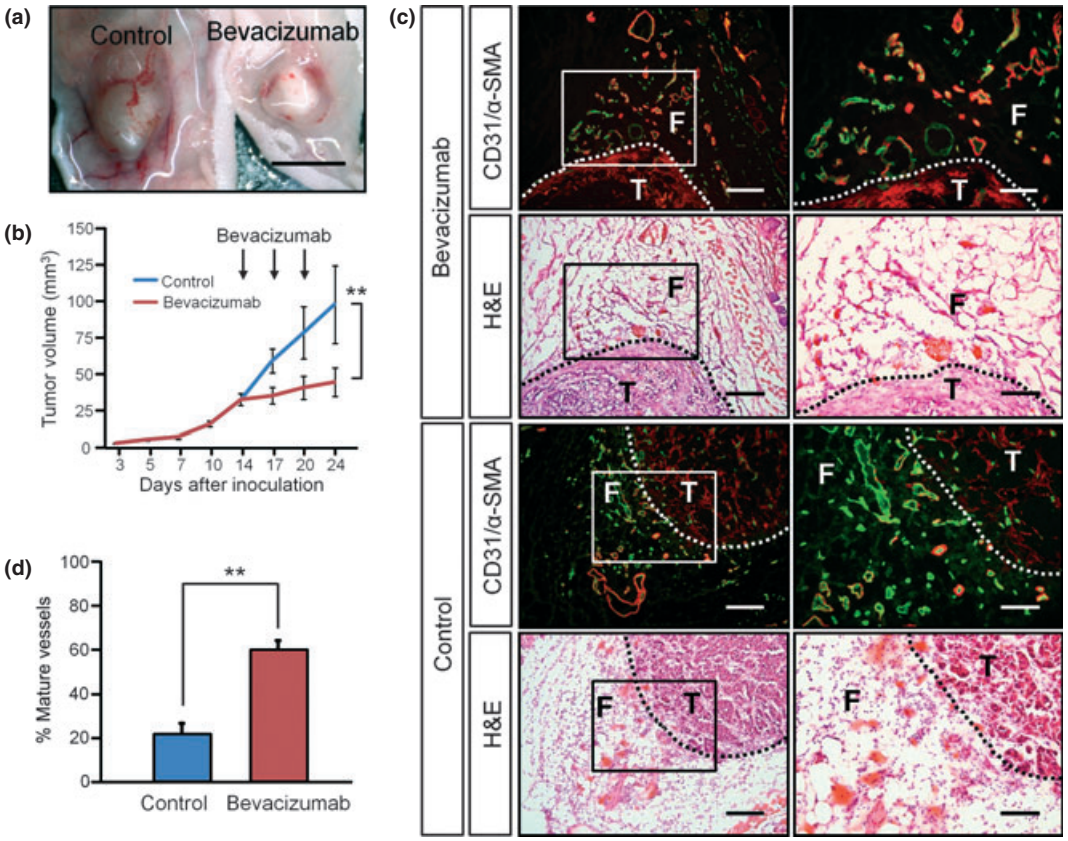

Fig. 2. Resistance of mature blood vessels in the fibrous cap to antiangiogenic therapy with anti-vascular endothelial growth factor (VEGF) neutralizing antibody. HT29 tumor cells were inoculated subcutaneously into mice, which were commenced on bevacizumab treatment 2 weeks later. (a) Gross appearance of the tumor after bevacizumab treatment. Scale lines: 5 mm. (b) Tumor volumes in untreated mice or mice treated with bevacizumab. Bevacizumab was injected when indicated (black arrows). Data are the mean ± SEM tumor volume ($n > 7$). $^{**}P < 0.01$. (c) Tumor sections, including the fibrous cap surrounding the tumor, 24 days after tumor cell inoculation were stained with anti-CD31 mAb (green) and anti-α-smooth muscle actin (α-SMA) Ab (red) or H&E. Higher-power views of the areas indicated by the boxes in the left-hand panels are shown in the right-hand panels. Dotted lines show the border between the fibrous cap surrounding the tumor mass (F) and the tumor mass (T). Scale lines: 200 μm (left-hand panels); 100 μm (right-hand panels). (d) Quantitative evaluation of the percentage of mature blood vessels, in which endothelial cells were covered with mural cells, of total blood vessels in the fibrous cap. More than six random fields of view were evaluated. Data are the mean ± SEM. $^{**}P < 0.01$.

