Evaluation of coronary collateral circulation in early ischemia in rat hearts
A morphological study

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Abstract
Histological changes were examined in the left ventricular free wall of the heart in 40 rats after ligation of the distal anterior descending coronary artery for 15, 30 minutes and 1, 2, 3, 4, 5, 6 hours. Auto-injection of tracers for light microscopy was used to examine the development of collateral circulation. Morphological changes of the ischemic myocardium were observed by PAS staining and transmission electron microscopy. Fifteen minutes after coronary occlusion, the dye was observed in the vein of the ischemic zone; however, 30 minutes after coronary occlusion, the dye appeared in the capillaries of the outer-third layer. These findings suggested that the collateral circulation becomes functional between 15 and 30 minutes after coronary occlusion. Collateral circulation increased gradually in the capillaries of the ischemic zone. Thirty minutes after coronary occlusion the dye was seen only in the outer-third layer of the left ventricular ischemic zone. One hour after occlusion, the dye appeared in the superficial space and the middle-third layer. Three hours after occlusion, the dye was seen in all layers of the ischemic zone. The dye appeared in the ischemic zone, where myocardium damage was not homogeneous. The positive reaction obtained by PAS staining corresponded with the capillaries, veins and superficial spaces in which the dye was evident. These results suggest that collateral circulation, venural back flow and superficial space flow are able to prevent myocardial infarction in early ischemia. (J Nippon Med Sch 1977; 64: 329–336)

Key words: myocardium, ischemia, coronary collateral development, venural back flow, ultrastructure

Introduction
The collateral vessels could provide a measure of protection against ischemia induced by coronary artery occlusion. Blungart et al. have suggested retrospectively that the loss of contractile myocardial tissue may be limited by the presence of a previously well-developed collateral network in man. The presence of collateral blood flow is associated with a lower incidence of ischemia, a smaller area of infarct size, a better left ventricular functional performance, a reduced long-term mortality and decreased frequency of sudden death in patients with coronary artery disease. Miles et al. have suggested that collateral blood flow is the most important determinant of the rate and extent of cell death within an ischemic zone. In species such as the pig, which has no intercoronary connections, coronary occlusion will lead to a zone of severe ischemia that, in the absence of reperfusion, will rapidly deteriorate to irreversible injury and
cell death. By contrast, in species such as the dog, extensive collateral connections may show cellular injury and may deliver sufficient blood to allow survival in the original ischemic zone. There are few reports concerning the study of the presence of collateral flow in ischemic myocardium by injecting dye. The purpose of the present study was to estimate the time course of development of collateral blood flow after coronary occlusion, and to examine the effect of collateral circulation on the ischemic myocardium in rats. Also, the relationship between collateral blood flow and ischemic myocardial injury is discussed.

**Materials and Methods**

Forty male rats (250-300 gm) of the Wistar strain were used. The left anterior descending coronary artery was tied under Diethyl ether and sodium pentobarbital anesthesia (2.8 mg per 100 gm), and a left thoracotomy was opened through the fourth or fifth intercostal space. The pericardium was exposed and incised. A small curved round needle carrying an artificial nonabsorbable suture was passed through the ventricular myocardium at the tip of the left anterior descending coronary artery. The thoracic wall was then closed with metal clips. During this procedure, the rate was maintained on intermittent positive pressure breathing with a Harvard respirator. Fifteen minutes to six hours later each rat was injected intraperitoneally with heparin (1000 units/kg body weight), the heart was excised, and autopsies were performed after ischemic intervals of 15, 30 minutes and 1, 2, 3, 4, 5, 6 hours. There were at least four rats in each group after coronary artery occlusion.

**Morphological analysis**

(1) **Auto-injection of tracer**

For identification of vascular flow in the ischemic and non-ischemic zones, a 0.1% silver nitrate solution mixed with 2% Chinese ink was injected before the end of the coronary occlusion period through the tail vein. The ischemic region was clearly demarcated by the absence of dye (Fig. 1). Tissue samples were taken from the center of the ischemic area in the left ventricular wall.

(2) **Light microscopy**

For the identification of myocardium damage, heart specimens for light microscopy (LM) were fixed with 10% neutral formalin and embedded, and specimens cut 2 μm thickness were stained with periodic acid Schiff's reagent (PAS). For identification of collateral flow, specimens cut 10 μm thickness were examined under the light microscope without staining.

(3) **Transmission electron microscopy**

Specimens obtained from the ischemic area were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for three hours, postfixed with 1% osmium tetroxide for two hours, dehydrated in a graded series of ethanol, and propylene oxide, and embedded in Epok 812. All of the thin sections were cut with a diamond knife on a Porter MT 5000 ultramicrotome and were doubly stained with uranyl acetate and lead citrate. The sections were examined with a Hitachi H 800 electron microscope at an accelerating voltage of 75 kv.

