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Effects of Transient Coronary Occlusion on the Capillary Network in the Left Ventricle of Rat

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Abstract: The objective was to examine the changes in the capillary network in the left ventricle of rats subjected to transient occlusion of the left coronary artery followed by reperfusion (I-R). Eighteen Wistar rats were divided into three groups and all rats were anaesthetized with ethyl ether and artificially ventilated. The I-R 1 rats were subjected to a 3 min occlusion followed by reperfusion; the I-R 3 rats had three 3 min occlusions separated by 3 min of reperfusion; the Sham-operated rats underwent surgery but the coronary artery was not occluded. The thorax was closed at the end of the procedures and the rats were sacrificed for isolation of the hearts 30 d after treatment. Frozen sections of the left ventricles were cut and differential staining was used to classify the capillary portions. Five additional rats treated as the I-R 1 group were sacrificed at 120 min after reperfusion. Their left ventricles were used for immunohistochemical investigation of the early expression of bFGF and VEGF. By comparison with the Sham-operated rats, both I-R groups showed increases in the capillary density of total and venular capillary portions, an increased capillary : myocyte (C : M) ratio and a decrease in the capillary domain area in the three capillary portions. The changes in the I-R 1 group were significantly greater than those in the I-R 3 group, suggesting that the frequent experience of ischemic attack reduces the capacity of angiogenesis. In the rats sacrificed 120 min after the start of reperfusion, bFGF and VEGF were expressed on capillaries and in some myocytes. Punctate bFGF or VEGF staining was observed even 30 d after the transient ischemia. One 3 min occlusion of the left coronary artery followed by reperfusion produced changes in capillarity that would increase the oxygen supply to ventricular tissues. These effects may be attributed to the bFGF and VEGF expressed around capillaries. Repeated occlusions interspersed with a short period of reperfusion reduced the advantageous effects on capillarity. [Japanese Journal of Physiology, 47, 537–543, 1997]

Key words: capillary remodeling, transient ischemia, bFGF, VEGF, heart.

The effects of coronary occlusion on ventricular vascularization have intensively been studied with respect to the formation of collaterals [1, 2]. In contrast, the effect on the capillary network in the ventricular wall has been studied very little. In our previous work on rats, coronary occlusion caused hypertrophy of cardiomyocytes in the non-ischemic region but did not cause significant changes in the capillary domain area [3]. This result suggests that there was neogenesis of capillaries in this region. Furthermore, intravenous injection of vasopressin, which caused only short-lasting hypoxic or ischemic changes (3–10 min) in the electrocardiogram, produced a marked increase in capillary density in hearts 30 d later, even though heart weight was unchanged [4]. These results encouraged us to study the effects of transient occlusion of coronary artery on the capillarity of the left ventricle.

Capillaries are non-uniform thin tubes and the capillary domain area (i.e., the cross-sectional tissue area supplied by a single capillary) is larger in arteriolar than venular capillary portions. The arteriolar and venular capillary portions express alkaline phosphatase (AP) and dipeptidylpeptidase IV (DPPIV), respectively [5, 6]. In this study, the double-staining

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method for AP and DPPIV was used to differentiate arteriolar and venular capillary portions.

In our earlier study [4], immunohistochemistry revealed a strong expression of angiogenic factor, basic fibroblast growth factor (bFGF), 24 h after a single injection of vasopressin and punctate staining of capillaries 30 d after the treatment. The expression of bFGF, and also vascular endothelial growth factor (VEGF) protein, was studied immunohistochemically in the experiment presented here.

MATERIALS AND METHODS

Eighteen male 8-week-old Wistar rats were used. They were provided with standard rat chow and water ad libitum, and all procedures were performed according to the institutional guidelines for care and use of laboratory animals. Rats were anaesthetized with ethyl ether, intubated with a polyethylene tube (outer diameter 2 mm), and mechanically ventilated with a rodent ventilator using room air containing a low concentration of ethyl ether. Groups of six rats were randomly subjected to one of the following treatments: one 3-min period of ischemia followed by reperfusion for the next 30 d (I-R 1); three 3-min periods of ischemia each separated by 3 min of reperfusion and followed by reperfusion for 30 d (I-R 3); a Sham-operation without ischemia. Five additional rats treated as I-R 1 were sacrificed at 120 min after the start of reperfusion and their hearts were used for the immunohistochemical localization of bFGF and VEGF proteins.

