Adaptive Changes in the Capillary Network in the Left Ventricle of Rat Heart

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Summary: Capillaries are nonuniform thin tubes: The arteriolar and venular capillary portions express alkaline phosphatase (AP) and dipeptidyl peptidase IV (DPPIV), respectively. Differences in enzyme activities between arteriolar and venular capillary portions could be shown by staining sections of cardiac tissues for AP and DPPIV after coronary infusion of microspheres and by staining cultured endothelial cells that had been collected from coronary microvessels. Through use of a double staining method for AP and DPPIV, adaptive changes in the capillary network were studied in rat hearts exposed to cold, exercise, hypertension, chronic coronary occlusion, and transient coronary occlusion followed by reperfusion. Two patterns could be seen in the adaptations of the ventricular capillary network. The increase in the venular capillary portions is accompanied by remarkable increases in capillary density and capillary-to-my-

ocyte ratio. The increase in the arteriolar capillary portion seemed to be accompanied by a decrease or only a limited increase in capillary density in stressed hearts. The increase in the total capillary density improves the capacity for oxygen transport to tissues with a high tissue perfusion and a short diffusion distance for oxygen. The increase in the arteriolar capillaries may also improve oxygen transport by increasing the arterial blood perfusing the tissue. This seems, however, a compensation for the limited angiogenesis: The alleviation of stresses, such as pharmacological treatment of the hypertrophied heart and reperfusion after transient ischemia, increases venular capillary portions and capillary density. These changes are discussed with immunohistochemical observations of rapid and prolonged expressions of angiogenic growth factors. [Japanese Journal of Physiology, 48, 229–241, 1998]

Key words: capillary network, left ventricular network, adaptive change, endothelial cell, differential enzyme distribution.

Capillary endothelial cells in rat hearts regenerate *in vivo* at a turnover rate of 140 to 400 d as estimated from the number of endothelial cells incorporating intraperitoneally injected ³H-thymidine [1]. On the other hand, capillary angiogenesis normally occurs only in young animals, more than half the capillaries present in cardiac tissues of adult rats being formed in the first 4 weeks after birth [2]. The question of whether cardiac capillary networks in adult rats can undergo adaptive changes in response to environmental or pathophysiological stimuli is of interest. Cardiac capillarity has been studied intensively, and many good

reviews are available on capillary angiogenesis [3–5]. However, capillaries are not merely thin uniform tubes, connecting arterioles to venules: They show local differences in function and characteristics. The oxygen tension in the blood in the arteriolar capillary portion is high, but in the venular portion it is low. On the arteriolar side, fluid passes from the blood through the intercellular junctions of endothelial cells into the interstitial space, then passes back to the blood on the venular side [6]. Although it has been shown that vascular smooth muscle cells proliferate in arterioles near their junction with capillaries [7], angiogenesis of

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capillaries occurs from the venular side [8]. Thus studies on any adaptive changes in the capillary network should recognize the differences between the arteriolar and venular portions. Since normal rat heart has minimal collateral circulation [9], this may be a useful animal model for the study of adaptive changes in capillary networks in response to various stimuli. This article reviews studies, mainly from our laboratory, on experimentally induced changes in the capillary network of left ventricular tissue in adult rat heart.

Enzymes as Markers for the Capillary Network and Their Functions

It is clearly demonstrable *in vitro* that the alkaline phosphatase (AP) present on endothelial cells functions as a phosphatase. Its physiological role *in vivo*, however, remains unclear. Adenosine is produced by the action of AP on ADP and AMP. Since adenosine is a potent vasodilator, AP might thus be involved in lowering microvascular tone [10, 11]. Adenosine is also known to reduce platelet aggregation. AP is distributed on both the luminal and abluminal membranes of endothelial cells [12], and it has been shown that ATP released from myocardial cells [13] reaches the abluminal membrane of endothelial cells and is degraded to adenosine. Another possibility is that AP may control endothelial cell proliferation: The specific substrate for AP *in vivo* is tyrosine phosphate [14], which is closely associated with growth factor receptors. Phosphorylation of the intracellular portion of the receptor stimulates cell proliferation; thus AP could probably regulate the growth factor receptors and control proliferation of endothelial cells [15] resulting in their stabilization. This hypothesis is consistent with the fact that AP is not expressed in growing tumor tissues and that in the proliferating endothelial cells its activity is reduced. Whatever its role, the widespread distribution of AP on endothelial cells of the microvasculature means it has been used as a histochemical marker for capillaries [16]. AP activity is not, however, expressed by all capillaries in normal adult rat hearts; therefore the validity of AP as the marker enzyme for capillaries has been questioned [3]. In fact, many capillaries in cross-sections of cardiac tissue show dipeptidylpeptidase IV (DPPIV) activity, but not AP activity. In normal adult rats, AP-sensitive, capillaries represent only 20 to 30% of the total number of capillaries having AP, DPPIV, or both enzymes; in human hearts, AP-reactive capillaries account for less than 10% of the total.

