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THREE-DIMENSIONAL CHANGES IN LYMPHATIC ARCHITECTURE AROUND VX2 TONGUE CANCER - DYNAMIC CHANGES AFTER ADMINISTRATION OF ANTIANGIOGENIC AGENT

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ABSTRACT

We examined the three-dimensional changes of the lymphatic architecture in the rabbit VX2 tongue cancer model after administration of an antiangiogenic agent, TNP-470.

TNP-470 at 30 mg/kg was administered via the auricular vein to the rabbit four times every other day from 3 days after transplantation of the tumor. The tongue and both sides of deep cervical lymph nodes of rabbit were observed at 10 days after transplantation. Lymph node metastasis was confirmed histopathologically. Morphological changes of collecting lymphatic vessels and lymphatic capillaries were observed, and the number and diameter of lymphatic vessels within 500 μm around the tumor were measured using the combined method with 5'-nucleotidase staining and three-dimensional reconstruction imaging.

Tumor growth and lymph node metastasis were suppressed by administration of TNP-470. In the TNP-treatment group, the mean number of lymphatic capillaries was significantly fewer than in the control group. The mean diameter of collecting lymphatic vessels was significantly smaller than in the control group.

In conclusion, our results suggest that cancer cell invasion into the lymphatics is probably decreased by inhibiting not only the growth of tumor but also new formation of lymphatic capillaries around the tumor by administration of TNP-470.

The dissemination of malignant cells from a primary tumor to local tissue or to distant organs via lymphatics or blood vessels is characteristic of cancer progression and is the major cause of mortality from malignant tumors (1). Cancer of the oral region tends to spread to regional lymph nodes relatively early (2,3). Therefore, it is important for the treatment of cancer to suppress not only the growth of the primary tumor but also the regional lymph node metastasis.

Inhibition of angiogenesis has been developed recently as a new anticancer therapy (4) because solid tumors initiate an angiogenic process to support their growth (5). Interestingly, some reports have shown that antiangiogenic agents inhibit not only tumor growth but also lymph node metastasis (6-8). It is necessary to assess the effect of antiangiogenic agents on lymphatic vessels around tumors because metastasis to the cervical lymph nodes in oral cancer is established by cancer cells disseminating via lymphatic vessels.

We have reported earlier that three-dimensional changes occur in lymphatic architecture around the tumor during the development of cancer using the combination

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method of 5'-nucleotidase (5'-Nase) staining and three-dimensional reconstruction imaging (9).

In this study, we examined the three-dimensional changes of the lymphatic architecture that occurred after administration of an antiangiogenic agent.

MATERIALS AND METHODS

Rabbits and Tumor Cells

Japanese white male rabbits (Oriental Bio Co., Ltd., Japan), weighing 2.0-2.5 kg, were used in this study. They were maintained in a standard environment (room temperature: 22±2°C, room humidity: 55±5%) in the vivarium of Iwate Medical University. The rabbits received a standard pellet diet with water ad libitum. VX2 cells were transplanted into the rabbit tongue and consistently spread to the regional lymph nodes (10). The VX2 cell line was maintained by successive transplantations onto the inside of the right leg muscle. All experiments were carried out according to the guidelines of the Animal Experiment Committee of our Institute.

Antiangiogenic Agent

TNP-470 (AGM- 1470), a synthetic analogue of fumagillin isolated from *Aspergillus fumigatus* (11), was used in this study. Takeda Chemical Industries (Osaka, Japan) generously provided us with TNP-470. TNP-470 was suspended in a vehicle of 7% ethanol in saline.

A Rabbit VX2 Tongue Cancer Model and Experimental Design

Rabbits were injected with $5x10^5$ VX2 cells (0.1 ml of the cell suspension) into the left lateral border of the tongue under sodium pentobarbital anesthesia.