Ischemia and reperfusion were achieved as described by Himori and Matsuura [7]. Briefly, a left thoracotomy was performed via the fourth intercostal space, and the pericardium opened. The left atrial appendage was raised and a small curved needle threaded with fine silk (Elp No.1) was passed through the ventricular myocardium just under the left atrial appendage to encompass the left coronary artery. The thread was then tied in an overhand knot (an occluder). Two other threads were tied to the main knot (releasers). The ligature could be tightened or loosened by pulling on the relevant threads. In the I-R 1 group, the left coronary artery was occluded for 3 min, and then the ligature was released and removed. In the I-R 3 group, the artery was subjected to three 3-min periods of ligation with 3 min of reperfusion, and the ligature was removed after the third occlusion. Occlusion produced a clearly demarcated cyanotic swollen area of acute ischemia corresponding to the distribution of the left coronary artery distal to the ligation. After the release of the ligature, reperfusion occurred and the reperfused area lost its cyanotic appearance. In the Sham-operated group, the thread was looped around the coronary artery but not tightened. After the treatments, the left lung was inflated, the chest closed and pneumothorax evacuated.

The rats were sacrificed by quick decapitation with a guillotine 30 d after the operation. This time point was selected, because significant changes in the cardiac capillarity were confirmed 30 d after coronary occlusion [3] or vasopressin injection [4] in our previous studies. The heart was removed, dipped in optimum cutting temperature compound (O.C.T. compound® Miles Inc., USA), frozen in liquid nitrogen and stored at −80°C.

Using a cryotome, tissue cross-sections were cut from the widest part of the ventricle at 10 or 16 μm. Four to six sections, each 80 μm apart, were obtained from each left ventricle. Double-staining of sections was performed to differentiate arteriolar and venular capillaries as described previously [5, 6], after which they were covered with crystal mount (Biomeda, CA, USA). The validity of the double-staining method for differentiating the capillary portions was confirmed in a previous study [8]. Arteriolar capillary portions in which the endothelial cells contain alkaline phosphatase were stained blue, venular portions containing dipeptidylpeptidase IV stained red, and the intermediate portions containing both enzymes stained violet. Capillaries stained, respectively, blue, violet and red are thus referred to as arteriolar, intermediate and venular capillary portions. Microscopic observations revealed cross-sections of capillaries in the outer and inner muscle layers and longitudinal ones in the middle layer. Visual fields were selected in the inner layer close to the endocardium (i.e., subendocardium). A morphological measurement was made in one arbitrarily selected microscopic visual field covering 0.0615 μm^2 in each section and four sections were evaluated. Since 100 to 150 capillaries were present in each visual field, more than 400 capillaries were examined for each rat.

The cross-sections of differently coloured capillaries depicted on sheets of paper using a drawing tube attached to a microscope were transmitted to a personal computer via an *X-Y* digitizer for counts of capillaries and measurement of the capillary domain area (CDA). Krogh's tissue cylinder radius was calculated from CDA values, the CDA being treated as circular. Cross-sections of subendocardial myocytes were sketched and their areas calculated using an image scanner and a personal computer on NIH image program 1.52. The capillary : myocyte ratio (C : M ratio) was calculated from the number of myocytes and capillaries counted under the microscope.

Table 1. Basic data from rats subjected to transient ischemia 30 d after treatment.

Group	BW (g)	HW (mg)	LVW (mg)	LVW/BW
Sham-operated (n=6)	306.3±11.1	758.3±25.8	583.3±28.2	1.91±0.15
I-R 1 (n=6)	306.5±32.5	729.8±76.7	542.0±59.0	1.78±0.05
I-R 3 (n=6)	297.5±20.6	740.5±42.6	555.8±28.6	1.87±0.07

BW, body weight; HW, heart weight; LVW, left ventricular weight.

