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

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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.

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

Cardiac myxoma is the most common primary tumor of the heart. 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. Although myxomas are generally considered to be benign, malignant tumors have been described. 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 and vascular endothelial growth factor (VEGF) is a multifunctional cytokine that has been shown to be an important regulator of tumor angiogenesis. 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. Variously differentiated vascular structures are frequently observed in the gelatinous matrix. 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. 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.

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 (yWF), 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.

Methods
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 5μm thick
were used for conventional light microscopy and immuno-
histochemistry.

Immunohistochemical Analysis
Immunoenzymatic staining was carried out using the
Vectastain Elite ABC Kit (Vector Laboratories, Burlingame,
CA, USA). Sections were preincubated in methanol con-
taining 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 (0.2mg/ml)
and hydrogen peroxide (0.015%). Sections were counter-
stained 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
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
passes 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 for
30 min. Peroxidase activity was visualized with 3,3'-
diaminobenzidine (DAB) tetrahydrochloride (0.2mg/ml)
and hydrogen peroxide (0.015%). Sections were counter-
stained with methyl green, dehydrated, and mounted.

Cell Proliferation Assay
Proliferation of cardiac myxoma cells induced by exog-
enously 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 (5Ci)/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.

ELISA
The VEGF concentrations in cell-free culture superna-
tantants from the 2 cardiac myxoma cell lines were deter-
mined using an ELISA kit. Cell supernatant was obtained
from each cell line (2,700,000 cells) in 25-cm² tissue cul-
ture flask after incubation with fresh full growth medium
and 6% fetal bovine serum for 7 days. We incubated 200μl
of cell supernatant with 50μl assay diluent for 2h 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 2h at room tem-
perature. After addition of color reagent, absorbance was
measured at 450 nm in a microplate reader. For standardi-
zation, 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 fibro-
blasts, 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 isothionate-phenol-
chloroform method11. First-strand cDNA synthesis was
performed by reverse transcription of 20μl of total RNA
using the First-Strand cDNA Synthesis kit (Pharmacica
Biotech) and 1μl of the cDNA reaction was used for PCR
as described previously. For the detection of VEGF expres-
sion, 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 CTG
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' cor-
responding to nucleotides +2,952 to +3,074, and the anti-
sense 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 GCC TCT TCA TGG TGA TTG T-3' cor-
responding to nucleotides +2,954 to +3,075, and the anti-
sense primer was 5'-TGC CAG CAG TAC AGC ATG GTC
TTG-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 exten-
sion 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 ß-actin.

Statistical Analysis
Results from the cell proliferation assay are expressed as
the mean±SEM for 3 wells. For multiple treatment groups,
one-way ANOVA followed by Fisher’s least significant
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 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 $\times 200$).

**Secretion of VEGF by Cardiac Myxoma Cells**

We next determined whether cultured cardiac myxoma cells could secrete VEGF protein. The 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
myxoma cells, HUVE cells, and TIG-7 fibroblasts. Cells were treated with recombinant 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. The proliferation of HUVE cells was also stimulated by recombinant VEGF165 in a dose-dependent manner (Fig. 3, Upper panel). However, no growth regulatory effects were observed in TIG-7 fibroblasts treated with recombinant VEGF165 (Fig. 3, Lower panel). Each point represents the mean ± SEM from 3 wells. *p<0.05, **p<0.01, ***p<0.001 compared with no VEGF treatment.

cpm, counts per min.

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 ± SEM from 3 wells. *p<0.05, **p<0.01, ***p<0.001 compared with no VEGF treatment.

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 μg/ml) for 48 h, and then cell proliferation was measured. The proliferation of myxoma and HUVE cells treated with recombinant VEGF165 was compared with the proliferation of myxoma and HUVE cells treated with the neutralizing VEGF antibody (Fig. 4, Upper and Lower panels, respectively). Each point represents the mean ± SEM from 3 wells. *p<0.05, **p<0.01 compared with no VEGF-antibody treatment.

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. 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. Those authors, more recently, demonstrated that monocyte chemotactic 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 chemotactic protein-1, was primarily expressed in the infiltrating macrophages. 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. Interleukin-8 is an inflammatory cytokine and a chemotactic factor for neutrophils. The production of interleukin-8 by neoplastic cells has the potential of enhancing tumor cell migration and vascularization. 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 differentiation, proliferation. On the other hand, interleukin-6 acts on a wide range of tissues and cell lines and promotes cell differentiation, proliferation, and migration of tumor precursor cells in the subendocardium, or overproduction of glycosaminoglycans, mainly chondroitin-6-sulfate, by myxoma cells. 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

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