We designed an experimental group that received TNP-treatment and a control group. In the TNP-treatment group, TNP-470 at 30 mg/kg was administered under anesthesia via the auricular vein four times every other day (total 120 mg/kg) from 3 days after transplantation. In the control group (n=5), the vehicle without TNP-470 was administered the same way as in the TNP-treatment group. The rabbits were killed by lethal injection of sodium pentobarbital 10 days after transplantation, and the tongues and the deep cervical lymph nodes were dissected. The primary tumors on the tongue were measured using a caliper, and the tumor volume was calculated according to the formula $a^2b/2$, where a and b are the shortest and the longest diameters of the tumor, respectively. The tongues were divided into two parts; an anterior part for histopathological examination by hematoxylin and eosin (H-E) staining and a posterior part for identification of the lymphatic vessels by 5'-Nase staining. The deep cervical lymph nodes were observed histopathologically for confirmation of metastasis by H-E staining.

Three-Dimensional Architecture of Lymphatic Vessels Around Tumor

We carried out a three-dimensional reconstruction of lymphatic vessels according to the method devised by Fujimura et al (12). Briefly, the specimens after immersion in fixative were embedded in 5% carboxymethyl cellulose (CMC) gel and frozen in cold hexane (-80°C). The frozen CMC block was cut using a cryostat (Bright Instrument. Co. Ltd., UK) and a cryo-film transfer kit (Finetec, Japan) devised by Kawamoto and Shimizu (13). We produced one hundred of the 10 μm cryo-serial sections. The sections were immersed in the 5'-Nase substrate solution and then in 1% ammonium sulfide solution (14). Two-dimensional image data of sections after 5'-Nase staining were input to a computer (Macintosh G4, Apple), and then 5'-Nase-positive lymphatic vessels were extracted from these images. After threshold treatment, these vessels were reconstructed to produce three-dimensional images by the volume rendering method (VoxBlast ver.

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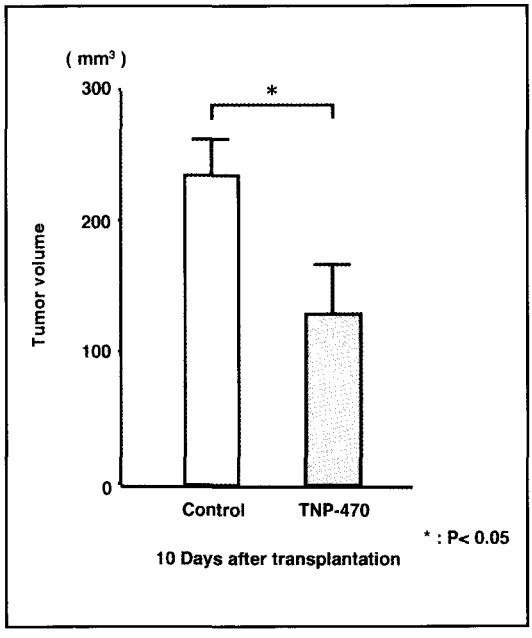

Fig. 1: Effect of inhibiting the growth of the VX2 tongue cancer after administration of TNP-470. Tumor volumes in the TNP-treatment group were significantly lower than those in the control group, Data are presented as mean ± SD.

2.3.3, VayTec, USA). A rotation image of the three-dimensional lymphatic architecture around the tumor was produced and observed from various perspectives.

Number and Diameter of Lymphatics

The superior longitudinal muscles accompanying collecting lymphatic vessels (SLCL) are among the main pathways of lymphatic vessels in the normal tongue of the rabbit (12). Fujimura et al suggested that there also were vertical muscles accompanying collecting lymphatic vessels (VL) joined to the SLCL (12). Furthermore, we showed that the number of lymphatic capillaries and the diameter of SLCL on lymphatic vessels around the tumor increased and enlarged, respectively, during the development of VX2 tongue cancer (9). In this study, we observed the SLCL, VL joining to SLCL and lymphatic capillaries, and measured the number and diameter of these lymphatic vessels within 500 μm around the tumor. We counted the number of lymphatic capillaries using rotation images. The largest diameter of the SLCL was confirmed on the three-dimensional image and then was measured at this position on the two-dimensional image.

For statistical evaluation of the data, we used the Mann-Whitney t-test, significant level of $P < 0.05$ by InStat (ver. 2.03).

