Table 1 Summary of autopsy cases

Cases	Age/ Sex	Cause of death	History of IHD	Pathology of OMI
1	69/M	Lung cancer	–	–
2	91/F	Pancreas cancer	–	–
3	57/M	Acute myelomonocytic leukemia	–	–
4	64/M	Esophageal cancer, laryngeal cancer	–	–
5	78/M	Subarachnoid hemorrhage	–	Posterior
6	74/F	Aortic dissection (DeBakey type I)	–	–
7	76/M	Cholangiocarcinoma	–	–
8	84/M	Pancreas cancer	–	Anteroseptal
9	69/F	Cancer, unknown origin	–	–
10	93/M	Brain infarction	–	–
11	91/F	Asphyxia	–	–
12	68/F	Malignant lymphoma	–	–
13	60/M	Pancreas cancer	–	–
14	64/F	Cancer, unknown origin	–	–
15	63/M	Esophageal cancer	–	–
16	68/F	Acute myeloblastic leukemia	–	–
17	85/M	Brain infarction	+	Anterior
18	85/M	Pneumonia	–	Septal
19	91/F	Amyloidosis	+	–
20	81/M	Aortic dissection (DeBakey type I)	+	Posterior
21	71/M	Dilated cardiomyopathy	–	Septal
22	78/M	Lung cancer	–	–
23	86/F	Brain infarction	–	Anteroseptal

IHD indicates ischemic heart disease; OMI, old myocardial infarction; M, male; F, female.

IgG1κ, Dako, Carpinteria, Calif) for monocytes/macrophages; mouse monoclonal antihuman α-SMA (1:1000, 1A4, isotype IgG2a, Sigma, St. Louis, Mo) for the smooth muscle cells (SMCs); rabbit polyclonal antihuman CD3 (1:200, Dako, Glostrup, Denmark) for T lymphocytes; and mouse monoclonal antihuman CD34 (1:50, isotype IgG1, Novocastra, Newcastle upon Tyne, UK) mainly for the endothelial cells and partly for the hematopoietic cells on the cell surface. Rabbit polyclonal antibody against human lymphatic vessel endothelial hyaluronan receptor-1 (LYVE-1) (1.60 μg/mL, author produced [24]) and mouse monoclonal antihuman podoplanin antibody (1:200, 11-003, AngioBio Co, Del Mar, Calif) were used for identification of lymphatic vessels.

2.3. Immunohistochemistry

Immunohistochemical examinations were performed as described previously [7]. In brief, deparaffinized sections were incubated with 3% nonfat milk to eliminate the nonspecific binding and with peroxidase-labeled secondary antibody (Envision System, Dako) following the primary antibodies. Horseradish peroxidase activity was visualized with 3,3′-diaminobenzidine tetrahydrochloride (Merck, Darmstadt, Germany) to give the reaction product a brown color, and then the sections were counterstained with hematoxylin. Either nonimmune rabbit IgG or nonimmune mouse IgG of each isotype was also used instead of the respective primary antibodies as a negative control. The tissue blocks retrieved from the human placenta or small intestine were used for the positive controls for immunohistochemistry in the case of the reactions for VEGF-C and VEGF-D or LYVE-1 and podoplanin, respectively.

2.4. Morphometric study

The total number of vascular (CD34) or lymph vessels (LYVE-1 and podoplanin) in the whole intima of each of the 169 sections was counted under a light microscope at high-power magnification (×200). The vessels were identified as morphologically circumferential brown products of 1 or more endothelial cells with counterstained nuclei. In this case, single-dot staining was not included. The number of VEGF-C–positive or VEGF-D–positive cells in the intima was counted for each section, and then a numerical grade was assigned as follows: according to the number of VEGF-C–positive cells: – indicates no staining, ±, 1 to 19 positive cells; +, 20 to 99; 2+, 100 to 499; and 3+, more than 500 in each section of coronary artery; according to the number of VEGF-D–positive cells: + indicates 1 to 499; 2+, 500 to 999; 3+, 1000 to 1499; and 4+, more than 1500 in each section of coronary artery. All counts were performed by 2 investigators (TN and YY) using a double-headed light microscope.

2.5. Statistical analysis

The data are presented as mean ± SEM unless otherwise stated. The data were statistically analyzed by the Mann-Whitney U test, Spearman's rank correlation analysis, the Bonferroni test, and the χ^2 test. A P value of less than .05 was considered statistically significant.

3. Results

3.1. American Heart Association classification of examined sections

One hundred sixty-nine coronary arterial specimens were carefully classified by 3 independent pathologists (TN, YN, and SS) according to the type of atherosclerotic lesion (AHA classification) [23] as follows: 12% (21/169) as diffuse intimal thickening (DIT); 33% (55/169) as types I and II, which are early lesions; and 35% (59/169) as types IV through VI, which are advanced lesions. The type III bridging lesion between early and advanced lesions comprised 20% (34/169) of the specimens. In the types III and IV lesions, the accumulation of macrophage-derived

1. Introduction

Emerging evidence supports the hypothesis that atherosclerosis is an inflammatory disease [1], and sustained inflammatory reaction of coronary atherosclerosis is suggested to be a cause of plaque instability, resulting in its rupture [2]. Therefore, investigation regarding the factors affecting the acceleration and/or maintenance of the inflammatory reaction in atherosclerotic plaque is required to understand the pathophysiology of coronary atherosclerosis and to develop an efficient strategy to prevent plaque rupture.

Angiogenesis is a common event occurring in the inflammatory foci, and newly formed blood vessels that originated mainly from the adventitia and rarely from the lumen have also been observed in the atherosclerotic plaques of human coronary arteries [3-5]. We previously demonstrated that the number of neovessels in the intimas of human coronary arteries was correlated with the degree of the inflammatory infiltration and the severity of the atherosclerosis [4], suggesting the essential contribution of plaque angiogenesis to the inflammatory reaction in, and progression of, atherosclerotic lesions. This was also supported by an experimental study indicating that systemic administration of angiogenic inhibitors, endostatin and TNP-470, resulted in suppression of the plaque progression in apolipoprotein E–deficient mice [6].

Recent studies suggest that vascular endothelial growth factor (VEGF; namely, VEGF-A) is a key regulator of angiogenesis in atherosclerotic lesions. We [7] and others [8] demonstrated that VEGF-expressing cells were distributed around the inflammatory foci of coronary atherosclerosis in human subjects, and experimental studies indicated that acceleration of intimal hyperplasia was associated with angiomatoid proliferation of leaky capillaries by arterial gene transfer of VEGF-A [9] and that VEGF-A accelerated the rate of lesion development in animal models of atherosclerosis [10-13], suggesting the essential contribution of VEGF-A to the progression of atherosclerosis.

In turn, a network of lymphatic vessels drains the extravasated bloodless fluid, protein, and inflammatory cells from the tissues [14]. Lymphatic vessels also serve an immune function by antigen-presenting cells that patrol the tissues to the various lymphoid organs. Congenital lymphedema (Milroy's disease) and lymphedema distichiasis are caused by impaired lymphatic drainage and have mutations in the gene of VEGF receptor (VEGFR)-3 (Flt-4) and FOXC2, respectively [15]. The lymphatic system may be implicated in numerous diseases including lymphedema, inflammation, autoimmune diseases, and malignancy [14]; however, very few studies have been done on lymphangiogenesis than those on angiogenesis.

Recent studies demonstrated that the relatively new members of the VEGF family, namely VEGF-C and VEGF-D, are ligands for both VEGFR-2 (Flk-1/KDR) and VEGFR-3 and promote lymphangiogenesis [16-19] as well as angiogenesis [20-22]. Very little information, however, is available regarding the expression and distribution of producing cells of these angiogenic and lymphangiogenic factors, which suggest the possible contribution of angiogenesis and lymphangiogenesis in the progression of human atheroma.

In this study, we immunohistochemically examined human coronary arteries obtained from autopsy cases to define the relationship among the coronary atherosclerotic lesion types on the basis of the American Heart Association (AHA) classification, intimal neovascularization (angiogenesis and lymphangiogenesis), and expression of VEGF-C and VEGF-D. We found that lymphangiogenesis was a relatively rare event compared with angiogenesis, although both VEGF-C and VEGF-D were abundantly expressed, suggesting the imbalance of angiogenesis and lymphangiogenesis in the atherosclerotic lesions of human coronary arteries.

2. Materials and methods

2.1. Sample processing

Within 16 hours of the death of 23 Japanese patients (14 men and 9 women), ranging in age from 57 to 93 years (mean ± SD, 76 ± 11 years), hearts were obtained at autopsy at the Kyushu University Hospital (Table 1). All patients were selected by randomized prospective sampling, and their deaths were not caused by acute coronary disease. Eight patients were clinically and microscopically revealed to undergo old and focal myocardial infarction. The coronary arteries were cannulated, washed with 0.1 mol/L phosphate-buffered saline (pH 7.4), and perfused with 1 L of freshly prepared 4% (wt/vol) paraformaldehyde in 0.1 mol/L sodium phosphate (pH 7.4) at 100 mm Hg. Then, each heart was immersed in 4% paraformaldehyde for at least 24 hours at 4°C. The right coronary artery and left anterior descending coronary artery were dissected free from the surface of the heart, cut perpendicular to the long axis at 3-mm intervals, processed according to standard methods, and then embedded in paraffin. One hundred sixty-nine blocks were obtained and cut into 3-μm-thick serial sections at once. Sections from each block were serially subjected to hematoxylin and eosin, elastica-van Gieson's and Masson trichrome stainings, as well as immunohistochemistry. In accordance with the definitions proposed by the Committee on Vascular Lesions of the Council on Arteriosclerosis (AHA) [23], the atherosclerotic lesion type of each section was carefully classified by 3 independent pathologists (TN, YN, and SS).

2.2. Antibodies

The following antibodies were used for immunohistochemical studies: rabbit polyclonal antihuman VEGF-C (2.1 μg/mL, Zymed Laboratories, Inc, South San Francisco, Calif); rabbit polyclonal antihuman VEGF-D (2.0 μg/mL, H-144, Santa Cruz Biotechnology, Santa Cruz, Calif); mouse monoclonal antihuman CD68 (1:100, KP-1, isotype

foam cells and CD3-positive T lymphocytes was frequently seen around the atherosclerotic lipid cores, especially in the fibrous cap and shoulder regions. Type V lesions were found in 4% (6/169) of the specimens in this study, representing cases of fibroatheroma. Two specimens of the type V lesion were associated with calcified plaque. Type VI lesions were in 2% (4/169) of the specimens and were associated with disruptions of the lesion surface, hematoma or hemorrhage, and thrombotic deposits.

3.2. Expression of lymphangiogenic factors

3.2.1. Vascular endothelial growth factor-C

The percentage of specimens associated with at least a few VEGF-C–positive cells was as follows: DIT, 81% (17/21); type I, 56% (9/16); type II, 95% (37/39); type III, 97% (33/34); type IV, 100% (49/49); type V, 100% (6/6); and type VI, 100% (4/4). Vascular endothelial growth factor-C–positive cells were more commonly observed in the advanced lesions than in the early lesions ($P < .0001$, Spearman's rank correlation analysis, Spearman's $\rho = 0.52267$; Table 2A). In DIT lesions, VEGF-C–positive cells showed a few macrophages in the intima and a few spindle-shaped SMCs in the intima and media (data not shown). In cases of early atherosclerotic lesions (types I and II), VEGF-C–positive cells were mainly some foamy macrophages in the intima and some spindle-shaped SMCs in the intima and media (Fig. 1C). In types III through VI

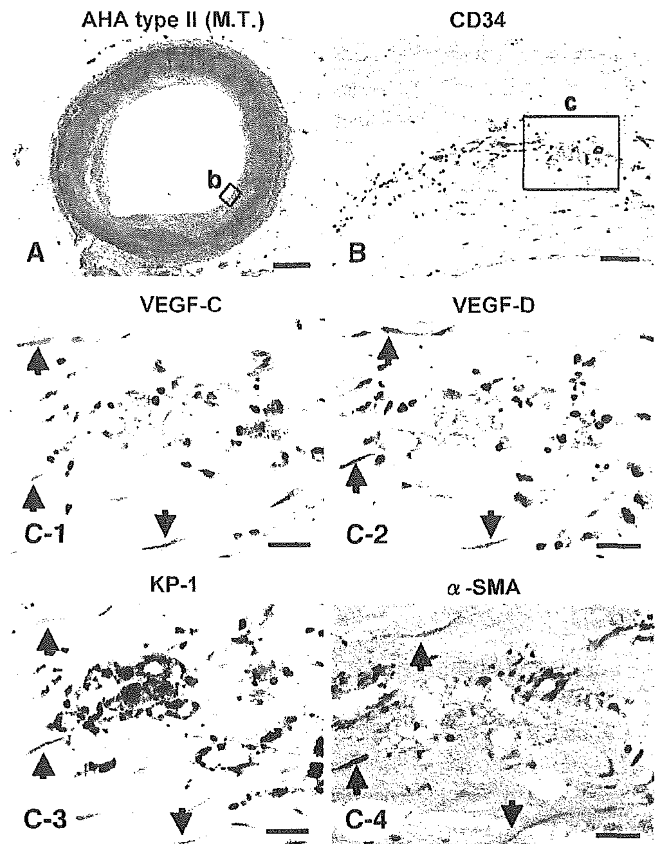

Fig. 1 Immunohistochemical identification of intimal neovascularization and expression of VEGF-C and VEGF-D in an early atherosclerotic lesion (AHA type II). Panels (B) and (C) are counterstained with hematoxylin. Scale bars: in (A), 500 μm; (B), 50 μm; and (C), 20 μm. A, A light microscopic view of a case of an AHA type II lesion (Masson trichrome staining). B, Corresponding lesion of the serial section as the boxed area indicated in panel (A) showing CD34-positive vessels in the intima (circumferential brown reaction products). C, C-1 through C-4 are the corresponding lesions of the serial sections from the boxed area in panel (B). Each serial panel shows the immunoreactivity for VEGF-C (C-1), VEGF-D (C-2), KP-1 (monocytes/macrophages, C-3), and α-SMA (SMCs, C-4). The arrows indicate α-SMA–positive SMCs. Note that most foamy macrophages (KP-1) are also positive for both VEGF-C and VEGF-D.