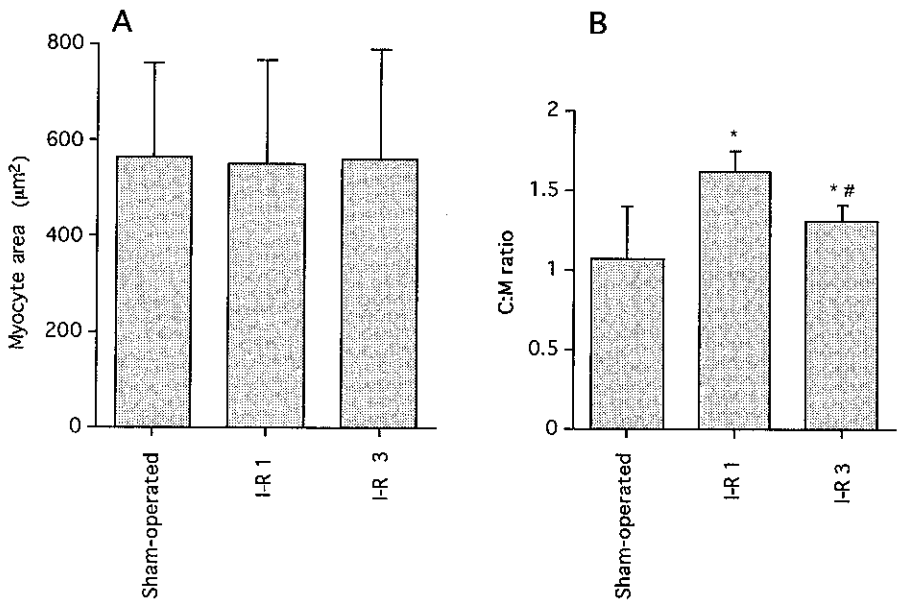

Fig. 1. Effects of transient ischemia on myocyte area (A) and C : M ratio (B) in the subendocardium of the left ventricle 30 d after the transient ischemia or Sham operation (n=6 for each group). Values are means±SD. * Significantly different from Sham-operated group (p<0.01). # Significantly different from I-R 1 group (p<0.01).

Immunohistochemical staining for bFGF protein was made on 10 μm frozen sections with anti-bovine basic FGF (Upstate Biotechnology, USA), which also reacts with rat bFGF. The bFGF was visualized with biotinized anti-mouse IgG and aminoethylcalbasol (AEC Substrate Kit, Nichirei, Tokyo, Japan) mainly according to the maker's instructions. The staining for VEGF protein was also performed on the frozen sections using anti-VEGF (A-20). Anti-VEGF (A-20) is an affinity-purified rabbit polyclonal antibody raised against a 20 amino acid synthetic peptide corresponding to residues 1–20 mapping at the amino terminus of human VEGF (Santa Cruz Biotech, CA, USA). The anti-VEGF (A-20) is reported to react with rat VEGF. VEGF was also visualized with aminoethylcalbasol (AEC Substrate Kit). The sections were counterstained with Mayer's hematoxylin solution, mounted with PBS containing glycerol, covered with cover glasses and attached with nail varnish.

All data are given as means±SD. Comparisons among multiple groups were carried out using ANOVA, and Kruskal-Wallis and Mann-Whitney U-tests. p values <0.05 were considered significant.

RESULTS

Basic physical data are listed in Table 1. No significant differences were found in any parameters among the groups.

As Fig. 1 indicates, the cross-sectional areas of myocytes showed no significant difference among the groups. In contrast, the C : M ratio was increased in the I-R groups as compared to the Sham-operated group (1.07±0.75). The C : M ratio of the I-R 1 group (1.62±0.15) was significantly higher than that of the I-R 3 group (1.31±0.12).

The CDA values of all three capillary portions showed significant decreases in the I-R groups as compared to those of the respective capillary portions in the Sham-operated group. The decrease in the I-R 1 group was greater than that in the I-R 3 group (Fig. 2). Figure 3 also shows that the total capillary density and density of the venular portions increased significantly in both I-R groups; again the changes in the I-R 1 group were significantly greater than those in the I-R 3 group. Krogh's tissue cylinder radius was decreased by the treatments of transient ischemia in all the capillary portions (Fig. 4).

Only faint staining of bFGF was obtained, confined

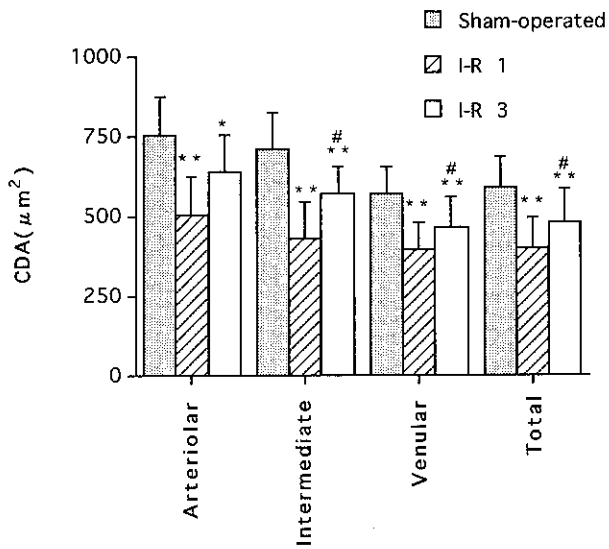

Fig. 2. Changes of CDA for arteriolar, intermediate and venular portions and average values for total capillary portions in the subendocardium of the left ventricle 30 d after the transient ischemia or Sham operation (*n*=6 for each group). Values are means±SD. Significantly different from Sham-operated group (* $p<0.05$, ** $p<0.01$) and from I-R 1 group (# $p<0.01$).

