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Process of Coronary Capillary Tube Formation by Mononuclear Cells Expressing β-Actin in the Ischemic Myocardium

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Abstract

Background: Capillary tube formation is essential for angiogenesis. It was previously thought that capillary tubes are formed through self-division of pre-existing endothelial cells (ECs) or folding of EC-progenitor cells. We found that circulating mononuclear cells that express β-actin (β-MNCs), participate in arteriogenesis, (formation of arterioles and arteries, and their growth) but it remained obscure whether and how they participate in capillary tube formation.

Methods: We induced acute myocardial infarction in beagles, and examined the process of capillary tube formation by β-MNCs in the infarcted myocardium.

Results: β-MNCs were recruited to coronary vessels, in particular to capillaries, in the border zone of infarcted myocardium. β-MNC nuclei were either round or flat, indicating existence of several species of β-MNCs. They sprouted out from a pre-existing coronary arteriole, capillary or venule into the interstitial space, and adhered to each other in tandem. An intracellular cavity was formed by the loss of granular cytoplasm in each cell. The intercellular junctions which connected cell-to-cell disappeared, and the cavities connected with each other to form a capillary tube. New β-MNCs moved to the distal end of the tube and migrated outside to extend the capillary tube. ECs of the capillary tubes thus formed possessed CD_{31}, a marker for EC, but not β-actin. Their nuclei were either round or flat, indicating existence of two different capillary tubes.

Conclusions: Coronary capillary tube formation in the ischemic myocardium is initiated by β-MNCs, not by their folding but by intracellular cavity formation through the loss of their granular cytoplasm and consequent tube formation by cavity-to-cavity connection.

Keywords: β-MNCs; Capillary tube; Intracellular cavity formation; Intracellular granules; Myocardial infarction

Introduction

Capillary tube formation is an essential process of angiogenesis. It was previously thought that differentiated endothelial cells (ECs) form capillary tubes by self-division or folding [1-5] (Figure 1).

Although a number of *in vitro* studies have been performed to identify substances that accelerate or inhibit capillary tube formation [6-12], there has been a dearth of *in vivo* investigations of the mechanisms of capillary tube formation in ischemic tissues. It therefore remains to be elucidated whether changing shape by ECs initiates capillary tube formation, as seen *in vitro*, and whether and in what way precursor cells participate in capillary tube formation, especially in ischemic tissues *in vivo*.

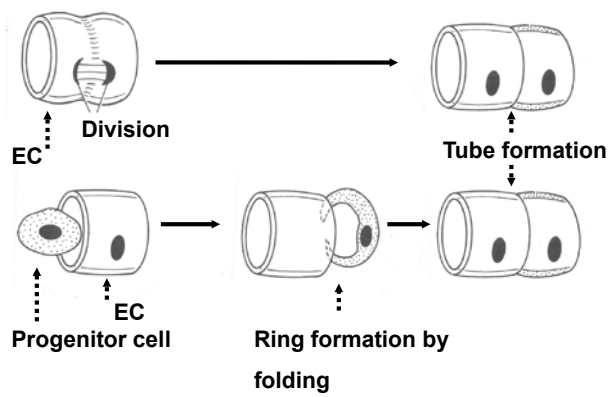

Figure 1: Schematic representation of previously proposed process of capillary tube formation. A: Self-division of a pre-existing endothelial cell (EC). B: Changing configuration of a progenitor cell by folding.

Angiogenesis is known to occur [13], and capillary tube formation has been confirmed, in the ischemic myocardium [14]. The exact mechanisms of coronary capillary tube formation in the ischemic myocardium are, however, not well understood.

In studies on therapeutic coronary angiogenesis in the ischemic myocardium [13,15-18], we found mononuclear cells (β-MNCs),

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which possess β-actin, a marker of the synthetic phenotype of vascular smooth muscle cells (SMCs) [19], but not α-smooth muscle actin (α-SMA), a marker of the contractile phenotype of VSMCs and myofibriblasts [20,21]. These β-MNCs were present not only in the coronary vessels, but also in the interstitial spaces of the ischemic myocardium. We found that β-MNCs participate in arteriogenesis (formation of arterioles and arteries, and their growth) [22]. Their role in angiogenesis (capillary formation) and how they form coronary capillary tube however remained to be elucidated.