RESULTS

Suppression of the Tumor Growth and Regional Lymph Node Metastasis

The mean volumes of the VX2 tumors in the TNP-treated and the control rabbits were 143.7±45.2 mm^3 and 233.1±24.5 mm^3, respectively. Tumor volumes in the TNP-treatment group were significantly lower than those in the control group *(Fig. 1)*. Histopathological findings of the tongue in the control group showed that the VX2 tumor proliferated, infiltrated and destroyed muscle bundles in the transplanted side of the animals. In the tumor stroma, there were thick blood capillaries and chronic inflammatory cell infiltrates *(Fig. 2)*. In the TNP-treatment group, the area infiltrated by the VX2 tumor was narrower than in the control group (data not shown).

VX2 cells were detected in the deep cervical lymph nodes of all rabbits in the control group. In contrast, lymph node metastasis was observed in only one of five rabbits in the TNP-treatment group. Thus, lymph node metastasis was suppressed by administration of TNP-470 *(Fig. 3)*.

Three-Dimensional Architecture of Lymphatic Vessels Around Tumor

5'-Nase-positive lymphatic vessels were not observed in the tumor area but were in the surrounding musculature, where VX2 cells did not invade *(Fig. 4)*. In the control

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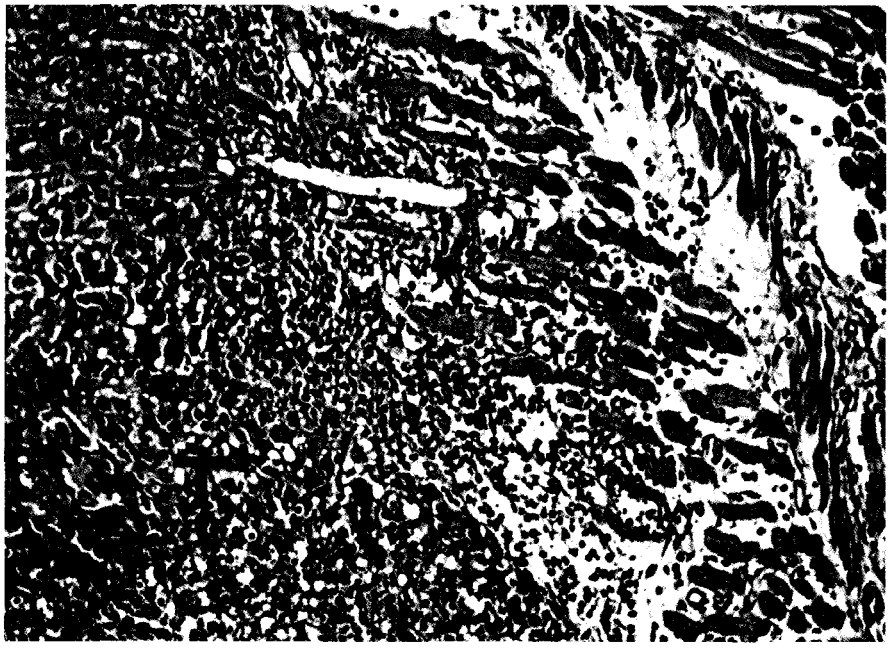

Fig. 2: Histological section of tongue at 10 days after transplantation of VX2 tumor cells. (H-E staining, x4). T: tumor cells.

group, VL and SLCL within 50 to 100 μm outside of the muscle bundles invaded by VX2 tumor, cells were pressed intensely to the dorsal side of the septum. There were a few lymphatic capillaries closer than 100 μm of the tumor periphery. Between 100 to 300 μm from the tumor periphery, lymphatic capillaries, about 30 to 50 μm long, were detected on VL *(Fig. 5)*. The mean number of these capillaries was 16.0±2.8. They became to form a tree-like branching pattern instead of a network pattern *(Fig. 5)*. The mean diameter of SLCL was 118.0±15.3 μm. The part of SLCL to which VL joined had the largest diameter.

In the TNP-treatment group, VL and SLCL within 50-100 μm from the tumor periphery were slightly pressed to the dorsal side of the septum compared to the control group *(Fig. 6)*. In the range of 100 to 300 μm from the tumor periphery, the mean number of lymphatic capillaries in endomysium joined to VL was 9.0 ± 3.3, significantly fewer than in the control group *(Fig. 7a)*. There were a few lymphatic capillaries that formed the tree-like branching pattern *(Fig. 6)*. The mean diameters of SLCL was 75.0±21.8 μm, significantly smaller than in the control group *(Fig. 7b)*. The part of SLCL to which VL joined had the largest diameter in both treatment and control groups.