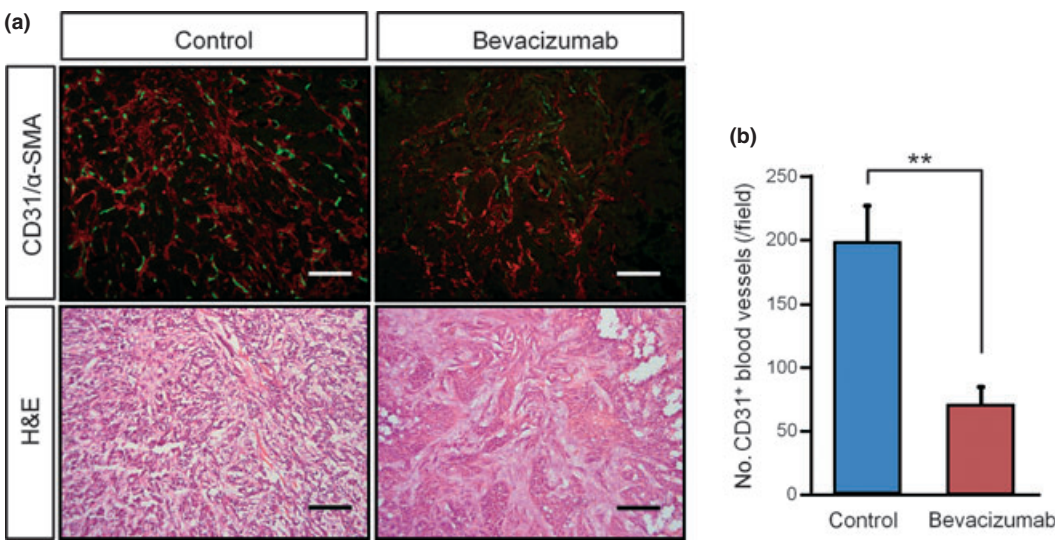

Fig. 3. Vascular density in the tumor parenchyma after bevacizumab treatment. (a) Tissue sections from the HT29 tumor mass in untreated mice or mice treated with bevacizumab were stained with anti-CD31 mAb (green) and anti-α-smooth muscle actin (α-SMA) Ab (red) or H&E. Scale lines: 200 μm. (b) Quantitative evaluation of the number of blood vessels in the tumor parenchyma. More than seven random fields of view were evaluated. Data are the mean ± SEM. $^{**}P < 0.01$.

(Fig. 2c). These dynamic phenotypic changes were not observed routinely in the tumor parenchyma (Fig. 3).

Because bevacizumab is specific inhibitor of human VEGF and does not neutralize murine VEGF[13] and, moreover, cells composing the fibrous cap are of murine origin, it is likely that human tumor cell-derived VEGF is involved in this blood vessel formation in mouse tissue (fibrous cap). Therefore, it is important to determine the degree to which tumor cell-derived VEGF drives vascular formation in the fibrous cap. To assess this, we used axitinib, a potent small molecule inhibitor for both human and mouse VEGFR and related receptor tyrosine kinases.

After 1 week treatment with axitinib, HT29 tumor growth was significantly reduced and, even more importantly, the volume of the tumor shrank such that is was less that the volume at the beginning of treatment (Fig. 4a,b). As observed in HT29 tumors treated with bevacizumab, immunohistological analysis of the fibrous cap after axitinib treatment revealed a decrease in immature vessels and an increase in the fraction of mature vessels:total blood vessels, from 28.6 ± 2.4% to 60.3 ± 5.0% after treatment (Fig. 4c,d). Shrunken vessels with reduced diameters were observed after axitinib treatment, similar to the observations after bevacizumab treatment (Fig. 4c). These data suggest that mature blood vessels in the fibrous cap are resistant to treatment with bevacizumab and other common angiogenesis inhibitors. Moreover, it is the tumor-derived VEGF that affects blood vessel formation in the fibrous cap, because attenuation of immature blood vessels was not significantly different following treatment with inhibitors specific for human, but not mouse, VEGF or inhibitors blocking both human and murine VEGFR.

Next, to confirm that these changes in vascularity in the fibrous cap are not specific to this one tumor cell line, we inoculated Colo320DM tumor cells into mice and treated them with bevacizumab. Three injections of bevacizumab suppressed tumor growth, as was observed with HT29 cells (Fig. 5a). On Day 24 after tumor cell inoculation, the vasculature of the fibrous cap was evaluated. As expected, the number of immature vessels was decreased and the ratio of mature vessels to total blood vessels increased significantly from 17.6 ± 2.0% to 53.0 ± 5.6% (Fig. 5b,c). The mature blood vessels also tended to be less dilated. Together, these results suggest that treatment with antiangiogenic agents not only affects the vasculature in the tumor parenchyma, but also the abnormal vasculature surrounding the tumor mass (i.e. causing a reduction of immature vessels and an induction of the mature squashed vascular phenotype).

