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Prevention of cancer recurrence in tumor margins by stopping microcirculation in the tumor and tumor–host interface

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Key words

Combretastatins, radiotherapy, tumor blood flow, tumor vessels, viable rim

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Combretastatins interrupt blood flow of solid tumor vascular networks and lead to necrosis by blocking nutrients. However, tumors recover from tumor blood flow interruption-induced damage and develop viable rims. To investigate why cancer recurs and its prevention, we used a combretastatin derivative, Cderiv (=AC7700), and analyzed changes in tumor–host interface (T-HI) vessels, which were closest to cancer cells in the tumor margin after tumor vessel disruption, and the microenvironment surrounding them. Treatment with Cderiv (10 mg/kg) interrupted tumor blood flow in all regions of LY80 (a variant of Yoshida sarcoma) tumor, but not T-HI vessel blood flow. The same Cderiv dose given 72 h after 5 Gy irradiation stopped T-HI vessel blood flow and prevented cancer recurrence. Treatment in the reverse order, however, did not affect T-HI vessel blood flow. The greatest difference between the two treatments was the occurrence of gradual T-HI edema with the former. Severe T-HI edema compressed T-HI blood vessels, so that circulation stopped. Thus, the distance between a tumor margin and its nearest functioning host vessel became much larger, and the tumor marginal region became a microenvironment that lacked a nutritional supply. Cancer cells in tumor margins received nutrients through two circulation routes: tumor vessels and T-HI vessels. Our starvation methods, which involved treatment with Cderiv 72 h after 5 Gy irradiation, blocked both circulation routes and may have great potential as a clinical strategy to prevent cancer recurrence.

Combretastatins[1] are vascular disrupting agents (VDAs), a new category of cancer therapeutic drugs.[2–4] In attempts to find clinical applications, many derivatives have been synthesized.[5–8] These compounds irreversibly interrupted tumor blood flow (TBF) at about one-fifth of the maximum tolerated dose and induced necrosis in almost all tumor tissue areas.[9–14] One combretastatin derivative, Cderiv (=AC7700 = ombrabulin), had particularly effective TBF interruption, but without the severe bone marrow toxicity or cumulative toxicity of cytotoxic anticancer drugs.[15] Combretastatins have thus attracted attention as agents to be used to starve solid tumors.

However, despite combretastatins' strong destructive effects on tumors, cancer cells often regrow in tumor margins and form viable tumor rims (i.e., recurrences).[3,13,16] One common feature of VDAs is that a viable rim occurs in tumor margins after treatment.[17–20] To improve VDA treatment efficacy, we must clarify the cause of cancer recurrence in such regions and prevent it. The reason that such viable rims occur involves two possibilities, related to a tumor's nutritional supply.[3,18] One possibility is an inherent difference in responses of the tumor periphery and those of the bulk of the tumor.[21] Vascular disrupting agent-induced TBF interruption may be strong in the intratumoral region but weak in the peritumoral region, so cancer cells growing in the latter region do not die. Another possibility is a difference in the blood supply resources of each location: cancer cells in the intratumoral region receive blood by tumor vessels alone, whereas cells in the peritumoral region are supplied by both tumor vessels and host vessels.[22] To date, no direct evidence indicates which possibility is primary, so we must clarify this issue to prevent recurrences.

We recently found that Cderiv inhibited cancer recurrences in tumor margins when given after irradiation.[23] Conversely, irradiation after Cderiv treatment did not inhibit cancer recurrence. We hypothesized that Cderiv treatment used after irradiation results in the tumor–host interface (T-HI) becoming a microenvironment in which cancer cells at tumor margins cannot grow, compared with irradiation given after Cderiv treatment or with Cderiv used alone. We therefore decided to clarify changes in these microenvironments that occur after these treatments.

We establish here that the TBF response to Cderiv did not differ in intratumoral or peritumoral regions and that cancer cells growing in tumor margins (outermost peritumoral regions) received nutrients through T-HI vessels even if tumor vessels were completely destroyed. In addition, we show that to prevent such recurrences, we must stop circulation in both tumor vessels and T-HI vessels.

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Materials and Methods

Complete materials and methods are described in Data S1.

Rats and tumor. Male Donryu rats were used. Animal care and all experimental procedures were approved by the Committee on the Ethics of Animal Experiments at Tohoku University (Sendai, Japan). The tumor cell line used was LY80.

Anesthesia. All animal experiments were carried out under anesthesia.

Vascular disrupting agent. We used combretastatin A-4 derivative Cderiv (=AC7700) as a VDA.

Combination of Cderiv and X-irradiation. In this study, 5 Gy72hCderiv indicates the group receiving i.v. Cderiv 72 h after 5 Gy irradiation; Cderiv72h5 Gy indicates the group receiving 5 Gy irradiation 72 h after i.v. Cderiv.

Tumor blood flow measurement. The hydrogen clearance method was used to measure TBF.[14]

Implantation of transparent chambers. To directly observe changes in tumor vessels and measure extravasation of sodium fluorescein (fluorescein sodium; Tokyo Chemical Industry Co., Tokyo, Japan), we implanted transparent chambers into the dorsal skin flaps of rats.[24]

Extravasation of fluorescein sodium and its half-life in tissue. To evaluate extravasation of fluorescein sodium into tumors and its clearance, we measured maximum fluorescence intensity, time to reach maximum intensity, and the half-life of fluorescence intensity.[25]

Quantification of changes in T-HI edema. We separated a tumor from the edema surrounding it with ophthalmic scissors and calculated the weight ratio.

Detection of T-HI edema and recurrent tumor foci by MRI. To produce *in vivo* images of T-HI regions after 5Gy72hCderiv and Cderiv72h5Gy treatments, we carried out MRI with a 7.0-T Bruker PharmaScan system (Bruker Biospin, Ettlingen, Germany).

Histology. Histological sections were stained by H&E.

Electron microscopy. Electron microscopic observation was carried out using a JEM-1400 electron microscope (JEOL, Tokyo, Japan).

Immunoblotting. The supernatant from tissue fragments was obtained by the Laemmli method.[26] For immunoblotting, separated proteins were electrophoretically transferred to PVDF membranes as described by Towbin *et al.*,[27] incubated with HRP-conjugated affinity-purified sheep IgG antibody against rat serum albumin, visualized by a chemiluminescence method, and analyzed using ImageQuant (GE Healthcare, Chalfont St Giles, UK).

Statistical analysis. All results are means ± SD. *P*-values of 0.05 or lower were significant.