Accordingly, we conducted this present study to examine whether and by what mechanisms β-MNCs form coronary capillary tubes in the ischemic myocardium *in vivo*.

Methods

Myocardial infarction model

We conducted animal experiments at the Jikei University School of Medicine Institute for Animal Experiments. The experiment protocol was approved by the University Administrative Panel on Laboratory Animal Care.

For the experiment, 28 beagles were anesthetized with pentobarbital sodium (30 mg/kg, i.v.). An 8-F catheter was introduced via the right common carotid artery into the left coronary ostium. After confirmation of the coronary anatomy by angiography, a self-expandable polymer ball (2x2 mm) was injected into the left anterior descending artery in order to occlude it [17,18]. After recovery from anesthesia, the animals were cared according to the University guideline.

After repeated coronary angiography and left ventriculography, the animals were sacrificed using intravenous pentobarbital sodium (100 mg/kg) and potassium chloride (10 mg/kg) each 7 animals at 1, 2, 4 and 8 weeks later. The heart was excised, and the border zones of the infarcted anterior wall of the left ventricle were excised and were stored in 20% formaldehyde solution. In other 6 control beagles in which myocardial infarction was not produced, the anterior wall was excised and was stored similarly.

Histology

The excised myocardium was sliced into successive 2.5μm-thick slices. By this procedure, a cell could be separated into 2 slices and therefore 2 different cell markers could be stained in the same call.

Immunohistolochemical staining: The excised border zone of the infarcted myocardium was cut into successive 2.5 μm-thick slices, and the slices were stained by immunohistochemical staining for various cell markers; β-actin (monoclonal anti-β-actin clone AC-15, mouse ascites fluid, Sigma Co, St Louis, USA) which had been considered to be a marker of synthetic phenotype of vascular smooth muscle cells [19], CD_{34} (rabbit monoclonal anti-human CD_{34} antibody, clone EP 373Y, Epitomics Inc., Burlingame, CA, USA) for bone marrow mesenchymal cells [20], α-smooth muscle actin (α-SMA; mouse anti-human smooth muscle action, clone/Klon 1A4, Code No M0851, Dako Epos, Glostrup, Denmark) and vimentin (rabbit anti-human vimentin antibody, clone Vim 3B4, Code No U7034, Dako Epos Sigma Co) for contractile phenotype of vascular smooth muscle cells or myofibroblasts [20,21,23], and CD_{31} (rabbit monoclonal anti-human CD_{31} antibody/PECAM-1, clone EP 3093, Abgent, SanDiego, CA, USA) for ECs [20,24]. Two adjacent slices were stained for different markers to clarify whether the same cell possessed more than one marker; one slice for β-actin staining and the anther adjacent slice for staining of other marker.

Counting of capillary tubes: The number of capillary tubes having β-MNCs/250x250 μm^2 of border zone, i.e., capillary tube-density was counted and was compared between control 1, 2, 4 and 8 weeks after infarction formation. Randomly selected 5 border zones of each beagle were used for counting.

Capillary tubes were classified into 1) those constituted by β-MNCs filled with granular cytoplasm and without cavity formation, i.e., immature capillary tube; 2) those constituted by β-MNCs with cavity and decreased cytoplasm and without loss or partial loss of intercellular junctions, i.e., maturing capillary tube; 3) and those constituted by β-MNCs with complete loss of granular cytoplasm and remnant of junctions, forming a tube, i.e., matured capillary tube.

Existence of remnant junction was considered as a marker of newly formed capillary tube and the remnant was used to discriminate them from the pre-existing capillary.

Statistical analyses

Data were expressed as mean ± SD and were tested by Student's "t" test. A $p<0.05$ was considered significant.

Results

Circulating β-MNCs in the coronary lumen

β-MNCs were abundant in coronary arterioles, capillaries and venules. They contained CD_{34}, indicating a bone marrow origin. β-MNCs however did not contain α-SMA, CD_{31}, and vimentin as shown in our previous study [22].