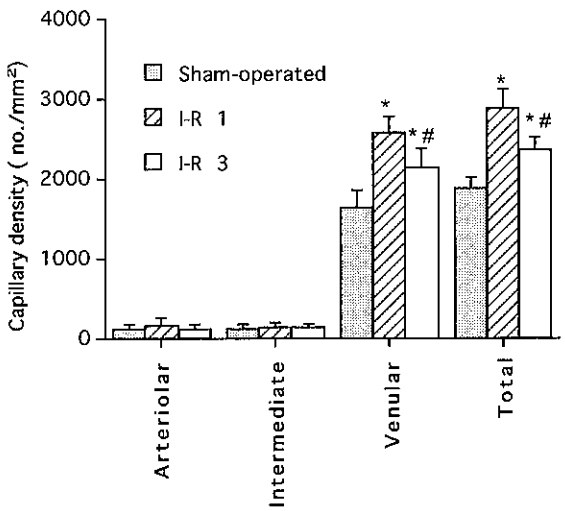

Fig. 3. Capillary density for arteriolar, intermediate and venular portions and total capillary number in the subendocardium of the left ventricle 30 d after transient ischemia or Sham operation (*n*=6 for each group). Values are means±SD. Significantly different from Sham-operated group (* $p<0.01$) and from I-R 1 group (# $p<0.01$).

in interstitial spaces, but no staining of VEGF was observed in the cardiac tissues obtained from 6 Sham-operated rats (micrographs are not shown). In sections obtained from 5 rats in the I-R 1 group, 120 min after reperfusion, bFGF was seen mainly within the cytoplasm of the myocytes (Fig. 5A). VEGF was visible on capillaries and other microvessels at this time (Fig.

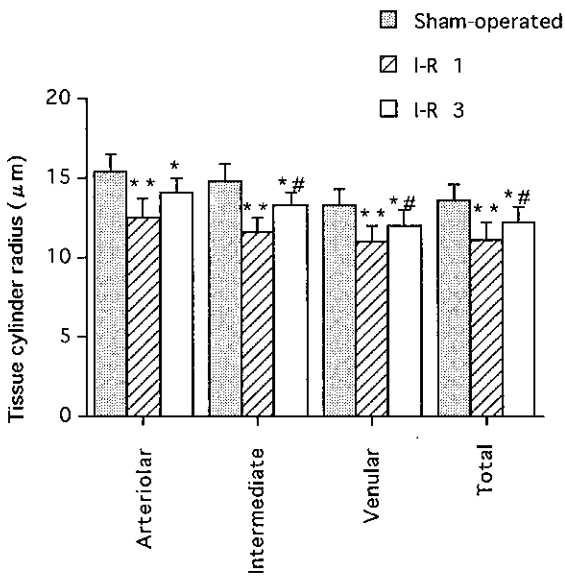

Fig. 4. Tissue cylinder radius for arteriolar, intermediate and venular portions and average values for total capillary portions in the subendocardium of the left ventricle 30 d after transient ischemia (*n*=6 for each group). Values are means±SD. Significantly different from Sham-operated group (* $p<0.05$, ** $p<0.01$) and from I-R 1 group (# $p<0.01$).

5B). Some capillaries were found stained for bFGF (Fig. 5C) and VEGF (Fig. 5D) 30 d after the transient occlusion.

DISCUSSION

Vasospastic angina pectoris causes transient ischemia usually lasting from 2 to 10 min. The intravenous injection of vasopressin, which caused only short-lasting (3–10 min) ischemic changes in the electrocardiogram, produced a marked increase in capillary density in the heart 30 d after treatment [4]. We therefore studied the effect of 3-min coronary occlusion on the capillarity of the left ventricle in the experiment presented here. The increase in the C : M ratio in both I-R groups, without any alteration in the area and density of myocytes, provides evidence that even a short period of ischemia stimulates capillary neogenesis. The increase in total capillaries following transient occlusion seemed to be due to an increase in the venular capillary portion. These results, together with those obtained previously after intravenous vasopressin injections, suggest that capillaries continue to increase long after a short period of coronary ischemia. This result seems consistent with the finding that the capillary density of ventricular tissue biopsy samples was significantly higher in patients with vasospastic angina than in patients reporting no ischemic chest pain [9].

