DISTRIBUTION OF OVEREXPRESSED VASCULAR ENDOTHELIAL GROWTH FACTOR IN THE LEFT VENTRICLE FOLLOWING MYOCARDIAL INFARCTION

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Abstract: Vascular endothelial growth factor (VEGF) mRNA is upregulated within a few hours after coronary artery ligation in pigs. However, it is unclear whether VEGF mRNA is overexpressed during the healing process following acute myocardial infarction (AMI). The aim of the present study was to investigate the relationship between serial changes in VEGF mRNA level in the left ventricle and angiogenic activity in the infarcted myocardium. Eighty male SD rats aged 8 weeks were used in this study. Left coronary artery ligation was performed in 40 rats, and a sham operation was performed in 40. The rats were sacrificed 3, 6, and 12 hours and 1, 2, 3, 7, and 14 days after the operation, and the hearts were collected. Northern blot was performed to assess the VEGF mRNA level in the left ventricle. Immunohistochemical staining for CD44 evaluated angiogenic activity in the infarcted area. VEGF mRNA levels on days 1 to 3 were greater in infarcted rats than in sham-operated rats. CD44-positive capillaries were detected from day 1 to 7 day in the infarcted area, and the number of CD44-positive capillaries was significantly greater on day 3 than at any other time points ($p<0.01$). I suggest that serial changes in the VEGF mRNA level parallel the angiogenic activity of the infarcted area.

Key words: angiogenesis, healing, myocardial infarction, vascular endothelial growth factor

INTRODUCTION

Angiogenesis rarely takes place in healthy adult individuals, but may be activated under certain pathologic circumstances, such as tumor growth[1-3], ischemia[4-6], and wound healing[7-10]. Repair of necrotic tissue follows acute myocardial infarction[11-13]. Angiogenesis is also upregulated during the healing period after acute myocardial infarction[14-15].

Vascular endothelial growth factor (VEGF) is a heparin binding, secreted growth factor with a unique target cell specificity for vascular endothelial cells[16-17]. VEGF regulates endothelial cell function, including enhancement of angiogenesis[18-21], and of capillary permeability[22-23], and transient induction of calcium uptake[24]. VEGF mRNA is upregulated in cultured cardiac myocytes subjected to hypoxia and in the pig left ventricle within 6 hours of coronary artery ligation[25]. These results suggest that VEGF mRNA is overexpressed in acute myocardial ischemia. However, it is unclear whether VEGF mRNA is upregulated in the subacute phase of myocardial infarction when the healing process occurs in the infarcted tissue. The relationship between VEGF mRNA level and angiogenic activity in the infarcted myocardium has not been identified. I hypothesized that VEGF may regulate angiogenesis directed towards healing in the infarcted myocardium. The present

study clarifies the serial changes in the level of VEGF mRNA, the localization of VEGF, and the relationship between VEGF mRNA levels and angiogenic activity in the infarcted myocardium.

MATERIALS AND METHODS

Protocols and procedures were approved by the animal welfare committee of our institution.

Animal model

Adult male Sprague-Dawley rats (SLC, Shizuoka, Japan) weighing 250 to 300 g at the start of the experiment were used. Animals were housed and given standard rat chow and water ad libitum. In total, 130 rats were used; 84 underwent coronary artery ligation, and 46 had a sham operation. The overall survival rate was 72%. After animals with an infarct size less than 20% were excluded, 40 rats with infarcts and 40 rats with sham operation remained, with 5 animals from each group examined at each of eight time points.

Surgery

Animals were anesthetized with sodium pentobarbital (50 mg/kg, IP) and ventilated by positive pressure through an endotracheal tube attached to a small animal respirator (Rodent ventilator model 683, Harvard Apparatus, South Natick, USA.) Following left thoracotomy and incision of the pericardium, the left anterior descending coronary artery was ligated near its origin with 6-0 silk suture. The thorax was closed, and the rats were returned to their cages. The sham-operated rats underwent the same surgical procedure except that the suture that passed under the left anterior descending coronary artery was not tied.