DISCUSSION

This report presents the dynamics of lymphatic architecture around a tumor after administration of an antiangiogenic agent.

There are few reports published on the three-dimensional architecture of lymphatic vessels *in vivo*. Fujimura et al have described the three-dimensional lymphatic architecture in the normal tongue of the rabbit using the combination method of 5'-Nase staining and three-dimensional reconstruction imaging (12). We also have studied the three-dimensional dynamics of the lymphatic

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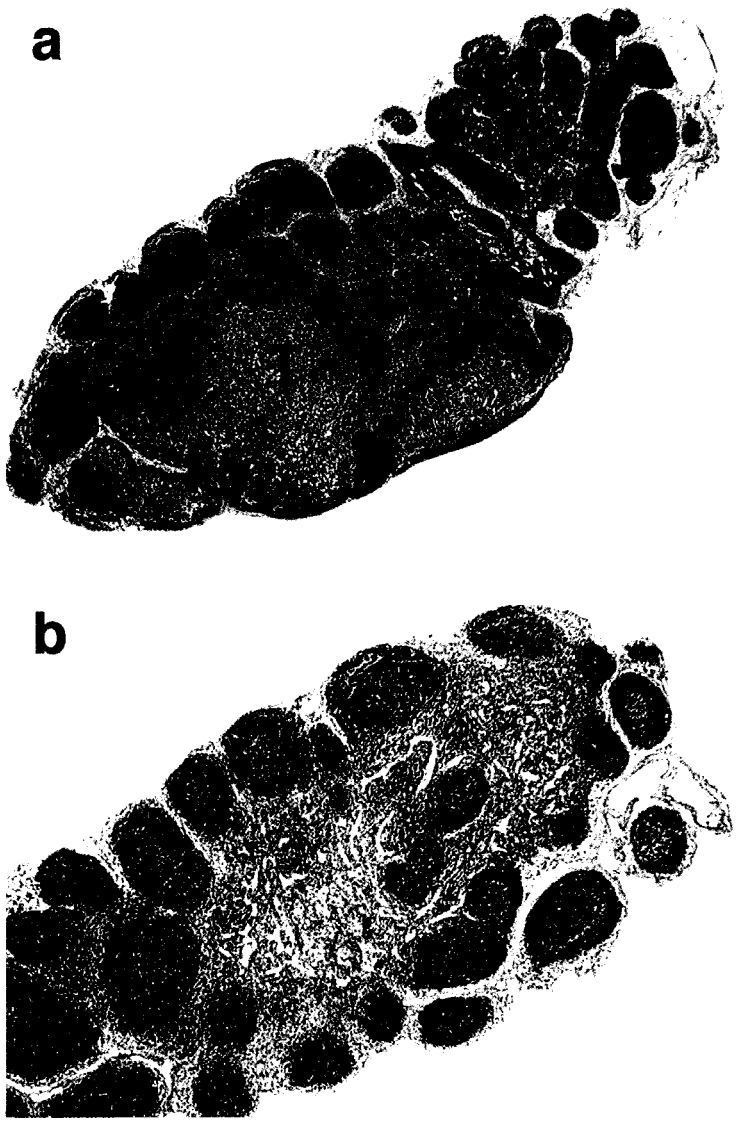

Fig. 3: Inhibition of cervical lymph node metastasis of the VX2 tongue cancer after administration of TNP-470. (H-E staining, a: control, x2; b: TNP-470 treatment, x4) T: tumor cells.

architecture around the tumor during VX2 tongue cancer growth (9) and found morphological changes that increased the number of lymphatic capillaries and enlarged the diameter of SLCL during tumor growth (9). The lymphatic capillaries around the tumor showed a tree-like branching pattern, not a network pattern (9). Our results in the control group confirm the observation in our previous report (9). We favor that lymphatic capillaries may be newly formed from preexisting lymphatic vessels, a process called tumor lymphangiogenesis, in normal tissue outside the tumor margin during cancer growth. Many questions remain regarding the correlation between tumor lymphangiogenesis and regional lymph node metastasis (15). Some reports have shown overexpression of