DPPIV, which is a serine protease widely distrib-

uted in mammalian tissues, has also been proposed as a marker enzyme for capillaries. It is one of the membrane-bound proteases that may modulate the effects of peptide hormones at the surface membrane of endothelial cells [17]. DPPIV releases peptides with proline residue in the penultimate position at the N-terminal. Regions of the brain with high DPPIV activities in the capillary endothelium contain high levels of substance P, a potential substrate for DPPIV. Circulating peptides may transmit signals to the central nervous system through conformation changes of the membrane-anchored enzyme without themselves crossing the endothelium. DPPIV may take part in controlling these processes. Other roles proposed for DPPIV include involvement in the transport of amino acids across the endothelium [17] and in collagen metabolism [18]. DPPIV has been shown to play a role in antigen-induced activation of T lymphocytes [19]. It has also been established that lung endothelial DPPIV, which is a fibronectin-binding protein [20], is an adhesion molecule for lung metastatic carcinoma cells carrying fibronectin [21]. Although the expression of DPPIV on capillaries and venules has been reported by several authors [22–24], it is difficult to be certain that the localization of DPPIV is unique to the venular side of capillaries.

Another marker for capillaries, the von Wilbrand factor (Factor VIII), which is widely used in capillarity studies in human cardiac tissues, is weakly expressed in a few capillaries in normal rat hearts. The number of endothelial cells expressing factor VIII is increased in rat cardiac tissues injured by irradiation [25].

Distribution of AP and DPPIV in Endothelial Cells

Lojda [22] showed AP to be distributed on the arteriolar capillary portion and DPPIV on the venular capillary portion in rat heart. Grim *et al.* [23], Lojda [22], Mrazkova *et al.* [24], who made comprehensive studies on species- and organ-specificities of AP and DPPIV, reported locally different expressions of AP and DPPIV in microvessels in rat, dog, and human hearts. Batra *et al.* [26] refined the histochemical staining technique as a double staining method for visualization of AP-reactive and DPPIV-reactive portions of rat heart capillaries. Assuming the AP- and DPPIV-reactive capillary portions to be arteriolar and venular capillary portions, respectively, they analyzed the distribution of arteriolar and venular capillary portions in rat hearts with hypertrophy [27] or volume overload [28] and during postnatal growth [29].

Fig. 1. Micrographs of double-stained cardiac tissue sections. **A:** A cross-section of the subendocardium of a rat heart 30 d after sham operation on the coronary artery. Capillaries with AP activity are stained blue and DPPIV red. Capillaries reactive to both enzymes are stained violet. **B:** A

cross-section of the subendocardium from a rat heart 30 d after a 3-min transient coronary occlusion. Red-stainable DPPIV-reactive capillaries are increased. Horizontal bar represents 20 μm .

Methodological Details

Frozen sections (16 μm) of cardiac tissues are placed on glass slides and encircled with Pap-pen (Daido Sangyo Co., Tokyo). A small amount of substrate solution specific for DPPIV (see below) is poured onto the sections that are kept at room temperature for 30 min. The sections are rinsed with phosphate-buffered salt solution (PBS, pH 9.2), then treated with the substrate solution specific for AP for 30 min. After being rinsed with distilled water, the sections are covered with Crystal Mount (Biomedica Corp., Foster City, CA); cover glasses are not used, since our experience indicates they cause color changes [30].

The solution for demonstrating DPPIV consists of 8 mg glycyl-L-proline-4-methoxy-beta-naphthylamine, which is a substrate for DPPIV, dissolved in 1 ml of N,N-dimethylformamide, and 10 mg fast blue B dissolved in 20 ml of 0.1 M acetate buffer (pH 7.4). The solution for AP consists of 20 mg naphthol AS-MX phosphate, which is a substrate for AP, dissolved in 1 ml N,N-dimethylformamide, and 20 mg variamine blue salt RT dissolved in 10 ml of 0.1 M Tris-HCl buffer (pH 9.2). The dimethylformamide in the solutions is responsible for the diazo-coupling of fast blue B and variamine blue with the reaction product of DPPIV and AP, respectively. This results in red staining of DPPIV-containing cells, and blue staining of cells with AP. Intermediate capillary portions expressing both AP and DPPIV are stained violet. Examples of micrographs of stained subendocardial cross-sections from rat left ventricle are shown in Fig. 1, where blue, red, and violet-stained capillaries are seen.

Fig. 2. Micrographs showing colored microspheres ($\phi=10\ \mu\text{m}$) in cardiac capillaries. First blue and then red microspheres were injected. Microspheres are retained in a blue-stained capillary portion and do not pass into the adjoining violet- and red-stainable portions. A retention of blue microspheres did not disturb the distribution of microspheres introduced by the following injection. The horizontal bar represents 25 μm .

