Quantification of tumour-induced angiogenesis by image analysis

MICHIO IWAHANA*†, YUTAKA NAKAYAMA†,
NORIKO G. TANAKA†, MASANOBU GORYO*,
AND KOSUKE OKADA*

*Department of Veterinary Pathology, Faculty of Agriculture, Iwate University, Ueda 3-18-8, Morioka 020, Japan, and †New Products Research Laboratories IV, Daiichi Pharmaceutical Co., Ltd., Kitakasai 1-16-13, Edogawa-Ku, Tokyo 134, Japan

Summary. Quantitative techniques for *in vivo* and *in vitro* angiogenesis were developed using an image analyser. In the *in vivo* study, a Millipore chamber filled with mouse sarcoma 180 (S180) cells was transplanted subcutaneously to the dorsal side of a mouse, and the area of neovascularization induced by the tumour cells was quantified by image analysis. Images of vascular networks with poor contrast had their contrast improved by Laplacean transformation. The area of vascular network was 16.9 mm^2 in the control group without tumour cells and 44.2 mm^2 in the group with tumour cells, demonstrating a significant increase in neovascularized area by tumour cells. In the *in vitro* study, migration of vascular endothelial cells was induced with conditioned media of S180 cells. Image analysis was used to count automatically the nuclei of migrated endothelial cells, which were stained violet with Giemsa's solution. This automated measurement by image analyser is expected to save labour and time. Checkerboard analysis revealed that the endothelial cell migration induced by S180-conditioned medium was due to chemotaxis. The quantitation method using an automated image analyser is valuable in evaluating the induction of neovascularization by tumours and the effect of pharmacological agents on tumour angiogenesis *in vivo* and *in vitro*.

Keywords: sarcoma 180, angiogenesis, cell migration, quantification, image analysis

In solid malignant tumours, angiogenesis is necessary for continued growth beyond a certain size so that sufficient nutrients can be supplied and waste products can be eliminated (Folkman 1985). Several studies have demonstrated that tumour cells produce and release angiogenic factors (Fenselau & Mello 1976; Folkman &

Correspondence: Michio Iwahana, New Products Research Laboratories IV, Daiichi Pharmaceutical Co., Ltd., Kitakasai 1-16-13, Edogawa-Ku, Tokyo 134, Japan.

Klagsbrun 1987). Tumour-induced angiogenesis has been studied *in vivo* by the following methods: chorioallantoic membrane (CAM) assay using fertilized eggs (Folkman & Cotran 1976), dorsal air sac assay using a transplantation technique with Millipore tubing on the dorsal side of rats (Folkman *et al.* 1971) or mice (Wakamatsu *et al.* 1990), the cheek pouch method using the transparent chamber technique in hamsters (Greenblatt & Schubik 1968) and the cornea method using rabbit

cornea (Gimbrone *et al.* 1974). Among these, the murine dorsal air sac method seems to be most suitable for evaluating the inducibility of angiogenesis, because varieties of tumour lines can be used and the efficacy of inhibitors can be determined using these tumour lines experimentally (Lee *et al.* 1987). In the present study, we investigated image analysis procedures for quantification of the subcutaneous vascular network induced by sarcoma 180 (S180), which grow as a solid form on subcutaneous injection. Subsequently, in order to assess the angiogenic factor production or release activity of S180 cells, we determined the effect of conditioned media of S180 cells on the growth and migration of vascular endothelial cells. Additionally, we tried to use an image analyser to count the number of migrating cells by the Boyden chamber method (Boyden 1962).

Materials and methods

Animals

Male ICR mice, 6 weeks of age, were purchased from Japan SLC, Inc. (Shizuoka, Japan) and maintained under SPF conditions at our animal facility. Five mice were used in the dorsal air sac method.

