Vascular Endothelial Growth Factor is an Autocrine Growth Factor for Cardiac Myxoma Cells

Hironosuke Sakamoto, MD; Tetsuo Sakamaki, MD*; Tsugiyasu Kanda, MD†;
Yoko Tsuchiya, BS††; Mahito Sato, MD; Hiroko Sato, MD**;
Yuko Oyama, MD**; Yoshie Sawada, MD*; Jun-ichi Tamura, MD;
Ryoza Nagai, MD*; Masahiko Kurabayashi, MD**

Background Cardiac myxomas are generally considered benign, but malignant tumors have been reported. Vascular endothelial growth factor (VEGF), an angiogenic factor, plays a role in the growth, progression, and metastasis of solid tumors and it has been reported that VEGF expression is upregulated in cardiac myxomas that have a high microvessel density. The purpose of this study was to determine whether cardiac myxoma cells possess a VEGF-autocrine system that regulates tumor growth.

Methods and Results Immunohistochemical analyses revealed the presence of VEGF and its receptors, VEGFR-1 (flt-1) and VEGFR-2 (KDR/flk-1), in the cytoplasm of tumor cells from 18 of 18 myxoma tissue specimens examined. Two different myxoma cell lines were established and constitutively secreted large amounts of VEGF as determined by enzyme-linked immunosorbent assay. The expression of VEGF, VEGFR-1, and VEGFR-2 mRNA was detected in both cell lines by reverse-transcriptase polymerase chain reaction. Myxoma cell proliferation, as determined by thymidine incorporation, was enhanced by the addition of VEGF in a dose-dependent manner, and cell proliferation was inhibited in a dose-dependent manner by the addition of a neutralizing VEGF antibody.

Conclusions These results indicate that cardiac myxoma cells possess a VEGF-autocrine system, which could contribute to the malignant potential of histologically benign myxomas through direct stimulation of tumor cell growth as well as through induction of angiogenesis. (Circ J 2004; 68: 488–493)

Key Words: Angiogenesis; Autocrine system; Cardiac myxoma; Cell proliferation; Vascular endothelial growth factor

Cardiac myxoma is the most common primary tumor of the heart.¹ Ultrasound technology has been constantly progressing and the quality of echocardiographic images has improved almost year by year, which has lead to both the early detection of myxomas and asymptomatic patients.² Although myxomas are generally considered to be benign, malignant tumors have been described.^{3,4} At present, little is known about the mechanism responsible for the malignant potential of cardiac myxomas. Angiogenesis plays an important role in the growth, progression, and metastatic spread of solid tumors^{5,6} and vascular endothelial growth factor (VEGF) is a multifunctional cytokine that has been shown to be an important regulator of tumor angiogenesis.⁷ Cardiac myxomas are characterized microscopically by polygonal or spindle-shaped tumor cells embedded in a gelatinous matrix rich in chondroitin-6-sulfate and hyaluronic acid.⁸ Various differ-

entiated vascular structures are frequently observed in the gelatinous matrix.⁹ Recently, it was reported that VEGF expression is upregulated in cardiac myxomas in association with a high microvessel density, which suggests that VEGF may play a key role in angiogenesis for myxomas.¹⁰ Therefore, we hypothesized that cardiac myxomas may have an autocrine system regulating angiogenesis and tumor growth. In this report, we present evidence that cardiac myxoma cells express high concentrations of VEGF and its receptors, VEGFR-1 (flt-1) and VEGFR-2 (KDR/flk-1), and that VEGF acts as an autocrine growth factor in vitro.

Methods

Materials

All chemicals were purchased from Sigma Chemical Co (St Louis, MO, USA) unless stated otherwise. Rabbit polyclonal antibodies against human VEGF (sc-152), VEGFR-1 (sc-316), and VEGFR-2 (sc-504) were purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, CA, USA) and were used for immunohistochemistry. Rabbit polyclonal antibody against human von Willebrand factor (vWF), an endothelial cell-specific marker, was obtained from Dako (Copenhagen, Denmark). A VEGF enzyme-linked immunosorbent assay (ELISA) kit was purchased from IBL Co, Ltd (Fujioka, Gunma, Japan). Recombinant human VEGF₁₆₅ and a neutralizing monoclonal antibody against human VEGF₁₆₅ (clone 16F1; mouse IgG) were from IBL and were used for a cell proliferation assay. ENDO Media