Results

Tumor blood flow interruption 72 h after irradiation completely prevents cancer recurrence in tumor margins, unless the interruption is inadequate. Figure 1(a) illustrates the experimental schedule. Figure 1(b) shows the therapeutic effect of 5 Gy irradiation plus 10 mg/kg Cderiv. The 5Gy72hCderiv group alone had complete tumor regression. Figure 1(c) provides the change in tumor volume for days 12–16 in the 5Gy72hCderiv group. Although tumor volume increased greatly at 24 h after TBF interruption, this increase was transient, then tumors shrank rapidly for 48–72 h. The body weights in the two groups did not differ significantly (Fig. 1d), so the combined treatments did not burden the rats. Evaluation of tumors removed from rats in each group at the end of the experiment (Fig. 1e) showed that all tumors in the 5Gy72hCderiv group had turned yellow, and no tumors had viable cells. Viable rims did not occur in this group (Fig. S1). In addition, when TBF was stopped after fractionated irradiation (2 Gy irradiation/day for 4 days), tumors had a transient increase in volume and thereafter rapidly regressed (Fig. S2a), just as for TBF interruption after a single 5-Gy irradiation dose. Tumor blood flow of non-irradiated tumors decreased gradually with tumor growth. Tumor blood flow of tumors receiving fractionated irradiation did not decrease, however, and tumor growth was significantly inhibited ($P < 0.0001$) (Fig. S2b). Figure 1(f) shows the therapeutic effect of 5 Gy irradiation plus 3 mg/kg Cderiv. With 3 mg/kg Cderiv, unlike 10 mg/kg Cderiv, even tumors in the 5Gy72hCderiv group regrew. We also analyzed TBF changes during 8 h after treatment with 0.9% NaCl, 3 mg/kg Cderiv, or 10 mg/kg Cderiv at 72 h after 5 Gy irradiation (Fig. 1g). In the group receiving 10 mg/kg Cderiv, TBF completely stopped 1 h later and did not recover. In the group receiving 3 mg/kg Cderiv, TBF decreased to 9.6 ± 7.7% 2 h later. However, 8 h later, TBF recovered to 52.8 ± 24.4%.

Some cancer cells survive along tumor margins after TBF interruption by Cderiv and form a viable rim. Cancer regrowth in tumor margins often occurred in the group given Cderiv alone. Figure 2(a) shows the tumor at 55 h after Cderiv treatment. Tumor degenerated, and all tumor blood vessels disappeared. The T-HI area, however, contained many relatively large blood vessels with circulatory functions. Figure 2(b), a typical histological section, shows the T-HI at 55 h after Cderiv treatment. The tumor became completely necrotic, but several cancer cells survived at the tumor–host tissue boundary. Figure 2(c) shows tumor tissue at 96 h after Cderiv treatment; Figure 2(d,e) shows enlargements of the boxed areas. All inner regions of the tumor remained necrotic, but cancer cells regrew at tumor margins and formed a viable rim (Fig. 2c).

Fine structure of microvessels 24 h after Cderiv treatment. As Figure 2 illustrates, all tumor vessels lost circulatory functions approximately 24 h after 10 mg/kg Cderiv treatment, but the function of vessels in the T-HI remained intact. To elucidate how the fine structure of microvessels changed at that time, we observed, using electron microscopy, vessels in different regions: a tumor region that was more than 200 μm away from the tumor boundary (Fig. 3a-I); a tumor margin within 200 μm of the boundary (Fig. 3a-II); a non-tumor region within 200 μm of the boundary, that is, the T-HI (Fig. 3a-III); and an s.c. region that was more than 200 μm away from the tumor boundary (Fig. 3a-IV). We obtained the typical microvessels in Figure 3(b) from the regions shown in Figure 3(a). In tumors, lumens of vessels in both the inner region (Fig. 3b-I) and the marginal region (Fig. 3b-II) disappeared after Cderiv treatment, and endothelial cells were in direct contact with residual erythrocytes. In contrast, lumens of vessels in the T-HI (Fig. 3b-III) and subcutis (Fig. 3b-IV) did not close. These observations support the vital microscopic findings that Cderiv stopped tumor blood circulation but did not stop blood flow in non-tumor blood vessels. Endothelial damage was more prominent in tumor vessels in the inner region (Fig. 3b-I) compared with tumor vessels in the marginal region (Fig. 3b-II). Tumor vessels in the inner region had many endothelial cells with gaps, deformed endoplasmic reticulum, and debris within the cytoplasm. These findings indicate that severe tumor vessel degradation occurred in the inner

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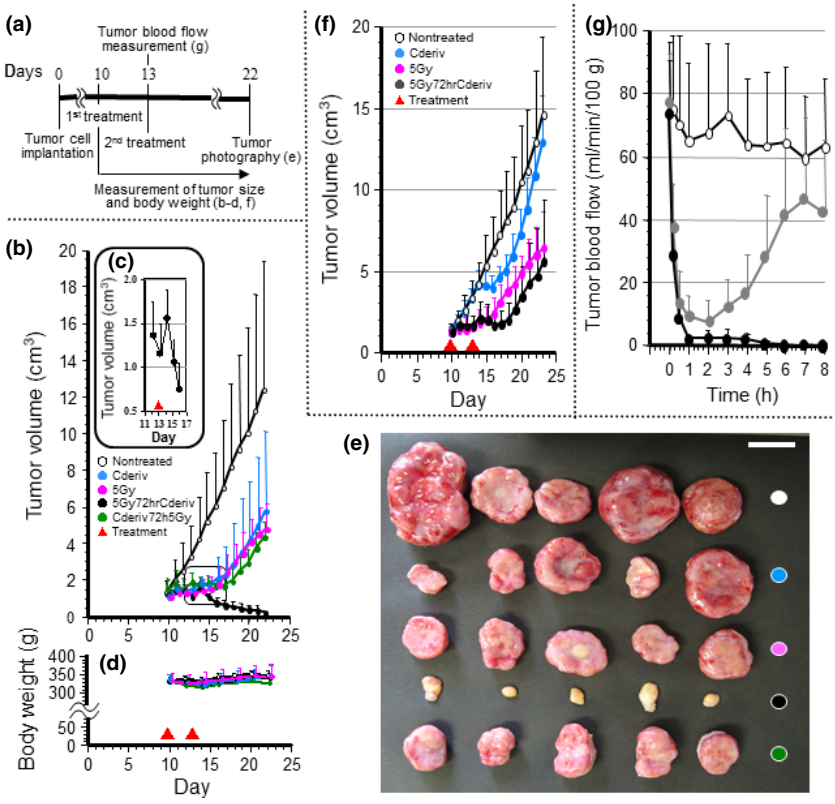