Cells

Ascitic sarcoma 180 (S180) cells were obtained from the National Cancer Center, Tokyo, Japan, and maintained in ICR mice by weekly i.p. inoculation. Cultured S180 cells were obtained from Dr J. Folkman (Children's Hospital Medical Center and Harvard Medical School, Massachusetts, USA) and were maintained *in vitro*. S180 cells were cultured *in vitro* with RPMI 1640 medium containing 10% fetal bovine serum (FBS, Lot. No. 111892; Hyclone, Logan, USA) at 37°C under a humidified atmosphere with 5% CO_2 and 95% air. S180 cells were used for experiments after the first passage in our laboratory. Human umbilical vein endothelial cells (HUVEC, Kurabo Co., Osaka Japan) were seeded in E-GM UV medium (modified MCDB 131 medium containing bovine brain extract, heparin, endothelial growth factor, hydrocortisone and antibiotics, Kurabo Co., Osaka Japan) supplemented with 10% fetal bovine serum (FBS), and maintained serially at 37°C under a humidified atmosphere with 5% CO_2 and 95% air. HUVEC was used for experiments after the third to sixth passage in culture.

Mouse dorsal air sac method and image analysis

S180 cells were harvested from ascites fluid washed with phosphate-buffered saline solution (PBS) and were suspended in PBS at a concentration of 1×10^8 cells/ml. A Millipore chamber (filter pore size 0.45 μm; Millipore Co., Bedford, MA, USA) was filled with 0.2 ml of either cell suspension or PBS and implanted subcutaneously into a dorsal side of mice. Four days after implantation, mice were anaesthetized with pentobarbital sodium and fixed in the prone position. A wide rectangular incision was made in the skin on the dorsal side, and the skin was carefully ablated. A silicon gasket (Millipore) with the same shape as the Millipore chamber was placed in the subcutaneous tissues adjacent to the Millipore chamber, and the area was photographed with a Medical Nikkor camera (Nikon, Tokyo, Japan).

Images of the vascular network on the subcutaneous tissue were taken with a monochromatic TV camera (Ikegami Tsushinki Co. Ltd, Tokyo, Japan) equipped with a blue filter (G545; Kenko, Tokyo), and the images were acquired through a digital signal and basic algorithms with an image analyser (Luzex 5000 X; Nireco Co., Hachioji, Tokyo, Japan). Image analysis was performed and the area of blood vessels was measured.

All procedures in this investigation were approved by the Animal Welfare Committee of our institution.

Preparation of S180-conditioned medium

Cultured 6×10^4 S180 cells were suspended in 15 ml of RPMI 1640 medium containing 10% FBS and seeded in a 75-cm^2 flask (Costar Co., Cambridge, MA., USA). After culture for 3 days, S180 culture supernatant was harvested and separated by centrifugation (745 ***g***, 5 min).

Assay for proliferation of HUVEC

HUVEC was trypsinized, suspended in E-GM UV medium and seeded on 96-well microtitre plates (Becton Dickinson, Lincoln Park, USA) at 1×10^3 cells/well. The following day, medium was replaced with 2% FBS-vascular endothelial cell base medium (E-BM: modified MCDB131; Kurabo Co.) containing 2.5–40% of S180 culture supernatant. As a control for S180 culture supernatant, RPMI 1640 medium containing 10% FBS was added at the same concentration (i.e. 2.5–40%) as S180 culture supernatant. After 3 days in culture, cell growth was monitored by a crystal violet staining method. Briefly, 100 μl of 0.025% crystal violet staining solution was added to each well. After standing

for 60 minutes, each well was rinsed with distilled water 5 or 6 times, and 100 μl of 5% SDS (sodium dodecyl sulphate) was added to each well. Optical density values (at 590 nm) were measured with a Titertek Multi-Scan (Dai-nippon Pharmaceuticals Inc., Tokyo, Japan). All experiments were performed in triplicate.

Assay for HUVEC migration and measurement with an image analyser

Migration of HUVEC was assayed according to the Boyden chamber method (Boyden 1962). After trypsinization, HUVEC was resuspended in E-BM medium containing 2% FBS and loaded into each upper well of the chambers (Chemotaxis Chamber; Ieda Trading Co. Ltd., Tokyo, Japan) at 1.2×10^4 cells/well. Various concentrations of S180-conditioned medium were loaded into each lower well of the chambers. The upper and lower wells were separated by a Nuclepore polycarbonate filter (8-μm pore; Costar Co., Cambridge, MA, USA) precoated with gelatin (300 μg/ml) dissolved in PBS. After 5 hours of incubation at 37°C with 5% CO_2, cells adhering to the upper surface of the filter were removed with a swab, the filters were fixed in 100% methanol, and the cells which had migrated through the pores and adhered to the lower filter surface were stained with Giemsa's solution (Merck, Darmstadt, Germany).