(Received December 2, 2003; revised manuscript received February 19, 2004; accepted February 27, 2004)

Departments of General Medicine, *Medical Informatics and **Medicine and Biological Science, Gunma University Graduate School of Medicine, Maebashi, †Department of General Medicine, Kanazawa Medical University, Ishikawa, ††IBL Co Ltd, Fujioka and ‡Department of Cardiovascular Medicine, Graduate School of Medicine, University of Tokyo, Tokyo, Japan

Mailing address: Hironosuke Sakamoto, MD, Department of General Medicine, Gunma University Graduate School of Medicine, 3-39-22 Showa-machi, Maebashi, Gunma 371-8511, Japan. E-mail: sakamoth@med.gunma-u.ac.jp

and TIL Media I were obtained from IBL. [Methyl-³H]-thymidine was obtained from ICN Pharmaceuticals, Inc (Irvine, CA, USA).

Tissue Preparation

Tissue specimens from cardiac myxomas were obtained from 18 patients who underwent cardiac surgery between 1978 and 1998 at the Gunma University Hospital and its affiliated hospitals. All patients gave informed consent, and the study protocol was approved by the Gunma University Hospital ethics committee. The myxomas were located in the left atrium in 17 patients and in the left ventricle in 1 patient. Tissue specimens were fixed in 4% buffered formalin (pH 7.2) and embedded in paraffin. Sections 5 µm thick were used for conventional light microscopy and immunohistochemistry.

Immunohistochemical Analysis

Immunoenzymatic staining was carried out using the Vectastain Elite ABC Kit (Vector Laboratories, Burlingame, CA, USA). Sections were preincubated in methanol containing 0.3% hydrogen peroxide for 30 min and blocked with protein-blocking serum-free solution (Dako) for 15 min to minimize nonspecific reactions. Antibody against VEGF (1:100), VEGFR-1 (1:100), VEGFR-2 (1:100), or vWF (1:50) was applied for incubation for 30 min at room temperature. Sections were incubated with biotinylated secondary antibody for 30 min and then incubated with horseradish-peroxidase-labeled streptavidin solution for 30 min. Peroxidase activity was visualized with 3,3'-diaminobenzidine (DAB) tetrahydrochloride (0.2 mg/ml) and hydrogen peroxide (0.015%). Sections were counterstained with methyl green, dehydrated, and mounted.

Cell Line Establishment and Maintenance

Human cardiac myxoma cell lines were originally isolated from 2 different patient samples by the enzymatic digestion method. The cell lines were maintained in 25-cm² tissue culture flasks (Costar, Cambridge, MA, USA) containing full growth medium composed of ENDO Media and TIL Media I (1:1) supplemented with 6% fetal bovine serum. The culture medium was changed weekly, and the cells were subcultured monthly. Myxoma cells between passages 6 and 10 were used for the present studies. A human umbilical cord endothelial (HUVE) cell line was established from human umbilical cords by collagenase dissociation and grown in 1% gelatin-coated flasks in ENDO Media I containing 1.5% fetal bovine serum. HUVE cells from passages 5–10 were used for the present study. The TIG-7 human fibroblast line was given to us by Dr Kazuhiko Kaji (Tokyo Metropolitan Institute of Gerontology, Tokyo, Japan) and was grown in F12/DMEM containing 10% fetal bovine serum.

ELISA

The VEGF concentrations in cell-free culture supernatants from the 2 cardiac myxoma cell lines were determined using an ELISA kit. Cell supernatant was obtained from each cell line (2,700,000 cells) in 25-cm² tissue culture flasks after incubation with fresh full growth medium and 6% fetal bovine serum for 7 days. We incubated 200 µl of cell supernatant with 50 µl assay diluent for 2 h at room temperature in a 96-well plate coated with a monoclonal antibody against VEGF. After 3 washing steps, a conjugate consisting of a polyclonal VEGF antibody and horseradish

peroxidase was added and incubated for 2 h at room temperature. After addition of color reagent, absorbance was measured at 450 nm in a microplate reader. For standardization, serial dilutions of recombinant human VEGF were assayed.