Fig. 1. Prevention of cancer recurrence in tumor margins by complete tumor blood flow (TBF) interruption at 72 h after 5 Gy irradiation. (a) The experimental schedule. (b) Effects of combination therapy (5 Gy + 10 mg/kg combretastatin derivative [Cderiv]) on s.c. LY80 tumors. $n = 5$, each group. (c) Enlarged view for the group receiving i.v. Cderiv 72 h after 5 Gy irradiation (5Gy72hCderiv) 2–6 days after the second treatment. (d) Body weight change after treatments. Red triangles, treatments. (e) Tumors in each group at 12 days after the first treatment. Scale bar = 2.0 cm. (f) Effects of 5 Gy + 3 mg/kg Cderiv on s.c. LY80 tumors. $n = 5$, each group. (g) Tumor blood flow change after different doses of Cderiv given after 5 Gy irradiation. Black circles, 10 mg/kg Cderiv ($n = 8$); gray circles, 3 mg/kg Cderiv ($n = 10$); white circles, 0.9% NaCl solution ($n = 8$). Cderiv was given at 0 h. Cderiv72h5 Gy, group receiving 5 Gy irradiation 72 h after i.v. Cderiv.

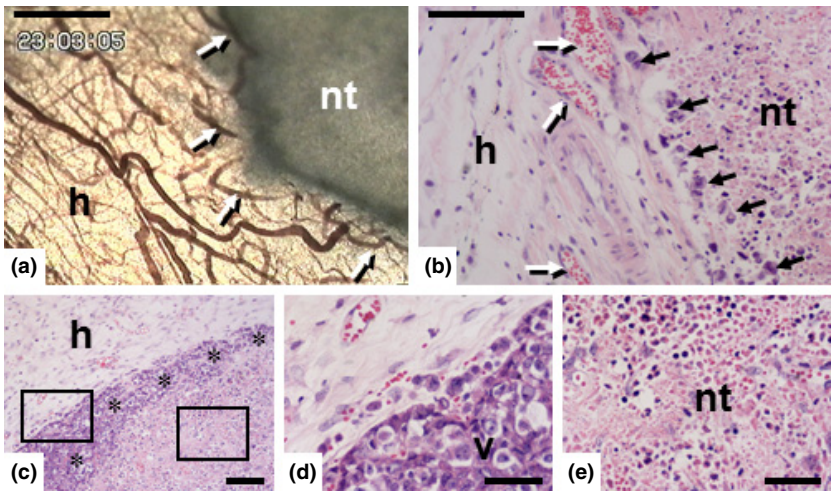

Fig. 2. Progression of a viable rim of recurrent cancer cells at a tumor margin after tumor blood flow interruption. (a) Vital microscopic findings of a tumor in a transparent chamber at 55 h after 10 mg/kg combretastatin derivative (Cderiv) treatment. h, host; nt, necrotic tumor; white arrows, tumor–host interface vessels. Scale bar = 50 μm. (b) Histology. Black arrows, residual cancer cells. Scale bar = 100 μm. (c) Viable rim at 96 h after 10 mg/kg Cderiv treatment. Asterisks, viable rim. Scale bar = 100 μm. (d) Magnified image of (c), left boxed area. v, viable cancer cells. Scale bar = 50 μm. (e) Magnified image of (c), right boxed area. nt, necrotic tumor. Scale bar = 50 μm.

tumor region. A characteristic finding for vascular endothelial cells in the T-HI was the presence of a large number of vesiculo-vacuolar organelles (VVOs) in the cytoplasm (Fig. 3b-III). The VVO is an intracellular organelle involved in endocytosis.[28]

Tumor blood flow response to Cderiv does not differ in intratumoral and peritumoral regions. We investigated whether the TBF response to Cderiv differed in peritumoral and intratumoral regions. The peritumoral region includes the marginal area shown in Figure 3, which was defined at the microscopic level. Figure 4(a) shows the positional relationship between the two electrodes that were inserted vertically from the same point at the tumor surface. The dorsal skin thickness of 7-week-old rats used was 1.5 ± 0.1 mm ($n = 20$), thus the sensor part (1-mm tip) of an inserted electrode at the depth of 2.5 mm always included the tumor margin. Figure 4(b) shows TBF before treatment with 1 mg/kg Cderiv. Tumor blood flow values at 2.5 and 6.0 mm from the surface were 49.4 ± 16.5 and 27.2 ± 13.4 mL/min/100 g, respectively. Tumor blood flow at the 2.5-mm depth was significantly (paired t-test) higher than that at the 6.0-mm depth. Figure 4(c) shows the rate of change in TBF at different times after 1 mg/kg Cderiv treatment. The TBF at each time point was normalized to the TBF value before Cderiv. For 1 mg/kg i.v. Cderiv, the TBF of both regions decreased to 20% 1 h later and recovered to 70–75% 6 h later. The TBF response to Cderiv was the same in the different regions measured ($P = 0.9046$). Figure 4(d) shows TBF before 10 mg/kg Cderiv treatment. The TBF

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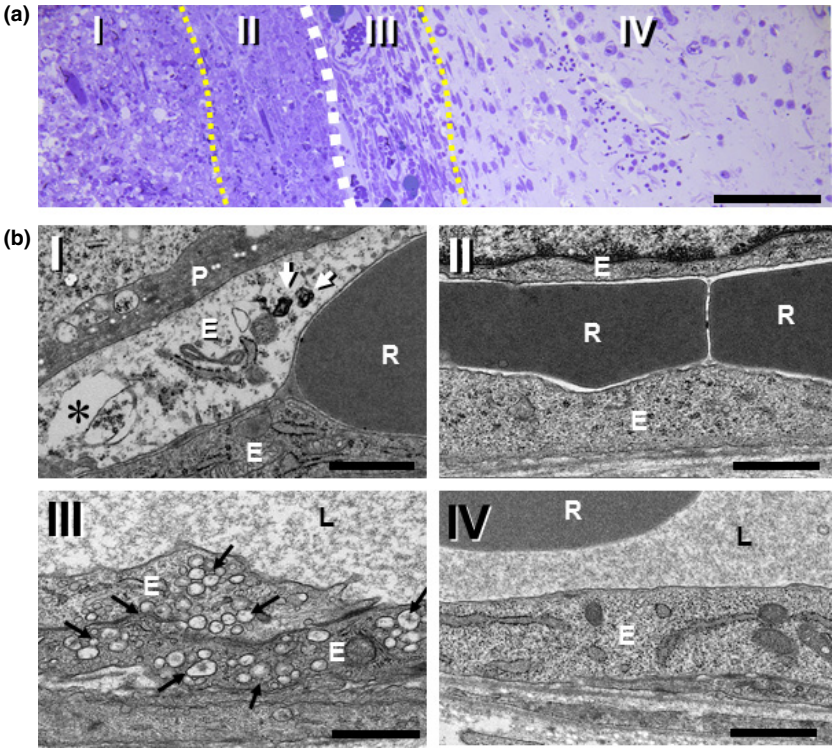