Tissue collection

Infarcted and sham-operated rats were reanesthetized and killed by excision of the heart at 3, 6, and 12 hours, and on 1, 2, 3, 7, and 14 days after the operation. Heart weight was measured. In infarcted rats, a coronal section was made at the point of the ligation, and the apical-side sections of the left ventricle, including the infarct zone, were subdivided coronally into 3 portions. The most basal portion including the infarct zone was immersed in a 2% solution of 2, 3, 5-triphenyltetrazolium chloride (TTC) in normal saline at 37℃ for 30 minutes for determination of infarct size. This portion was also fixed in 10% phosphate-buffered formalin for photography. The mid portion was fixed in 10% phosphate-buffered formalin and embedded in paraffin for histologic evaluation and immunohistochemistry. The most apical portion was rapidly frozen in liquid nitrogen and stored at −85℃ for total RNA isolation and Northern blotting. In sham-operated rats, a coronal section was made at the point near the origin of the left anterior descending coronary artery, and tissue was collected similarly to the infarcted rats.

Determination of infarct size

Ventricular sections encompassing the entire left ventricle circumference of each sample stained with TTC were photographed. Myocardial infarction was defined as the corresponding pale region of the left ventricular wall. Epicardial circumferential lengths of both infarcted and noninfarcted regions were measured using an image analysis processor

(Cardio 500™, Kontron Elektronik, Eching, Germany). Infarct size was then calculated as the percentage of infarct length relative to the entire left ventricular circumference of each section.

RNA preparation and Northern blotting

Myocardial total RNA was isolated from frozen heart tissue using the isogen kit. The concentration of RNA was calculated from the absorbance at 260nm prior to size fractionation. Agarose gel (1% seakem GTG) electrophoresis of RNA (total 15μg RNA per lane) and transfer to nylon membranes (Amersham, Buckinghamshire, UK) were carried out. After transfer, RNA blots were exposed to shortwave UV light to cross-link RNA to the membrane and to visualize the major ribosomal RNA bands. Each membrane was hybridized using the cDNA probes of rat VEGF and rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) labeled with [^{32}P]dCTP by random primer extension (Amersham)[26]. Membranes were exposed to X-ray film. Results of autoradiographs from Northern blot analysis were quantified by densitometry. The signals of specific mRNAs were normalized to those of GAPDH mRNA to normalize for differences in loading and transfer of mRNA[27].

Immunohistochemistry

To determine cell specificity, the following primary antibodies were used: rabbit polyclonal antibody to human VEGF (Takara, Ohtsu, Japan) and mouse monoclonal antibody to rat CD44 (Pharmingen, San Diego, CA, USA). As a nonimmune primary antibody, rabbit normal serum (Nichirei, Tokyo, Japan) was used for a negative control. To block endogenous peroxidase, the paraffin-embedded sections of the heart tissue were immersed in 3% hydrogen peroxidase in methanol for 10 minutes and in 10% normal serum for 20 minutes. The specimens were incubated with each primary antibody at 37℃ for 60 minutes. The streptavidin-biotin method was performed with a Histofine SAB-PO(R) kit (Nichirei) for VEGF staining and a SAB-AP(M) kit (Nichirei) for CD44 staining. The sections were incubated with 50μg/ml of 3', 3'-diaminobenzidine (DAB) in phosphate buffered saline (PBS) for 10 minutes for VEGF staining, and with 2% Vector Red (Vector Laboratories, Burlingame, CA, USA) in 100 mM Tris-Hcl for 30 minutes for CD44 staining. Sections were counterstained lightly with hematoxylin.

The distribution of VEGF in non-ischemic myocytes, border zone myocytes, infarcted myocytes, and macrophages in the infarcted area was graded on a four-point scale (0, absent; 1, mild; 2, moderate; and 3, severe).

Capillary density

A total of 10 different fields under total magnification X 400 from endocardium to epicardium in the infarcted area were randomly selected, and the number of CD44-positive capillaries per field was counted to determine semiquantitatively the activity of neocapillary formation at each infarct time point.

Histopathology

Paraffin-embedded sections of the heart, including the infarct zone, were stained with hematoxylin and eosin, and Masson's trichrome, as described elsewhere[28-31]. Features of myocardial edema or hemorrhage, necrosis, infiltrating cells, and fibrosis were graded on a four-point scale (0, absent; 1, mild; 2, moderate; and 3, severe).