Reverse Transcription and Polymerase Chain Reaction (RT-PCR)

In cardiac myxoma cells, HUVE cells, and TIG-7 fibroblasts, expression of mRNA for VEGF and its receptors, VEGFR-1 and VEGFR-2, was assessed by RT-PCR. Total RNA was isolated by the guanidinium isothiocyanate-phenol-chloroform method.¹¹ First-strand cDNA synthesis was performed by reverse transcription of 20 µl of total RNA using the First-Strand cDNA Synthesis kit (Pharmacia Biotech) and 1 µl of the cDNA reaction was used for PCR as described previously. For the detection of VEGF expression, 2 primers were synthesized from the coding region. The sense primer was 5'-CGA AGT GGT GAA GTT CAT GGA TG-3' corresponding to nucleotides +170 to +192, and the antisense primer was 5'-TTC TGT ATC AGT CTT TCC TGG TGA G-3' corresponding to nucleotides +681 to +705. For the detection of VEGFR-1 expression, 2 primers were synthesized from the coding region. The sense primer was 5'-CAA GTG GCC AGA GGC ATG GAG TT-3' corresponding to nucleotides +3,262 to +3,284, and the antisense primer was 5'-GAT GTA GTC TTT ACC ATC CTG TTG-3' corresponding to nucleotides +3,736 to +3,759. For the detection of VEGFR-2 expression, 2 primers were synthesized from the coding region. The sense primer was 5'-GAG GGC CTC TCA TGG TGA TTG T-3' corresponding to nucleotides +2,954 to +2,975, and the antisense primer was 5'-TGC CAG CAG TCC AGC ATG GTC TG-3' corresponding to nucleotides +3,640 to +3,662. The PCR protocol consisted of denaturation at 94°C for 5 min, 30 cycles of annealing at 55°C for 30 s, and extension at 72°C for 1 min, followed by an additional 7 min of extension at 72°C. A positive reaction resulted in 403-bp and 535-bp amplified products, which were visualized on a 1.5% agarose gel containing ethidium bromide. A positive reaction for the PCR for VEGFR-1 and VEGFR-2 showed amplified products of size 498 bp and 709 bp, respectively. The integrity of mRNA for all samples was confirmed by amplification of β -actin.

Cell Proliferation Assay

Proliferation of cardiac myxoma cells induced by exogenously added VEGF was measured using the [methyl-³H]-thymidine incorporation method. Myxoma cells (6,000 cells/well), HUVE cells (6,000 cells/well), or TIG-7 fibroblasts (3,000 cells/well) were seeded in triplicate in 96-well plates in full growth medium. After 48 h, the cells were made quiescent by incubation with serum-free F12/DMEM for 16 h. The quiescent cells were treated with various concentrations of recombinant human VEGF₁₆₅ (0, 2.5, 10, 40, and 160 ng/ml) in F12/DMEM for 48 h, and labeled with 185 kBq (5 µCi)/ml [methyl-³H]-thymidine for a further 18 h. The cells were washed, harvested, and processed for counting in a liquid scintillation counter. Within each assay, triplicate determinations were made.

Statistical Analysis

Results from the cell proliferation assay are expressed as the mean $\pm$ SEM for 3 wells. For multiple treatment groups, one-way ANOVA followed by Fisher's least significant

Fig 1. Immunostaining of VEGF, VEGFR-1, VEGFR-2, and vWF in cardiac myxoma tissues. Hematoxylin and eosin-stained section (A) of a myxoma shows the gelatinous matrix with tumor cells, occurring singly and in multilayered, concentric vascular structures. These cells have a polygonal or spindle shape. Positive immunoreaction for VEGF (B), VEGFR-1 (C), and VEGFR-2 (D) can be seen as the accumulation of DAB reaction products in the cytoplasm of tumor cells. In contrast, there is a positive reaction for vWF (E) in the inner cell layer of the vascular structures (arrows), but staining of myxoma cells is rarely observed (Magnification $\times 200$).

difference test was performed. Statistical significance was accepted at a value of $p < 0.05$. All statistical comparisons were performed using Stat View-J 5.0 (Abacus Concepts Inc, Berkley, CA, USA).