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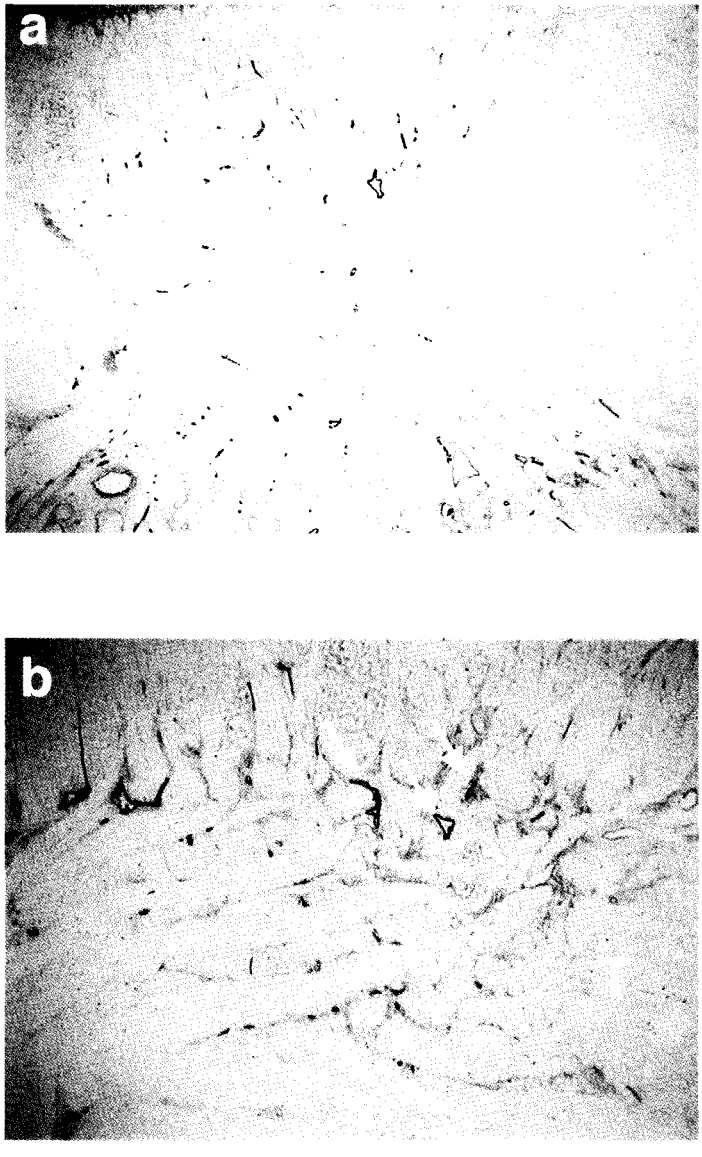

Fig. 4: Two-dimensional images of the lymphatic vessels around tumor in TNP-treatment group. (5-Nase staining, a: x2; b: x4) arrow: SLCL, T: tumor.

the lymphangiogenic molecule of vascular endothelial cell growth factors C (VEGF-C) and D (VEGF-D), and the VEGF receptor 3 (VEGFR-3), which is a receptor for VEGF-C and -D in various tumors (16,17). A correlation between expression of these factors and their receptor with mortality has been reported (18,19). We showed an increase in the number of lymphatic capillaries and lymph node metastasis of all rabbits in the control group. We think that increasing the number of lymphatic capillaries probably enhances the opportunity for cancer cell invasion into the lymphatics (10). Nevertheless, it remains to be seen whether antilymphangiogenic therapy prevents

