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Novel Methods to Study Angiogenesis Using Tissue Explants

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Abstract

Tissue explants of skeletal muscles, brain, kidney, liver and spleen from mice were cultured using collagen gel. Electron microscopic observation revealed that formation of capillary tubes with pericyte-like cells occurred only from the tissue explant of skeletal muscles. The capillary tubes formed in the collagen gel were positive for tomato lectin and platelet/endothelial cell adhesion molecule (PCAM)-1 antibody. Formation of capillary tubes in the rat was more predominant than in the mouse. Plasmalemmal vesicles were clearly observed in the capillary tubes from rat tissue explant. Muscle fiber-type differences were also observed. In the soleus muscle, the formation of capillary tubes was predominant than the tibialis anterior muscle. Using this culture model from the rat soleus muscle, effects of α -isoproterenol (β -adrenergic receptor agonist) and low-frequency electrical stimulation were examined on the formation of capillary tubes and fine structures of skeletal muscle explant. The formation of capillary tubes was promoted by α -isoproterenol administration. At low-frequency electrical stimulation, the formation of capillary tubes was inhibited. Both α -isoproterenol and electrical stimulation reduced the degeneration of skeletal muscles. This culture method of skeletal muscles may provide a useful model that can examine the effects of various drugs and physical stimulations.

Keywords: angiogenesis, skeletal muscles, collagen gel culture, α -isoproterenol, low-frequency electrical stimulation

1. Introduction

To study the process of angiogenesis and test new agents with angiogenic or anti-angiogenic potential, suitable assays are essential [1]. For in vitro studies of angiogenesis, several culture techniques have been developed. The most commonly used in vivo assays are the

chorioallantoic membrane (CAM) assay, the corneal micropocket assay and the dorsal skin-fold assay [2–5]. The corneal micropocket assay, an analysis conducted within an avascular environment, is often used to study the efficacy of angiogenic compounds. The efficacy of anti-angiogenic compounds in inhibiting growth factor-induced vascularization and spontaneous vascularization is usually studied in vascular environments such as the CAM assay or the dorsal skin-fold assay. These assays have proven useful and have dramatically advanced our knowledge of angiogenesis. However, they are also limited in several respects (for a detailed review see Refs. [6, 7]) : (a) complicated surgical techniques are required; (b) only a limited number of test compounds can be assayed (e.g., in the case of the micropocket assay); and (c) simultaneous assessment of both angiogenic and anti-angiogenic compounds in the same assay is not feasible without the addition of exogenous growth factors. Fortunately, the method using collagen gel is free from these limitations. Collagen gel culture has been widely used for analyzing the biological process of angiogenesis [8–11]. Using the collagen gel culture, we have conducted electron microscopic studies and immunohistochemical studies during angiogenesis. In the collagen gel culture of aortic explants, capillary tubes with pericyte-like cells were observed [12]. As a source of angiogenesis, however, aortic explants were generally used in the collagen gel culture. Capillary permeability varies greatly among tissues, and this can be correlated partly with the type of endothelium. For simulation of angiogenesis, establishment of suitable in vitro model of capillaries might be effective. The endothelial cells of some capillaries have fenestrations or pores. For instance, fenestrated capillaries occur in renal glomeruli. Capillaries without fenestrations in the brain and skeletal muscle are known as continuous capillaries. As discontinuous capillaries, there are sinusoids. Sinusoids occur in large numbers in the liver and spleen [13].

In this study, we report the formation of capillary tubes from tissue explants of skeletal muscle, brain, kidney, liver and spleen from mice was cultured using collagen gel. Electron microscopic observation revealed that the formation of capillary tubes with pericyte-like cells occurred only from the skeletal muscle explant. Lectin and immunohistochemical studies showed that the capillary tubes formed in the collagen gel have architecture of capillary in vivo. There were some differences regarding the formation of capillary tubes among animal species and fiber types. We demonstrated that the soleus muscle from rats was most suitable model to study angiogenesis. Using tissue explant from the rat soleus muscle, effects of α -isoproterenol and low-frequency electrical stimulation were examined.