Sections from cardiac tissues treated by the double staining method, thus show changes in coloring from blue, to violet, and to red along the capillary paths from the arteriolar side to the venous side, as illustrated previously [31].

Validation of the Double Staining Method

A direct validation of the double staining method was made by using colored microspheres and endothelial cell cultures [32]. Microspheres with a diameter of 10 μm were injected into the coronary artery of Langendorff-perfused rat hearts, which were then placed in OCT compound (Ekhard, USA) and frozen in liq-

Fig. 3. Blue- and red-stained endothelial cells collected from the coronary system during the primary culture on a plastic disc. **A:** Four days after the seeding of freshly collected endothelial cells showing elongated, bamboo leaflet-shaped blue- and red-stained cells. **B:** Proliferating endothelial cells on the seventh day of primary culture

showing a colony of one elongated and 9 polygonal blue-stained cells and 2 red-stained elongated cells. **C:** A colony of red-stained endothelial cells forming a fold (some material is excreted underneath, as seen by a perpendicular cross-section in [32]). Horizontal bars represent 100 μm .

uid nitrogen. The left ventricular wall was sectioned with a cryotome and stained as described above, without acetone fixation. Micrographs illustrating differently colored microspheres in cardiac capillaries are shown in Fig. 2. All microspheres were confined to the blue-stained, i.e., AP-reactive, capillaries. The blue-stained capillaries branched and further along the capillary path stained violet, and finally red. Thus it seems probable that AP-reactive capillaries are situated on the inflow side, i.e., the arteriolar side, and that DPPIV-reactive capillaries are on the venular side.

Endothelial cells for culture were obtained from nembutal-anesthetized laparatomized 6-week-old male rats. A thin polyethylene tube was placed in the abdominal aorta, and the vena cava was incised as an outlet for the blood and perfusate. The cardiac tissues were flushed with PBS to remove the blood; then the polyethylene tube was advanced so that its tip was situated at the origin of the aorta, and sutured. PBS containing 0.1% trypsin was then infused for 3 min. The perfusate from the right atrium was collected and centrifuged at 2,000 rpm for 7 min at 4°C. Cells in the pellet were quickly dispersed in RMPI 1640 culture solution (Gibco Inc., USA), supplemented with 10% fetal calf serum, and plated on uncoated Falcon 60 mm tissue-culture dishes. These dishes were incubated in an atmosphere of 5% CO₂:95% air at 37°C with 90% humidity. Low-density lipoprotein (LDL) uptake, which is specific to endothelial cells, was confirmed by application of acetylated LDL labeled with a fluorophore (DiI) (Biomedical Technologies Inc., Stoughton, MA, USA) to cells cultured for 10 d in the primary culture. Most cells emitted fluorescent light

after a 4 h incubation following the addition of the fluorescent LDL to the culture medium. Seeded endothelial cells slowly proliferated on the primary culture dishes, and most cells became elongated during the first stage of the primary culture.

The double staining technique was applied to cultured cells. Although blue-stainable endothelial cells elongated during the first stage of primary culture (Fig. 3A), the cells proliferating from them became short, flat, and polygonal (Fig. 3B). It seems probable that immediately after isolation and settling on the culture dishes, the endothelial cells retained their elongated shape as in microvessels. Cells newly proliferating on dishes, however, lost this shape and became polygonal, they continued to stain blue over five passages, although their stainability became gradually weak. The cells that stained red remained rather elongated after proliferation and continued to stain red on passaging five times. The red-stainable cells appeared to excrete some material from the lower side of the monolayer and became folded, suggesting that they had a stronger tendency to form structures than the blue-stainable polygonal cells did [32]. This observation may be related to the angiogenic activity that had been observed on the venular side [8].

Thus cultured endothelial cells express AP or DPPIV, or both. On the basis of the results of a microsphere test, the AP-stainable cells probably originate from arterioles or arteriolar capillary portions, and DPPIV-stainable cells from venules or venular capillary portions. (Note that arterioles and venules seen in cross-sections of cardiac tissues are easily distinguished from capillaries because they feed or collect several capillaries.) Although the functional basis

for the differential expressions of AP and DPPIV and any possible relation with oxygen tension remain unknown, it seems justifiable to assume that arteriolar, venular, or intermediate capillary portions can be distinguished by the double staining method.

Parameters for Morphometric Analysis of Capillary Network

Various morphometric parameters for different types of capillaries have come from studies [27] on cross-sections of capillaries stained differentially by the double staining method. Capillaries of the three types are intermixed and do not form separate clusters. The number of different capillary portions per unit area of the tissue cross-sections has been counted separately in the subendocardium and subepicardium; the middle layer of the ventricle contains circularly arranged capillaries. Since changes in capillarity occurring in response to stimuli are most marked in the subendocardium, capillaries are often counted in this layer to obtain the relative density of the different capillary portions, expressed as the number of respective capillary portions per unit area or as a percentage of the total number of portions. The capillary density, however, depends on the myocyte area and provides no information on capillary proliferation. A better index of capillarization is the number of capillaries assigned to one myocyte (number of capillaries divided by the number of myocytes in a unit area, C:M ratio) [3]. This ratio may still fail to give a true measure of capillarity because the fate of capillaries remains unknown when myocytes disappear by apoptosis.