Statistical analysis

All values are expressed as mean ± SD. Fisher's least significant difference procedure was performed for the comparison of VEGF mRNA level and capillary density between each time point. $P<0.05$ was considered statistically significant.

RESULTS

VEGF mRNA expression in the heart

The level of VEGF mRNA in the infarcted hearts was significantly elevated from day1 to day 3 after the operation compared to sham-operated rats. The peak level of VEGF mRNA was attained on day 2 (Fig. 1, 2).

Immunohistochemical localization of VEGF

Cardiac myocytes did not immunostain for VEGF in sham-operated rats. In infarcted rats, necrotic cardiac myocytes were negative for VEGF. VEGF was initially detected in cardiac myocytes adjacent to the infarct zone (border zone) 6 hours after infarction. The distribution of VEGF was spread to the non-ischemic myocytes from hour 6 to day 14, and then to infiltrating cells and endothelial cells of neocapillaries in the infarcted zone from day 1 to day 7 (Fig. 3). The distribution of immunostaining for VEGF in myocytes and infiltrating cells was prominent from day 1 to day 3 (Table 1).

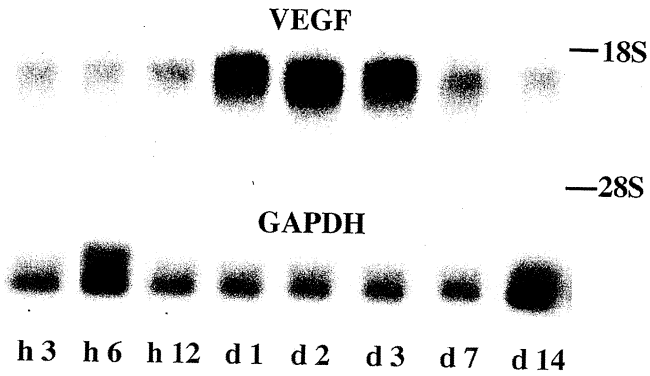

Fig. 1. Representative example of Northern hybridization for vascular endothelial growth factor (VEGF) mRNA in infarcted rats.

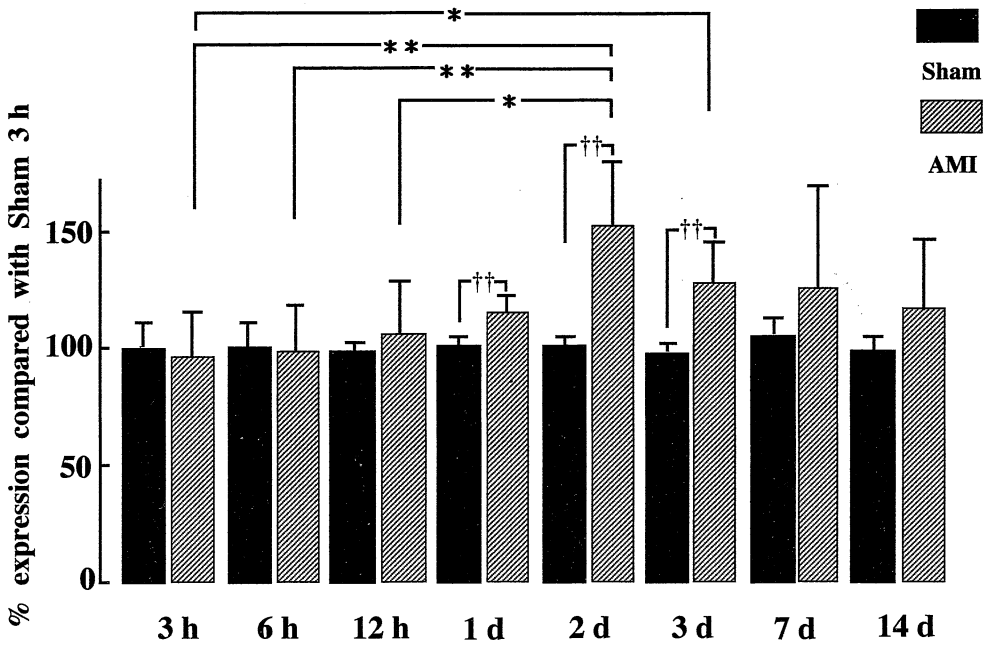

Fig. 2. Serial changes in vascular endothelial growth factor (VEGF) mRNA level in infarcted and sham-operated rats. **P < 0.01,*P < 0.05, † † p < 0.01. Data are mean ± SD.
AMI : acute myocardial infarction.