Table 2 Relationship between the AHA lesion type and VEGF-C and VEGF-D expression in the intima

(A) VEGF-C expression in the intima

AHA classification								
VEGF-C	DIT	I	II	III	IV	V	VI	Total
–	4	7	2	1				14
±	11	3	9	2	5	2		32
+	5	4	20	13	18	2		62
2+	1	2	8	16	18	1		46
3+				2	8	1	4	15
Total	21	16	39	34	49	6	4	169

(B) VEGF-D expression in the intima

VEGF-D	DIT	I	II	III	IV	V	VI	Total
+	5	6	10	7	15	4	1	48
2+	7	2	9	12	16	1	1	48
3+	5	4	10	6	6			31
4+	4	4	10	9	12	1	2	42
Total	21	16	39	34	49	6	4	169

(2A) The number of VEGF-C–positive cells: – indicates no staining; ±, 1 to 19 positive cells; +, 20 to 99; 2+, 100 to 499; and 3+, more than 500. According to the atherosclerotic lesion types advanced, the number of VEGF-C–positive cells in the intima was increased ($P < .0001$, Spearman's rank correlation analysis, Spearman's $\rho = .52267$).

(2B) The number of VEGF-D–positive cells: + indicates 1 to 499; 2+, 500 to 999; 3+, 1000 to 1499; and 4+, more than 1500. No significant correlation was observed between the number of VEGF-D–positive cells and the atherosclerotic progression ($P = .3507$, Spearman's rank correlation analysis, Spearman's $\rho = -0.07223$).

atherosclerotic lesions, immunoreaction of VEGF-C was found in the accumulation of numerous foamy macrophages in the intima and some spindle-shaped SMCs in the intima and media (Fig. 2C and D). Vascular endothelial growth factor-C–positive cells were mainly located around the atheromatous plaque, particularly in the shoulder, the cap region, and the deeper portion of the intima. A few endothelial cells were also positive for VEGF-C (data not shown).

3.2.2. Vascular endothelial growth factor-D

All 169 specimens including DIT and advanced atherosclerotic lesions showed a VEGF-D–positive reaction. The VEGF-D was abundantly expressed in many SMCs and macrophages, including foamy macrophages, in the intima

and media (Fig. 1C and Fig. 2C and D). We could not prove a significant correlation between the number of VEGF-D–positive cells and the atherosclerotic progression (P = .3507, Spearman's rank correlation analysis, Spearman's $\rho = -0.07223$; Table 2B). Some endothelial cells were also positive for VEGF-D (data not shown).

3.3. Progression of atherosclerosis and intimal angiogenesis and lymphangiogenesis

3.3.1. Angiogenesis

No intimal neovascularization, labeled by CD34, was found in any coronary artery with DIT at all, and inversely, all coronary arteries of type VI lesions were associated with angiogenesis in the atherosclerotic intimas. From types I to VI, the more the atherosclerotic lesions advanced, the more often the neointima contained newly formed blood vessels, as follows: DIT, 0% (0/21); type I, 38% (6/16); II, 54% (21/39); III, 79% (27/34); IV, 82% (40/49); V, 83% (5/6); and VI, 100% (4/4) (P < .0001, Mann-Whitney U test).

3.3.2. Lymphangiogenesis

In almost all cases, the LYVE-1 and podoplanin immunoreactivities could differentiate lymphatic vessels from CD34-positive blood vessels in the human small intestine and the adventitia of coronary artery as control (Fig. 3). In the corresponding atherosclerotic intima, on the other hand, LYVE-1–positive or podoplanin-positive lymphatic vessels were partly negative for CD34 in the intima of 169 coronary arteries (Fig. 4). Six intimal vessels were positive for both LYVE-1 and CD34 (Fig. 4C and D; summarized in Table 3A). Similar findings were obtained in using antipodoplanin antibody (data not shown).

Quantitative study revealed that only 13 vessels were positive for LYVE-1 in all samples, whereas 3955 vessels were positive for CD34 in the intima (Table 3B). On the other hand, 360 vessels were positive for LYVE-1 and 6921 vessels were positive for CD34 in the adventitia of 20 coronary arteries. Thus, there were far fewer lymphatic vessels in the intima than in the adventitia (P < .0001, χ^2 test).

3.4. Progression of atherosclerosis and lymphangiogenesis in the intima

Because of the very small number of positive vessels, statistical analysis regarding the relationship between

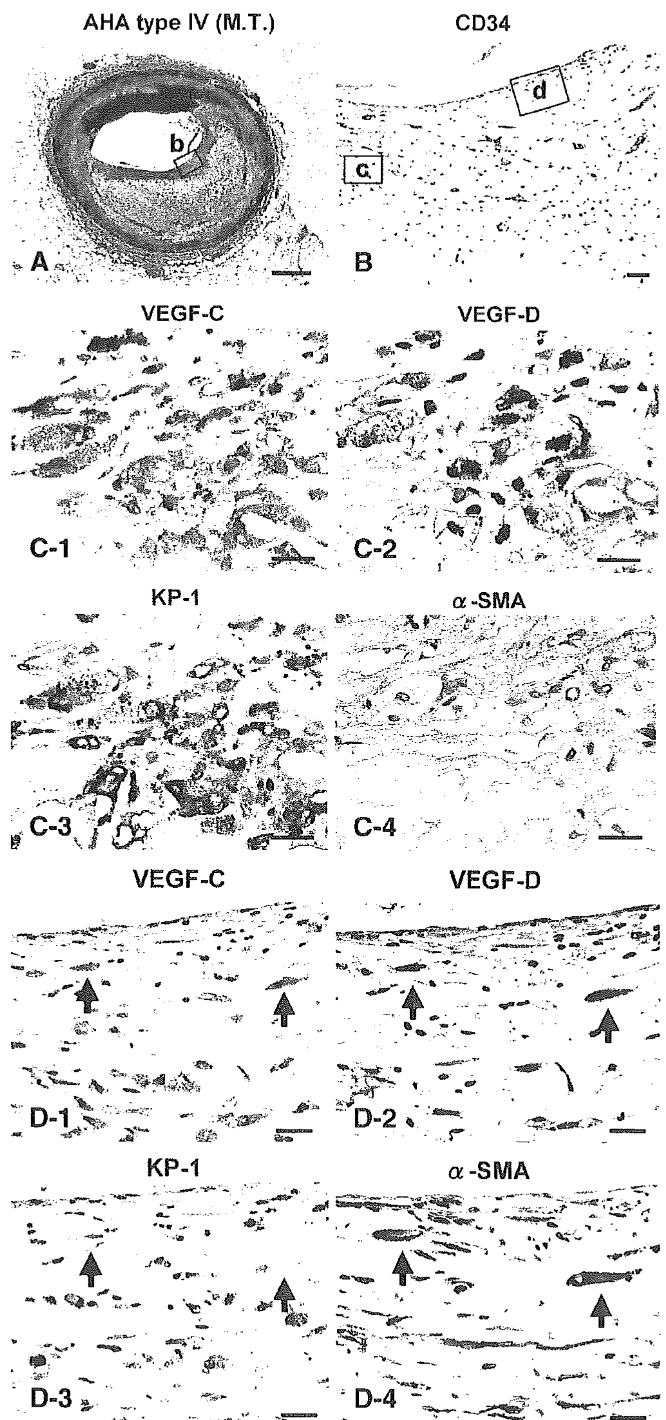

Fig. 2 Immunohistochemical identification of intimal neovascularization and expression of VEGF-C and VEGF-D in an advanced atherosclerotic lesion (AHA type IV). Panels (B) through (D) are counterstained with hematoxylin. Scale bars: in (A), 500 μm; (B), 50 μm; and (C) and (D), 20 μm. A, A light microscopic view of a case of an AHA type IV lesion (Masson trichrome staining). B, Corresponding lesion of the serial section from the boxed area indicated in panel (A) showing CD34-positive vessels in the intima (circumferential brown reaction products). C, C-1 through C-4 are the corresponding lesions of the serial sections from the boxed area in panel (B). Each serial panel shows the immunoreactivity for VEGF-C (C-1), VEGF-D (C-2), KP-1 (monocytes/macrophages, C-3), and α-SMA (SMCs, C-4). The arrows indicate α-SMA–positive SMCs. Note that most foamy macrophages (KP-1) are also positive for both VEGF-C and VEGF-D. D, D-1 through D-4 are the corresponding lesions of the serial sections from the boxed area in panel (B). Each serial panel shows the immunoreactivity for VEGF-C (D-1), VEGF-D (D-2), KP-1 (monocytes/macrophages, D-3), and α-SMA (SMCs, D-4). The arrows indicate α-SMA–positive SMCs. Note that most foamy macrophages (KP-1) and some SMCs (α-SMA, arrows) are positive for both VEGF-C and VEGF-D.

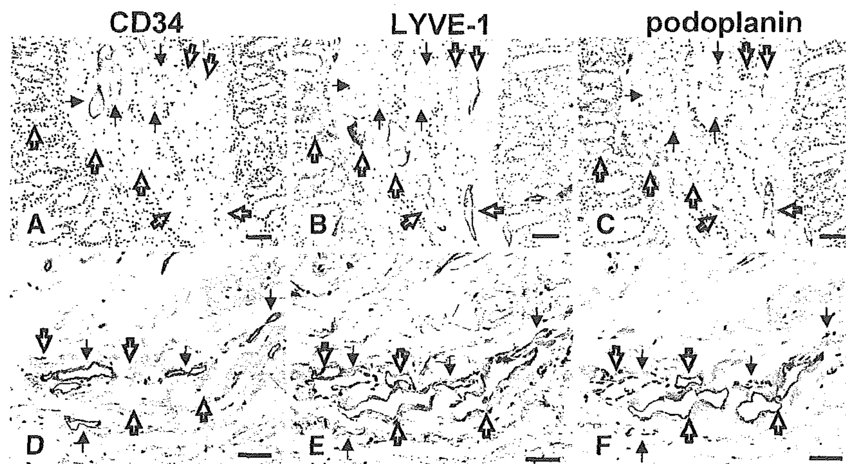

Fig. 3 Immunohistochemical identification of blood (CD34) and lymphatic (LYVE-1 and podoplanin) vessels in the human small intestine (A-C) and the adventitia of human coronary artery (D-F). All sections were counterstained with hematoxylin. Scale bars = 50 μm. Immunohistochemical labeling of blood (CD34, panels A and D) and lymphatic (LYVE-1, panels B and E; podoplanin, panels C and F) vessels in the human small intestine (A-C) and the adventitia (D-F) of serial sections. Note that no apparent double labeling of CD34 (arrows) and LYVE-1 or podoplanin (open arrows) was found in vessels located in the controls.

LYVE-1–positive vessels and atherosclerotic lesion types could not show a positive correlation (P = .1616, Mann-Whitney U test). Similar findings were seen in using antipodoplanin antibody (data not shown).