Results

Immunostaining of VEGF and its Receptors

For cardiac myxoma cells to utilize VEGF for autocrine growth, it is necessary that they VEGF and its receptors. We first examined the expression of VEGF and its receptors, VEGFR-1 and VEGFR-2, at the protein level by immunohistochemistry. Hematoxylin and eosin-stained sections of a myxoma showed a gelatinous matrix with polygonal or spindle-shaped tumor cells, occurring singly and in multilayered, concentric vascular structures (Fig 1A). In all 18 tissue specimens from cardiac myxomas, positive immunoreactions for VEGF, VEGFR-1, and VEGFR-2 were observed in the cytoplasm of tumor cells occurring singly, in small cords and in the multilayered, concentric vascular structures (Fig 1B–D). In contrast, a positive reaction for vWF was observed only in the inner cell layer of vascular structures, and staining of tumor cells was rarely observed (Fig 1E).

Fig 2. Detection of VEGF, VEGFR-1, and VEGFR-2 mRNAs by RT-PCR in cultured cardiac myxoma cells, HUVE cells, and TIG-7 fibroblasts. Reverse-transcribed cDNA was subjected to VEGF-, VEGFR-1-, and VEGFR-2-specific PCR amplification (403/535-, 498-, and 709-bp products, respectively). cDNA from all samples was also subjected to β -actin-specific PCR amplification (548-bp product). Positive controls were TIG-7 fibroblasts for VEGF and HUVE cells for VEGFR-1 and VEGFR-2.

Secretion of VEGF by Cardiac Myxoma Cells

We next determined whether cultured cardiac myxoma cells could secrete VEGF protein. The 2 myxoma cell lines, maintained in full growth medium with 6% fetal bovine serum for 7 days, constitutively secreted large amounts of VEGF protein (899 and 3,000 pg/ml, respectively) into the culture medium.

Expression of VEGF and its Receptor mRNAs by Cardiac Myxoma Cells

We next examined the expression of VEGF and its receptors, VEGFR-1 and VEGFR-2, at the mRNA level by RT-PCR in the cardiac myxoma cells, HUVE cells, and TIG-7 fibroblasts. VEGF mRNA was expressed in myxoma cells and TIG-7 fibroblasts, but not in HUVE cells (Fig 2). In contrast, both VEGFR-1 and VEGFR-2 mRNA were expressed in myxoma cells and HUVE cells, but not in TIG-7 fibroblasts.

VEGF as a Putative Growth Factor for Cardiac Myxoma Cells

The co-expression of VEGF and its receptors, VEGFR-1 and VEGFR-2, in cardiac myxoma cells suggests the possibility of an autocrine system for growth stimulation. To determine whether exogenous VEGF₁₆₅ can stimulate cell proliferation, we measured the effect of recombinant human VEGF₁₆₅ on thymidine incorporation by cardiac

Fig 3. Effects of exogenously added VEGF₁₆₅ on cell proliferation. Cells were treated with recombinant VEGF₁₆₅ (0, 2.5, 10, 40, or 160 ng/ml) for 48 h, and then cell proliferation was measured by thymidine incorporation. The proliferation of both myxoma (Upper panel) and HUVE (Middle panel) cells was enhanced by recombinant VEGF₁₆₅ in a dose-dependent manner. No growth regulatory effects were observed in TIG-7 fibroblasts treated with recombinant VEGF₁₆₅ (Lower panel). Each point represents the mean $\pm$ SEM from 3 wells. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared with no VEGF treatment. cpm, counts per min.

myxoma cells, HUVE cells, and TIG-7 fibroblasts. Cells were treated with recombinant human VEGF₁₆₅ (0, 2.5, 10, 40, or 160 ng/ml) for 48 h, and cell proliferation was measured. The proliferation of myxoma cells was enhanced by recombinant VEGF₁₆₅ in a dose-dependent manner (Fig 3, Upper panel). The proliferation of HUVE cells was also stimulated by recombinant VEGF₁₆₅ in a dose-dependent manner (Fig 3, Middle panel). However, no growth regulatory effects were observed in TIG-7 fibroblasts treated with recombinant VEGF₁₆₅ (Fig 3, Lower panel).