The area to which one capillary provides oxygen is called the capillary domain area (CDA) [33]. The CDA may seem to be equivalent to the reciprocal of capillary density. However, it is more informative if the individual tissue area supplied by a particular type of capillary portion is measured separately. Since arteriolar and venular capillary portions are intermixed, the CDAs must be measured by a somewhat elaborate procedure. Microscopic images of cross-sections showing the capillaries are transmitted to a personal computer via an image scanner, and localizations of the capillary portions are saved in the memory. The distances from capillary to capillary are calculated. The midpoints of these distances are joined by straight lines to form polygons whose areas are calculated separately, by an appropriate software program, for different capillary portions. A large value for the CDA indicates that the area supplied by that capillary is large and the diffusion distance for oxygen is long. Smaller CDAs are found for the venular type of capillary than

for the arteriolar type under various conditions. This must mean more branching exists in the venular than in the arteriolar capillary portions. If CDAs were similar for both capillary portions, the arteriolar capillary portion would be more efficient than the venular one in supplying oxygen because of the higher oxygen tension in the arteriolar portion. In cardiac tissues, however, CDAs for the arteriolar capillary portions are larger than for the venular ones, suggesting that oxygen may be supplied to cardiac tissues with a similar efficiency in both portions.

This advantageous morphological feature of the capillary network is further strengthened by erythrocyte distribution. In quickly frozen rat heart, more erythrocytes were found in the venular capillary portion than in the arteriolar capillary portion [34].

In experiments by Batra and Rakusan [27], longitudinal sections were made by cutting the myocardial wall parallel to the long axis of the left ventricle. Capillaries were traced from feeding arterioles to collecting venules to measure various parameters. These included (1) mean capillary path length, which represents the mean lengths of all capillary pathways connected to the same feeding arteriole and collecting venule; (2) capillary set length, which is the total length of all capillaries fed by one arteriole; (3) capillary portion length, i.e., the length of blue-stained arteriolar, violet-stained intermediate, or red-stained venular capillary portions (it is probable that the arterIALIZATION of capillaries produces an elongation of the arteriolar capillary portion and a shortening of the venular capillary portion); (4) segment length, which is the distance between two ensuing branch points on a given capillary portion (if branching increases in a given capillary portion, the interval of capillary branching decreases); (5) segment numbers, per portion, which indicates the frequency of branching in the capillary portion; (6) segment numbers per path length; (7) segment number per set, which represents the number of segments of all capillaries fed by one arteriole.

Cold

Nonshivering thermogenesis enables animals exposed to low temperatures to maintain the homeostasis of body temperature and physiological functions. Blood flow is increased in liver, kidney, brown adipose tissues, and thyroid gland [35]. To support the increase in blood flow in various organs, there are increases in heart weight, cardiac output, cardiac tissue blood flow [35], and oxygen consumption of the heart [36]. An increase in capillary density in the ventricular walls may be expected to underlie such adaptations, but in-

creased capillarity has not been reported in cardiac tissues of cold-acclimated rats. Heroux and Pierre [37] wrote, "It is surprising to find that cold acclimation did not change the number of opened capillaries in the heart." Their experiments, however, were made on adult rats exposed to 6°C for 5 weeks, and they mentioned that capillaries in cardiac tissues in young rats can grow as an adaptive response to various stimuli. Young rats, however, easily die when exposed to low temperatures. To surmount this problem, a study [38] was made using young adult rats (15.9 weeks old) from stock reared at 5°C for 68 generations [39–41]. Newborn rats were to some extent protected from the low temperature in the cold room (5°C) by their mothers, but were directly exposed to it after weaning.

Some offspring from the original stock had been returned to a temperature of 25°C at the 12th generation, and their offspring were reared under the warm temperature thereafter. These rats were designated as nonacclimated control rats.

In cold-reared rats, as compared with control rats, the heart and cardiac cells were hypertrophied, total capillary density had increased, and the capillary domain areas were reduced along the capillary path from the arteriolar to the venular capillary portions. The number of the venular capillary portions did not change significantly, but the arteriolar and intermediate capillary portions showed a significant increase. These changes suggest that the cardiac capillary network was better developed in the cold-reared rats than in the control rats. In the cold-adapted rats, the hypertrophic changes in cardiac cells are probably accompanied by improvements in the capacity for oxygen delivery [38]. Similar changes were observed in some skeletal muscles from rats reared at the low temperature [42]. Small improvements in capillarity have also been reported in oxidative skeletal muscles after a 4-week exposure to low temperature [43].