3.5. Relationship between neovascularization and VEGF-C and VEGF-D expression

3.5.1. Blood vessels ($CD34^+$/LYVE-1^-)

The degree of VEGF-C–positive cell occurrence, but not that of VEGF-D, in the atherosclerotic intima was positively correlated with the number of intimal blood vessels ($CD34^+$/LYVE-1^-; Fig. 5) (Spearman's rank correlation analysis - VEGF-C: $P < .0001$, Spearman's ρ = 0.52491 [Fig. 5A]; VEGF-D: P = .0789, Spearman's ρ = 0.13556 [Fig. 5B]).

3.5.2. Lymphatic vessels (LYVE-1^+)

The degree of VEGF-C–positive cells in the atherosclerotic intimas was positively correlated with the number of intimal LYVE-1^+ (Fig. 6) (Spearman's rank correlation analysis - LYVE-1: P = .0322, Spearman's ρ = 0.16487 [Fig. 6A]). No significant correlation was observed between the degree of VEGF-D–positive cells and the number of lymphatic vessels in the atherosclerotic intima (Fig. 6B).

4. Discussion

We immunohistochemically examined the expression of VEGF-C and VEGF-D, intimal neovascularization, and atherosclerotic progression in human coronary arteries. Key observations made in this study are as follows: (1) the frequency of VEGF-C–expressing cells, but not that of VEGF-D–expressing cells, was positively correlated with the number of neovessels as well as the severity of atherosclerosis; (2) VEGF-C was expressed mainly in foamy macrophages and in some SMCs; and (3) although intimal angiogenesis was frequently observed, lymphangiogenesis was a very rare event (even when there was frequent expression of VEGF-C and VEGF-D) in the atherosclerotic intima of human coronary arteries. To the best of our knowledge, this is the first report assessing lymphangiogenesis and the expression of lymphangiogenic factors in human atherosclerotic lesions, and findings obtained in this study imply an unusual development of neovessels (namely, an imbalance of angiogenesis and lymphangiogenesis) in coronary atherosclerosis.

4.1. Selection for markers that label blood and lymphatic endothelial cells

The reason why we used CD34 to distinguish vascular endothelial cells from lymphatics is that LYVE-1 is a lymphatic-specific receptor for hyaluronan and is expressed on lymphatic but not blood vascular endothelium [24-26], with the possible exception of liver sinusoids. Podoplanin, a 38-kDa membrane glycoprotein originally identified on podocytes, is expressed on the endothelium of lymphatic capillaries but not in the quiescent or proliferating blood vascular endothelium [27]. A recent important study demonstrated that podoplanin$^+$/$CD34^{low}$ endothelial cells were identified as lymphatic endothelial cells; in contrast, podoplanin$^-$/$CD34^{high}$ endothelial cells were vascular endothelial cells using flow cytometry [28]. They also revealed that CD31 and von Willebrand factor, which have been used as markers for endothelium of blood vessels, were stably expressed by both lymphatic and vascular endothelial cells [28]. Therefore, we concluded that CD34

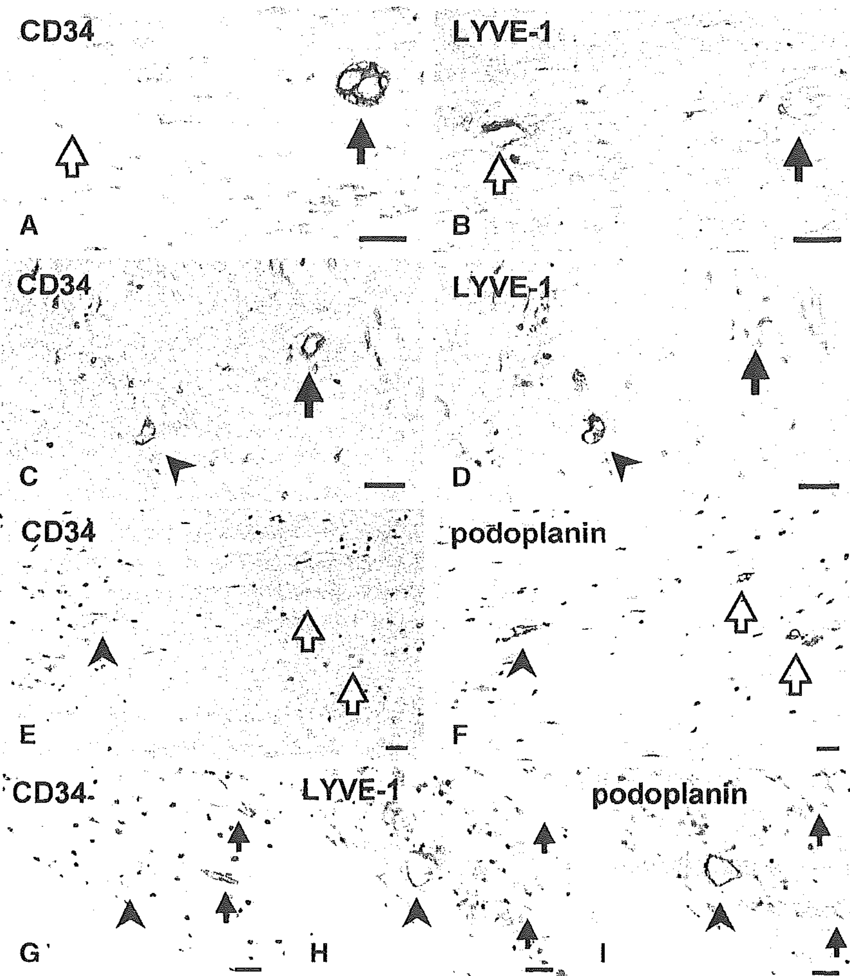

Fig. 4 Immunohistochemical identification of blood (CD34) and lymphatic (LYVE-1 and podoplanin) vessels in the intima of human coronary artery. All sections were counterstained with hematoxylin. Scale bars = 20 μm. A-D, Immunohistochemical labeling of blood (CD34, panels A and C) and lymphatic (LYVE-1, panels B and D) vessels in the atherosclerotic intima. Panels (A) and (B) and panels (C) and (D) are serial sections of different tissue samples. In panels (A) and (B), no apparent double labeling of CD34 (arrows) and LYVE-1 (open arrows) was found in vessels. In panels (C) and (D), the arrowhead indicates a double-positive vessel. Note that these panels also contain a single-labeled (CD34) vessel (arrow). E-F, Immunohistochemical labeling of blood (CD34, panel E) and lymphatic (podoplanin, panel F) vessels in the atherosclerotic intima. Panels (E) and (F) are serial sections. Panel (F) shows 3 podoplanin-positive vessels (2 open arrows and 1 arrowhead). The arrowhead indicates a double-positive vessel. G-I, An example demonstrating the LYVE-1 and podoplanin doubly labeled vessel in the intima. Panels (G) to (I) are serial sections (panel G, CD34; panel H, LYVE-1; panel I, podoplanin). CD34-positive vessels (arrows) are almost negative for LYVE-1 and podoplanin. The LYVE-1 and podoplanin doubly labeled vessel is weakly positive for CD34 (arrowhead, panel G).

is, at present, the best marker to distinguish vascular endothelial cells from others, including lymphatic vessels.

4.2. Role of expression of lymphangiogenic factors in atherosclerosis

Recent emerging evidence strongly suggest and have established an essential contribution of VEGF-A to the intimal neovascularization as well as the progression of atherosclerosis in human [7,8] and animal [9-13] subjects; however, the information regarding the roles of lymphangiogenesis and expression of lymphangiogenic factors have been very limited. Similar to VEGF-A, VEGF-C and VEGF-D also stimulate angiogenesis in animal models (ie, in the mouse and rabbit corneas) as well as in the hind limb ischemia model [20-22]. On the other hand, unlike VEGF-A, the expression of VEGF-C is not regulated by hypoxia [29] but is increased in response to proinflammatory cytokines [30], and, furthermore, VEGF-C has synergistic effects with VEGF-A regarding the induction of angiogenesis [31].

In this study, we identified KP-1–positive macrophages as a major source of VEGF-C in human coronary atherosclerosis, a finding supported by some recent reports indicating that VEGF-C was expressed in tumor-associated macrophages in human subjects [32] and that monocytes derived from human peripheral blood expressed VEGF-C when they were stimulated by tumor necrosis factor-α or

Table 3 The number of blood and lymphatic vessels in atherosclerotic coronary arteries

(A) The number of CD34, LYVE-1–positive vessels classified according to the atherosclerotic lesion types

AHA classification								
Endothelial marker	DIT	I	II	III	IV	V	VI	Total
$CD34^+$	0	63	392	980	1832	259	429	3955
$LYVE\text{-}1^+$	0	0	4	3	1	0	5	13
($CD34^+/LYVE\text{-}1^+$)			(1)		(1)		(4)	(6)

(B) Comparison between the number of LYVE-1–positive and CD34-positive vessels in the intima and the adventitia of coronary arteries

	Intima (n = 169)	Adventitia (n = 20)
LYVE-1–positive vessels	13*	360
CD34-positive vessels	3955	6921

* $P < .0001$ (χ^2 test).

lipopolysaccharide. Vascular SMCs were also reported to express VEGF-C [33], also supporting our current findings. Not only the cell sources and their distribution of VEGF-C but also their close relationships with the degree of atherosclerosis and neovessels clarified in this study were very similar to those of VEGF-A in our previous study [7]. Together, it may be possible that VEGF-A and VEGF-C might synergistically contribute to angiogenesis in coronary atherosclerotic plaques.

An alternative factor, VEGF-D, also stimulates lymphangiogenesis, which was shown by adenoviral delivery of the short form of human VEGF-D [34]. Our study showed VEGF-D abundantly in both macrophages and SMCs throughout all phases of coronary atherosclerosis in the intima and media, without significant correlation to the severity of the plaque or to the intimal neovascularization, suggesting that VEGF-D is not likely to be an important regulator for plaque progression in human coronary arteries. These results are supported by a previous study [35]. The exact reason why the expression of VEGF-D was not correlated to the atherosclerotic lesions and to neovascularization in our samples is unknown; a possible explanation may include the lack of some proteases that could sufficiently activate VEGF-D in atherosclerotic lesions, because VEGF-D requires proteolytic digestion after secretion to be an active form [36]. To clarify this hypothesis, further studies are called for to detect such enzymes and to confirm whether VEGF-D in human coronary arteries is biologically active.

4.3. Significance of loss of lymphangiogenesis in coronary atherosclerosis

At present, to our best knowledge, no published report can be found regarding lymphangiogenesis in atherosclerotic coronary arteries in human subjects. An experimental study demonstrated that the blockage of cardiac lymphatic drainage promoted subendothelial and medial edema of the coronary artery in a dog [37], suggesting that lymphatic vessels play an important role in the efflux of interstitial fluid from the wall of the coronary artery. Here we demonstrated that lymphangiogenesis, labeled by LYVE-1 and podoplanin, was rare in human coronary atherosclerosis compared with that seen in the adventitia, even where angiogenic neovessels labeled by CD34 were frequently observed. These results suggest that the imbalance of angiogenesis and lymphangiogenesis may result in disturbed lymphatic drainage in atherosclerotic plaque. Paavonen et al [38] reported that there were few VEGFR-3–positive lymphatic vessels in chronic wounds such as ulcers and decubitus wounds of human legs. They suggested that the relative absence of lymphatic vessels in the chronic wounds may be one of the reasons for their impaired healing. Lymphedema due to insufficient lymphatic drainage can be caused by inflammatory or neoplastic obstruction of the lymphatic vessels including nonhealing wounds, lymphatic filariasis, peritoneal carcinomatosis, or surgery for breast cancer [14]. In case of tumors, absence of functional lymphatics within tumors may contribute to interstitial hypertension and interfere with the delivery of therapeutic agents [39]. The unfavorable effect of the disturbance of lymphatic drainage may include delay of the healing process of inflammatory reaction (eg, the cardiac lymphatic blockade model also showed degenerative changes and fibrosis in adventitia [37]) and infiltration with mononuclear cells in adventitia [37]. Loss of lymphatic vessels, therefore, implies an increased amount of interstitial fluid and pressure, which may affect the oxygenation, microcirculation, and rupture of plaques. Further experimental studies are called for to certify that these explanations may be significant in plaque progression.