Cytological pictures ($\times 100$) of the lower surface of the filter were taken with a colour TV camera (Nikon, Tokyo, Japan) attached to the microscope, and the images were acquired with an image analyser. Using nuclei stained violet with Giemsa's solution as indicators of migration, the number of nuclei was counted with the image analyser. Checkerboard analysis (Zigmond & Hirsch 1973) was performed to evaluate whether vascular endothelial cell migration in response to sarcoma 180-conditioned medium was by chemotaxis or chemokinesis.

Results

*Quantification of changes in subcutaneous blood vessels induced by S180 cells*

A typical initial image of area adjacent to the subcutaneously implanted PBS chamber on the dorsal side is shown in Figure 1a. The image of blood vessels with poor contrast showed improved contrast following Laplacean transformation (Figure 1b), and the binary image (superimposed on the initial image) reflected the actual distribution of blood vessels (Figure 1c). Laplacean

Figure 1. Image processing of vascular network. a, Initial monochromatic image; b, image after Laplacean transformation of initial image; c, binary image of the blood vessels superimposed on the initial image.

transformation was therefore performed in all analyses.

Compared with the distribution pattern of blood vessels of mice with the PBS chamber (Figure 2a), mice implanted with a Millipore chamber containing S180 cells showed characteristically convoluted blood capillaries as well as dilation of existing blood vessels
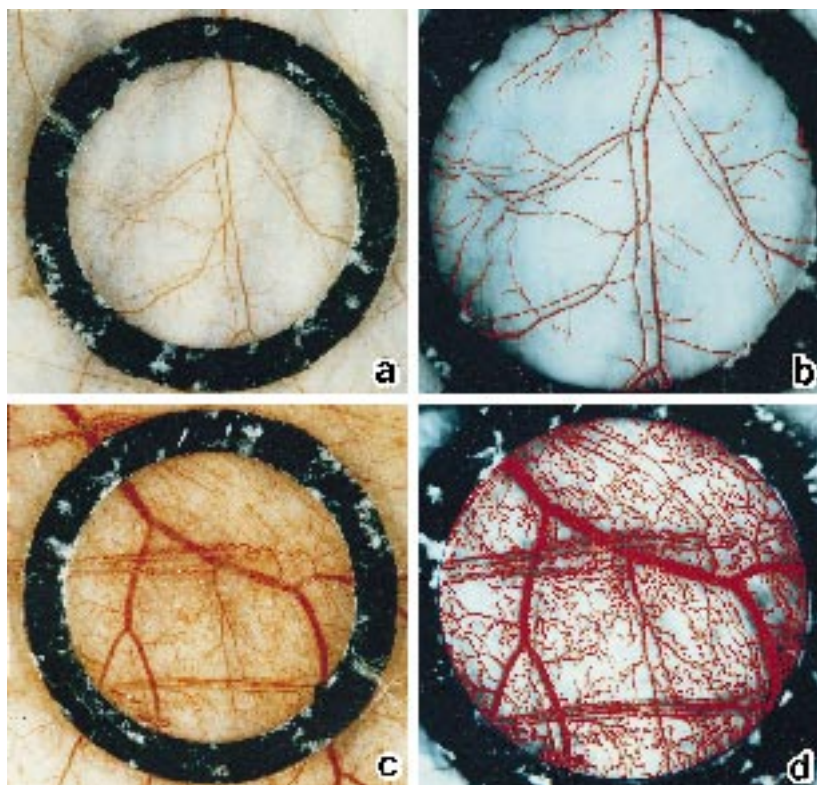
Figure 2. Comparison of initial and binary images of dorsal sides of mice with PBS and S180. a, Initial image of subcutaneous blood vessels of an ICR mouse implanted with a Millipore chamber containing PBS; b, binary image of subcutaneous blood vessels superimposed on the initial image (a); c, initial image of subcutaneous blood vessels of an ICR mouse implanted with a chamber containing S180 cells; d, binary image of subcutaneous blood vessels superimposed on the monochromatic initial image (c).