**Results**

1. **Light microscopic observations**

(1) **PAS staining**

The non-ischemic zone and the outer third layer of the ischemic zone at 15 minutes after coronary occlusion were positive by PAS staining. The middle-third and inner-third layers were moderately positive by PAS staining (Fig. 2A). Thirty minutes after coronary occlusion, the outer-third layer of the ischemic zone was positive, and the middle-third
and inner-third layers were moderately positive by PAS staining; however, a part of the ischemic zone was negative (Fig. 2B). One hour after coronary occlusion, a positive reaction by PAS staining was seen in the outer-third layer and a part of the middle-third layer in the ischemic zone, but the other regions of the ischemic zone were stained negative by PAS staining (Fig. 2C). Three hours after coronary occlusion, a part of the inner-third and middle-third layers also appeared positive by PAS staining (Fig. 2D). Four hours after coronary occlusion the distribution of positive reactions obtained by PAS staining was the same as that at three hours after coronary occlusion, while the negative reactions increased to more than those observed at three hours after coronary occlusion (Fig. 2E).

2) The dye distribution
Fifteen minutes after coronary occlusion, the dye was seen in the veins of the outer-third layer of the ischemic zone (Fig. 2F). Thirty minutes after coronary occlusion, the dye appeared in the capillaries of the outer-third layer (Fig. 2G). One hour after coronary occlusion, in addition to prestage, the dye appeared in the middle-third layer and the superficial space (Fig. 2H). After three hours of coronary occlusion, in addition to prestage, the dye appeared in the capillaries of the inner-third layer (Fig. 2I). Four hours after coronary occlusion, the dye was distributed in a scattered manner throughout the ischemic ventricular wall (Fig. 2J). Five and six hours after coronary occlusion, the dye distribution was the same as that observed at four hours coronary occlusion. Fig. 3A shows the distribution of the dye at three hours after coronary occlusion. The dye was seen in the superficial space (arrow head), the veins (V) and the capillaries (arrow), but not seen in the coronary artery of the ischemic zone. Fig. 3B shows the distribution of positive reactions obtained by PAS staining. A positive reaction of myocytes was seen in the region beneath the epicardium in the neighborhood of capillaries and veins that contained the dye.

2. Electron microscopic observations
When electron microscopic photographs were taken, we always compared light and electron microscopic findings in adjacent sections of each tissue block. The histological appearance of the ischemic myocardium showed that there was not a homogeneous change in the ischemic zone. Two different forms (cell edema and non-cell edema) of degeneration of the myocytes were observed (Fig. 4). So we prepared photographs showing both of these forms of degeneration by electron microscopy. Fifteen minutes after coronary occlusion, the myocytes showed mild clumping of chromatin in the nucleus, dilation of the sarcoplasmic reticulum, a mild decrease in the number of glycogen granules and a short I-band (Fig. 5A). Thirty minutes after coronary occlusion, the myocytes showed moderate clumping of chromatin in the nucleus, a moderate
Fig. 3 Light micrographs showing the positive-PAS staining and the dye in ischemic zone at three hours after coronary occlusion
A: The dye is seen in superficial space (arrow head), the veins (V) and some capillaries (arrow).
B: The myocytes by Positive-PAS staining are seen in the outer third layer or around the vein and some capillaries.

Fig. 4 Light micrographs showing the myocyte damage in ischemic zone by staining with toluidine blue. It was not homogeneous. Minimal and severe changes such as cell edema are seen.
A: At 30 minutes. B: At three hours.

decrease in the number of glycogen granules, swollen mitochondria, a short I-band and moderate interfibrillar edema (Fig. 5B). Three hours after coronary occlusion, a different form of degeneration of the myocytes was observed. The myocytes with minimal damage showed mild clumping of chromatin in the nucleus, and swollen mitochondria (Fig. 5C). The moderately damaged myocytes showed a decrease in the number of glycogen granules, a wide I-band, swollen mitochondria, and moderate interfibrillar edema (Fig. 5D). Four hours after coronary occlusion, the myocytes with mild damage showed mild clumping of chromatin in the nucleus, a moderate decrease in the number of glycogen granules, a short I-band and moderate interfibrillar edema (Fig. 6A). The severely dama-
Fig. 5 Electron micrographs showing the myocyte damage in ischemic zone
A: Fifteen minutes after coronary occlusion, dilation of sarcoplasmic reticulum (arrow) and mild decrease in glycogen granule are seen. B: Thirty minutes after coronary occlusion, swollen mitochondria, short I-band, moderate interfibrillar edema and moderate decrease in glycogen granules are seen. C: Three hours after coronary occlusion, many glycogen granules (arrow) and swollen mitochondria are seen. D: Three hours after coronary occlusion, decrease in glycogen granules, moderate interfibrillar edema and wide I-band are seen.

Discussion

The development of collateral circulation and myocardial damage in the left ventricular wall after ligation of the distal anterior descending coronary artery were investigated using light and electron microscopy. Myocardial infarction does not result in a homogeneous change in the ischemic zone during the period from 30 minutes to six hours after coronary occlusion (Table 1). The ischemic zone contains myocytes which show the two different forms of degeneration. In the present study, the collateral circulation, back flow from the vein and the flow through superficial spaces were determined after coronary occlusion (Table 2).