To confirm the specificity of growth stimulation by VEGF, we carried out additional experiments using neutralizing antibodies. Cardiac myxoma cells (high endogenous VEGF content) were treated with the neutralizing anti-human VEGF₁₆₅ mouse monoclonal antibody (0, 0.19, 0.56, 1.67, or 5.00 μ g/ml) for 48 h, and cell proliferation

Fig 4. Effects of the VEGF₁₆₅ antibody on cell proliferation. Cardiac myxoma cells (high endogenous VEGF content) were treated with the neutralizing anti-human VEGF₁₆₅ mouse monoclonal antibody (0, 0.19, 0.56, 1.67, or 5.00 μ g/ml) for 48 h, and then cell proliferation was measured by thymidine incorporation. HUVE cells were cultured at the optimal concentration of VEGF₁₆₅ (100 ng/ml) in 1.5% fetal bovine serum containing F12/DMEM and various concentrations of VEGF antibody for 48 h, and then cell proliferation was measured. The proliferation of both myxoma and HUVE cells was inhibited by the neutralizing VEGF antibody in a dose-dependent manner (Fig 4, Upper and Lower panels, respectively). Each point represents the mean $\pm$ SEM from 3 wells. * $p < 0.05$, ** $p < 0.01$ compared with no VEGF-antibody treatment.

was measured. HUVE cells were cultured in the optimal concentration of VEGF₁₆₅ (100 ng/ml) in 1.5% fetal bovine serum containing F12/DMEM and various concentrations (0, 0.19, 0.56, 1.67, or 5.00 μ g/ml) of VEGF₁₆₅ antibody for 48 h, and cell proliferation was measured. The proliferation of cardiac myxoma cells was inhibited by the neutralizing VEGF₁₆₅ antibody in a dose-dependent manner (Fig 4, Upper panel). The proliferation of HUVE cells was also inhibited by the neutralizing VEGF antibody in a dose-dependent manner (Fig 4, Lower panel). These findings confirm the specificity of the VEGF-induced proliferation of cardiac myxoma cells.

Discussion

To our knowledge, this is the first documentation that VEGF is an autocrine growth factor for cardiac myxoma cells. Cardiac myxoma cells constitutively expressed VEGF and its receptors, VEGFR-1 and VEGFR-2, at the mRNA and protein levels. Cardiac myxoma cells secreted large amounts of VEGF protein into the culture medium. Myxoma cell proliferation was enhanced by exogenous VEGF and inhibited by the addition of neutralizing VEGF antibodies in a dose-dependent manner.

Our results are supported by the recent findings of Kono et al who analyzed myxomas and adjacent atrial myocardium from 15 patients, each with a solitary tumor (13 left atrial, 2 right atrial), for VEGF protein expression and microvessel density.¹⁰ All tumors showed a positive immunohistochemical reaction for VEGF, whereas the adjacent atrial myocardium exhibited a negative reaction, indicating that expression of VEGF gene was confined to the tumor. Tumors with the highest expression of VEGF showed the greatest density of microvessels (angiogenesis), suggesting that VEGF expression by the myxoma induces and sustains angiogenesis for tumor growth.¹⁰ Those authors, more recently, demonstrated that monocyte chemoattractant protein-1, a potent monocyte chemoattractant, and thymidine phosphorylase, an angiogenic enzyme, were mainly located in myxoma cells and infiltrating macrophages, and CC chemokine receptor-2, a receptor for monocyte chemoattractant protein-1, was primarily expressed in the infiltrating macrophages.¹² However, no previous studies have demonstrated the expression of the VEGF receptors, VEGFR-1 and VEGFR-2, in cardiac myxomas.

We recently reported that cardiac myxoma cells can secrete abundant amounts of interleukin-8 as well as interleukin-6 in vitro.^{13,14} Interleukin-8 is an inflammatory cytokine and a chemotactic factor for neutrophils.¹⁵ The production of interleukin-8 by neoplastic cells has the potential of enhancing tumor cell migration,¹⁶ vascularization,^{17,18} and proliferation.¹⁹ On the other hand, interleukin-6 is a multi-functional cytokine that is produced by many cells and has pleiotropic effects. Interleukin-6 acts on a wide range of tissues and cell lines and promotes cell growth and differentiation on the one hand and growth arrest on the other hand.^{20,21} Cardiac myxomas produce and secrete interleukin-6, which may explain the inflammatory or autoimmune features observed in most patients affected by these tumors.^{22–24} Recently, interleukin-6 was recognized as an indirect inducer of angiogenesis, exerting its activity through the induction of VEGF in other cell lines.²⁵ Thus, both interleukin-6 and interleukin-8 may cooperate with VEGF in the angiogenesis in cardiac myxomas.