(Figure 2c). As assessed by the image analyser, the area of blood vessels in the group implanted with chambers containing S180 cells (Figure 2d) was significantly larger than that in the control group implanted with chambers containing PBS (Figure 2b); the area of vascular network was 16.9 mm^2 in the PBS control group and 44.2 mm^2 in the group with S180 cells, demonstrating a significant increase in neovascularized area by S180 cells (Table 1).

Growth-promoting activity of S180-conditioned medium on HUVEC

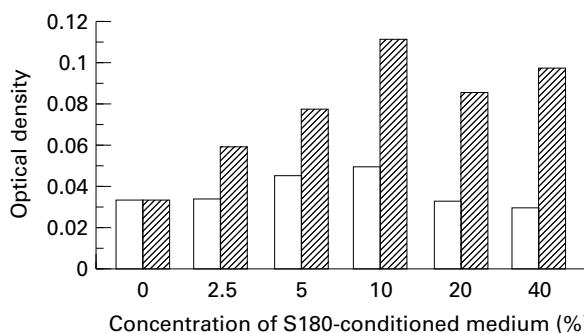S180-conditioned medium stimulated *in vitro* proliferation of HUVEC in a dose-dependent manner by between 2.5 and 10% (Figure 3). An approximately threefold increase in the *in vitro* proliferation of HUVEC was observed in the presence of 10% S180-conditioned medium, as compared with the control group. Concentrations greater than 10% gave the same results as the 10% medium.

Table 1. Area of vascular network induced by S180 cells

Content in the chamber	Area mean $\pm$ s.d. (mm^2)
S180 cells	44.2 $\pm$ 10.7**
PBS	16.9 $\pm$ 2.7

Millipore chambers were filled with either 2×10^7 S180 cells or PBS and implanted subcutaneously into the dorsal side of ICR mice on day 0. On day 4, the image of the vascular network in contact with the Millipore chamber was binarized, and the area was measured.

** $P < 0.01$ *vs* the group implanted with chambers containing PBS (Student's *t*-test).

Figure 3. Growth promoting activity of a sarcoma 180-conditioned medium on HUVEC. Various concentrations of ▨, S180-conditioned medium or ☐, RPMI 1640 medium containing 10% FBS were added to the culture at 1 day after seeding HUVEC (1×10^3 cells/well). After 3 days culture, the number of proliferated cells was counted by a crystal violet staining method.

Stimulation of HUVEC migration by S180-conditioned medium

HUVEC loaded into the upper wells of the Boyden chamber migrated through the pores of the filter and adhered to the lower surface after 5 hours incubation. Nuclei of migrated HUVEC were stained with Giemsa's solution. The migrated cells were distinguished from the surrounding areas in the initial image (Figure 4a). Threshold levels were set by the total summation of the vectors at each level, namely, intensity, hue and purity. Image analysis revealed excellent binary images of migrated cells with dark violet nuclei (Figure 4b), making it possible to count the number of migrated cells accurately with the image analyser in automated operation. Checkerboard analysis revealed that the HUVEC migration induced by S180-conditioned medium was due to chemotaxis and not to chemokinesis (Table 2).
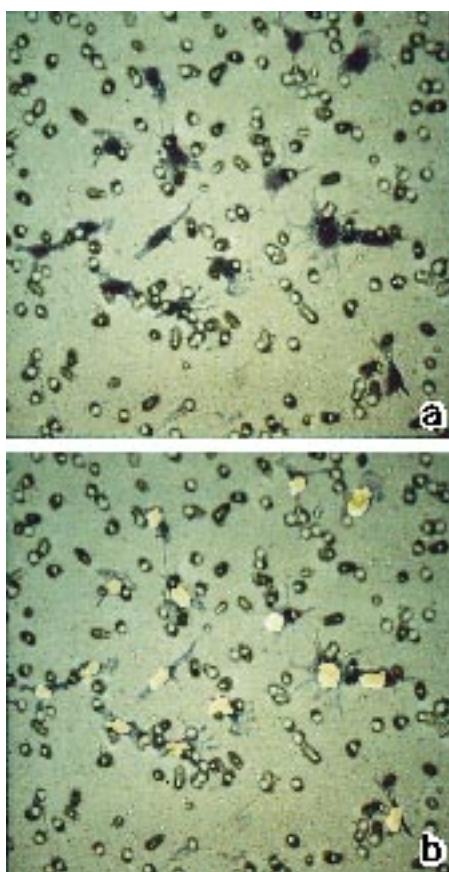
Figure 4. Picture of the microscopic image of migrated HUVEC. a, Initial image: nuclei of migrated and adhered cells were stained dark violet with Giemsa's solution; b, binary image of cell nuclei superimposed on the initial image: the light yellow dot shows the binary image of cell nuclei.