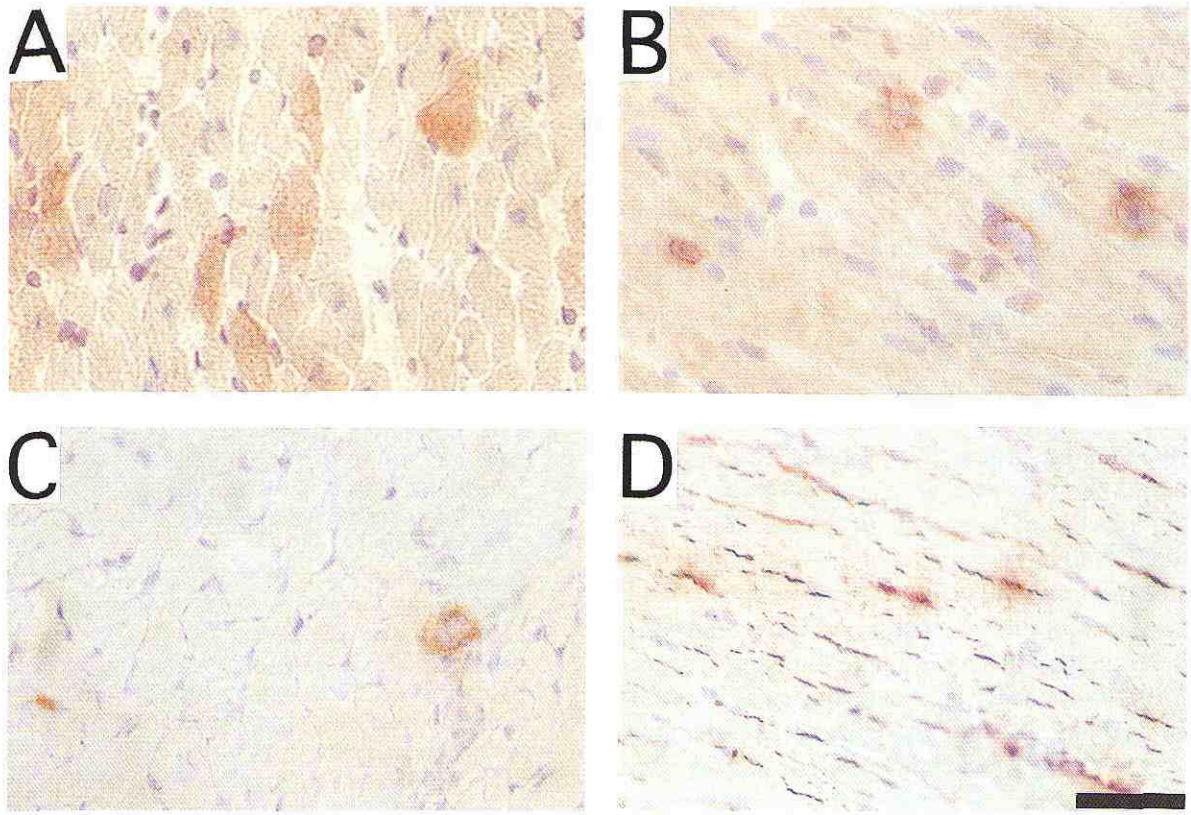

Fig. 5. Micrographs of immunohistochemically stained cardiac tissue sections. (**A**) A cross-section of the subendocardium of a rat heart 120 min after the start of reperfusion following a 3-min coronary occlusion. The section was stained immunohistochemically for bFGF visualized brownish red with anti-mouse IgG and aminoethylcalbasol. bFGF is seen within the cytoplasm of the cardiomyocytes. (**B**) A similar section stained immunohistochemically for VEGF visualized brownish red with anti-rabbit IgG and aminoethylcalbasol. VEGF is seen on the capillaries. (**C**) A cross-section of the subendocardium of a rat heart obtained 30 d after ischemia reperfusion. bFGF staining is found in some capillaries. (**D**) VEGF staining can be seen along some capillaries in the middle layer of the left ventricular wall. All of the sections are counterstained with hematoxylin to show the nuclei. Scale bar represents 40 μm.