2. Materials and methods

2.1. Collagen gel culture

This collagen culture technique is a modification of our previous works [14, 15]. Samples (soleus muscles, cerebral cortex of frontal lobe, liver, cortex of kidney and spleen) were obtained from 1-month-old ICR male mice ($n = 5$). The samples were cut into small pieces ($2 \times 2 \times 2$ mm) under a stereoscopic microscope. The small pieces were placed at the bottom of tissue culture dish (35 mm; $n = 25$). Each tissue culture dish consists four pieces. An even layer of reconstituted collagen solution (0.3% Cellmatrix type IA, Nitta Gelatin, Tokyo, Japan) was

overlaid and gelled at 37°C for about 10 min. After gelation, Ham's F-12 medium (Invitrogen Corp., Carlsbad, CA, USA) containing 20% fetal bovine serum (FBS), 1% nonessential amino acids, 100 units/ml of penicillin and 100 mg/ml of streptomycin (Invitrogen Corp., Carlsbad, CA, USA) was added. Cultures were performed for 14 days in an incubator (95% air/5% CO₂). During the culture period, a phase contrast microscope was used to observe the capillary tube formation. These experiments were made three times. To examine the differences in animal species and fiber type, explants from skeletal muscles (soleus and tibialis anterior muscles) from rats were cultured as described above. All animal experiments were approved by the Committee on Animal Experimentation, Saitama Medical University (Permission No. 851 for mice and No. 934 for rats) and carried out in accordance with the "Guidelines for Animal Experimentation at Saitama Medical University."

2.2. Electron microscopy

The cultured materials were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for 1 h and then fixed with 1% OsO₄ in 0.1 M phosphate buffer (pH 7.2) for another hour. After dehydration with ethanol, samples were embedded in epoxy resin. After preparation of ultrathin sections, ultrathin sections were stained with uranyl acetate and lead citrate. Kajikawa's tannic acid stain was also used for demonstrating of elastic fibers [16]. The stained ultrathin sections were observed under a transmission electron microscope (JEOL JEM-1010, Tokyo, Japan). When capillary-like structures were observed, following lectin and immunohistochemistry were performed.

2.3. Lectin and immunohistochemistry

After fixation with cold 80% ethanol, capillary tubes were observed with FITC-conjugated endothelial cell-specific tomato lectin (*Lycopersicon esculentum*; EY Labo, CA, USA), a lectin that selectively binds to fucose residues on the endothelial cell surface [17]. Cold 80% ethanol-fixed, collagen gel-embedded specimens were stained by streptavidin/peroxidase immunohistochemistry technique for intercellular adhesion molecule-1 (ICAM-1), platelet/endothelial cell adhesion molecule-1 (PCAM-1) and integrin β_2 detection. The specimens were treated with 0.3% H₂O₂ in methanol to block endogenous peroxidase activity and then incubated with the polyclonal rabbit anti-rat ICAM-1, PCAM-1 or integrin β_2 antibody (Santa Cruz Biotechnology, Inc. California, USA). Biotinylated anti-rabbit immunoglobulin was added as a secondary antibody. The horseradish peroxidase labeled streptavidin-biotin complex was then used to detect the second antibody. Finally, the specimens were stained with 3,3'-diaminobenzidine, which was used as a chromogen. The brown or dark brown stained cells were considered as positive. Some specimens were stained with Giemsa before being examined under a light microscope.

2.4. Effects of α -isoproterenol and low-frequency electrical stimulation

Excised material was divided into three groups as follows:

- a. α -isoproterenol administration group (n = 48): To the culture medium, 10 μ M α -isoproterenol was added [18].

- b. low-frequency electrical stimulation group (n = 24): Using C-Dish (ION Optix, Milton, MA, USA), electrical stimulation (50 Hz, 2 h at 1.0 V; [19]) was performed by Ohm Pulser LFP-4000 A (Zen Iryoki corp, Fukuoka, Japan).
- c. control group (n = 48): without α -isoproterenol and low-frequency electrical stimulation.