Table 1. Serial changes in the distribution of vascular endothelial growth factor following acute myocardial infarction

localization	3h	6h	12h	1d	2d	3d	7d	14d
Cardiac myocytes								
Necrotic zone	0	0	0	0	0	0	0	0
Border zone	1	1	2	3	3	3	2	2
Non ischemic zone	0	1	2	3	3	2	2	1
Infiltrating cells								
Necrotic zone	0	0	1	2	3	3	2	0

0 ; absent, 1 ; mild, 2 ; moderate, 3 ; severe.

Semiquantification of neocapillary formation

Only a few capillaries demonstrated CD44 in the hearts of sham-operated rats. In infarcted rats, the number of CD44-positive capillaries on day 3 was significantly greater than at all other time points. The number of CD44-positive capillaries on day 2 was significantly greater than at hours 3, 6, and 12 (Fig. 4, 5).

Histopathologic parameters of healing in the heart

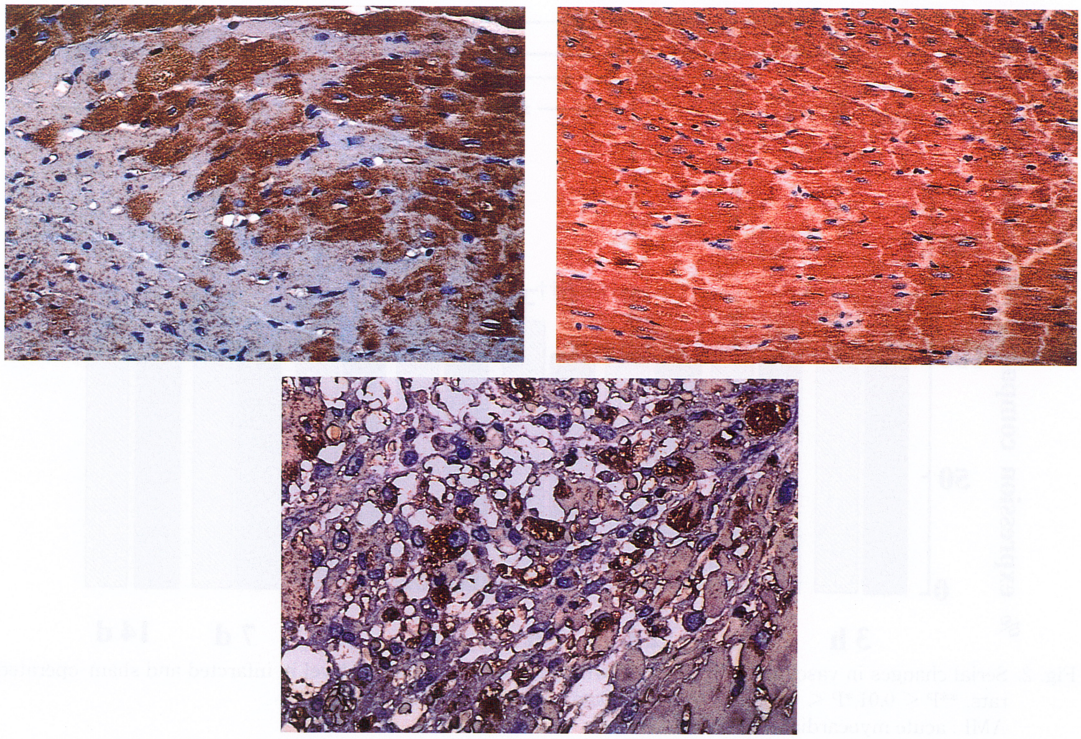

Fig. 3. Immunohistochemical distribution of vascular endothelial growth factor (VEGF) in infarcted myocardium.
(A) VEGF was initially detected in the border zone and non-ischemic cardiac myocytes on hour 6 following acute myocardial infarction (×100).
(B) Non-ischemic cardiac myocytes were positive for VEGF on hour 12 following acute myocardial infarction (×100).
(C) Infiltrating cells and endothelial cells in infarcted area were positive for VEGF on day 3 following acute myocardial infarction (×400).