Fig. 3. Fine structure of microvessels at 24 h after 10 mg/kg combretastatin derivative (Cderiv) treatment. (a) Light microscopic images of tissue in and around the tumor–host interface (T-HI) in a LY80 tumor. White dashed line, tumor boundary. Scale bar = 200 μm. (I) Inner region; (II) marginal region; (III) T-HI; and (IV) s.c. tissue. (b) Electron microscopic images of typical endothelial cells (E) in a vessel in four regions. (I) Endothelial cells in the inner region. P, pericyte; R, red blood cell. Arrows, debris from intracellular organelles; asterisk, gap formed in the cytoplasm. (II) Endothelial cells in the tumor margin. (III) Endothelial cells in the T-HI. L, vascular lumen. Arrows, vesiculo-vacuolar organelles. (IV) Endothelial cells in s.c. tissue. Scale bar = 1 μm.

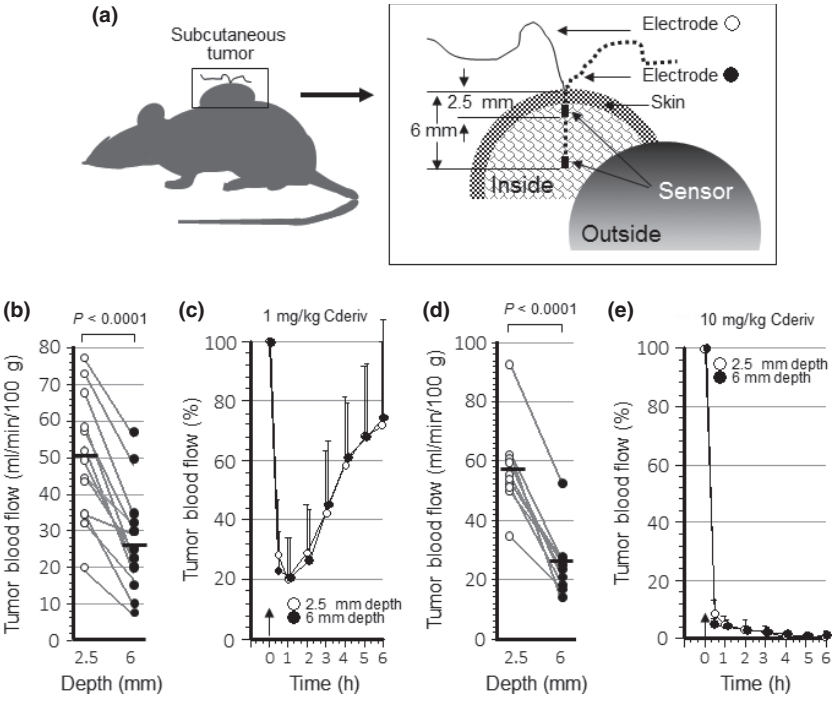

Fig. 4. Tumor blood flow (TBF) response in intratumoral and peritumoral regions to combretastatin derivative (Cderiv). (a) Positions of electrodes inserted into the tumor. Initial TBF (b) and its change after 1 mg/kg Cderiv treatment (c). Black circles, intratumoral region (n = 13); white circles, peritumoral region (n = 13). Lines of (b) connect corresponding regions of the same tumor. Cderiv was given at 0 h. Initial TBF (d) and its change after 10 mg/kg Cderiv (e). Black circles, intratumoral region (n = 10); white circles, peritumoral region (n = 10). Lines of (d) connect corresponding regions of the same tumor. Cderiv was given at 0 h.

values at 2.5 and 6.0 mm from the surface were 57.2 ± 14.8 and 25.8 ± 10.9 mL/min/100 g, respectively. The TBF at the 2.5-mm depth was significantly (paired t-test) higher than that at the 6.0-mm depth. Figure 4(e) shows the rate of change in TBF at different times after 10 mg/kg Cderiv treatment. For 10 mg/kg i.v. Cderiv, the TBF of both regions decreased to 5% or less at 1 h later and did not recover during the measurement period (6 h). The TBF response to 10 mg/kg Cderiv was the same despite the use of different measuring regions (P = 0.7747).

Extravasation of sodium fluorescein before and after TBF interruption, and movement of fluorescein to tumor marginal and intratumoral regions. To confirm hemodynamically that nutritional vessels contributing to cancer recurrence in tumor margins are T-HI vessels, not tumor vessels, we studied how transport of a substance into tumor tissues changed before and

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after Cderiv treatment. Figure 5(a) shows an LY80 tumor growing in a transparent chamber before Cderiv treatment. Tumor tissue lost circulation and became necrotic at 29 h after Cderiv treatment (Fig. 5b). Vessels in the T-HI and normal tissue remained intact, apparently because their circulatory functions were not destroyed. However, T-HI vessels were the most permeable, and the fluorescence intensity of sodium fluorescein around those vessels peaked within 5 min of treatment (Fig. 5c,d). Before Cderiv treatment, sodium fluorescein appeared throughout the tumor at a high concentration, because all tumor vessels functioned (Fig. 5c). In contrast, transport of sodium fluorescein into necrotic regions markedly decreased, because tumor vessels had been destroyed (Fig. 5d). Regions around T-HI vessels received high concentrations of sodium fluorescein even after Cderiv treatment, however, because T-HI vessel blood flow was not blocked (Fig. 5d). Figure 5(e) presents a typical analysis of sodium fluorescein movement into a tumor region in which TBF was interrupted. A functioning vessel in a T-HI region was chosen, and four points on a straight line were selected from the vessel toward the necrotic tumor area. As the distance from the T-HI vessel increased, the maximal value of fluorescence intensity decreased, and the time to reach this maximal value lengthened. In addition, half-life values for sodium fluorescein in each region were 15.3, 31.0, 40.3, and 48.6 min, respectively. Thus, the half-life was longer in proportion to the distance. Figure 5(f–h) provides results of the analysis of the relationship between the distance from a vessel to the tissue and circulatory parameters at 30 regions in three transparent chambers. As the distance from a functioning blood vessel increased, the amount of fluorescent dye reaching the tissue decreased (Fig. 5f, $r = -0.7693$), and the time to reach maximal fluorescence intensity increased (Fig. 5 g, $r = 0.9498$). The half-life increased in proportion to the distance from a vessel (Fig. 5 h, $r = 0.7805$). Figure 5(i,j) presents results for tumors that had no Cderiv treatment. Such tumors had extremely high vascular density, and the distance from any point in the tissue to the nearest vessel was, at most, 60 μm. The distance from a vessel