4.4. Study limitation

A limitation of the current study is that we still cannot assess why the expression of VEGF-C contributed mainly to angiogenesis but not to lymphangiogenesis in human coronary atherosclerosis. Using commercially available antibodies against VEGFR-1/FLT-1, VEGFR-2/FLK-1/

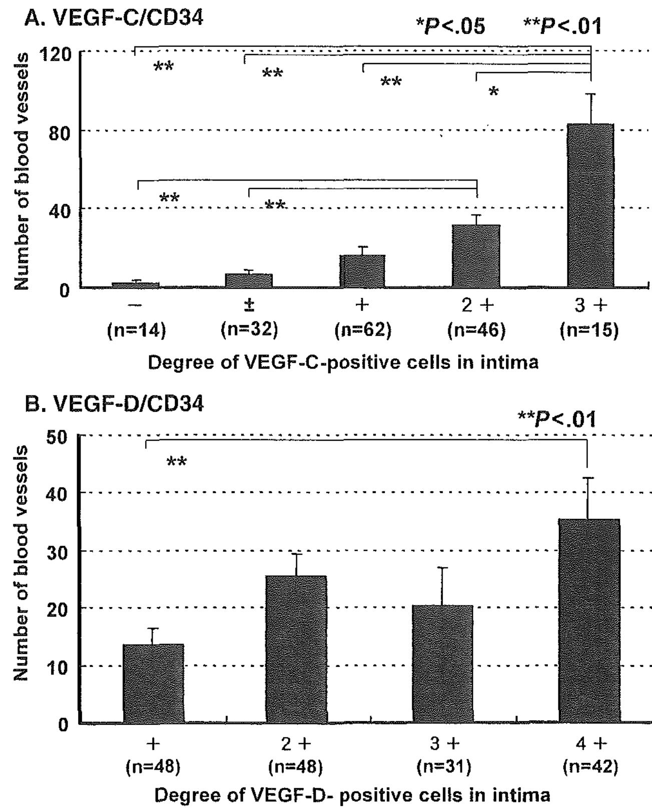

Fig. 5 Bar graphs indicating the relationship between neovascularization and VEGF-C and VEGF-D expression in the intima. The number of intimal blood vessels was counted as CD34-positive lumens. Each bar represents the mean value of the intimal blood vessel number ± SEM in all sections examined in each group. The differences among the groups were analyzed by the Bonferroni test (asterisk indicates $P < .05$; double asterisk, $P < .01$). A, The number of VEGF-C–positive cells in the intima of each section was categorized into 5 grades as follows: – indicates no staining; ±, 1 to 19 positive cells; +, 20 to 99; 2+, 100 to 499; and 3+, more than 500 in each section of coronary artery. The correlation of the degree of VEGF-C–positive cells in the intima and the number of newly formed blood vessels in the atherosclerotic intima was found to be statistically significant ($P < .0001$, Spearman's rank correlation analysis, Spearman's $\rho = .52491$). B, The number of VEGF-D–positive cells in the intima of each section was categorized into 4 grades as follows: + indicates 1 to 499; 2+, 500 to 999; 3+, 1000 to 1499; and 4+, more than 1500 in each section of coronary artery. No significant correlation was observed between the degree of VEGF-D–positive cells and the number of newly formed blood vessels in the atherosclerotic intima ($P = .0789$, Spearman's rank correlation analysis, Spearman's $\rho = .13556$).

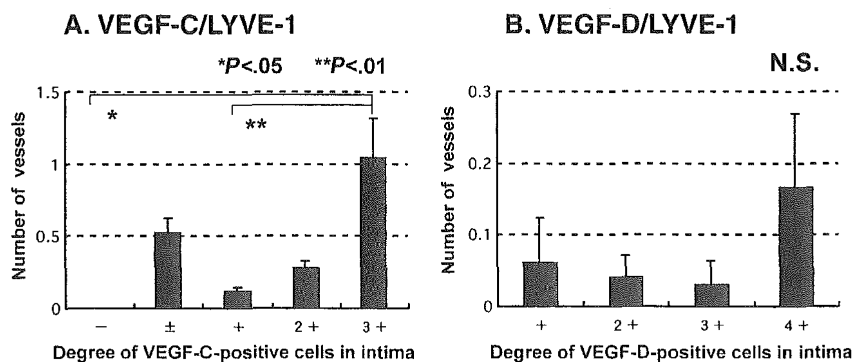

Fig. 6 Bar graphs indicating the relationship between lymphangiogenesis and VEGF-C and VEGF-D expression in the intima. Each bar graph represents the mean value of the intimal blood vessel number ± SEM in all sections examined in each group. The differences among each group were analyzed by the Bonferroni test (asterisk indicates $P < .05$; double asterisk, $P < .01$). A, The relationship between VEGF-C expression and LYVE-1–positive vessels in the intima. The correlation of the degree of VEGF-C–positive cells in the intima and the number of lymphatic vessels in the intima was found to be statistically significant ($P = .0322$, Spearman's rank correlation analysis, Spearman's $\rho = 0.16487$). B, The relationship between VEGF-D expression and LYVE-1–positive vessels in the intima. No significant correlation was observed between the degree of VEGF-D–positive cells and the number of LYVE-1–positive vessels in the intima ($P = .1439$, Spearman's rank correlation analysis, Spearman's $\rho = 0.11290$).

KDR, and VEGFR-3/FLT-4, we investigated the expression patterns of these receptors; however, we concluded that patterns of labeling of these antibodies were not definitively reliable in our 169 sections as well as in the human small intestine as a positive control. A recent report indicated that VEGF-C/FLT-4 signals negatively modulated FLK-1/KDR signals to maintain endothelial cell integrity [40]; thus, the imbalance between VEGFR-3/FLT-4 and FLK-1/KDR expressions, if reliable antibodies were available, might clarify part of the evidence of disturbed lymphangiogenesis in coronary atherosclerosis. Thus, further studies are required in this regard.

5. Conclusions

In summary, we demonstrated that the number of VEGF-C–expressing cells well correlated with the amount of intimal neovascularization as well as the types of atherosclerotic lesions advanced in human coronary arteries associated with atherosclerosis. This may be an unsuitable circumstance for the occurrence of lymphangiogenesis in the atherosclerotic intima of the coronary artery; therefore, VEGF-C may act as an endogenous regulator for angiogenesis rather than lymphangiogenesis in the atherosclerotic intima, which may also participate in coronary plaque progression.

Acknowledgments

We thank Hiroshi Fujii for his excellent technical assistance. This work was supported in part by a Grant in Aid (YY and KS) from the Japanese Ministry of Education, Culture, Sports, Science, and Technology, Tokyo, Japan, and by a Grant of Promotion of Basic Science Research in Medical Frontier from the Organization for Pharmaceutical Safety and Research, Tokyo, Japan (YY and KS; project no.MF-21).

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Platelet-Derived Growth Factor-AA Is an Essential and Autocrine Regulator of Vascular Endothelial Growth Factor Expression in Non–Small Cell Lung Carcinomas

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Abstract

It is widely accepted that angiogenesis is required for tumor progression. Vascular endothelial growth factor (VEGF) is a key molecule for tumor angiogenesis; however, its expressional regulation is not well understood during all stages of tumorigenesis. Using cell lines and surgical specimens of human non–small cell lung cancers (NSCLCs), we here show that platelet-derived growth factor-AA (PDGF-AA) is an essential autocrine regulator for VEGF expression. To directly assess the expression of PDGF-AA–dependent VEGF and its roles in tumorigenesis, we stably transfected established cell lines with their antisense genes. In addition, the levels of PDGF-AA and VEGF expression in surgical sections were measured and compared with clinicopathologic findings such as tumor size and patient prognosis. PDGF-AA tightly regulated VEGF expression and had a greater effect on tumor size and patient prognosis than did VEGF in both cell lines and surgical sections. PDGF-AA expression was not seen in the atypical adenomatous hyperplasia at all, whereas VEGF was occasionally seen. Furthermore, the frequency of VEGF expression was higher in advanced NSCLCs than in precancerous lesions, which was tightly correspondent to the results for PDGF-AA. These results indicate that PDGF-AA is an important regulator of the frequency and level of VEGF expression during the transition from a precancerous lesion to advanced cancer. The PDGF-AA/VEGF axis, therefore, may be a ubiquitous autocrine system for enhancing angiogenic signals, and PDGF-AA, and its related pathways could be a more efficient target of antiangiogenic therapy for cancers than VEGF and its pathways. (Cancer Res 2005; 65(16): 7241-8)

Introduction

Angiogenesis is required for tumor progression, as supported by a number of studies showing a reduction in tumor growth by antiangiogenic agents (1–3). Folkman et al. proposed the concept of an "angiogenic switch" that is required for the progression to advanced cancer from an occult tumor (4). It is now widely accepted that the angiogenic switch is "off" when the effect of proangiogenic molecules is silent and "on" when the net balance is tipped in favor of angiogenesis (5, 6). Relatively less attention, however, has been paid to defining which molecule is the angiogenic switch or how it acts.

According to the original proposal (1), a molecule must have the following characteristics before it can be considered as a possible angiogenic switch: (a) it must be expressed specifically in advanced cancer but not in precancerous or early cancerous lesions of <2 mm^3; and (b) it must determine the size, and probably the malignant potentials, of cancers by controlling the expression of angiogenic growth factors. In the last decade, various signals that trigger tumor angiogenesis, including angiogenic factors, have been identified (7), and among these, vascular endothelial growth factor-A (VEGF-A, commonly called VEGF) has been recognized as one of the most potent mediators of tumor angiogenesis (8). A logical question followed: is VEGF an angiogenic switch for tumors? Unfortunately, this is not likely, because VEGF is abundantly expressed in cancers, precancerous lesions, and their originating tissue, although VEGF is essential for tumor angiogenesis and its frequency of expression is higher in advanced malignancies than in noncancerous tissue (9, 10). These theoretical considerations suggest the existence of upstream regulator(s) that mediate the expression of VEGF in tumors as molecular candidates for the angiogenic switch; however, the regulational mechanism of the expression of VEGF in each tumor has not been fully elucidated.

Several important studies have indicated that some oncogenes determining the malignant potentials of cancer, including Ras (11) and HER-2/*neu* (12), up-regulate VEGF expression, suggesting that the "angiogenic switch" might contain multiple molecules and signal transduction pathways. Furthermore, knowledge of important related mechanisms, such as that of the extracellular regulatory system for enhancing the expression of VEGF, is also limited.

Recently, we showed that the platelet-derived growth factor-AA (PDGF-AA)/PDGFα-receptor (PDGFRα)/p70S6K pathway in mesenchymal cells (fibroblasts and vascular smooth muscle cells) was essential for therapeutic and tumor angiogenesis *in vivo* (13). We also showed that PDGF-AA, which does not target the vascular endothelial cells, dominantly regulates the expression of VEGF, essentially contributing to the angiogenic process *in vivo* (13).

A previous study revealed that the expression of PDGF-AA is strictly limited in mesenchymal cells but not in cells of epithelial lineage, because of the methylation of GC-rich sequences of the *PDGF-A* promoter in epithelial cells (14). Demethylation can result in dysregulated expression of multiple genes and is now

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

The authors declare that they have no competing financial interests.

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doi:10.1158/0008-5472.CAN-04-4171

suggested to be an essential step for carcinogenesis (15, 16). These facts may support the hypothesis that a gain-of-function in cancers causes the dysregulated expression of PDGF-AA, which in turn causes the enhanced expression of VEGF by an autocrine mechanism, a system would be similar to that observed in mesenchymal cells. Using established cell lines and surgical specimens of non–small cell lung cancers (NSCLCs), which are a major cause of death in Western countries and Japan, we here show that PDGF-AA is a critical autocrine regulator for VEGF, which may be involved in the "angiogenic switch"–related pathways in malignancies.

Materials and Methods

Cells. The cancer cell lines, QG56 (human lung squamous cell carcinoma), A549 (human lung adenocarcinoma), and SAS and TF (human oral squamous cell carcinoma), were maintained in continuous culture in RPMI 1640 supplemented with 100 units/mL penicillin/streptomycin and 10% FCS. These cell lines were washed, and cell pellets were collected and then frozen in liquid nitrogen.

Reverse transcriptional-PCR. The gene expression of full-length *PDGF-A* was determined from MRC5 cells (a human fibroblast cell line). Total RNA of cultured cells growing exponentially was extracted using ISOGEN (Nippon Gene, Inc., Toyama, Japan) according to the manufacturer's protocol. Total RNA of clinical tissue was isolated using the same method. Synthesis of cDNA was done with 5.0 μg total RNA using a first-strand cDNA Synthesis Kit (Invitrogen Corp., Carlsbad, CA).