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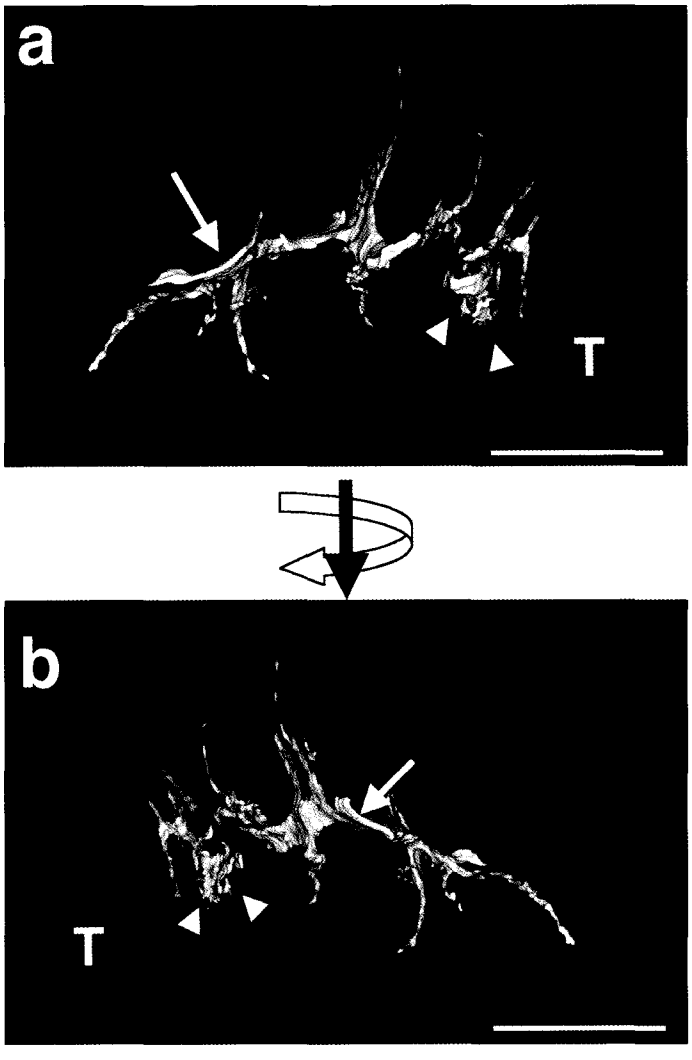

Fig. 5: Three-dimensional lymphatic architecture around tumor in the control group. The lymphatic capillaries of tree-like branching pattern were confined on VL (b is a 180 degree rotation image of a). SLCL (arrow), Lymphatic capillaries on VL (arrow head), T: tumor, Bar: 500 μm.

lymphatic metastasis (20), but we suggest that suppressing the increase in the number of lymphatic capillaries around a tumor may restrict metastasis to the regional lymph node.

Antiangiogenesis agents suppress tumor growth by inhibiting tumor angiogenesis (21, 22). Although TNP-470, an antiangiogenic agent, promotes anti-tumor effects against a variety of tumors in animal models (6-8,23), its mechanism of action has not yet been elucidated. Kusaka et al reported that this agent selectively inhibited the capillary-like tube formation of endothelial cells with a minimal effect on non-endothelial cell growth (24). TNP-470 prevented the entry of endothelial cells into the G_1 phase of the cell cycle (25) and inhibited the cell growth induced by both VEGF and basic fibroblast growth factor (26). The number of lymphatic capillaries in the TNP-treatment group was

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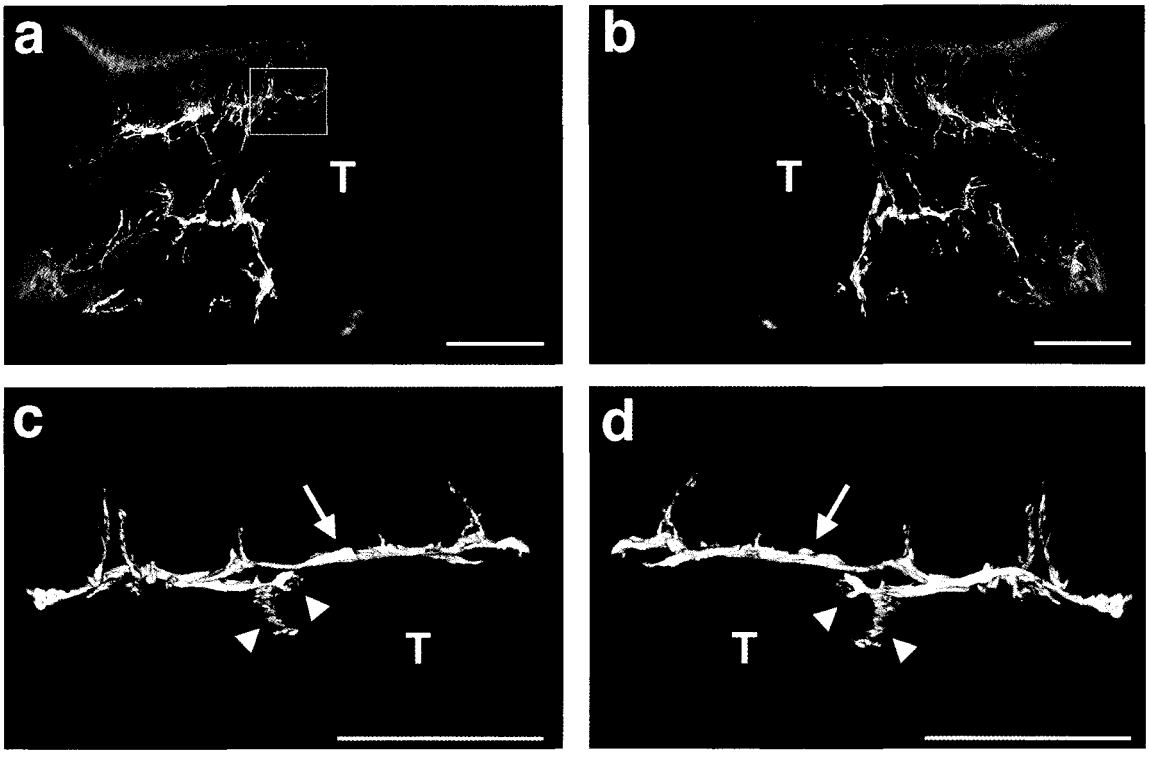