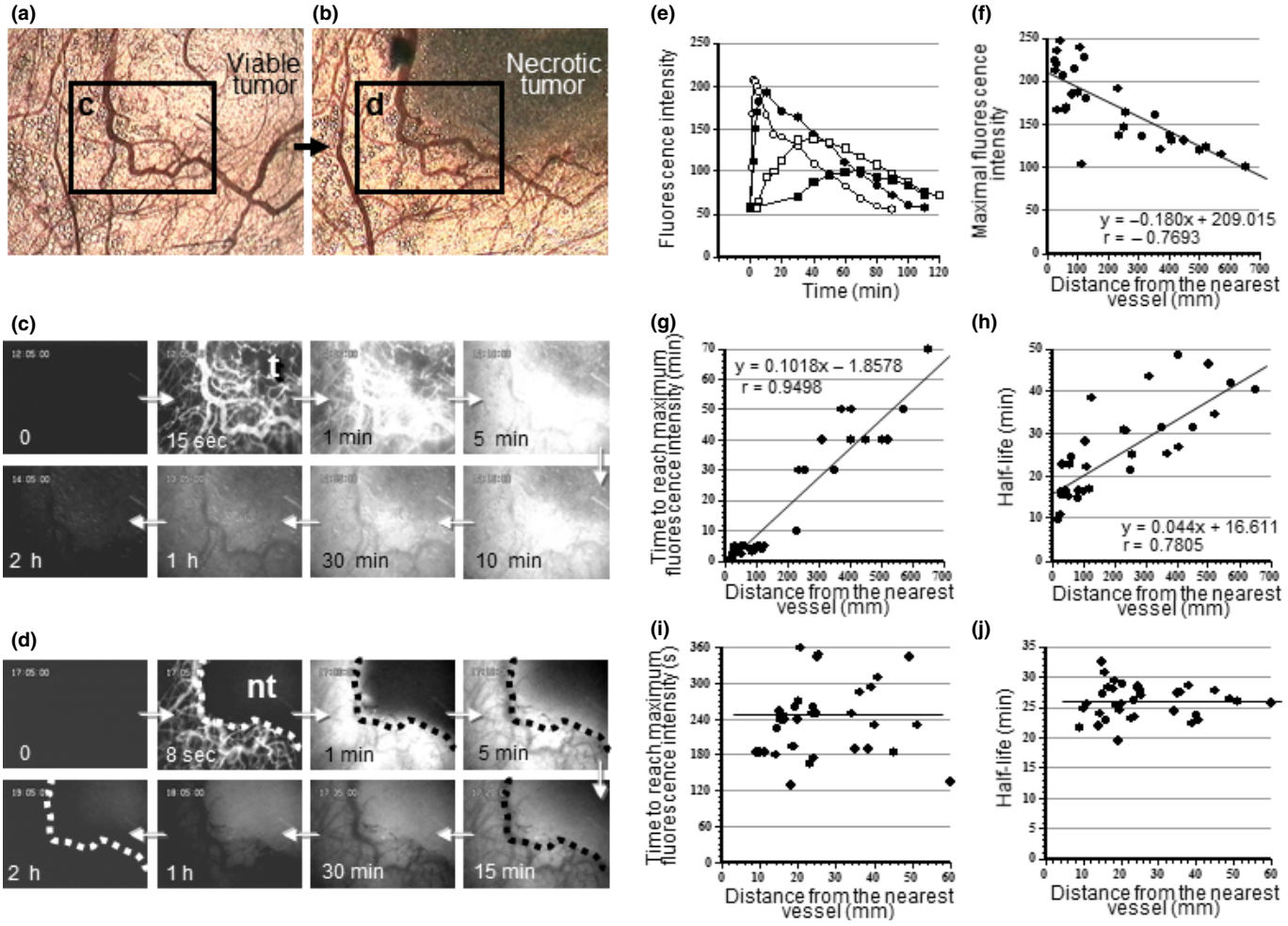

Fig. 5. Transport of sodium fluorescein into tissues around the tumor–host interface before and after 10 mg/kg combretastatin derivative (Cderiv) treatment. (a) A tumor growing in the transparent chamber. (b) At 29 h after 10 mg/kg Cderiv treatment. (c) A representative of extravasation of sodium fluorescein (1 mg/kg) before Cderiv treatment. The observation area is the black rectangle in (a). t, viable tumor; arrows, direction of time. (d) A representative of transport of sodium fluorescein (1 mg/kg) into necrotic tumor (nt) after Cderiv treatment. The observation area is the black frame in (b). Dashed line, tumor boundary; arrows, direction of time. (e) Representative changes in maximal fluorescence intensity, time to reach maximal fluorescence intensity, and half-life over time. The distance from a vessel to a tissue: black circles, 230 μm; black squares, 650 μm; white circles, 50 μm; white squares, 400 μm; (f–h) Maximal fluorescence intensity, time to reach maximal intensity, and half-life (washout of sodium fluorescein from tissues), respectively, related to distance from a vessel to tissues. (i, j) Time to reach maximal fluorescence intensity and half-life, respectively, did not relate to distance from a vessel to tissues in tumor tissues without Cderiv treatment.

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and each parameter showed no correlation. In 35 regions in four transparent windows, the average time to reach to maximal fluorescence intensity and average half-life were 3.9 ± 1.0 and 26.0 ± 2.8 min, respectively. That is, in a tumor tissue with abundant blood vessels, circulation parameters were almost constant in any region.