Exercise

The effects of exercise on cardiac capillaries have been intensively studied. However, controversial results have been reported [4, 44]. It seems probable that capillaries proliferate only when exercise training started when animals are young [3–5]. In our laboratory, exercise training of weaned 3-week-old male Wistar rats was started on a horizontally rotating rat treadmill [29]. The speed of the treadmill was gradually increased to 25 m/min over 4 weeks; then the treadmill was tilted 7%. This exercise load was maintained during the final 2 weeks. The training took place for 60 min/d, 6 d/week. Control rats were put on the treadmill every training day, but the treadmill was

not switched on. Body weight was lower in the exercise group than in the sedentary control group. The ratio of the left ventricular weight to the body weight was increased by the exercise. The proportions of the three capillary types were in the order of venular>intermediate>arteriolar in the control group, and in the order of arteriolar>intermediate>venular in the exercise group. The total capillary density was increased by exercise training, but the capillary domain areas showed significant decreases in the exercise group compared with those for the respective capillary portions in the control group. The exercise training caused clear effects on the capillary geometry in the longitudinal sections of left ventricular walls in support of the results from the cross-sections. Segment lengths decreased in all three capillary portions, suggesting increases in capillary branching, and the segment numbers per portion increased in the arteriolar capillary portion, but decreased in the venular capillary portion. Arteriolar capillary portion length increased and the venular capillary portion length decreased, suggesting a trend toward arterialization of the capillary network. The capillary path length showed a small decrease in the exercise group. Similarly, increases in total capillary and arteriolar capillary portion were observed in soleus muscle from exercise-trained rats [45].

An expression of angiogenically active growth factors, bFGF and VEGF, could be seen immunohistochemically in heart tissues [46]. The capillary arterialization in the heart may be related to bFGF expression, since this growth factor stimulates proliferation of vascular smooth muscle cells [47]. bFGF is mainly induced by the disruption of cell membranes. The exercise training may possibly cause small and reversible disruption of cardiomyocytes. Whatever their cause, these changes in the capillary network improve the capacity for oxygen delivery to cardiac tissues in young rats.

In contrast, exercise training of adult rats (started at 18 months of age), failed to cause any significant advantageous changes in the capillary network, although the C:M ratio increased moderately, compared with sedentary adult rats. CDAs increased because of the hypertrophy of cardiomyocytes (to be published) caused a lengthening of the distance for the oxygen diffusion. The mechanism for the hypertrophy in exercised adult rats remains unknown. The involvement of angiotensin II, which is known to cause hypertrophic changes in myocardial cells [48], may, however, be mentioned as a possible cause, since plasma angiotensin II is increased by exercise [49]. Its relation to age should be studied in the future.

Hypertension and Cardiac Hypertrophy

The effects of hypertension on the capillary network in cardiac tissues can be clearly seen in spontaneously hypertensive rats (SHR). The capillary density, which is not especially low in young SHR, decreases with age because of the rapid growth of the cardiomyocytes. The angiogenesis of capillaries probably fails to keep pace with the hypertrophy of cardiomyocytes. The arterial blood pressure attains a steady state in 7 months in SHR, and the capillary density gradually increases, eventually reaching values similar to those in normotensive rat. The relationship between capillary density and age gives a U-shaped curve with a minimal value at the age of 7 months [50]. The high cardiac weight, low capillary density, and large CDAs in growing SHR were partially attenuated by treatment with nifedipine, but segment lengths were greater than in the nontreated SHR [51].

The stroke-prone strain of spontaneously hypertensive rats (SHRSP) shows changes that are more marked than in SHRs. Since the blood pressure steadily increases, most SHRSP die within 40 weeks from a stroke. In this strain, the myocyte cross-sectional area continues to increase throughout the life span, and a low capillary density is observed at the age of 30 weeks. CDAs are accordingly significantly larger than those in age-matched normotensive rats, as is the proportion of arteriolar capillary portions. Even at 40 weeks, the C:M ratio is increased despite the decrease in capillary density, suggesting that capillary angiogenesis is still occurring, but is outstripped by cardiac hypertrophy [52].

Neonatal hyperthyroidism is known to produce cardiac hypertrophy. In 2-d-old rats treated for 12 or 28 d with triiodothyronine, arteriolar and capillary densities were similar to those of control rats, but the total lengths were greater, suggesting a concomitant proliferation of capillaries [53]. Immunocytochemistry confirmed in hyperthyroidic cardiac tissues that proliferating cell nuclear antigen (PCNA) was present in endothelial cells, but not in hypertrophic cardiomyocytes [54].