The malignant potential of cardiac myxomas remains controversial, although they do occasionally exhibit behavior more characteristic of malignant tumors, including local recurrence after excision and distant metastasis with vessel invasion and independent growth.^{3,4} The reported recurrence rate for cardiac myxoma ranges from 1 to 22%, with a rate approximately 12–22% for familial myxoma and Carney complex (multiple myxomas), whereas it is only 1–3% for sporadic tumors.²⁶ Mutations of the gene encoding the protein kinase A type 1- regulatory subunit have recently been identified in patients with familial or multifocal tumors.^{27,28} However, the cause of recurrence in patients with sporadic cardiac myxomas is not clear. Many factors are considered to be related to the risk of recurrence and metastasis, including inadequate resection, incomplete resection of the site of attachment, embolization and intra-operative displacement of tumor material, the presence of tumor precursor cells in the subendocardium, or overproduction of glycosaminoglycans, mainly chondroitin-6-sulfate, by myxoma cells.⁸ The existence of a VEGF-autocrine system in cardiac myxoma, as shown in this study, may explain the malignant potential of histologically benign myxomas, especially the remote growth of myxomatous material that has embolized.

Study Limitations

The first concerns the sample size, which was relatively small, so the findings should be interpreted cautiously. Second, whether interleukin-6 and/or interleukin-8 can enhance the expression of VEGF in cardiac myxoma cells remains to be determined. Finally, it is necessary to compare the co-expression of VEGF and its receptors in myxoma tissues from patients with and without malignant features. If the expression of VEGF and its receptors is higher in tumor tissues from patients with malignant features than in those from patients without malignant features, doctors will be able to utilize the measurement of VEGF and its receptors for prognosis. Furthermore, anti-VEGF therapy may be more effective in cardiac myxomas compared with other tumors. Further studies are needed to confirm the role of the VEGF-autocrine system in cardiac myxomas.

Conclusions

We have demonstrated for the first time that VEGF is an autocrine growth factor for cardiac myxoma cells. In conjunction with previous reports, the present findings suggest that a VEGF-autocrine system could contribute to the malignant potential of histologically benign myxomas through direct stimulation of tumor cell growth as well as through induction of angiogenesis.

References

1. Pinede L, Duhaat P, Loire R. Clinical presentation of left atrial cardiac myxoma: Series of 112 consecutive cases. *Medicine* 2001; **80**: 159–172.
2. Yuda S, Nakatani S, Yutani C, Yamagishi M, Kitamura S, Miyatake K. Trends in the clinical characteristics of cardiac myxoma: 20-year experience of a single large tertiary referral center in Japan. *Circ J* 2002; **66**: 1008–1013.
3. Wada A, Kanda T, Hayashi R, Imai S, Suzuki T, Murata K. Cardiac myxoma metastasized to the brain: Potential role of endogenous interleukin-6. *Cardiology* 1993; **83**: 208–211.
4. Read RC, White HJ, Murphy ML, William D, Sun CN, Flanagan WH. The malignant potentiality of left atrial myxoma. *J Thoracic Cardiovasc Surg* 1974; **68**: 857–868.
5. Folkman J. What is the evidence that tumors are angiogenesis dependent? *J Natl Cancer Inst* 1990; **82**: 4–6.
6. Folkman J, Shing Y. Angiogenesis. *J Biol Chem* 1992; **267**: 10931–10934.
7. Plate KH, Breier G, Weich HA, Risau W. Vascular endothelial growth factor is a potential tumour angiogenesis factor in human gliomas in vivo. *Nature* 1992; **359**: 845–848.
8. Negishi M, Sakamoto H, Sakamaki T, Ishikawa O, Kanda T, Tamura J, et al. Disaccharide analysis of glycosaminoglycans synthesized by cardiac myxoma cells in tumor tissues and in cell culture. *Life Sci* 2003; **73**: 849–856.
9. Ferrans VJ, Roberts WC. Structural features of cardiac myxomas. *Hum Pathol* 1973; **4**: 111–146.
10. Kono T, Koide N, Hama Y, Kitahara H, Nakamo H, Suzuki J, et al. Expression of vascular endothelial growth factor and angiogenesis in cardiac myxoma: A study of fifteen patients. *J Thorac Cardiovasc Surg* 2000; **119**: 101–107.
11. Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 1987; **162**: 156–159.
12. Zhang T, Koide N, Wada Y, Tsukioka K, Takayama K, Kono T, et al. Significance of monocyte chemoattractant protein-1 and thymidine phosphorylase in angiogenesis of human cardiac myxoma. *Circ J* 2003; **67**: 54–60.
13. Sakamoto H, Sakamaki T, Wada A, Nakajima T, Kanda T, Murata K. Dexamethasone inhibits production of interleukin-6 by cultured cardiac myxoma cells. *Am Heart J* 1994; **127**: 704–705.
14. Sakamoto H, Sakamaki T, Kanda T, Hirao Y, Ohyama Y, Ogishi K, et al. Immunosuppressive drugs inhibit the production of interleukin-6 and interleukin-8 in cultured cardiac myxoma cells. *Res Commun*