Construction of AS-PDGF-A/AS-VEGF plasmid. Primers incorporating the *Eco*RI site for PCR of PDGF-A were as follows: forward 5′-aaGAATTCatgaggaccttggcttgcctgc and reverse 5′-ttGAATTCttaggtgggttt-taacctttttctttt. PCR amplicons were directly inserted into a plasmid vector (PCR II) by the TA cloning method according to the manufacturer's protocol (Invitrogen). Gene expression was compared using the CEQ 2000 Sequence Detection System (Beckman Coulter, Inc., Fullerton, CA). The whole sequence was determined to be completely matched to a reported sequence (Genbank accession no. X03795). The amplicons were abstracted using an *Eco*RI restriction enzyme and then inserted into the vector for mammalian cell expression, pcDNA3.1(+) (Invitrogen).

The human VEGF165 cDNA expression plasmid vector was previously obtained (17). The *AS-VEGF* gene expression plasmid based on pcDNA3.1(+) was constructed as described above.

Establishment of stable transformant. Stable transfections were done using LipofectAMINE 2000 reagent (LF2000; Invitrogen) according to the manufacturer's instructions. Briefly, logarithmically growing cells were transfected with plasmid-LF2000 complex at a 1:2 weight ratio. Forty-eight hours later, the selective medium containing 0.5 mg/mL geneticin antibiotics (G418; Promega Corp., Madison, WI) was replaced, and the cell clones were selected and identified. Next, the transfected cells were spread onto 96-multiwell plates and cultured to confluence, and the cell clones that reduced PDGF-AA or VEGF expression were selected using the ELISA kit included in the Human PDGF-AA Immunoassay System and the ELISA kit included in the Human VEGF Immunoassay System, which detect VEGF121 and VEGF165 (both from R&D Systems, Inc., Minneapolis, MN).

The cell cloning was done thrice, and the cell clone showing the lowest PDGF-AA or VEGF expression in each cell line was used for experiments.

Immunoprecipitation. For immunoprecipitation, cells were lysed with 500 μL of cell lysis buffer (Promega) containing a cocktail of protease inhibitors (1.5 mmol/L pepstatin, 4 mmol/L leupeptin, 0.01 mol/L aprotinin, and 500 mmol/L phenylmethylsulfonyl fluoride), and the supernatant of the lysed cells was recovered for immunoprecipitation. The amount of protein was determined with a Protein Assay kit (Bio-Rad Laboratories, Inc., Hercules, CA), and aliquots of 500 μg of proteins were used for immunoprecipitation.

Nonspecific proteins bound to magnetic beads were eliminated by exposure to protein-G magnetic beads (Pharmacia Biotech AB, Uppsala, Sweden). For immunoprecipitation, 3 μg of anti-human PDGFRα antibody (R&D Systems) was added to the tube, bead pellets were heat treated in 20 mL mercaptoethanol sample buffer, and the samples were loaded onto 10% SDS-polyacrylamide gel. After transfer to the nitrocellulose membrane, immunoprecipitated proteins were determined by immunoblotting with polyclonal anti-phosphorylated-PDGFRα antibody (p-PDGFRα; Tyr^{720}, 1:100; Santa Cruz Biotechnology, Inc., Santa Cruz, CA).

Tissue samples. For immunohistochemistry, tissue samples were derived from 128 Japanese patients with NSCLC who underwent surgical resection at the Department of Surgery, Kyushu University Hospital (Fukuoka, Japan) between January 1990 and April 1995; for real-time quantitative PCR, samples were taken from 60 Japanese patients with NSCLC who underwent surgical resection in the same department between January 1996 and April 2000. No patient had received any antitumor treatment, including anticancer drugs or radiation therapy, before surgery.

Tumor and adjacent normal lung tissue samples were freshly obtained from resected lobes, immediately frozen in liquid nitrogen, and stored at −80°C for real-time PCR and reverse transcription-PCR (RT-PCR), or embedded in paraffin for immunohistochemistry after formalin fixation. All patients gave their informed consent before the tissue sampling.

Real-time quantitative PCR. Gene expression was measured using the ABI Prism 7000 Sequence Detection System (Perkin-Elmer Corp., Foster City, CA). The primer sequences were as follows: human PDGF-A, 636 bp, forward 5′-TCCACGCCACTAAGCATGTG-3′; reverse 5′-TCGACCTGACTCC-GAGGAAT-3′; probe 5′-FAM-CTGCAAGACCAGGACGGTCATTTACGA-TAMRA-3′; human VEGF, 574 bp, forward 5′-GAAGTGGTGAAGTTCATG-GATGTCTAT-3′; reverse 5′-TCAGGGTACTCCTGGAAGATGTC-3′; probe 5′-FAM-ACTGCCATCCAATCGAGACCCTGG-TAMRA -3′.

The coefficient of correlation was $\rho = 0.97$, and the slope was constant in each experiment. As internal controls, the same samples were tested for 18S rRNA (Perkin-Elmer) in the same manner. Each sample was analyzed in duplicate.

Immunohistochemistry. Formalin-fixed and paraffin-embedded tissue sections (diameter, 5 mm) were reacted overnight at 4°C with goat anti-PDGF-A antibody (1:65 in PBS, R&D Systems) or mouse anti-VEGF antibody (1:20 in PBS, R&D Systems) as primary antibodies. Isotype-matched nonimmune antibodies were used for negative controls. The rinsed sections were subjected to peroxidase-labeled secondary antibody (1:250 in PBS) at room temperature for 30 minutes. PDGF-A or VEGF protein was visualized using diaminobenzidine, and nuclei were counterstained with hematoxylin. A positive reaction was defined as immunohistochemical positivity in cells that showed a stronger reaction than that seen in arterial vascular smooth muscle cells in the same specimen, and in at least 30% of tumor cells at a ×200 high-power field for five randomly selected areas. All sections were examined by two independent investigators (Y.S. and T.K.) who were blinded to the clinical data.

For evaluation of angiogenesis, the dehydrated slides were treated with 40 mg/mL of proteinase K (DAKO, Carpinteria, CA) for 10 minutes at room temperature. After washing in PBS, they were treated with 3% H_2O_2 and then 5% nonfat dry milk, and incubated with von Willebrand factor (vWF, 1:800; DAKO) overnight at 4°C. The following procedures and visualization were done using the same methods described above. The microvessels in the tumor and adjacent mesenchyma, which was margined at 2-mm distant from the tumor periphery, were labeled with vWF under light microscopy.

Animals. Male BALB/c *nu/nu* mice (5 weeks old) were from Kyudo Co., Ltd. (Tosu, Saga, Japan). All animal experiments were done under approved protocols and in accordance with recommendations for the proper care and use of laboratory animals by the Committee for Animal, Recombinant DNA, and Infectious Pathogen Experiments at Kyushu University and according to the law (no. 105) and notification (no. 6) of the Japanese Government.

Tumor implantation model. With mice under sufficient anesthesia by an i.p. injection of sodium pentobarbital, 1×10^6 tumor cells were injected s.c. into the abdominal region. Measurement of the tumor was done thrice per week. Tumor volumes were estimated by the formula $V = \pi/6 \times a^2 \times b$, where a was the short and b was the long axis (11).

Statistical analysis. Results are presented as the mean ± SE. Differences between groups were determined by one-way ANOVA followed by an unpaired Student's t test with Bonferroni correction for multiple comparisons. The correlation between *PDGF-A* and *VEGF* was analyzed using Spearman's correlation analysis and κ-statistic analysis. Survival curves were plotted using the Kaplan-Meier's analysis, and the log-rank test was used to determine the statistical differences between life curves. The relationship between the expression of PDGF-AA and clinicopathologic factors was examined using the Mann-Whitney U test and Fisher's exact test, and a positive ratio with respect to tumor size was examined using the Cochran-Armitage linear trend test. The relationship between the microvessel count was also examined using the Mann-Whitney U test. $P < 0.05$ was considered significant.

Results

Platelet-derived Growth Factor-AA Is an Autocrine Regulator of Vascular Endothelial Growth Factor Expression in Human Non–Small Cell Lung Cancers

Established cell lines. To obtain direct evidence that PDGF-AA is an autocrine regulator for VEGF in human NSCLCs, we first established independent cell lines [i.e., QG56 (human lung squamous cell carcinoma) and A549 (human lung adenocarcinoma)] which were stably transfected with plasmid pcDNA3.1(+) expressing full-length antisense human *PDGF-A* cDNA (*AS-PDGFA*). Control lines transfected with an empty vector or full-length

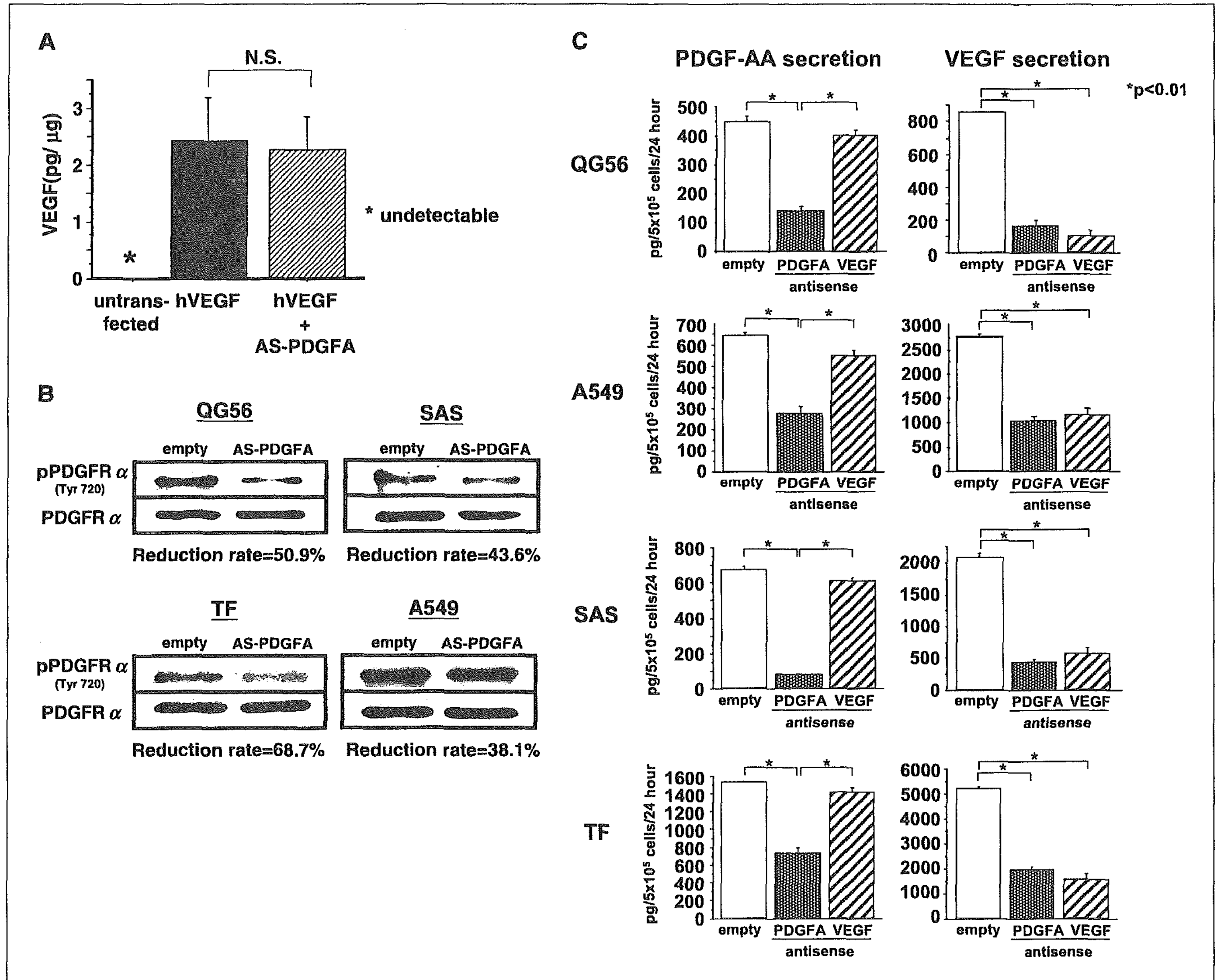

Figure 1. Specificity of *AS-PDGFA* for reduction of PDGFRα activity. *A, AS-PDGFA* transfection does not affect the expression of human *VEGF165* gene expression. Murine fibroblast (NIH3T3) was transfected with human *VEGF165* cDNA (hVEGF, *closed column*) or plasmid expressing antisense *PDGF-A* chain (AS-PDGFA), and culture medium 48 hours after gene transfer was subjected to human VEGF-specific ELISA. No reduction of VEGF expression by cotransfection of *AS-PDGFA* indicated any direct effect of *AS-PDGFA* to VEGF expression. *Columns*, averages of three independent experiments. *B*, reduction of phosphorylated PDGFRα caused by stable *AS-PDGFA* gene transfer in established cancer cell lines (QG56, A549, SAS, and TF). Stably transfected cells with *AS-PDGFA* or empty vector were established by the procedure described in Materials and Methods. Whole PDGFRα was immunoprecipitated and blotted by antibody for PDGFRα-specific phosphotyrosine (Tyr^{720}). The expression level of pPDGFRα was quantified by densitometry and standardized by nonphosphorylated PDGFRα, and the reduction rate was calculated. These experiments were done in duplicate and showed similar results. *C*, reduction of PDGF-AA secretion reduces the expression of VEGF in established cell lines. Establishment of cell lines (QG56, A549, SAS, and TF) with stably reduced PDGF-AA expression was described in Materials and Methods. After 24 hours of incubation without serum at 5×10^5 cells per well, the culture medium was subjected to ELISA for human PDGF-AA or VEGF. Each group contained $n = 3$. *Columns*, means; *bars*, ±SE. One-way ANOVA followed by an unpaired Student's t-test with Bonferroni correction for multiple comparison was done for statistical differences. *, $P < 0.01$.

antisense human *VEGF165* cDNA (AS-VEGF) were simultaneously established. In addition, two other cell lines, SAS and TF (human oral squamous cell carcinoma lines), were also included to confirm the findings. These cells were maintained in G418, and single-cell cloning was done thrice using 96-well plates. The clone that showed the lowest secretion of the target protein in each cell line was used for the following experiments.