Cardiac hypertrophy without hypertension and afterload can be induced by the administration of clenbuterol, an adrenergic beta receptor agonist originally designed as an antiasthma agent [55]. In our laboratory, 10- or 37-week-old Wistar rats were fed with a powdered diet containing 2 mg clenbuterol/kg. The average clenbuterol intake estimated from food intake was 169 $\mu\text{g/kg/d}$ in one rat group and 102 $\mu\text{g/kg/d}$ in the other [56]. Significant hypertrophy of cardiomyocytes and also of skeletal muscles was observed

within 10 d. Capillary density and number of venular capillary portions decreased, accompanied by increased CDAs in cardiac tissues and skeletal muscles. It seems probable that the rate of capillary angiogenesis failed to keep pace with the rate of hypertrophy [56].

Involvement of Angiotensin

Experiments in this laboratory have examined the involvement of angiotensin in the decrease in ventricular capillarity in hypertension. The effect of the angiotensin-converting enzyme inhibitor (ACEI) was studied in experimental hypertension produced by constriction of the renal artery. The right renal artery was partially constricted by a silver clip in 4-week-old Wistar rats. Arterial systolic blood pressure was elevated within 1 month and was accompanied by significant cardiomyocyte hypertrophy. The capillary density was notably decreased, although the C:M ratio was notably increased. The CDAs of the arteriolar, intermediate, and venular capillary portions were all increased. The ACEI derapril was administered to experimental rats for 1 month after confirmation of the development of the hypertension; it was dissolved at a concentration of 500 mg/l in the drinking water, which was available ad libitum (average dose was about 100 mg/kg/d). Within 1 month significant differences were observed between the derapril-treated group and the control group, which had been subjected only to constriction of the renal artery. In the treated group, the myocyte cross-sectional areas were smaller, and an increase was seen in capillary density and venular capillary portions with a significant decrease in arteriolar capillary portions [57].

In further experiments in this series, two other ACEIs, temocapril and cilazapril, were administered as above to SHRSP. In the drug-treated group, compared with untreated controls, the capillary density and venular capillary portion were significantly increased with a decrease in CDAs of all three capillary portions (in preparation).

ACEIs suppress both the synthesis of the vasoconstrictor angiotensin II and the degradation of the vasodilator bradykinin. To elucidate which pathway is more important in the beneficial effects of ACEIs on the ventricular capillarity, the actions of the angiotensin II receptor AT1 antagonist, TCV116 [58], were also studied in SHRSP [59]. TCV 116 dissolved in ethyl alcohol was dispersed in water and sonicated. This solution was added to powdered rat chow, which was then kneaded, formed into short rods, and dried. After 1 month on the chow containing TCV116 (the

average dose, based on daily food intake, was calculated to be 0.96 mg/kg/d), the systolic blood pressure, left ventricular weight, and cross-sectional myocyte area were significantly smaller than in the untreated group. Total capillary density and venular capillary portions were increased by more than 20% (in preparation). These results suggest at least a partial involvement of angiotensin II in the reduction in capillarity following renal constriction and in SHRSP.

Because left ventricular hypertrophy is often considered to be secondary to hypertension, the effect of lowering blood pressure by the administration of hydralazine to SHRSP has also been investigated. Hydralazine dissolved in the drinking water (14.0 mg/kg/d) significantly lowered systolic blood pressure to the level found in the cilazapril-treated SHRSP. A continuous intake of hydralazine failed, however, to reduce heart weight even after 1 month's treatment. The low capillary density and the increase in arteriolar capillary portions characteristic of SHRSP were also unaffected by hydralazine. Thus the recovery in capillarity in cardiac tissues appears to be more related to the decrease in hypertrophy than in blood pressure.

Chronic Coronary Artery Occlusion

Cardiomyocytes become hypertrophic following coronary artery occlusion; those in the transition zone immediately outside the ischemic zone undergo especially marked hypertrophic changes. They are still alive, but in danger of becoming ischemic [60–62]. To elucidate the changes in the capillary network resulting from coronary occlusion, the effects of chronic coronary artery occlusion on the capillary network of left ventricular tissue were studied in rat hearts; Sham-operated rats served as control [63]. A polyethylene tube was inserted into trachea of anesthetized rats under transcutaneous illumination. The heart was exposed through a left-sided thoracotomy in ethyl ether-anesthetized and artificially ventilated rats. The left coronary artery was occluded with a thin suture and the thorax closed. Thirty days after surgery, the heart was removed and cross-sections of the left ventricle were analyzed morphometrically. The cross-sectional areas of cardiomyocytes in the nonischemic zone were greatly increased with an even more pronounced hypertrophy in the transition zone. The hypertrophy of cardiomyocytes is closely related to the increased ACE activity in the coronary occluded cardiac tissue following coronary occlusion [64].