Improvement in blood vessel function in the fibrous cap following treatment with bevacizumab. We next evaluated whether the increased numbers of blood vessels covered by MCs in the fibrous cap resulted in any functional changes. First, we performed vascular permeability assays with dextran. Although the fibrous cap is not composed of tumor tissue, the permeability of the blood vessels in it was significantly increased (Fig. 6a). After treatment with bevacizumab, this

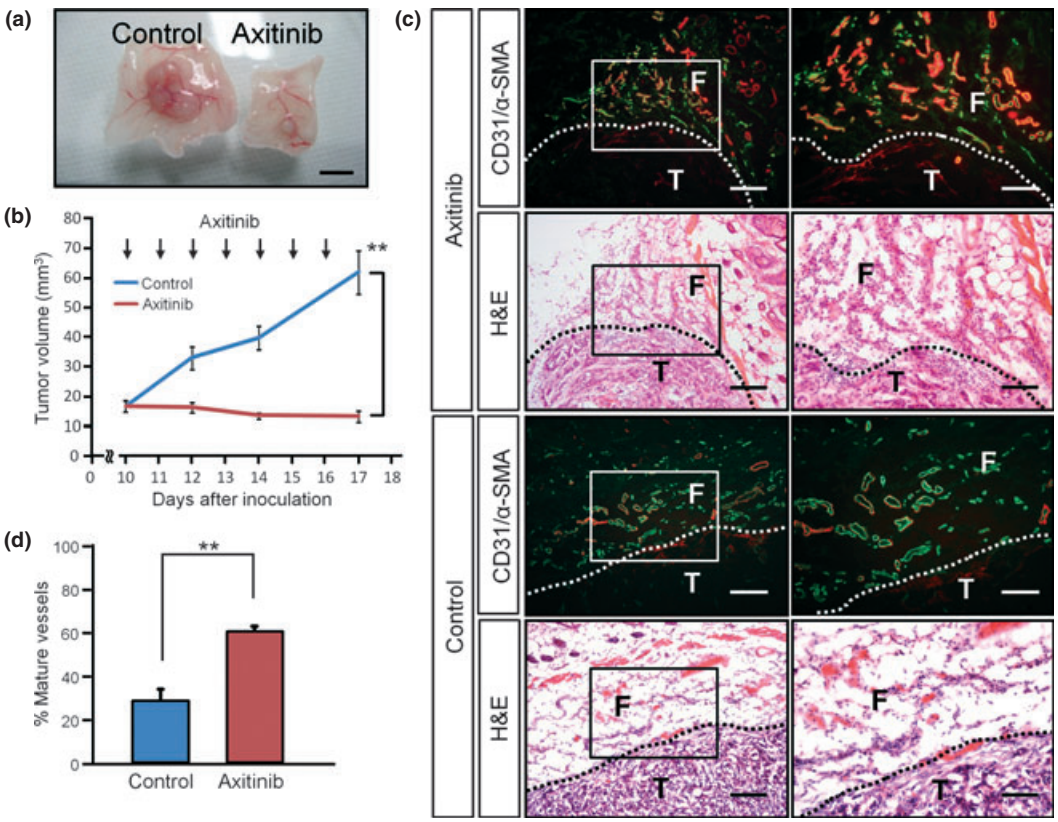

Fig. 4. Resistance of mature blood vessels in the fibrous cap after treatment with the potent, small molecule vascular endothelial growth factor receptor inhibitor, axitinib. HT29 tumor cells were inoculated subcutaneously into mice, which were commenced on axitinib treatment 10 days later. (a) Gross appearance of the tumors after treatment. Scale line: 5 mm. (b) Axitinib was injected as indicated (black arrows). Data are the mean ± SEM tumor volume ($n > 9$). $^{**}P < 0.01$. (c) Tumor sections, including the fibrous cap surrounding the tumor, 17 days after tumor cell inoculation were stained with anti-CD31 mAb (green) and anti-α-smooth muscle actin (α-SMA) Ab (red) or H&E. Higher-power views of the areas indicated by the boxes in the left-hand panels are shown in the right-hand panels. Dotted lines show the border between the fibrous cap surrounding the tumor mass (F) and the tumor mass (T). Scale lines: 200 μm (left-hand panels); 100 μm (right-hand panels). (d) Quantitative evaluation of the percentage of mature blood vessels, in which endothelial cells were covered with mural cells, of total blood vessels in the fibrous cap. More than seven random fields of view were evaluated. Data are the mean ± SEM. $^{**}P < 0.01$.