A preliminary experiment showed high expressions of PDGF-AA and VEGF in all cell lines subjected to the ELISA (data not shown). Using murine fibroblasts (NIH3T3), we next examined whether *AS-PDGFA* might be cross-reactive for human VEGF expression, because the nucleotide sequence of *PDGF-A* showed ~30% homology to that of *VEGF*. *AS-PDGFA* transfection did not affect the protein secretion level from cotransfected sense human *VEGF165* expression vector [pcDNA3.1(+)/hVEGF165)] in the culture medium of NIH3T3 (Fig. 1*A*), indicating that *AS-PDGFA* did not seriously affect the expression of human VEGF.

All cell lines expressed the known receptor of PDGF-AA, PDGFRα, which was detected by RT-PCR (data not shown). This was also confirmed by immunoprecipitation to PDGFRα, and as expected, the phosphorylation of PDGFRα seemed lower in cells that were stably transfected with *AS-PDGFA* than in those transfected with an empty vector in repeated experiments; this finding was later confirmed by densitometry (Fig. 1*B*).

Furthermore, all cell clones stably transfected with *AS-PDGFA* showed significantly reduced PDGF-AA secretion, as well as reduced VEGF. On the other hand, transfection of AS-VEGF contributed only to the reduction of VEGF and not to the expression of PDGF-AA (Fig. 1*C*), indicating that PDGF-AA is an autocrine regulator for the expression of VEGF in these cell lines.

Surgical specimens. For further evidence regarding the role of PDGF-AA in VEGF expression, we did real-time quantitative RT-PCR to test the correlation of mRNA levels between *PDGF-A* chain and *VEGF* in surgically resected fresh NSCLC samples from 60 patients (adenocarcinoma, n = 32; squamous cell carcinoma, n = 20; others, n = 8). The expressions of *PDGF-A* mRNA and *VEGF* mRNA in the tumor were tightly correlated (Spearman's correlation test, ρ = 0.838, P < 0.001), and there was a similar finding in adjacent normal lung tissue (Spearman's correlation test, ρ = 0.705, P < 0.001; Fig. 2*A*), suggesting that similar to our previous findings in noncancerous mesenchymal cells, the expression of *PDGF-A* was correlated to that of *VEGF* in NSCLCs.

Further retrospective analysis by immunohistochemistry using 128 formalin-fixed tissue sections (adenocarcinoma, n = 68; squamous cell carcinoma, n = 55; others, n = 5) also supported these findings; the immunohistochemically positive reaction of PDGF-AA was tightly correlated with that of VEGF (Fig. 2*B* and *C*; κ-statistic analysis, κ = 0.325; P = 0.0002).

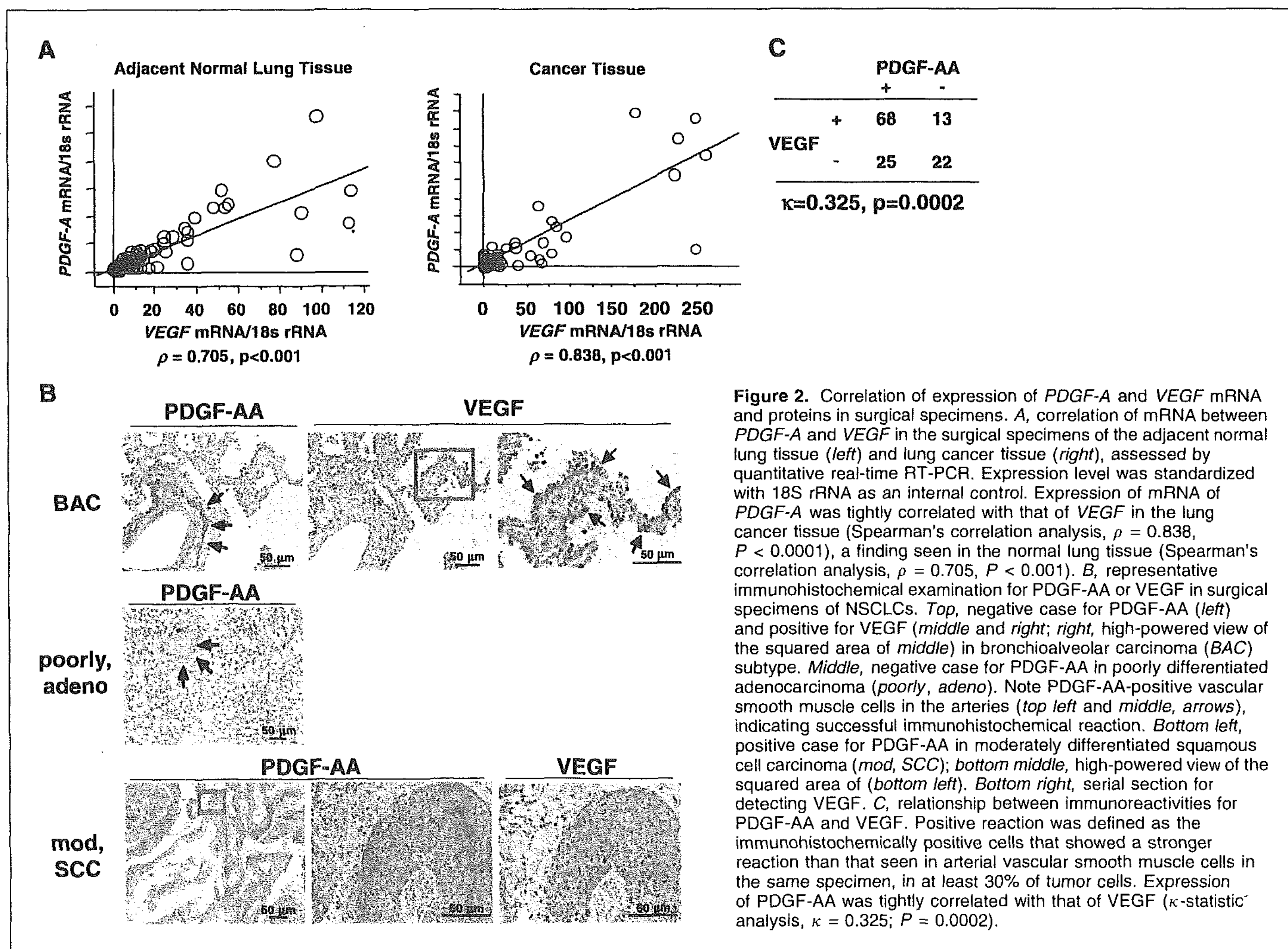

Figure 2. Correlation of expression of *PDGF-A* and *VEGF* mRNA and proteins in surgical specimens. *A*, correlation of mRNA between *PDGF-A* and *VEGF* in the surgical specimens of the adjacent normal lung tissue (*left*) and lung cancer tissue (*right*), assessed by quantitative real-time RT-PCR. Expression level was standardized with 18S rRNA as an internal control. Expression of mRNA of *PDGF-A* was tightly correlated with that of *VEGF* in the lung cancer tissue (Spearman's correlation analysis, ρ = 0.838, P < 0.0001), a finding seen in the normal lung tissue (Spearman's correlation analysis, ρ = 0.705, P < 0.001). *B*, representative immunohistochemical examination for PDGF-AA or VEGF in surgical specimens of NSCLCs. *Top*, negative case for PDGF-AA (*left*) and positive for VEGF (*middle* and *right*; *right*, high-powered view of the squared area of *middle*) in bronchioalveolar carcinoma (*BAC*) subtype. *Middle*, negative case for PDGF-AA in poorly differentiated adenocarcinoma (*poorly*, *adeno*). Note PDGF-AA-positive vascular smooth muscle cells in the arteries (*top left* and *middle, arrows*), indicating successful immunohistochemical reaction. *Bottom left*, positive case for PDGF-AA in moderately differentiated squamous cell carcinoma (*mod, SCC*); *bottom middle*, high-powered view of the squared area of (*bottom left*). *Bottom right*, serial section for detecting VEGF. *C*, relationship between immunoreactivities for PDGF-AA and VEGF. Positive reaction was defined as the immunohistochemically positive cells that showed a stronger reaction than that seen in arterial vascular smooth muscle cells in the same specimen, in at least 30% of tumor cells. Expression of PDGF-AA was tightly correlated with that of VEGF (κ-statistic analysis, κ = 0.325; P = 0.0002).