In the nonischemic zone, total capillary density and the C:M ratio increased, and CDAs in all capillary portions decreased, compared with values in the sham-operated group. Angiogenesis probably oc-

curred in the ventricular tissues subjected to the coronary artery occlusion in response to the raised oxygen demand of surviving cardiomyocytes. Concomitantly, the proportion of arteriolar capillary portions increased in this order: transition zone > nonischemic zone > sham-operated tissue [63]. These changes were more marked in the subendocardium than in the subepicardium. Ventricular tissues showed a widespread and strong immunohistochemical reaction for basic fibroblast growth factor protein (bFGF). This protein is said to stimulate proliferation of endothelial cells and vascular smooth muscle cells. The appearance of bFGF may be time-dependent: bFGF could not be found at 30 min after surgery, but diffuse staining was clearly seen at 24 h. Clear punctate staining for bFGF was observed on capillaries 30 d after surgery. Staining for vascular endothelial growth factor (VEGF) protein was seen 24 h after coronary occlusion (unpublished data). Thus stimuli for capillary proliferation and arterialization must persist for a long time after coronary occlusion [63].

The effects of coronary occlusion on local capillarization have also been studied by Saladek *et al.* [65], who administered losartan, an angiotensin II receptor antagonist. In comparison with nontreated coronary-occluded rats, the decrease in capillary density in the transition zone was less pronounced after losartan treatment and the C:M ratio was increased. This result suggests that angiotensin II may be involved in the effects of coronary occlusion and that treatment with an antagonist may aid the formation of new capillaries in the transition zone.

Pharmacologically Induced Transient Ischemia

Intravenous injection of vasopressin causes transient ECG changes similar to those in patients suffering from angina pectoris. A single bolus injection of vasopressin causes vasoconstriction in coronary microvessels with a diameter of less than 100 μ m [66]; thus vasopressin administration can be used as a model of coronary vasospasm [67]. Changes in capillary architecture have been studied in the left ventricular wall 1 month after the transient ischemia [68]. Donryu rats were injected via the tail vein, once daily for 4 d, with saline containing vasopressin at a dose of 0.0, 0.5, or 1.0 U/kg. The electrocardiogram showed transient changes characteristic of ischemia after the end of the each vasopressin injection, but they returned to the initial pattern within 10 min. Rats were sacrificed 30 d after the last injection. Body weight, left ventricular weight, and the cross-sectional area of cardiomyocytes showed no significant differences between experimen-

tal and saline-injected rats. The total capillary density and C : M ratio were increased in vasopressin groups, compared with the saline control group. The number of venular and arteriolar capillary portions significantly increased and the CDA values significantly decreased in the three capillary portions. SDlog, an index of the uneven distribution of capillaries, tended to decrease in all capillary portions at both vasopressin-dose levels. In ventricular tissues obtained 1 d after the last injection of vasopressin, widespread and strong immunohistochemical staining for bFGF was obtained in almost all capillaries. The same technique showed bFGF to be present in many capillaries 30 d after final injection. An application of the double staining to the adjacent section suggested that bFGF was present on both venular and arteriolar capillary portions.

The increase in capillary density and C : M ratio together with the arterialization would improve oxygen transport to cardiac tissues and thus meet the transiently increased oxygen demand produced by vasopressin injection. This result seems to be consistent with the statement that the "blood vascular system adapts its structure to meet the maximal oxygen needs of the tissues met rather than the average oxygen needs" [69].

Transient Coronary Occlusion

The effects of transient coronary occlusion on ventricular vascularization have been studied intensively with respect to preconditioning and the formation of collaterals that occurs within a few minutes after the start of reperfusion [70, 71]. In contrast, few studies examined the long-term effect of transient coronary occlusion on the capillary network in the ventricular wall. Besides the experiments described above on the pharmacological induction of transient ischemia, the effects on the capillary network of mechanical occlusion and release were investigated by using a suture [72]. Three groups of rats were subjected to one of the following treatments under artificial ventilation described above: 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 reperfusion, then with reperfusion continuing for 30 d (I-R 3); and a sham operation without ischemia. An additional group of six rats was subjected to I-R 1 and sacrificed at 30, 60, or 120 min after the start of reperfusion for the immunohistochemical localization of bFGF and VEGF proteins.

A curved needle threaded with fine silk was passed through the ventricular myocardium 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) [73]. 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 the ligature was then released. In the I-R 3 group, the artery was subjected to three 3-min periods of ligation with 3 min of reperfusion between each, and the ligature was removed after the third occlusion. The occlusion produced a well-demarcated swollen cyanotic area of acute ischemia corresponding to the distribution of the left coronary artery distal to the ligation. In the sham-operated group, the thread was looped round the coronary artery, but not tightened. After the treatments, the left lung was inflated, the chest closed, and the pneumothorax evacuated. Thirty days later, the hearts were removed, placed in O.C.T. compound, and frozen with liquid nitrogen.