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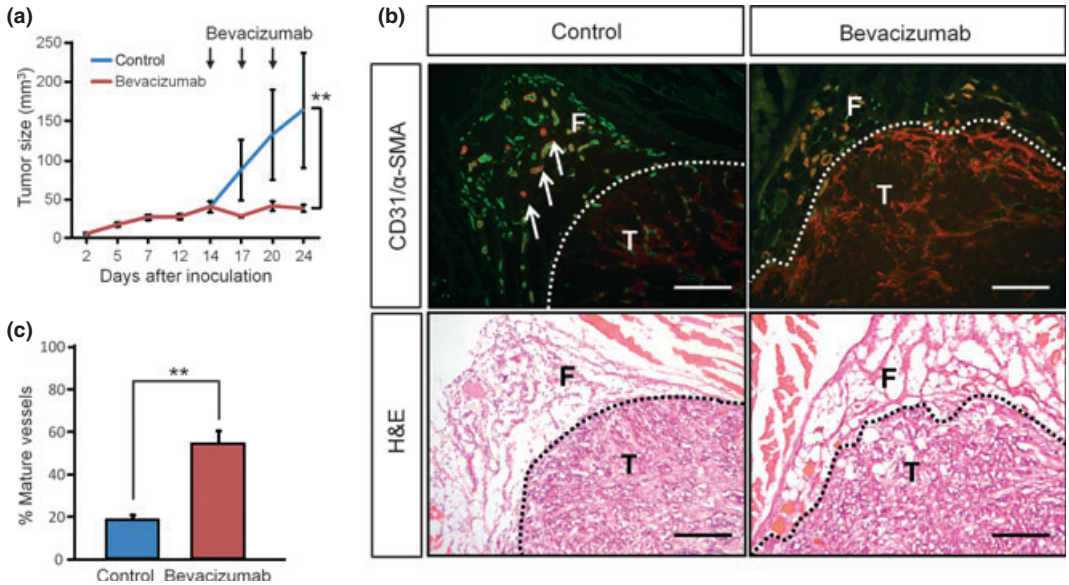

Fig. 5. Mature blood vessels in the fibrous cap of Colo320DM tumors show resistance to bevacizumab. Colo320DM tumor cells were inoculated subcutaneously into mice, which were commenced on bevacizumab treatment 2 weeks later. (a) Bevacizumab was injected as indicated (black arrows). Data are the mean ± SEM tumor volume ($n > 5$). $^{**}P < 0.01$. (b) Tumor sections, including the fibrous cap surrounding the tumor, 24 days after tumor cell inoculation were stained with anti-CD31 mAb (green) and anti-α-smooth muscle actin (α-SMA) Ab (red) or H&E. Dotted lines show the border between the fibrous cap surrounding the tumor mass (F) and the tumor mass (T). Scale lines: 200 μm (c) Quantitative evaluation of percentage of mature blood vessels, in which endothelial cells were covered with mural cells, of total blood vessels in the fibrous cap. More than seven random fields of view were evaluated. Data are the mean ± SEM. $^{**}P < 0.01$.

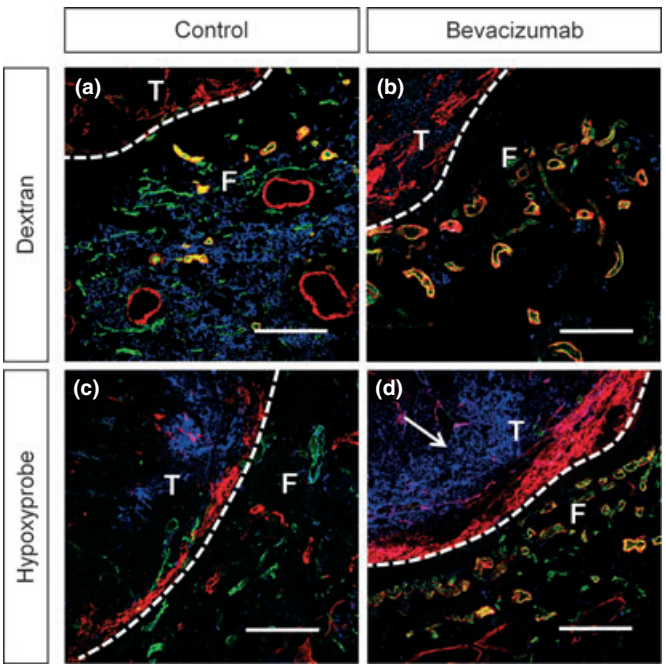

Fig. 6. Improvement in blood vessel function following bevacizumab treatment. (a,b) Microscopic images of FITC-dextran (blue) angiography and the vasculature (CD31, green; α-smooth muscle actin (α-SMA), red) of the fibrous cap from the control (a) and bevacizumab-treated (b) groups. (c,d) Hypoxia in the fibrous cap was detected by immunofluorescence staining of pimonidazole (blue), triple-stained with anti-CD31 mAb (green) and anti-α-SMA mAb (red). The arrow indicates a hypoxic area observed in the tumor parenchyma. Scale lines: 200 μm.