2.5. Semiquantitative enzyme-linked immunosorbent assay (ELISA)

Eight different cytokines involved in angiogenesis in the culture medium were semiquantified by ELISA (Signosis; Angiogenesis ELISA Strip, Santa Clara, CA, USA). Tumor necrosis factor (TNF)- α , vascular endothelial growth factor (VEGF), interleukin (IL)-6, fibroblast growth factor (FGF)-2, interferon (IFN)- γ , leptin, insulin-like growth factor (IGF)-1 and epidermal growth factor (EGF) were tested. ELISA was carried out according to the manufacturer's instructions. Outline is as follows. Eight wells were coated with eight different primary antibodies against a specific angiogenesis cytokine. The test sample was sandwiched between the primary antibody and enzyme-linked antibody. After incubation, unbound-labeled antibodies were washed out. TMB (3,3',5,5'-tetramethylbenzidine) is added, and the color developed. After addition of stop solution, absorbance is measured spectrophotometrically at 450 nm. The concentrations of the test samples are directly proportional to the color intensity. Data were shown as fold change relative control group.

2.6. Measurement of the length of the capillary tubes

Digital photography equipment (FUJIX DIGITAL CAMERA HC-2500, 3CCD, FUJI PHOTO FILM, Tokyo, Japan) was used with an optical microscope (objective lens $\times 5$). Because capillary tubes were overlapped in the vicinity of the explant, it is impossible to identify each other. Length of capillary tubes was measured from a distance of 200 μm of the outer edge of the explant. Using six culture dishes (four pieces of the explant per dish), capillary tubes captured by the objective lens $\times 5$ (1262 $\times$ 991 pixel). It was taken four places of one explant.

2.7. Statistical analysis

Tube formation from the soleus and tibialis anterior muscles of rats is quantified by measuring the length of these capillary tubes in two-dimensional microscope images of six culture dishes. Data were shown as means $\pm$ standard error of the mean. Statistical analysis for the data represented was conducted using two samples, with Mann-Whitney U test. A particular result was considered significant if the p value was <0.01 for a two-tailed test.

3. Results

3.1. Collagen gel culture

3.1.1. Skeletal muscles

After 2 days, some cells were migrating in the collagen gel in the vicinity of the explants (**Figure 1a**). Spindle-shaped cells were orientated radially to the explant. After 6–7 days, the cell strands were recognized. At this time, lumen formation could not be clearly demonstrated

Figure 1. Mouse soleus muscle. (a) After 2 days of cultivation, phase-contrast microscopy reveals fibroblastic cells outgrown from a mouse skeletal muscle explant (*) into a three-dimensional collagen gel. (b) After 6 days of cultivation, phase-contrast microscopy shows a tubular structure protruding (arrow) from a mouse skeletal muscle explant (*) into a three-dimensional collagen gel. Scale bar:90 μm .

(**Figure 1b**). After 10 days, the capillary tube formation with lumen was well demonstrated by electron microscopic observation. As revealed by cross section, several endothelial cells with pericyte-like cells were involved in the composition of capillary tubes (**Figure 2a, b**). The structure as capillary tubes was maintained at least 2 weeks. When the culture was continued, degradation of the capillary tubes was observed.

Figure 2. Mouse soleus muscle. (a, b) Electron micrographs of capillary tubes. In a cross section of capillary tube, the lumen is surrounded by two to three cells. Pericyte-like cells (*) are associated with the capillary tube. Scale bar: 2 μm .

3.1.2. Cerebral cortex of frontal lobe

After 3–4 days, spindle-shaped cells were migrated from the explants. The number was quite few. The strand of spindle-shaped cells was elongated (**Figure 3a**). The longitudinal axis was orientated radially to the stump. As revealed by cross section, several cells surrounded a cell with elastic membrane-like structure. Tube formation with lumen was not recognized (**Figure 3b**).

Figure 3. Mouse brain. (a) After 12 days of cultivation, a phase-contrast microphotograph shows tube formation (arrow) from mouse cortex of brain into a three-dimensional collagen gel. Scale bar: 50 μm . (b) An electron microphotograph of the collagen gel-induced tube formation. A cross section shows no lumen. Kajikawa's tannic acid stain for elastic fibers. Electron dense materials are defined among cells. Scale bar: 1 μm .

3.1.3. Cortex of kidney

After 3–4 days, spindle- and cobblestone-shaped cells were migrated from the explants. The strands of spindle-shaped cells were elongated (**Figure 4a**). As revealed by cross section, several cells were attached each other and formed cellular mass (**Figure 4b**). Some area, a single cell, formed the outline of a tube with a lumen. A cell of the wall was consisted of microvilli (**Figure 4c**).