β-MNC nuclei were round or flat in configuration, suggesting existence of several different cell species of β-MNCs (Figure 2).

Process of capillary tube formation by β-MNCs

At ≥1 week after myocardial infarction, migration of β-MNCs, amebic in configuration, was observed across the wall of arterioles, capillaries and venules into the interstitial space (Figures 3A, 3B). Crowding of β-MNCs into a confined potion of the external surface of these vessels was observed, suggesting it was the site of sprouting (Figures 3C, 3D).

At 1-2 weeks, β-MNCs adhered to each other in tandem fashion and extended from arterioles or venules into the interstitial space.

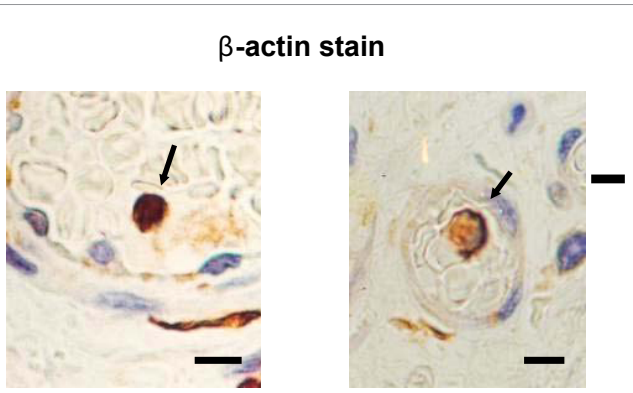

Figure 2: β–Actin-positive mononuclear cells (β-MNCs) circulating in the coronary vessels at one week after infarction production A: A β-MNC (arrow) with a round nucleus. B: A β-MNC (arrow) with a flat nucleus. Bars: 10 μm in this and following figures.

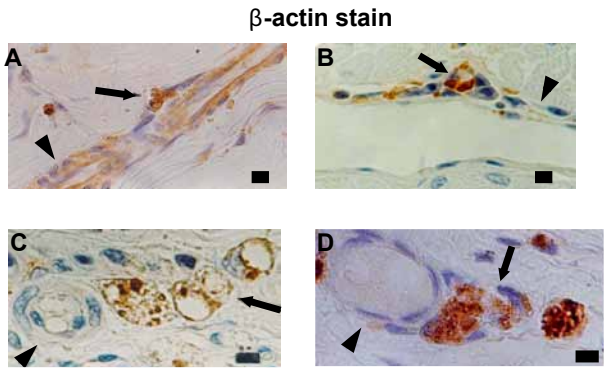

Figure 3: β-MNCs sprouted out from arterioles and venules and adherent to each other in tandem at one week after infarction production A: β-MNCs gathered in a portion of an arteriole (arrow), suggesting this area was the site of sprouting. Arrowhead: pre-existing arteriole. B: β-MNCs gathered in a portion of a pre-existing venule (arrow). Arrowhead: pre-existing venule. C: β-MNCs arising from a pre-existing arteriole (arrowhead) and adherent to each other in tandem (arrow). D: β-MNCs arising from a pre-existing venule (arrowhead) and adherent to each other in tandem (arrow).

These β-MNCs were filled with β-actin-positive granular cytoplasm but had no intracellular cavities, indicating that they were immature capillary tubes (Figure 4A, 4B). These immature capillary tubes were observed most frequently at 2 weeks (Table 1).

At 2-4 weeks, capillary tubes constituted by β-MNCs with decreased granular cytoplasm, intracellular cavities and intact intercellular junctions, i.e., maturing capillary tubes were observed. Red blood cell drained into the segment of the tubes where intercellular junctions disappeared (Figure 4B, 4C).

Capillary tubes formed as a consequence of complete loss of granular cytoplasm and remnant of intercellular junctions, linking up of cavities, and drainage of red blood cells into the lumen, i.e., matured capillary tubes, were most frequently observed at 8 weeks (Table 1).

Various stages of intracellular granular cytoplasm loss and cavity formation were observed throughout the 8 weeks of observation (Figure 5). β-SMA positive granular cytoplasm loss and consequent capillary tube formation was considered to occur in the order of B to F in Figure 5.