Treatment with Cderiv 72 h after irradiation induces substantial T-HI edema fluid, which contains much albumin. Figure 6(a) presents the experimental schedule. Figure 6(b,c) shows a change in the weight ratio of tumor-to-interface edema before (0 h) and after (12, 24, 48, and 72 h) the second treatment, for Cderiv72h5 Gy and 5 Gy72hCderiv, respectively. Slight edema initially occurred in the interface of LY80 solid tumors. For the Cderiv72h5 Gy group (10 mg/kg Cderiv), the interface edema tended to decline and reached a minimum after 48 h, although the decrease was not significant (Fig. 6b). In contrast, for the 5Gy72hCderiv group, T-HI edema significantly increased at 24 h. However, edema almost disappeared 72 h later, and the weight ratio returned to the pretreatment level (Fig. 6c). To visualize T-HI edema, MRI was carried out every 24 h for 72 h after the second treatment in the same tumor-bearing rats. Figure 6(d,e) provides typical T2-weighted images of a tumor from the Cderiv72h5Gy and 5Gy72hCderiv groups, respectively. For the MRI parameters here, water was reflected in pure white (Fig. S3). The pure white layer surrounding a tumor indicated by arrows in Figure 6(e-II) is therefore T-HI edema containing a large amount of water. The asterisks in Figure 6(d) clearly show recurrent foci, because we had correlated macroscopic findings with images from MRI by i.v. lissamine green given to tumor-bearing rats after the experiment (Fig. S3).

We next determined the major protein in the edema fluid. At 24 h after 5Gy72hCderiv treatment, we removed edema fluid in the T-HI, obtained an extract of the fluid, and carried out

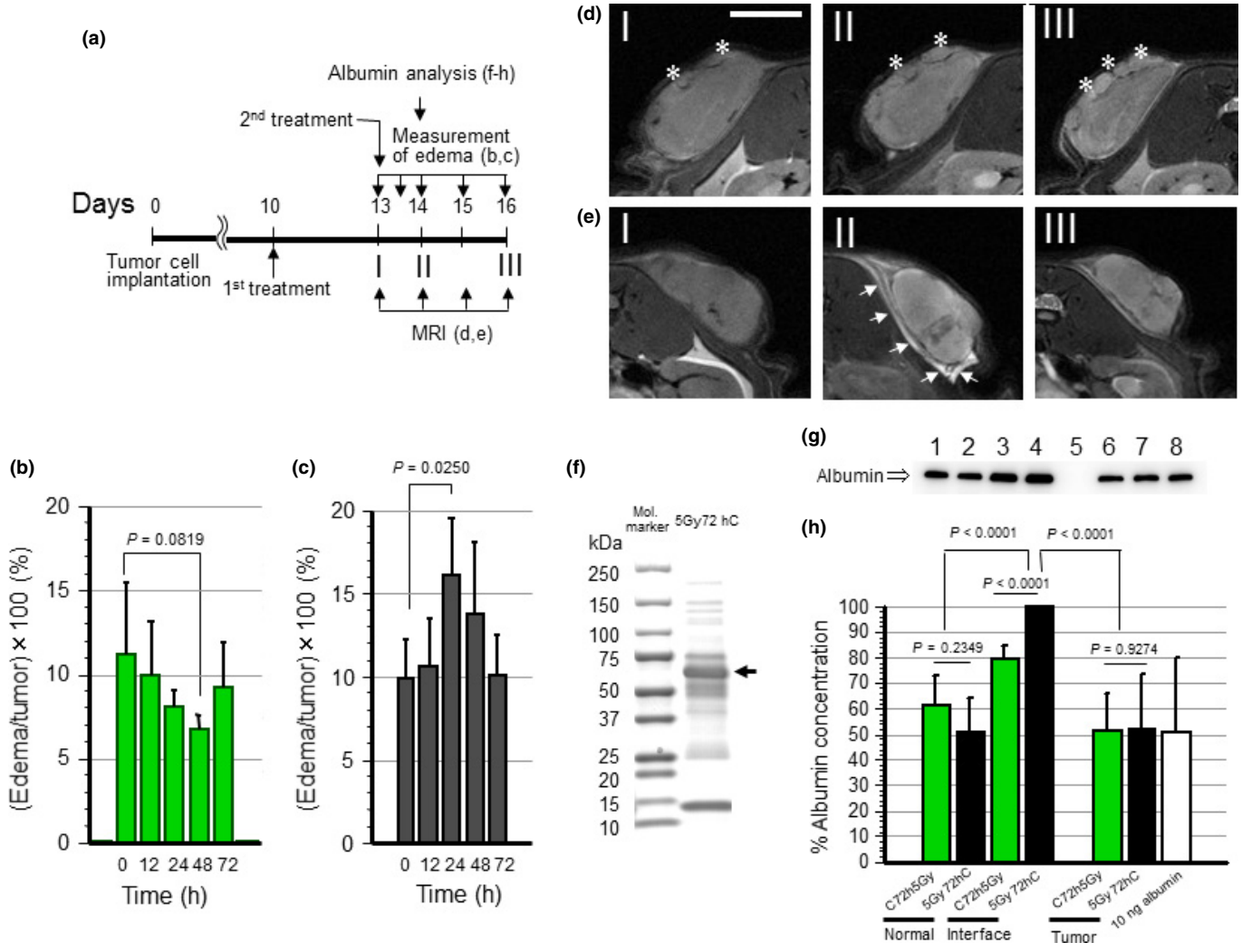

Fig. 6. Tumor–host interface (T-HI) edema containing a large amount of albumin induced by i.v. combretastatin derivative (Cderiv) given 72 h after 5 Gy irradiation **(**5 Gy72hCderiv). (a) The experimental schedule. Cderiv dose, 10 mg/kg. (b, c) Time course of edema in T-HI in treatment groups receiving 5 Gy irradiation 72 h after i.v. Cderiv (Cderiv72h5 Gy) and 5 Gy72hCderiv. $n = 5$ for each time point. (d) MRI over time after Cderiv72h5 Gy treatment. (I) Before 5 Gy irradiation; (II) 24 h after irradiation; (III) 72 h later. Image slice thickness, 1.0 mm. Asterisks, recurrent tumors. Scale bar = 1.0 cm. (e) MRI over time after 5Gy72hCderiv treatment. (I) Before 10 mg/kg Cderiv treatment; (II) 24 h after Cderiv treatment; (III) 72 h later. Image slice thickness, 1.0 mm. Arrows, T-HI edema. (f) Bio-Safe CBB staining of a gel after SDS-PAGE. Left, molecular (Mol.) mass marker; right, 5Gy72hCderiv group. Arrow, approximately 70 kDa. (g) Identification of albumin by anti-albumin antibody. Lanes 1, 3, and 6, Cderiv72h5Gy (C72h5Gy) group; lanes 2, 4, and 7, 5Gy72hCderiv (5Gy72C) group; lane 5, molecular marker; lane 8, 10 ng albumin. (h) Concentration in each tissue after Cderiv72h5Gy and 5Gy72hCderiv treatments. Each bar shows the mean ± SD of five samples. Each albumin concentration is shown as relative to the albumin value in T-HI induced after 5Gy72hCderiv treatment.