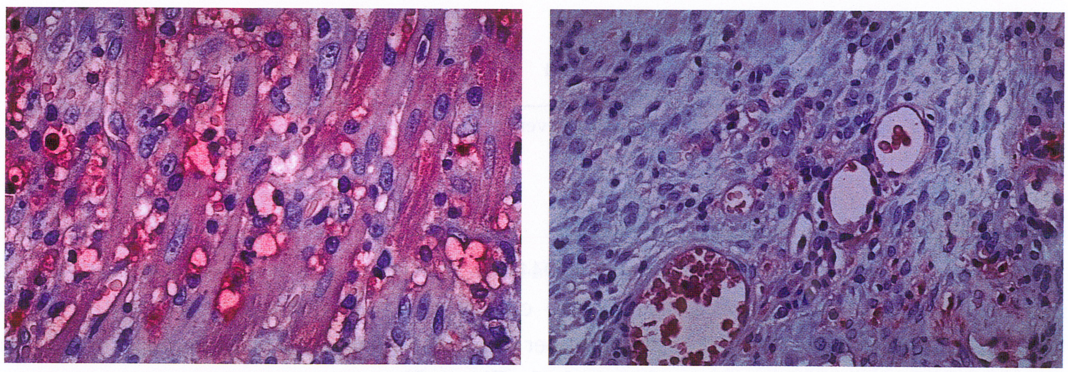

Fig. 4. Immunohistochemical distribution of CD44 in infarcted area on day 3 (×400, A) and on day 14 (×400, B).

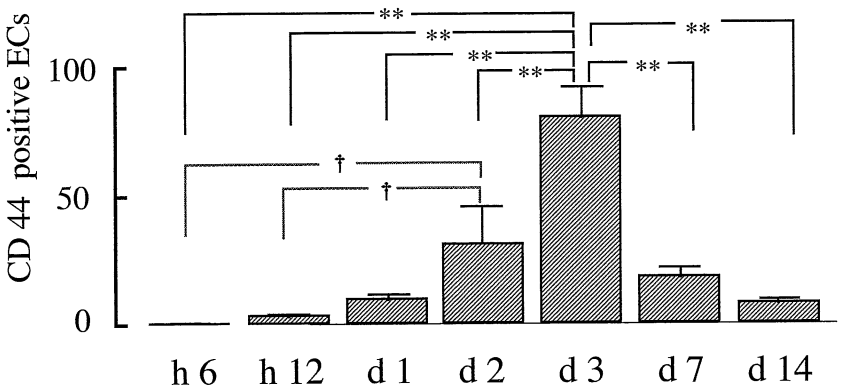

Fig. 5. Serial changes in CD44-positive capillary density following acute myocardial infarction. **p < 0.01, † p < 0.05 . Data are mean ± SD.

Table 2. Histopathological parameters of healing in infarcted area

Parameters	3h	6h	12h	1d	2d	3d	7d	14d
Myocardial edema or hemmorhage	2	3	3	3	2	1	0	0
Myocardial necrosis	0	0	1	2	2	2	1	0
Infiltrating cells	0	0	1	2	3	3	2	1
Fibrosis	0	0	0	0	1	2	3	3

0 ; absent, 1 ; mild, 2 ; moderate, 3 ; severe.

The histologic changes after acute myocardial infarction are presented as the average grades of 5 infarcted rats (Table 2). Infiltrating cells were prominently detected on days 1 to 3, when necrotic cardiac myocytes were replaced by fibrotic tissues.