Appearance of CD31 in granular cytoplasm and cellular membrane

The β-MNCs circulating in the coronary vessels did not possess CD_{31}, a marker for ECs, but CD_{31} appeared in the cytoplasm after β-MNCs were arranged in tandem (Figure 6A, 6B)

When capillary tube formation was completed, granular cytoplasm positive for β-actin and CD_{31} were no longer observed in the lumen, whereas CD_{31} but not β-actin appeared in the cellular membrane (Figure 6A-1, A-2, B-1, B-2).

Endothelial cells with either round or flat nuclei

Capillary tubes were constituted by ECs with either round or flat nuclei. These nuclei resembled those of matured arterial and venous ECs, respectively (Figure 6A-1, A-2, B-1, B-2). It remained unclear whether the differences in nuclear configuration of ECs were predetermined by the differences in nuclear configuration of β-MNCs.

Discussion

The aortic lumen develops extracellularly between adjacent ECs. ECs adhere to each other in circle, and change in shape and form a lumen [25]. In contrast, it was previously thought that capillary tubes are formed by folding of precursor cells, forming a ring-like configuration, or by self-division of the pre-existing ECs.

Folkman and Haudenshild conducted an *in vitro* experiment with cultured ECs, finding that a capillary tube began as a longitudinal vacuole in one cell, and appeared to be extruded and connect from one cell to the next [26]. Meyer, et al. reported that in vitro experiment using cultured ECs, excretion of intracellular vacuoles and cell death causes expansion of the intercellular space into a lumen [27]. Neither study, however, was able to elucidate the mechanisms of vacuole formation. In addition, because they studied capillary tube formation using cultured ECs in vitro, they could not examine the participation of EC-precursors/progenitors in the genesis of capillary tubes.

In a previous study using the same infarction model in beagles, we found that β-MNCs migrate into the interstitial space and then from

Time after induction of myocardial infarction (weeks)	Control	1	2	4	8
A. Immature capillary tubes	0.3±0.5	1.9±0.9**†††	4.3±1.0****†††	3.7±0.8****	1.3±0.8
B. Maturing and matured capillary tubes	0.1±0.4	0.4±0.5	2.0±1.0**	3.7±0.8****	7.3±1.4****††
C. Pre-existing capillaries	2.9±0.7	3.2±0.6	3.4±1.0	3.8±1.1	4.1±1.1

Control: beagles without myocardial infarction. * p<0.05, ** p<0.01, *** p<0.001 vs Control. †† p<0.01, ††† p<0.0001 vs B

Table 1: Number of capillary tubes formed by β-MNCs /250x250μm^2 of myocardial area.

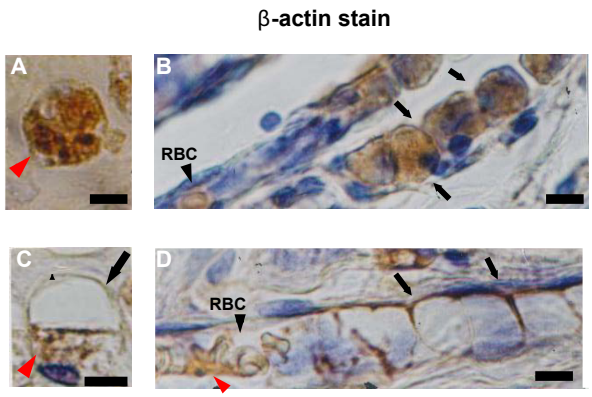

Figure 4: β-MNCs adherent to each other in tandem. A: Transected view of a β-MNC. The cell was filled with β-actin-positive granules (red arrowhead). B: Longitudinal view of β-MNCs filled with β-actin-positive granules and adherent to each other in tandem (arrows), indicating an immature capillary tube. Black arrowhead: A red blood cell (RBC) drained into a proximal segment of the capillary tube where intercellular junctions were lost. Two weeks after infarction production. C: Transected view of a β-MNC with a cavity (arrow) and residual granular cytoplasm (red arrowhead). D: Longitudinal view of a capillary tube in which granular cytoplasm were lost almost completely. Arrows: persistent intercellular junctions. RBCs appeared in a proximal portion of the tube where the intercellular junctions had disappeared (black arrowhead). Red arrowhead: residual β-actin-positive granular cytoplasm. Four weeks after infarction production.