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electrophoresis. We found a strong band at approximately 70 kDa (Fig. 6f). From the molecular mass, we estimated that this band included a large amount of albumin, which we showed by using anti-albumin antibodies (Fig. 6g, a representative example). We also obtained tissues from three regions (normal subcutis, T-HI, and tumor) at 24 h after the second treatment in both 5 Gy72hCderiv and Cderiv72h5 Gy groups and compared the amount of albumin in those tissues. Figure 6(h) shows the amount of albumin per unit volume in normal s.c. tissue, T-HI, and tumor tissue. The albumin content in the T-HI edema in the 5 Gy72hCderiv group was significantly higher than that in any other tissue.

Severe edema destroys T-HI vessels and keeps feeding vessels away from a tumor margin. We hypothesized that marked edema induced in the T-HI blocked the circulation of T-HI vessels and stopped the supply of nutrients to cancer cells in tumor margins. To indicate this, we treated a tumor growing in a transparent chamber with 5 Gy72hCderiv, and 24 h later, we analyzed sodium fluorescein diffusion to tumor margins. Figure 7(a) shows a tumor and nearby host tissue before irradiation. Figure 7(b) shows a change in the area shown in Figure 7(a) at 73 h after 5 Gy irradiation. Figure 7(c) is another change in the same area at 25 h after 10 mg/kg Cderiv treatment. The Cderiv promptly blocked TBF, and 24 h later tumors were completely necrotic. As expected, the swollen tumor and T-HI edema compressed the T-HI blood vessels, which stopped circulation. This result differed from that for intact T-HI vessels remaining after treatment with Cderiv alone. Figure 7(d) shows representative results of studies of sodium fluorescein transport to a tissue (the framed area in Fig. 7c) in the 5 Gy72hCderiv group. At 24 h after 10 mg/kg Cderiv treatment, sodium fluorescein was injected i.v. We made serial observations for 15 min, but the fluorescent dye did not appear in the observation field. This result is definitely due to complete destruction of the circulation function of T-HI vessels (Fig. 7c). We had the same results with all five rats. Figure 7(e) compares the distance between a tumor margin and its nearest functioning vessel before and after 10 mg/kg Cderiv treatment. Although the distance before Cderiv was 66.4 ± 73.7 μm, the distance after Cderiv was 637.5 ± 272.9 μm, a significant difference. Thus, the tumor marginal region completely lost its functioning vessels and became a microenvironment without a nutritional supply.

Discussion

The present study clearly showed that cancer cells growing in tumor margins receive nutrients needed for survival from both tumor vessels and T-HI vessels. Therefore, to prevent cancer recurrence in tumor margins, we must block both nutritional supply sources. This point is the most important conclusion of our study (Fig. 8).

Before reaching this conclusion, we investigated whether the TBF response to Cderiv differs in intratumoral and peritumoral regions, because if the TBF response to Cderiv was weaker in the peritumoral region, cancer cells in such regions would be less impaired. However, the response rate of TBF to Cderiv in the peritumoral region corresponded exactly to that in the intratumoral region. These results led us to exclude the possibility that the TBF responses to Cderiv in the peritumoral and intratumoral regions differed.

When TBF was blocked insufficiently by Cderiv at 3 mg/kg or less, the tumor began to regrow quickly, although tumor growth was transiently suppressed. Treatment with Cderiv at 10 mg/kg completely stopped all TBF within tumors. However, although complete TBF blocking was a prerequisite for strong suppression of tumor growth, it was not sufficient to eliminate all cancer cells. In this case, surviving cancer cells in tumor margins clearly received nutrients not from those tumor vessels but from T-HI vessels, whose blood flow was not interrupted by 10 mg/kg Cderiv.

When 10 mg/kg Cderiv was given at 72 h after 5 Gy irradiation, blood flow in T-HI vessels decreased gradually over several hours and stopped completely at 24 h. Treatment in the reverse order, however, did not affect blood flow in T-HI vessels. Several reports showed that although VDA treatment after irradiation enhanced radiation therapy effects, treatment in the reverse order did not enhance therapeutic efficacy.[23,29–33] Although the reason for this difference has not

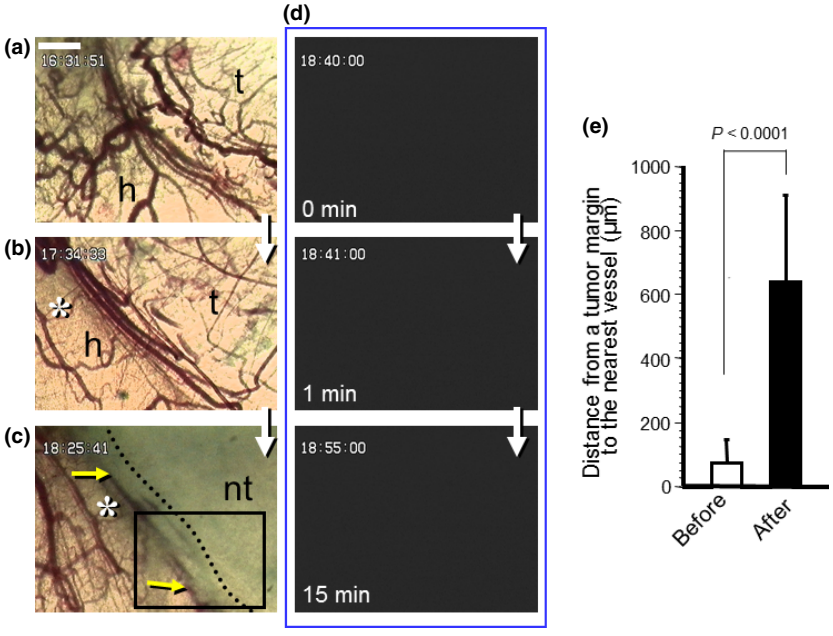

Fig. 7. Loss of circulation function of tumor–host interface vessels after treatment with i.v. combretastatin derivative (Cderiv) given 72 h after 5 Gy irradiation (5Gy72hCderiv). Representative vital microscopic findings of the effects of 10 mg/kg 5Gy72hCderiv on a tumor growing in a transparent chamber. (a) At 1 h before 5 Gy irradiation. (b) At 72 h after 5 Gy irradiation (just before Cderiv treatment). (c) At 25 h after 10 mg/kg Cderiv treatment. Dotted line, boundary of tumor before 25 h. h, host; nt, necrotic tumor; t, tumor; yellow arrows, collapsed tumor–host interface blood vessels. Asterisks in (b) and (c) indicate the same area. Arrows, direction of time. (d) Lack of sodium fluorescein delivery into tissues after 5Gy72hCderiv treatment. The observation area is the black frame in (c). Arrows, direction of time. (e) Distance from a tumor margin to the nearest host blood vessel after 5Gy72hCderiv treatment. Before 10 mg/kg Cderiv, $n = 12$; 24 h after Cderiv, $n = 11$.