Neither the cross-sectional areas of cardiomyocytes nor myocyte density showed significant difference between groups. In contrast, C : M ratio, the total capillary density, and density of the venular portions increased in both I-R groups, as seen in Fig. 1, compared with those of the sham-operated group, suggesting a proliferation of capillaries following transient coronary ischemia. These results seem to be consistent with the increased capillary density of the left ventricular wall in vascular spastic angina patients [74]. The CDAs of all three capillary portions significantly decreased in the I-R groups. The C : M ratio of the I-R 1 group was significantly higher than that of the I-R 3 group. The decrease in CDAs in the I-R 1 group was greater than in the I-R 3 group. Again the changes in the total capillary density and the venular capillary portion in the I-R 1 group were significantly greater than those in the I-R 3 group. It appears that increased ischemic episodes may reduce the angiogenic activity of cardiac tissues.

In sham-operated rats, only limited faint staining for bFGF was seen in the interstitial spaces in cardiac tissues without staining for VEGF. Rats killed immediately after a 30-min reperfusion following a 3-min coronary occlusion showed no clear reaction for bFGF. In contrast, after 120 min reperfusion there was clear staining for bFGF mainly within the cytoplasm of myocytes and also around scattered capillaries; VEGF was also visible on and around capillaries and other microvessels. In the rats examined 30 d after the occlusion, some capillaries were found clearly stained for bFGF or for VEGF.

Growth Factors

Stainings for bFGF protein in cardiac sections have

thus been observed in rats exposed to different kinds of external stresses that probably evoked capillary angiogenesis or capillary arterialization. Four types of bFGF proteins differing in molecular sizes have been reported. The smallest one is slowly released through intact cell membranes, but has no secretory sequences. Larger molecules are said to be released mainly through damaged cell membranes [75, 76]. Accordingly, the stresses of exercise and transient ischemia may have caused small and reversible disruption of cell membranes. Since bFGF also stimulates proliferation of smooth muscle cells, it seems likely that the clear expression of bFGF is related to the increase in arterioles and in capillary arterialization in the stress model. Consistent with this idea is the finding that in dogs whose coronary arteries had been occluded, the intravenous injection of bFGF produced significant increases in arterioles and capillaries in the left ventricular border zone of ischemia [77].

VEGF is reported to be rapidly induced by hypoxia *in vitro*, and it is a candidate for stimulating the proliferation of capillary endothelial cells and angiogenesis during hypoxia [78, 79]. VEGF mRNA is induced in 15 min after transient ischemia in isolated perfused rat hearts [80], and protein synthesis proceeds quickly once mRNA is induced [81]. The rapid syntheses of angiogenic growth factor proteins after transient ischemia are advantageous for the maintenance of the ventricular capillary network. The reason why these growth factors could still be stained 30 d after transient ischemic assault remains for future study.

Conclusion

Adaptative changes in the cardiac capillary network readily occur in young rats, for example, after low-temperature exposure and exercise training. These changes can also take place in adult rats in response to various conditions. Two patterns can be seen in these adaptations. The increase in the venular capillary portions is accompanied by a remarkable increase in capillary density, and the increase in the arteriolar capillary portion seems to be accompanied by a decrease, or only a limited increase, in capillary density. The increase in the total capillary density improves the capacity for oxygen transport to tissues with a high tissue perfusion. Furthermore, the increase in the arteriolar capillaries based on the increase in arterioles improves the oxygen transport capacity by increasing the amount of arterial blood perfusing the tissue. Both types of change in capillarity thus contribute to an increase in oxygen transport to tissues.

Stresses such as low temperature, exercise, and coronary occlusion increase the AP-expressing arteri-

olar capillary portions. Arterialization proceeds from arterioles toward the capillaries, a process stimulated by the stress-related humoral factor, angiotensin II. On the other hand, the alleviation of stress, such as pharmacological treatment of the hypertrophied heart and reperfusion after transient ischemia, induce DPPIV-expressing capillaries resulting in an increase in capillary density. However, the functional significance of different localizations of AP and DPPIV is unknown, and so is their relation to growth factors. Moreover, the question whether the expression of AP and DPPIV is reversibly changeable in endothelial cells of existing capillaries and in cultured cells remains for future study.

Although biological functions for AP and DPPIV have been suggested on the basis of *in vitro* studies, they have not been confirmed in *in vivo* experiments. Studies using AP-knockout animals might contribute to the understanding of the control and remodeling of capillary network in cardiac tissues. Furthermore, the effects of apoptosis on capillarity and the fate of capillaries surrounding myocytes that have undergone apoptosis should be studied. Thus the true nature of adaptive changes can be elucidated only by wide biological studies covering morphometry, enzyme syntheses, and stimulation of oncogenes.

The rapid and long persistent expression of bFGF and VEGF after brief ischemia suggests a biological response that ensures the maintenance of the oxygen supply to cardiac tissues: the mechanism for such a prolonged effect should be studied in the future. Similarly, the time course of the increase in capillaries produced by transient coronary ischemia in adult rats and their eventual long-term fate are important study questions.

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