Fig. 6: The three-dimensional lymphatic architecture around tumor in the TNP-treatment group. (b and d are a 180 degree rotation image of a and c, respectively.) SLCL (arrow), Lymphatic capillaries on VL (arrow head), T: tumor, Bar: 500 μm.

fewer than in the control group. This result suggests that TNP-470 may have a similar inhibitory effect on the proliferation of the lymphatic endothelial cells as it has on blood vascular endothelial cells. Inhibition of lymph node metastasis by TNP-470 was confirmed in the HT-1080 mouse model (6), VX2 rabbit tongue cancer model (7), and murine renal cell carcinoma model (8). Cervical lymph node metastasis was suppressed by administration of TNP-470 in this study.

In conclusion, our results suggest that, by administration of TNP-470, cancer cell invasion into the lymphatics is decreased by inhibiting not only the growth of cancer cells but also the new formation of lymphatic capillaries around the tumor.

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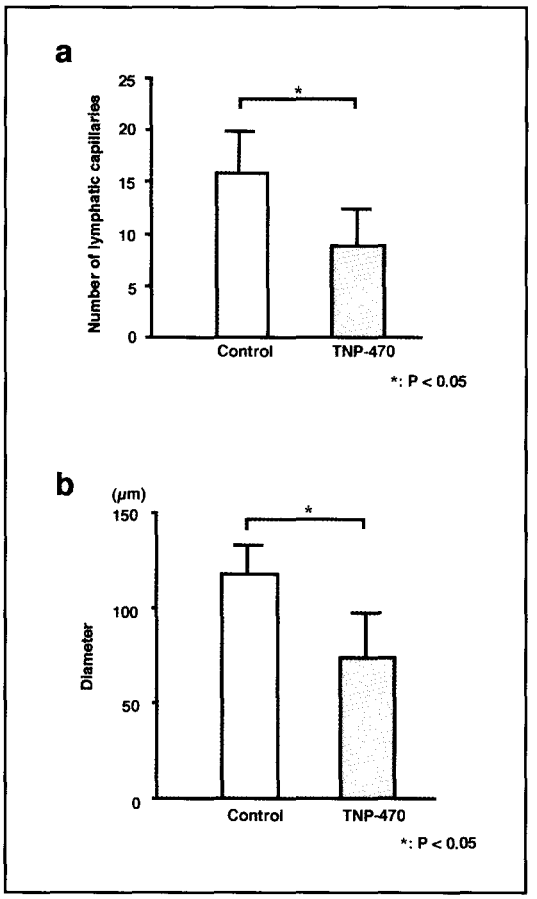

Fig. 7. Number of lymphatic capillaries and diameter of SLCL. a: Number of lymphatic capillaries; b: diameter of SLCL. Data are presented as mean±SD.

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