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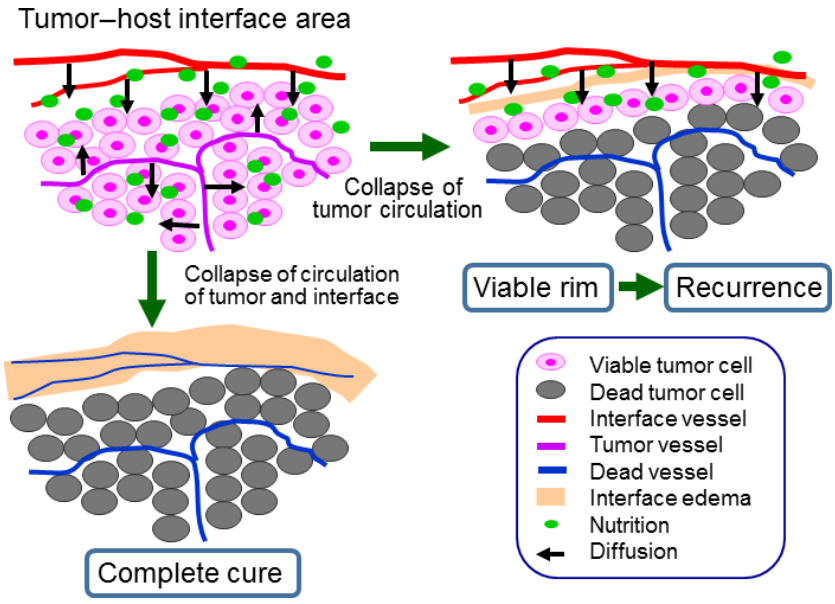

Fig. 8. Summary of results from this study.

been fully elucidated, one logical explanation is that because VDAs act strongly at the hypoxic tumor center and irradiation is effective at the well-oxygenated tumor periphery, the combination treatment provides an enhanced effect.[2,34] From this finding, we can understand why VDAs given before irradiation compromises the efficacy of radiotherapy, because the response to irradiation would be decreased by VDA-induced tumor hypoxia when a VDA is given before irradiation. However, this description cannot explain the strong synergistic effect of the combination that we obtained when VDA was given after irradiation.

Here, we compared changes in T-HI microenvironments induced by 5Gy72hCderiv and Cderiv72h5Gy treatments. The greatest differences between the treatments were that, with 5Gy72hCderiv, severe T-HI edema occurred at 24 h after the second treatment and T-HI blood vessels collapsed at that time, but with Cderiv72h5Gy the T-HI vessels remained functionally intact and no edema occurred. Unlike rapid blood flow interruption in tumor vessels, blood flow interruption in T-HI vessels caused by 5Gy72hCderiv proceeded slowly and was accompanied by gradual development of T-HI edema. These findings suggest that a close relationship exists between the two processes.

We next investigated why marked edema occurred in the T-HI after 5Gy72hCderiv treatment. One primary factor that is needed for edema formation is enhanced vascular permeability. Our electron microscopic observations confirmed that unlike endothelial cells in blood vessels of other regions, endothelial cells that constitute T-HI vessels contained many VVOs. This observation was consistent with that of Dvorak *et al.*[28,35] In fact, permeability of T-HI vessels to macromolecules (FITC-albumin and FITC-micelles) was quite high.[25] The fact that 5Gy72hCderiv treatment led to leakage of a large amount of albumin, and mostly in the T-HI region, shows that T-HI vessels are hyperpermeable and that VVOs had been functioning until blood flow in T-HI vessels stopped.

Drainage dysfunction in the T-HI region is another important factor needed for edema formation. As described above, a large amount of albumin moved into the extravascular space after 5Gy72hCderiv treatment. Thus, a large amount of water had clearly moved from T-HI blood vessels to the T-HI region at that time, because albumin is associated with strong water retention. If the drainage function was operating well, however, edema would not occur. The situation after Cderiv72h5Gy treatment seems to correspond to such a situation. Perhaps the primary reason for severe edema after 5Gy72hCderiv treatment may be failure of drainage in the T-HI region.

We believe that halting the blood flow in T-HI vessels as caused by severe edema resulted in the following alterations in the exchange of substances in the tumor margin: the average distance between one arbitrary point in the tumor margin and the blood vessel closest to it was approximately 70 μm before Cderiv treatment at 72 h after 5 Gy irradiation. For the region whose distance from a blood vessel was within 200 μm, each hemodynamic parameter was not so different between the two arbitrary regions (Fig. 5e–h). We thus concluded that 5 Gy irradiation had no negative effect on substances exchanged in the tumor margin. However, T-HI vessels lost circulatory function after 5Gy72hCderiv treatment, and the mean distance between the tumor margin and the vessel closest to it was 650 μm. This distance is sufficient for impairing the exchange of substances at the tumor periphery and causing cancer cell death, because the present study determined that the tissue concentration of a substance and its delivery rate were markedly reduced when the distance to a blood vessel from a point in the tissue was more than 400 μm. Thus, growth conditions for cancer cells in the tumor margin should worsen. Deenkamp *et al.*[36] reported that when blood circulation in a tumor-feeding vessel was stopped for more than 15 h, all cancer cells lost the ability to grow. Dysfunction of both tumor vessels and T-HI vessels after 5Gy72hCderiv treatment satisfies requirements for inducing death of cancer cells. Although it is not clear what molecule in the microenvironment is relating to cancer recurrence in tumor margins after TBF interruption, our starvation method that involved Cderiv treatment given 72 h after 5 Gy irradiation may have great potential as a clinical strategy to prevent cancer recurrence.

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Disclosure Statement

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Supporting Information

Additional supporting information may be found in the online version of this article:

Fig. S1. Histological effects of combination therapy (5 Gy + 10 mg/kg combretastatin derivative [Cderiv]).

Fig. S2. Prevention of cancer recurrence in a tumor margin by combretastatin derivative [Cderiv] treatment after fractionated irradiation.

Fig. S3. Water retention detected by MRI in tumor-bearing rats.

Data S1. Materials and methods.

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