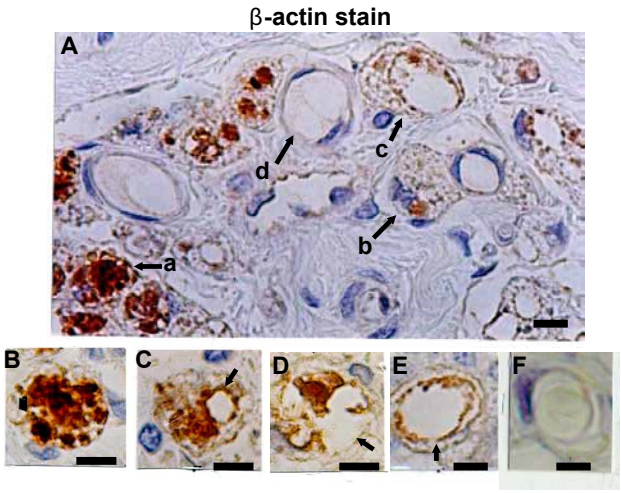

Figure 5: Various stages of cavity formation in β-MNCs. A: β-actin stain 4 weeks after myocardial infarction. a: A β-MNC filled with β–actin-positive granules. b: Formation of a small cavity. c: Loss of granular cytoplasm and formation of a large cavity. d: Complete loss of granular cytoplasm, with a smoothed luminal surface. Degranulation, cavity formation and consequent maturation were considered to occur in the order of B to F. Four weeks after infarction production.

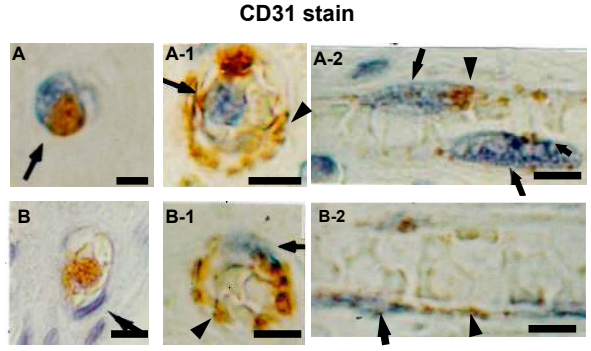

Figure 6: Two different configurations of nuclei of CD31-positive MNCs that formed capillary tube. A: A MNC containing a round nucleus and CD31-positive granular cytoplasm (arrow). A-1: An EC forming a tube. CD31 was present in the cell membrane (arrowhead). The cell possesses a round nucleus (arrow). The tube contained red blood cells. A-2: Longitudinal view of a capillary tube. The ECs possess round nuclei (arrows) and CD31 in the cell membrane. B: A MNC containing a flat nucleus (arrow) and CD31-positive granular cytoplasm (arrowhead). B-1: An EC with a flat nucleus (arrow) forming a tube. CD31 was present mainly in the cell membrane (arrowhead). A-2: Longitudinal view of a capillary tube. The ECs have flat nuclei (arrow) and CD31 in the cell membrane (arrowhead).

outside of the adventitia into the media of the existing arterioles or arteries to become SMCs by loosing β-actin and acquiring α-SMA, and into the intima to become ECs by acquiring CD_{31}, indicating that the β-MNCs are multipotent vascular progenitor cells [22].

Although the present study was a spot-to-spot observation and not the observation of the time-course changes in the same β-MNCs, the following process is considered to take place in capillary tube formation: on myocardial infarction production, β-MNCs are recruited to the coronary vessels, especially to capillaries [22]; they sprout out from the pre-existing coronary vessels, adhering to each other in tandem; an intracellular cavity is formed as a consequence of loss of intracellular β-actin-positive granular cytoplasm; intercellular junctions disappear and the cavities connect with each other to form a capillary tube; new β-MNCs are recruited to the terminal end of the capillary tube, migrate outside and adhere to each other in tandem; blood cells drained into the lumen, and these processes are repeated to extend the capillary tube (Figure 7).