DISCUSSION

In the present study, Northern hybridization demonstrated that the levels of VEGF mRNA on days 1 to 3 were significantly greater in the infarcted rats than in sham-operated rats. Previous studies revealed that VEGF mRNA is overexpressed within a few hours of acute myocardial ischemia in the pig heart[25]. However, our results suggest that the level of VEGF mRNA may be greater in the subacute phase of myocardial infarction such as on days 1 to 3 than in the acute phase such as within a few hours. Serial changes in the localization of VEGF after acute myocardial infarction have not been examined. Our immunohistochemical study revealed that cardiac myocytes were initially immunostained for VEGF only in the border zone at hour 6 after coronary artery ligation. Thereafter, the distribution of VEGF spread to nonischemic myocytes and infiltrating cells such as monocytes and macrophages in the infarcted area. The distribution of VEGF was most prominent on days 1 to 3. These immunohistochemical findings are consistent with the results of the Northern hybridization.

Angiogenesis is induced in wound healing. Various growth factors such as epidermal growth factor, basic fibroblast growth factor (bFGF), VEGF, and heparin-binding epidermal growth factor are upregulated in wound healing[7-10]. Among these factors, VEGF and bFGF are angiogenic peptides. The inflammatory response plays an important role in the healing of the infarcted myocardium. Inflammatory cytokines, such as interleukin 1β, interleukin 6, and tumor necrosis factor-alpha induce expression of VEGF[32-35]. Inflammatory cytokines secreted in the wound tissue may upregulate VEGF expression and activate angiogenesis directed towards wound healing. This inflammation-induced angiogenesis may occur in infarcted myocardium. Previous studies have revealed that VEGF and bFGF were upregulated after focal brain infarction, and their expression paralleled angiogenic activity[36-38]. The increase in mitogenic activity of bFGF parallels collateral development following coronary artery ligation in the dog[39]. A recent report revealed that hepatocyte growth factor mRNA (an angiogenic growth factor) is upregulated by myocardial ischemia and reperfusion[40]. However, the relationship between VEGF expression and angiogenesis directed towards healing after acute myocardial infarction has not been identified. In the present study, histopathologic parameters of healing and activity of neocapillary formation in the infarcted area were evaluated. CD44 is an adhesion molecule of vascular endothelial cells that is upregulated in neocapillary formation. CD44 is a histologic marker of newly formed endothelial cells[41-43]. The distribution of infiltrating cells and CD44-positive capillaries in the infarcted area was prominent on days 1 to 3, and diminished thereafter. The serial changes in the activity of healing and angiogenesis paralleled the levels of VEGF mRNA. These results suggest that VEGF mRNA may be upregulated after acute myocardial infarction for the purpose of angiogenesis directed towards healing the infarcted area. The present study provides information about a possible role of VEGF in angiogenesis during the healing process following acute myocardial infarction.

A recent clinical study indicated that serum concentrations of VEGF 7 to 28 days after acute myocardial infarction were greater than in the chronic stage following myocardial infarction[44]. The mechanism of increased serum VEGF in the subacute phase of myocardial infarction is unclear. In the present study, I did not measure serum VEGF. However, these results suggest that serum VEGF concentration in the subacute phase of myocardial infarction may reflect VEGF mRNA synthesis in the left ventricle following acute myocardial infarction.

Therapeutic angiogenesis for ischemia has been attempted with administration of angiogenic growth factors. Administration of VEGF enhances collateral circulation in rabbit hind limb ischemia[45]. Intracoronary administration of VEGF and bFGF before coronary artery ligation enhances collateral circulation in the dog[46-47]. However, serial changes in the mRNA levels of these angiogenic factors and their role after acute myocardial infarction are not fully established. Such information would be essential in the application of therapeutic angiogenesis. The present results suggest that administration of VEGF on days 1 to 3 after acute myocardial infarction may accelerate the healing process and shorten the duration of remodeling in the rat infarcted myocardium. VEGF may shorten the duration of cardiac rehabilitation and inhibit left ventricular remodeling in humans with acute myocardial infarction. However, VEGF may also enhance hyperemia and hemorrhage in the infarcted

area. It is unclear whether exogenous VEGF administration would be beneficial or harmful to the healing process after acute myocardial infarction. Further studies are needed to clarify the usefulness of therapeutic angiogenesis following acute myocardial infarction.

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