dextran extravasation decreased to a level comparable to that in normal tissue (Fig. 6b). These results indicate that the vasculature in the fibrous cap is influenced by VEGF secreted from the tumor cells and is functionally abnormal. Therefore, administration of bevacizumab not only normalizes blood vessels in the tumor parenchyma, but also in the fibrous cap. Moreover, it has been reported that treatment with bevacizumab induces hypoxia in the tumor parenchyma;[14] therefore, we evaluated the oxygenation level in the fibrous cap. Without bevacizumab treatment, the fibrous cap was not hypoxic (Fig. 6c) but, unlike the tumor parenchyma, which became hypoxic after treatment, the oxygenation level of the fibrous cap did not change after treatment (Fig. 6d).

Discussion

In the present study, we found that most blood vessels in the fibrous cap that formed at the tumor rim were immature, lacking MC coverage of the ECs. Upon treatment with angiogenesis inhibitors, these immature blood vessels were destroyed and only blood vessels covered with MCs remained. Moreover, despite MC coverage, dilated blood vessels were characteristically less dilated after treatment with angiogenesis inhibitors. These responses of blood vessels to angiogenesis inhibitors were quite similar to those observed in the parenchyma of the tumor itself. Therefore, these observations suggest that the fibrous cap is an environment mimicking the tumor microenvironment. Recently, it was reported that treatment with angiogenesis inhibitors induces normalization of tumor vascular function.[15] Indeed, in the present study the permeability of the blood vessels in the fibrous cap was significantly increased, as in the tumor parenchyma. After bevacizumab treatment, we found that the permeability of the vasculature in the fibrous cap was decreased. This functional change to the vasculature in the fibrous cap seems to be caused by the destruction of immature blood vessels not covered by MCs. However, when a hypoxic situation was established, hypoxia was not induced in the fibrous cap, suggesting a different mechanism underlying oxygenation in the fibrous cap compared with that in the tumor parenchyma. At present, the reasons underlying these differences in leakiness and hypoxia between the fibrous cap and the tumor parenchyma are

not known and further analysis of oxygenation in the fibrous cap is required.

It is interesting to know how angiogenesis inhibitors affect vascular maturation in the fibrous cap. Previously, we reported that hematopoietic stem cells (HSCs) promote angiogenesis,[16] that c-Kit$^+$ hematopoietic stem/progenitor cells accumulate around the edge of the tumor,[17] and that HSCs differentiate into MCs through a CD45$^+$ CD11b$^+$ stage.[18] Moreover, another group has reported that bone marrow-derived α-SMA-positive fibroblasts promote tumor growth.[19] Therefore, it is possible that bone marrow-derived cells more effectively contribute to the maturation and coverage of blood vessels in the fibrous cap after treatment with angiogenesis inhibitors.

Previous studies have suggested that well-matured blood vessels are sheathed in ECM and that cancer cells migrate along tracks made of ECM collagen fibers.[20] In terms of tumor invasion, it has been suggested that antiangiogenic therapy may be involved in this process.[21] Vascular remodeling induced by angiogenesis inhibitors leads to a more hypoxic tumor microenvironment, which results in enhanced tumor cell invasion into normal tissue.[22] Mature vessels are fully covered by ECM and it is therefore possible that tumor cells invade into the normal tissues along these ECM-rich vessels that are induced by angiogenesis inhibitors. In addition, regarding lymphatic metastasis, development of lymphatic vessels in the fibrous cap must be involved in the process. Further analysis of lymphangiogenesis in the fibrous cap is required.

How to destroy mature vessels may be the next task for treating cancer after the induction of vascular maturation by angiogenesis inhibitors. Moreover, the relationship between cancer stem cells and the fibrous cap after antiangiogenic therapy remains of great interest. We have reported that malignant cancer cells exhibiting high tumorigenic and metastatic ability locate in the vascular region at the edge of the tumor.[23] Therefore, a drug delivery system that targets mature vessels only in the tumor rim, and not in normal tissue, will be required in future to destroy vascular niches for malignant cancer cells.

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Disclosure Statement

The authors have no conflict of interest.

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