These findings indicate that not differentiated ECs but rather β-MNCs, multipotent precursor cells [22], form capillary tubes, and that loss of intracellular granular cytoplasm is the initiating mechanism of capillary tube formation. However, the mechanism of loss of granular cytoplasm remains unclear. It also remains to be elucidated how β-MNCs adhere to each other in tandem. A pressure gradient between pre-existing vessels and the interstitial space, and/or the substances that adhere to cells such as VE-cadherin [28] may contribute to this phenomenon.

Maturing or matured capillary tubes were sparse at 1 week, and increased in number at 2 weeks or more after myocardial infarction, indicating that at least 2 weeks are required for capillary tube maturation, and consequently for maturing of ECs.

In the embryo, common precursors differentiate to angioblasts, and the latter to pre-arterial and pre-venule cells. Pre-arterial and pre-venous cells differentiate to arterial and venule ECs, respectively [29]. It is not known whether pre-arterial and pre-venule cells exist in adults.

In this study, we observed circulating β-MNCs with round and flat nuclei in pre-existing arterioles, capillaries and venules, as well as immature capillary tubes that were constituted either by β-MNCs with round or flat nuclei and matured capillary tubers constituted either by ECs with round or flat nuclei. These findings suggest the existence of pre-arterial and pre-venule EC-precursor β-MNCs in adult beagles.

Acceleration of capillary formation as a whole has been targeted for therapeutic angiogenesis. Based on the results of this study, angiogenic

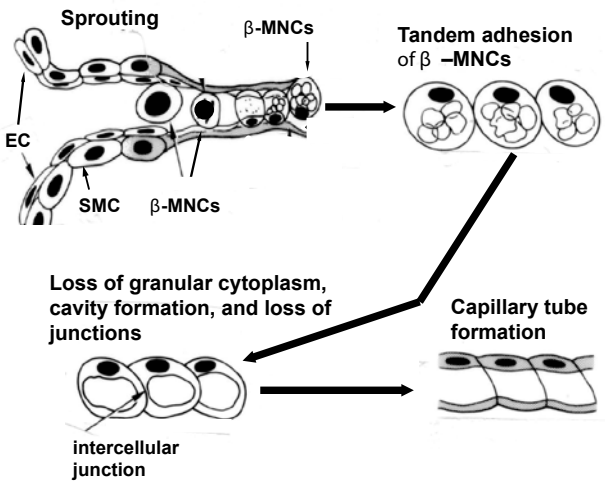

Figure 7: Schematic depiction of the process of capillary tube formation by β-MNCs in the ischemic myocardium. Circulating β-MNCs are recruited to a specific portion of a pre-existing vessel and initiate sprouting. Other β-MNCs then migrate through the vessel thus created into the interstitial space and adhere to each other in tandem. An intracellular cavity is formed in each cell by the loss of β-actin-positive granular cytoplasm. Intercellular junctions disappear and cavities connect with each other forming a capillary tube. New β-MNCs are recruited to the end of the newly formed capillary tube, migrate outside and adhere to each other in tandem. This process is repeated to extend the tube.

therapy targeting not only the recruitment and sprouting of β-MNCs, but also acceleration of their adhesion to each other in tandem, loss of granular cytoplasm and consequent cavity formation and loss of intercellular junctions shows promise in the treatment of ischemic heart disease.

Study Limitations

It remains to be examined whether the β-MNCs also participate in capillary tube formation in man.

Conclusions

β-MNCs were recruited to coronary vessels in the ischemic myocardium of beagles. They sprouted out from pre-existing coronary vessels into the interstitial space and adhered to each other in tandem. An intracellular cavity was formed by the loss of granular cytoplasm in each cell. The intercellular junctions which connected cell-to-cell disappeared, and the cavities connected with each other to form a capillary tube. New β-MNCs drained into the distal end of the tube and migrated outside, and these processes were repeated to extend the capillary tube.

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