The observation that the increases in capillarity were greater in the I-R 1 gruop than those in the I-R 3 group has implications with regard to VEGF production. VEGF functions as an endothelial cell-specific mitogen and a potent angiogenic factor [10]. Multiple isoforms of VEGF, generated by different mRNA splicing [11], have similar activities. VEGF has been demonstrated to be induced in the heart by hypoxia and ischemia *in vivo* [12]. These observations support the hypothesis that ischemia induces VEGF mRNA in cardiac myocytes, leading to increased VEGF protein production and thereby contributing to ischemia-mediated angiogenesis. Intramuscular administration of VEGF could induce dose-dependent collateral artery augmentation in a rabbit model of chronic limb ischemia [13]. It was reported that repetitive 5-min ischemia with an interval of 10–30 min of reperfusion did not have an additional effect on the induction of VEGF mRNA; its increase, on the contrary, was only about half that of a single 5–10 min of ischemia [14]. These observations may suggest that increased ischemic episodes with intervals of reperfusion may reduce the extent of the increase in VEGF protein synthesis and decrease the angiogenic activity of cardiac tissues.

The size of the capillary domain areas of the different capillary portions reflects the P_{O_2} of the blood they carry [6]. As in our previous study [4], the CDA values were in the order arteriolar>intermediate>venular portions in all three groups. In hypoxia, CDAs might be expected to decrease to ensure adequate tissue oxygenation. The present finding that the CDAs of all capillary portions decreased significantly 30 d after 3 min of ischemia seems to be consistent with this consideration.

In our previous study, the presence of the angiogenically active growth factor bFGF was demonstrated immunohistochemically along capillaries in the ventricular tissue 24 h after a single injection of vasopressin [4]. We have also shown bFGF to be strongly expressed within 24 h of coronary occlusion [3]. Hashimoto *et al.* reported that VEGF mRNA in-

creased at 15 min and peaked at 30 min after the onset of transient ischemia, but bFGF mRNA was detected only after prolonged ischemia in isolated and perfused rat hearts [14]. To further understand the regulation and roles of VEGF and bFGF in ischemic hearts, it is necessary to study the early expression of VEGF and bFGF at protein levels. In a preliminary experiment, cardiac tissues obtained at 30, 60 and 120 min after the start of reperfusion were stained for growth factor proteins. The earliest expression of the growth factors was observed only at 120 min.

In this study, only faint staining with bFGF antibody was observed between cardiomyocytes in the Sham-operated rat hearts. Strong bFGF staining was obtained in the cytoplasm of some myocytes 120 min after the release of coronary occlusion (Fig. 1A). Clear bFGF staining was still seen on some capillaries 30 d after the transient ischemia, in consistence with the observation in the vasopressin injection study [4]. It has been shown [15] that a single intravenous injection of bFGF produces significant increases in arterioles and capillaries in the left ventricular border zone of ischemia in dogs whose one branch of the left coronary artery had been occluded 30 d earlier. Thus, the present finding together with our previous study using vasopressin injection [4] is consistent with these studies, and increased capillary neogenesis seems to be present 30 d after the transient ischemia.

VEGF staining was clearly seen on many capillaries 120 min after the end of a 3-min occlusion (Fig. 1B). VEGF is a candidate for stimulating the proliferation of capillary endothelial cells and neoangiogenesis during hypoxia. To induce VEGF in HeLa cells in a culture system, it was necessary that the cells were continuously exposed to hypoxia or cobalt ions for up to 3 h [16]. Nevertheless, in this study, a transient ischemia of only 3 min led to marked staining for VEGF in the capillaries. Clearly it is advantageous to the maintenance of the ventricular capillary network that the expression of angiogenic factors can be triggered by such a short ischemic period. Similarly, in rabbits whose femoral arteries had been removed, a single intra-arterial bolus injection of VEGF resulted in significant improvements in blood flow and capillary density of the lower limb [17]. The present result that VEGF staining was found on some capillaries and the capillary density was increased 30 d after the transient ischemia is consistent with these studies.

In conclusion, one 3-min occlusion of the left coronary artery followed by reperfusion produced an improvement in capillarity that would increase the oxygen supply to ventricular tissues. These effects may be attributed to the bFGF and VEGF expressed around the capillaries. Repeated occlusions interspersed with a short period of reperfusion reduced the advantageous effects on the capillary.

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