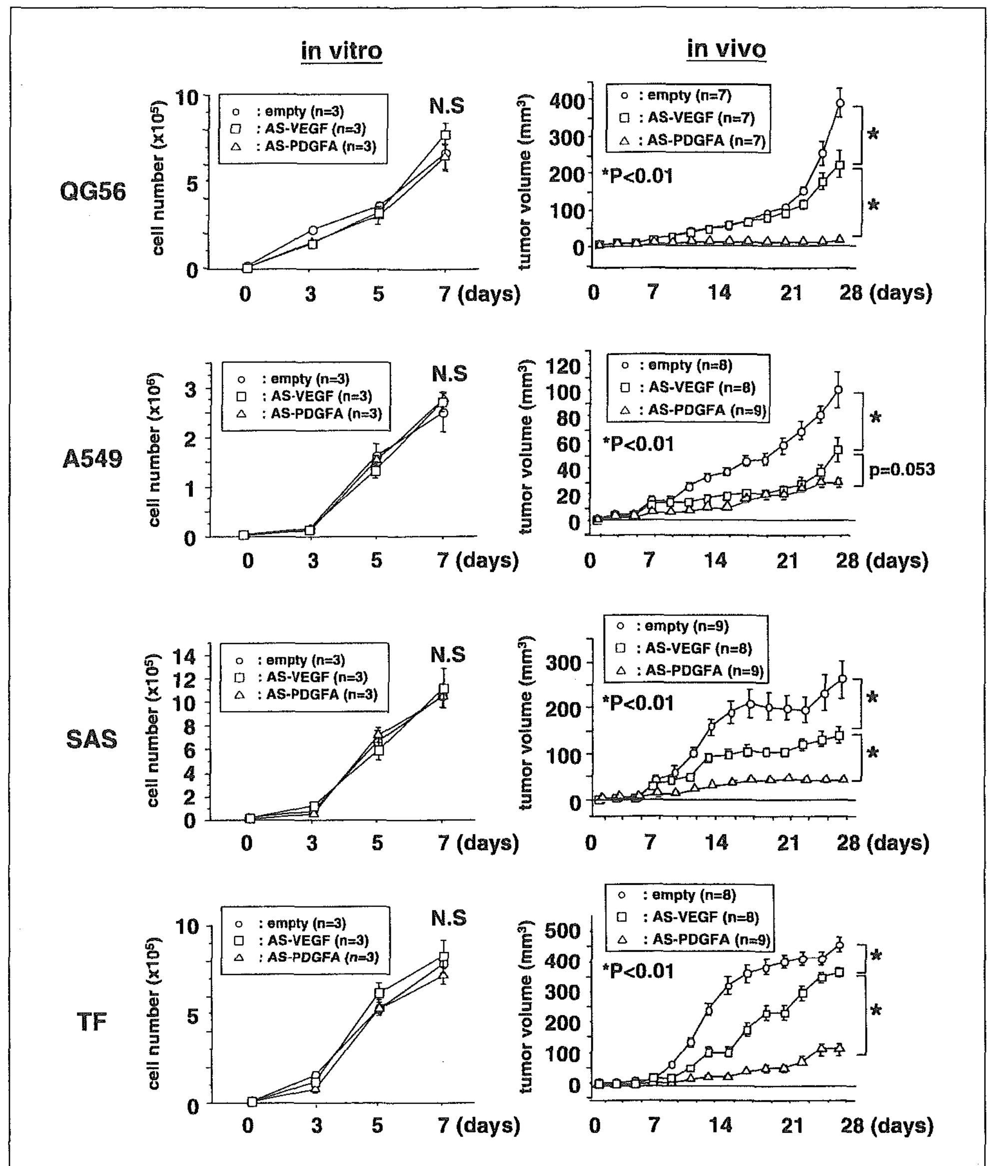

Figure 3. Significance of PDGF-AA and VEGF expression in tumor proliferation *in vitro* and in a tumor implantation model *in vivo*. *Left, in vitro*. Tumor cells (5×10^4 cells; QG56, A549, SAS, and TF) stably transfected with an empty plasmid, antisense *VEGF* (*AS-VEGF*), or antisense *PDGF-A* (*AS-PDGFA*) were spread on dish plate, and the number of tumor cells were counted at days 3, 5, and 7. No significant difference on the tumor proliferation was found in all groups. *Right, in vivo*. In turn, these tumor cells were s.c. injected into the abdominal wall, and the tumor growth was measured at each time point. *Points*, mean; *bars*, ±SE. Differences between the groups were compared using a one-way ANOVA followed by an unpaired Student's *t* test. *, $P < 0.01$.

Significance of Platelet-Derived Growth Factor-AA and Vascular Endothelial Growth Factor Expression in Human Non–Small Cell Lung Carcinomas

Established cell lines. We next returned to animal studies to examine the tumorigenesis in established cell lines. As a first step, we examined the proliferative activities of each cell transfected with an empty plasmid, *AS-PDGFA* or *AS-VEGF* and found no significant difference among the activities in any of the cell species (Fig. 3, *in vitro* experiment).

Reduction of VEGF expression resulted in mildly to moderately disturbed tumorigenesis in *nu/nu* mice in all four cell lines tested, as expected. On the other hand, stable transfection of *AS-PDGFA* caused considerably and significantly disturbed tumor growth in three of four cell lines, compared with those with an empty vector or *AS-VEGF* (Fig. 3, *in vivo* experiment). These results indicate that tumor growth is more dependent on the expression of PDGF-AA than that of VEGF.

We next assessed the tumor-induced angiogenesis *in vivo*, visualized by labeling with vWF, using tumor sections obtained at 28 days after tumor implantation (Fig. 4*A*, *arrows*, and *B*). None of the sections from QG56 tumor transfected with *AS-PDGFA* showed any histopathologic evidence of solid tumor; thus, these sections were excluded from the evaluation. In all tumor types, the number of intratumor microvessels was significantly decreased in the *AS-VEGF* group and even more significantly decreased in the *AS-PDGFA* group (Fig. 4*B*, *intratumor*). Similar findings were also observed for angiogenesis at the tumor periphery of SAS and TF tumors, but the effect was rather mild in A549 tumors (Fig. 4*B*, *peripheral*), probably reflecting the milder antitumor effect of antisense gene expression seen in Fig. 3.

Surgical specimens. Using 128 tissue sections immunohistochemically labeled for PDGF-AA (93 positive cases and 35 negative cases), we investigated the clinicopathologic role of PDGF-AA expression in NSCLCs. Among the clinicopathologic variables, only the tumor diameter was significantly larger in PDGF-AA-positive cases than in PDGF-AA-negative cases (Supplementary Table S1). Furthermore, as tumor size increased, the PDGF-AA-positive ratio was raised significantly (Cochran-Armitage test for linear trend, $P < 0.0001$), as was the VEGF-positive ratio (Cochran-Armitage test for linear trend, $P < 0.0001$;

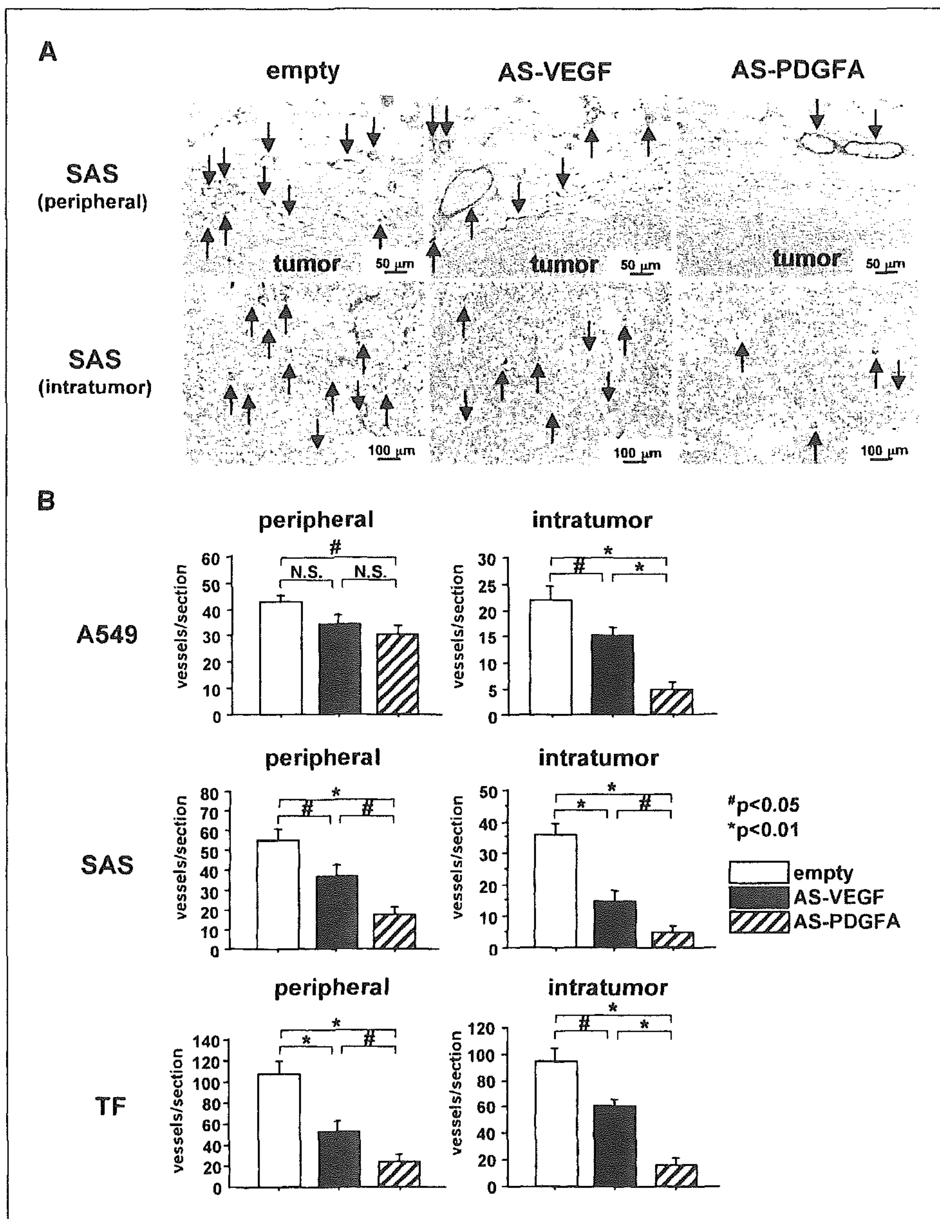

Figure 4. Immunohistochemical detection and quantification of microvessels in distinct part of tumors (tumor periphery and intratumor). Tumors shown in Fig. 4 were obtained at 28 days after implantation, and the sections from these were subjected to immunohistochemical examination for von Willebrand factor (*vWF*). QG56 was excluded from this analysis, because no apparent viable tumor could be found in this type of tumor transfected with *AS-PDGFA*. *A,* representative immunohistochemical findings of microvessels (*arrows*) in SAS tumors located at <2 mm from tumor periphery (*peripheral, top*) and at intratumor (*intratumor, bottom*). Note the marked reduction of the number of microvessels, especially in the tumor with *AS-PDGFA*. Counterstained with hematoxylin. *B,* quantitative analysis of the number of microvessels located at the tumor periphery or intratumor in each tumor type.

Table 1). Interestingly, all tissue sections of noninvasive adenocarcinoma, bronchioalveolar carcinoma (n = 7; all cases were included in $20 \leq \phi < 30$), and high-grade atypical adenomatous hyperplasia (AAH, n = 13), which are now considered to be precancerous lesions (18, 19), were negative for PDGF-AA. The seven cases of bronchioalveolar carcinoma included three VEGF-positive cases (42.9%). These results thus indicate that the expression of PDGF-AA may be essentially related to the size and progression of tumors.

Finally, a comparison of the 5-year survival rates of patients with NSCLCs who were VEGF positive (81 cases) or negative (47 cases) did not show a significant difference between the two groups (Fig. 5*A*; 38.6%, confidence interval [CI] = 15.6 versus 43.6%, CI = 11.6, respectively), whereas that of patients who were PDGF-A positive was significantly lower than that of those with a negative reaction ($P < 0.05$; Fig. 5*B*; 36.1%, CI = 17.8 versus 56.5%, CI = 11.0, respectively).

Discussion

The key observations obtained in the present study were as follows: (a) similar to our earlier observations in noncancerous mesenchymal cells, PDGF-AA was found to be an autocrine regulator for VEGF in NSCLCs, indicating that the PDGF-AA/VEGF axis may be a ubiquitous autocrine system for enhancing angiogenic signals; (b) the expression level of PDGF-AA was more critical for experimental tumor growth than that of VEGF *in vivo*, and the expression of PDGF-AA was rarely seen in precancerous or early cancer lesions of surgical sections; and (c) PDGF-AA expression was a prognostic indicator for individuals with NSCLCs. These results strongly suggest that PDGF-AA and its related pathways may be a more efficient target of antiangiogenic therapy for cancers than VEGF and its related pathways.

Recent studies have identified various signals related to tumor angiogenesis, including metabolic and/or mechanical stress, immune/inflammatory response, and factors that genetically

Table 1. Relationship between PDGF-AA/VEGF expression and tumor diameter of human NSCLCs

Diameter	AAH (n = 13)	Tumor diameter (ϕ) of NSCLCs (n = 128)				
		ϕ < 20 (n = 5)	20 ≤ ϕ < 30 (n = 21*)	30 ≤ ϕ < 40 (n = 31)	40 ≤ ϕ < 50 (n = 30)	50 ≤ ϕ (n = 41)
PDGF-AA-positive rate (%)[†]	0 (0.0)	2 (40.0)	12 (57.1)	19 (61.3)	24 (80.0)	36 (87.8)
VEGF-positive rate (%)[‡]	4 (30.7)	2 (40.0)	9 (42.8)	18 (58.0)	21 (70.0)	31 (75.6)

*Includes seven bronchioaveolar carcinomas.
†P < 0.0001, Cochran-Armitage test for trend.
‡P < 0.001, Cochran-Armitage test for trend.

activate oncogenes and control the production of angiogenic regulators such as VEGF, angiopoietins, fibroblast growth factors, hepatocyte growth factor, etc. (7). There is no longer doubt that these signals contribute to tumor progression; however, relatively less attention has been paid to the critical question of which molecules and signal transduction pathways are critically involved in the angiogenic switch and how they function.

VEGF is an essential mediator for tumor angiogenesis, and that conclusion has been supported by the findings of a number of experimental and clinical studies, including promising early results of a humanized anti-VEGF antibody, Avastin (20–22). VEGF, however, is not likely to meet the definition of an angiogenic switch, and this assertion was supported by the present finding that AAH occasionally expressed VEGF (Table 1). Therefore, we should be able to identify an upstream regulator that controls the expression of VEGF in tumors. In the current study, we identified PDGF-AA as a possible candidate for one of the molecules involved to the angiogenic switch that meets the definition noted above, and the relationship between the role of PDGF-AA and tumorigenesis has been summarized in Supplementary Fig. S1A.