- Mol Pathol Pharmacol* 1997; **97**: 60–66.
15. Matsushima K, Morishita K, Yoshimura T, Lavu S, Kobayashi Y, Lew W, et al. Molecular cloning of a human monocyte-derived neutrophil chemotactic factor (MDNCF) and the induction of MDNCF mRNA by interleukin-1 and tumor necrosis factor. *J Exp Med* 1988; **167**: 1883–1893.
 16. Wang JM, Tarabozetti G, Matsushima K, Damme JV, Montovani A. Induction of hepatotactic migration of melanoma cells by neutrophil activating protein/IL-8. *Biochem Biophys Res Commun* 1990; **169**: 165–170.
 17. Koch AE, Polverini PJ, Kunkel SL, Harlow LA, DiPietro LA, Elner VM, et al. Interleukin-8 as a macrophage-derived mediator of angiogenesis. *Science* 1992; **258**: 1798–1801.
 18. Stietter RM, Kunkel SL, Elner VM, Mortonyi CL, Koch AE, Polverini PJ, et al. Interleukin-8: A corneal factor that induces neovascularization. *Am J Pathol* 1992; **141**: 1279–1284.
 19. Schadendorf D, Moller A, Algermissen B, Worm M, Sticherling M, Czarnecki B. IL-8 produced by human malignant melanoma cells in vitro is an essential autocrine growth factor. *J Immunol* 1993; **151**: 2667–2675.
 20. Heinrich PC, Castell JV, Andus T. Interleukin-6 and the acute phase response. *Biochem J* 1990; **265**: 621–636.
 21. Graeve L, Baumann M, Heinrich PC. Interleukin-6 in autoimmune diseases: Role of IL-6 in physiology and pathology of the immune defense. *Clin Invest* 1993; **71**: 664–671.
 22. Hirano T, Taga T, Yasukawa K, Nakashima K, Nakano N, Takatsuki F, et al. Human B-cell differentiation factor defined by an anti-peptide antibody and its possible role in autoantibody production. *Proc Natl Acad Sci USA* 1987; **84**: 228–231.
 23. Jourdan M, Bataille R, Seguin J, Zhang XG, Chaptal PA, Klein B. Constitutive production of interleukin-6 and immunologic features in cardiac myxomas. *Arthritis Rheum* 1990; **33**: 398–402.
 24. Kanda T, Umeyama S, Sasaki A, Nakazato Y, Morishita Y, Imai S, et al. Interleukin-6 and cardiac myxoma. *Am J Cardiol* 1994; **74**: 965–967.
 25. Cohen T, Nahari D, Cerem LW, Neufeld G, Levi B-Z. Interleukin-6 induces the expression of vascular endothelial growth factor. *J Biol Chem* 1996; **271**: 736–741.
 26. McCarthy PM, Piehler JM, Schaff HV, Pluth JR, Orszulak TA, Vidaillet HJ, et al. The significance of multiple, recurrent, and 'complex' cardiac myxomas. *J Thoracic Cardiovasc Surg* 1986; **91**: 389–396.
 27. Casey M, Mah C, Merliss AD, Kirshner LS, Taymans SE, Denoi AE, et al. Identification of a novel genetic locus for familial cardiac myxomas and Carney complex. *Circulation* 1998; **98**: 2560–2566.
 28. Kishner LS, Carney JA, Pack SD, Taymans SE, Giatzakis C, Cho YS, et al. Mutation of the gene encoding the protein kinase A type I- α regulatory subunit in patients with the Carney complex. *Nat Genet* 2000; **26**: 89–92.