1. Collateral circulation development

Fifteen minutes after coronary occlusion, there was only a small amount of dye in the vein of the outer-third layer of the ischemic zone. Thirty min-

ged myocytes showed moderate clumping of chromatin, loss of glycogen granules, a wide I-band, swollen mitochondria with electron-dense deposits, and cell edema (Fig. 6B, C). Six hours after coronary occlusion, the ischemic zone contained varying amounts of reversibly and irreversibly injured myocardium. The necrotic myocytes showed clumping of chromatin in the nucleus, loss of glycogen granules and rupture of mitochondria (Fig. 6D).
Fig. 6 Electron micrographs showing the myocyte damage in ischemic zone

A: Four hours after coronary occlusion, short I-band and mild interfibrillar edema are seen. B: Four hours after coronary occlusion, swollen mitochondria have electron dense deposits (arrow). C: Four hours after coronary occlusion, loss of glycogen granules and cell edema are seen. D: Six hours after coronary occlusion, marked clumping of chromatin in nucleus, loss of glycogen granules and rupture of mitochondria are seen.

Table 1  PAS staining after coronary occlusion

<table>
<thead>
<tr>
<th></th>
<th>15 min</th>
<th>30 min</th>
<th>1 hour</th>
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<td>Outer-third layer</td>
<td>2+</td>
<td>2+</td>
<td>2+</td>
<td>2+spotty</td>
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<td>+spotty</td>
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<tr>
<td>Inner-third layer</td>
<td>+</td>
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Abbreviations: 2+: positive; +: moderate, -: negative

utes after coronary occlusion, the dye appeared in the veins and capillaries of the outer-third layer. This result suggest that collateral circulation in the ischemic zone becomes functional between 15 and 30 minutes after occlusion. One hour after occlusion, the dye was seen in the outer-third layer, and the amount of dye in the vein and within capillaries increased to more than that 30 minutes after occlusion. Three hours after coronary occlusion, the dye also appeared in the middle-third layer. Four to six hours after occlusion, the dye was found in the inner-third layer of the ischemic zone (Table 2).

There are many reports about the occurrence of collateral circulation and its distribution. Bloor and White showed experimentally in dogs that collateral circulation was of little significance under...
normal conditions and in the early stage of acute myocardial infarction without therapeutic intervention, since retrograde flow through collateral was always low during the first four days after occlusion\textsuperscript{19}. On the other hand, there is some evidence that coronary collateral circulation becomes functional very soon after coronary occlusion\textsuperscript{11}. Williams et al.\textsuperscript{12} suggested that many collaterals were more effective than fewer or no collateral in the acute stage of myocardial infarction in humans. Sanford et al.\textsuperscript{13} suggested that after coronary occlusion, there was a significant reduction in blood flow to the affected region that was most marked in the central subendocardial core of the ischemic tissue, and a slight reduction in blood flow to the epicardial marginal zone. This alteration in blood flow distribution produces ischemic necrosis of the region, necrosis that, in general, shows most severe cell damage in the inner-third layer of the myocardial wall\textsuperscript{14}. During the occlusion period there was gradual recovery of blood flow to the ischemic region. Moir and DeBra\textsuperscript{15}, using 36 Rb clearance methods, observed a disproportionate underperfusion of the endocardial region when coronary perfusion pressure or coronary blood flow was reduced. Becker et al.\textsuperscript{16}, using 15 μm radioactive microspheres, observed an endocardial epicardial (endo/epi) ratio of radioactivity of 0.76 ± 0.30 in the ischemic region after complete coronary occlusion in the canine. Opening of existing channels or a very rapid response to ischemia was related to the release of adenosine from the ischemic myocardial cells.

In the present study, firstly dye was deposited in the outer-third layer, and next in the middle-third and inner-third layers of the ischemic zone. As ischemia advanced, collateral circulation gradually increased from 15 minutes to four hours after coronary occlusion. The quantity of dye deposited was greater in the outer-third layer than in the inner-third layer. These findings indicate that collateral circulation was unevenly distributed in the ischemic zone.

### Table 2 Dye distribution after coronary occlusion

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<td>Superficial space</td>
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<tr>
<td>Outer-third layer</td>
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<td>+</td>
<td>+</td>
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<td>+</td>
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<tr>
<td>Capillary</td>
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<td>+</td>
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<tr>
<td>Vein</td>
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<td>Middle-third layer</td>
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<td>Capillary</td>
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<td>Inner-third layer</td>
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<td>Capillary</td>
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Abbreviations: –: absent, +: present.
have suggested that collateral circulation does not protect from myocardial infarction, but may be able to prevent the extension of myocardial infarction, thus reducing infarct size. Fujiwara et al. reported that thin layers immediately beneath the epicardium show minimal ischemic changes detectable by light and electron microscopy. In accordance with our results, a highly positive reaction by PAS staining was seen in the region beneath the epicardium. The dye was also seen in the superficial space at one hour after coronary occlusion. These results suggest that the presence of superficial space flow may be able to prevent ischemic damage of superficial myocardial cells.

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