Table 2. Checkerboard analysis of HUVEC migration induced by S180-conditioned medium

		Concentration of conditioned medium in each upper well of the chamber (%)			
		0	12.5	25	50
Concentration of conditioned medium in in each lower well of the chamber (%)	0	100	88	53	37
	12.5	174	89	105	51
	25	534	480	117	109
	50	580	516	307	96

Various concentrations of S180-conditioned medium were loaded into each upper and lower well of the chambers. HUVEC was loaded into each upper well. After the chambers were incubated at 37°C with 5% CO_2 for 5 hours, the number of cells which had migrated through the pores to the lower filter surface was counted with an image analyser. The assay was constructed by examining the migration of cells in the presence of conditioned media of increasing concentrations in the upper or lower wells. The results are shown as relative values, assuming that the number of migrated cells was 100 when only RPMI 1640 medium containing 10% FBS was loaded into each upper and lower well of the chambers.

Discussion

Among the various methods used to evaluate the angiogenic potential of malignant tumours, the use of *in vivo* assay systems such as the CAM method (Folkman & Cotran 1987) and the rabbit cornea method (Gimbrone *et al.* 1974) is often hindered by difficulty in controlling non-specific reactions such as inflammation. In ICR mice subcutaneously implanted with Millipore chambers containing PBS, subcutaneous blood vessels attaching to the chambers showed a similar distribution pattern as that of surrounding blood vessels distant from the chambers. This finding indicates that the Millipore chambers used in the present study do not induce inflammation.

It is often difficult to quantify the distribution of capillary network on the subcutaneous fascia. Lee *et al.* (1987) demonstrated that the degree of angiogenesis using the mouse dorsal air sac method could be only semiquantitatively scored by an operator blinded to the treatment. Wakamatsu *et al.* (1990) were also unable to obtain precise quantitation. In contrast, using our image analysis technique, we were able to both qualify the degree of neovascularization induced by tumour *in vivo* and automatically count the number of migrated cells. Although the Boyden chamber method (Boyden 1962) has been commonly used to confirm cell migration due to chemotaxis, this is a very labour-intensive and time-consuming method because it requires counting of individual cells under a microscope. We therefore sought to develop a more effective method. Automating the counting of cells with an image analyser has allowed us to reduce the possibility of operator asthenopia during measurement, and thereby improve the efficiency of cell counting.

However, it has been reported that S180-conditioned medium had the disadvantage of stimulating the motility of endothelial cells (Mullins & Rifkin 1984), and the S180-conditioned medium used in our experiment definitely stimulated the proliferation and chemotactic migration of HUVEC. The two mouse sarcoma 180 cell lines were of the same origin (the gift of Dr Folkman), but the conditioned medium of Mullins and Rifkin was prepared by incubation of S180 cells for 48 hours, whereas our incubation time was 72 hours. The difference in proliferation and migration stimulating activity seems to be the reason for the difference in the amount of angiogenic factor in the conditioned medium.

In conclusion, the quantitation method using an image analyser described here is valuable in evaluating the induction of neovascularization by tumours and the effect of pharmacological agents on tumour angiogenesis *in vivo* and *in vitro*.

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