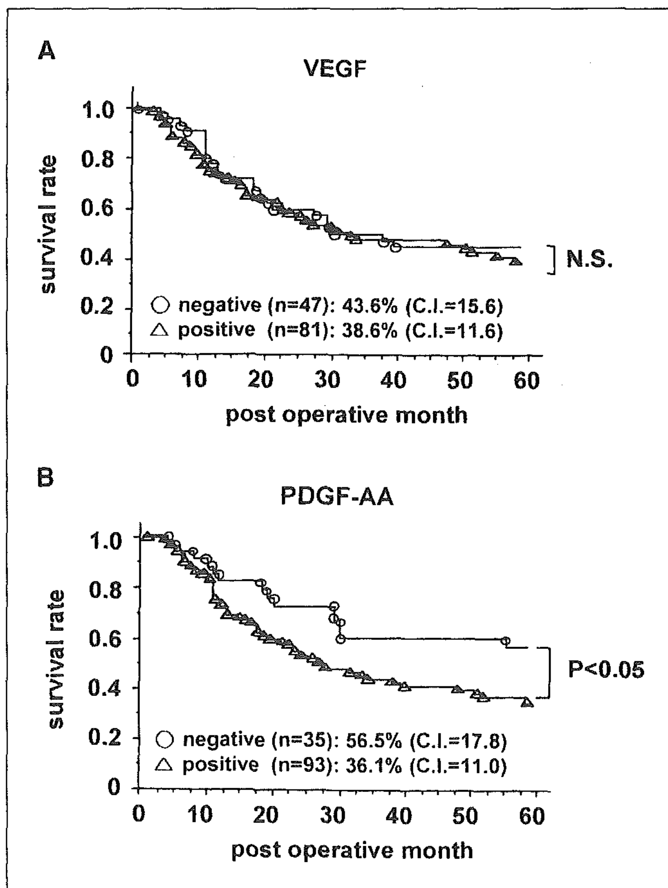

Figure 5. Comparison of 5-year survival of patients with NSCLCs. Kaplan-Meier curves indicating a 5-year survival of patients with VEGF-positive or -negative cases (*A*), PDGF-A-positive or PDGF-A-negative cases (*B*). The log-rank test was used to determine the statistical differences between life curves. A probability value of P < 0.05 was considered significant.

A tumor implantation assay indicated that inhibition of tumor growth was more pronounced in *AS-PDGFA* than in *AS-VEGF*, suggesting that the expression of PDGF-AA may not only contribute to the regulation of VEGF but also exert other effects on tumor progression. With regard to tumor angiogenesis, we hypothesize that (a) in addition to VEGF, PDGF-AA may regulate other factors advantageous to tumor growth, including other angiogenic factors; and (b) in addition to the indirect angiogenic effect using VEGF, PDGF-AA itself may directly contribute to the angiogenic responses. At present, little is known regarding the former point, which we are currently investigating via microarray analysis. The latter point may be supported by our previous study indicating that PDGF-AA stimulates and maintains the local VEGF expression in mesenchymal cells (13). Such a paracrine mechanism of PDGF-AA for angiogenesis, which is supported by several studies (23–27), may reflect the difference of tumor growth between *AS-PDGFA* and *AS-VEGF*. From this point of view, PDGF-AA is likely to be an autocrine and paracrine angiogenic switch in solid tumors (Supplementary Fig. S1*B*). Regarding the nonangiogenic actions, the paracrine mode of PDGF-AA causes a desmoplastic reaction (28), which is an important feature of advanced NSCLCs (29), by activating mesenchymal myofibroblasts.

Might a PDGF-AA-related signal transduction pathway be a more effective molecular target for antitumor therapy than a pathway related to VEGF? Although it seems premature to draw such a conclusion, the current study suggests this possibility, and some recent studies may also support it. For example, in a previous study that measured the levels of various angiogenic factors, including VEGF, in the tumors of neuroblastoma patients, only the expression level of PDGF-AA was significantly correlated to patient survival, even when a high level of expression of various angiogenic factors was detected (30). On the other hand, an inhibitor of tyrosine kinases, including PDGFRs, is likely to be effective in patients with malignant tumors (31), suggesting that PDGF-AA and its related pathways may be important targets for tumor

angiogenesis. Among these tyrosine kinases, it has been suggested in a recent experimental study that PDGF-AA and PDGFRα might be one of the essential regulators for tumor angiogenesis (32); some thalidomide analogues markedly inhibited tumor growth and angiogenesis *in vivo* via a marked reduction of PDGF-AA without apparent changes of the expression levels of other angiogenesis-related factors. Together, these findings strongly suggest that the PDGF-AA/PDGFRα signal transduction pathway warrants further study for the potential treatment of patients with intractable malignancies.

An important limitation of the present study was the lack of direct evidence of the demethylation status of the *PDGF-A* promoter in NSCLCs; because of this, the scheme shown in Supplementary Fig. S1*A* is still hypothetical, not definitive. For precise assessment regarding this issue, careful laser dissection of cancer cells should be done without contamination of mesenchymal cells, because the *PDGF-A* promoter of nontumorous mesenchymal cells should be demethylated. We have started this delicate assessment, and the data will be available in the near future.

In conclusion, we identified PDGF-AA as an autocrine, and probably a paracrine, regulator that made an essential contribution to the expression of VEGF in NSCLCs, affecting tumorigenesis revealed by experimental and clinicopathologic studies. Therefore, PDGF-AA and its related pathways, which determine the expression of VEGF in tumors, may have potential as molecular targets in an antitumor strategy for disrupting tumor angiogenesis.

Acknowledgments

Received 11/22/2004; revised 5/23/2005; accepted 5/31/2005.

Grant support: Japanese Ministry of Education, Culture, Sports, Science, and Technology grant-in-aid (Y. Yonemitsu and K. Sueishi) and Organization for Pharmaceutical Safety and Research Grant for the Promotion of Basic Science Research in Medical Frontiers project No. MF-21 (Y. Yonemitsu and K. Sueishi).

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We thank Drs. N. Kinukawa and J. Tsuchihashi for their help with the statistical analysis and Chie Arimatsu for her help with the animal experiments.

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Human Pathology (2005) 36, 357–363

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Genetic and epigenetic alterations of the *PTEN* gene in soft tissue sarcomas☆

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Received 9 September 2004; accepted 10 January 2005

Keywords:
PTEN gene;
Soft tissue sarcoma;
Promoter methylation;
Homozygous deletion

Summary The *PTEN/MMAC1* (*PTEN*) gene was identified as a tumor suppressor gene encoding a cytoplasmic protein that controls cellular processes. To investigate the potential role and the alteration of the *PTEN* gene in soft tissue sarcomas (STSs), we searched for homozygous deletion and promoter hypermethylation in a series of 48 STSs that was composed of malignant fibrous histiocytoma, leiomyosarcoma, malignant peripheral nerve sheath tumor, including 2 cases with a mutation that we previously reported; differential polymerase chain reaction and methylation-specific polymerase chain reaction, respectively, were used for the analyses. Furthermore, to determine whether *PTEN* gene alterations are involved in the down-regulation of PTEN expression, we examined the expression of PTEN protein in 38 cases in which paraffin-embedded tissues were available for immunohistochemical analysis. In addition to our previous results showing that 2 (4%) of 51 cases had a *PTEN* mutation, promoter methylation was recognized in 6 (13%) of 48 cases, and homozygous deletion was detected in 1 (2%) of 48 cases in the current study. Of 6 cases with promoter methylation of *PTEN* gene, 5 were malignant peripheral nerve sheath tumor. Decreased expression of PTEN protein was recognized in 11 (29%) of 38 STS cases. Of 9 cases with *PTEN* alterations (6 cases with promoter methylation, 2 with mutation, and 1 with homozygous deletion), 3 (33%) showed decreased expression of PTEN protein. Furthermore, decreased expression of the *PTEN* gene showed a statistically significant correlation with high MIB-1 labeling index in 38 STS cases examined (P = .0441). In conclusion, promoter methylation and homozygous deletion of the *PTEN* gene were found to be relatively rare events in cases of STS, as is mutation of the gene. Of 9 cases with a *PTEN* alteration, 3 (33%) showed a decrease in PTEN expression, indicating that *PTEN* gene alterations seem to play a minor role in the inactivation of *PTEN* in these tumors. Furthermore, although a further detailed analysis of a larger

☆ This study was supported by a Grant-in-Aid for General Scientific Research (12670167) from the Ministry of Education, Science and Culture, Tokyo, Japan.
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0046-8177/$ – see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.humpath.2005.01.017

number of cases is still necessary, the present results suggest that PTEN expression may be a useful indicator of cell proliferation in patients with STS.
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1. Introduction

The *PTEN* gene, located on chromosome 10q23.3, is a tumor suppressor gene that encodes a cytoplasmic protein with a protein tyrosine phosphatase domain and a domain extensively homologous to the cytoskeletal proteins tensin and auxilin [1]. Gene silencing by genetic and epigenetic alteration of the *PTEN* gene has been reported in a subset of malignancies [2-13]. Somatic mutations and deletion have both been reported in many types of sporadic tumors [2-9]. Loss of heterozygosity of chromosome 10q has been described in bone and soft tissue sarcomas (STSs) [14-18]. With regard to STSs, we reported previously that mutation of the *PTEN* gene was observed in only 2 cases of leiomyosarcoma (LMS) (3.9%) among 51 cases of STS; the results of that study suggested that those cases derived from the intraabdominal cavity might have undergone a different type of tumorigenesis compared with those derived from an extremity or the trunk [19]. Recent studies have reported that promoter methylation is a representative example of the transcriptional silencing of tumor suppressor genes. *PTEN* gene silencing by promoter methylation has been also demonstrated in some types of malignancy [10-13]. However, in the context of STS, the frequency of homozygous deletion and the methylation status of the *PTEN* gene remain unknown, as does the association between *PTEN* gene alteration and PTEN expression in cases of STS.

To clarify the alteration of the *PTEN* gene in STSs, we investigated the methylation status, as well as the homozygous deletion status of the *PTEN* gene in STSs cases that we had previously examined for *PTEN* mutation. Furthermore, to better understand the nature of PTEN alterations involved in the down-regulation of PTEN expression, we also examined the expression of PTEN protein by immunohistochemistry.

2. Materials and methods

2.1. Tumor samples and DNA extraction

Snap-frozen tumor samples from 51 cases of STS were obtained from the collection of soft tissue tumors registered in the Department of Anatomic Pathology, Pathological Sciences, Graduate School of Medical Sciences Kyushu University, Japan. Fresh samples were carefully dissected from the tumors to avoid including the surrounding normal tissue, and the samples were immediately frozen in liquid nitrogen and stored at −80°C. In addition, we confirmed by hematoxylin-eosin staining that normal tissue had not contaminated any of the samples. Diagnosis in each of the 51 cases was based on light microscopic examinations with hematoxylin-eosin staining of paraffin blocks. Immunohistochemical analysis using conventional markers of differentiation routinely used for the diagnosis of soft tissue neoplasms was performed in almost all of the cases. We excluded 2 cases of solitary fibrous tumor and 1 of low-grade fibromyxoid sarcoma from the study because the focus of the investigation is on malignant fibrous histiocytoma (MFH), LMS, and malignant peripheral nerve sheath tumor (MPNST). Histologic diagnosis of the 48 cases of STS in this study revealed 22 cases of MFH (16 cases of the storiform pleomorphic type and 6 cases of the myxoid type), 13 cases of LMS, and 13 cases of MPNST (Table 1). If possible, histologic tumor grade was evaluated according to the grading system of the French Federation of Cancer Centers (FNCLCC) [20]. Furthermore, cases were also evaluated according to the new American Joint Committee on Cancer (AJCC) staging system. Genomic DNA was

Table 1 Clinicopathologic parameters in 48 STS cases

Age (y)	≥60	27
	<60	21
Sex	Male	24
	Female	24
Location	Lower extremity	25
	Upper extremity	8
	Buttock	6
	Retroperitoneum	5
	Shoulder	1
	Posterior mediastinum	1
	Inferior vena cava	1
	Spinal cord	1
Histologic subtype	MFH	22
	Storiform pleomorphic type	16
	Myxoid type	6
	LMS	13
	MPNST	13
Size (cm)	≥10	17
	<10	31
Tumor type	Primary	40
	Recurrent	8
FNCLCC grading[a]	2	12
	3	26
AJCC stage[a]	I	1
	II	20
	III	16
	IV	1

[a] $n = 38$.