Figure 4. Mouse kidney. (a) After 7 days of cultivation, a phase-contrast microphotograph shows tube-like structure (arrow) from mouse cortex of kidney into a three-dimensional collagen gel. Scale bar: 50 μm . (b) An electron microphotograph of the collagen gel-induced tube-like structure. A cross section shows lumen (*). Scale bar: 2 μm . (c) Enlarged electron microphotograph of the cells showed in (b). Arrows indicate microvilli. Scale bar: 500 nm.

3.1.4. Liver

After 3–4 days, spindle-shaped cells were migrated from the explants. The strands of spindle-shaped cells were elongated (**Figure 5a**). As revealed by cross section, several cells were attached each other and formed cellular mass (**Figure 5b**).

Figure 5. Mouse liver. (a) After 10 days of cultivation, a phase-contrast microphotograph shows tube-like structure (arrow) from mouse liver into a three-dimensional collagen gel. Scale bar: 50 μm . (b) An electron microphotograph of the collagen gel-induced tube-like structure. A cross section shows tightly connected cells. Scale bar: 1 μm .

Figure 6. Mouse spleen. (a) After 7 days of cultivation, a phase-contrast microphotograph shows tube-like structure (arrow) from mouse spleen into a three-dimensional collagen gel. Scale bar: 50 μm . (b) An electron microphotograph of the collagen gel-induced tube-like structure. A cross section shows loosely contacted cells. Scale bar: 1 μm .

3.1.5. Spleen

After 2 days, numerous cells were migrated from the explants. Numerous cell strands of spindle-shaped cells were elongated (**Figure 6a**). Some cells were partly contacted, but distinct lumen was not observed (**Figure 6b**).

3.2. Lectin and immunohistochemistry

Since capillary tubes were observed only from the skeletal muscle explants, lectin and immunohistochemistry with antibodies were performed only for the cultured materials from skeletal muscle explants. Capillary tubes formed in the collagen gel were strongly positive for tomato lectin (**Figure 7**). Capillary tubes formed from the explants of muscles showed clearly

Figure 7. Mouse soleus muscle. After 11 days of cultivation, a fluorescent microphotograph of capillary tube (arrows). Capillary tube is strongly positive for FITC-conjugated endothelial cell-specific tomato lectin. Asterisk shows a muscle tissue explant. Scale bar: 50 μm .

Figure 8. Mouse soleus muscle. Capillary tubes (arrows) showed clearly immunoreactivity of PCAM-1. Asterisk shows a muscle tissue explant. Scale bar: 50 μ m.

immunoreactivity of PCAM-1 (**Figure 8**). ICAM-1 and integrin β_2 positive cells were sparsely distributed, but capillary tubes were almost negative (figure not shown).

3.3. Species difference in capillary tube formation from the skeletal muscle explant

In rats, numerous and long capillary tubes were observed from an explant. In mice, capillary tubes were few in number and short. Even without the statistical analysis, the difference is apparent (**Figure 9a, b**). Capillary tubes from rats were also positive for tomato lectin (**Figure 9c**) and PCAM-1 antibody (figure not shown). By electron microscopic observation, plasmalemmal vesicles or caveolae were clearly observed (**Figure 10a, b**). Typical gap junctions and tight junctions were not observed. Larger capillary tubes were also observed (**Figure 11**).

3.4. Difference in capillary tube formation between the soleus and tibialis anterior muscles

In the soleus muscle containing a large amount of red muscle fibers, the formation of capillary tube was predominant than the tibialis anterior muscle containing a large amount of white muscle fibers (**Figure 12a, b**). **Figure 13** indicates the results of statistical analysis.

3.5. Effects of α -isoproterenol and low-frequency electrical stimulation

3.5.1. α -Isoproterenol

Fine structures of explant: compared with the control group, the α -isoproterenol administration group was kept striated pattern (**Figure 14a, b**).

Formation of capillary tubes: in the α -isoproterenol administration group was seen promoting effect on the formation of capillary tubes (**Figure 15a, b**).

3.5.2. Low-frequency electrical stimulation

Fine structures of explant: compared with the control group, the low-frequency electrical stimulation group was kept striated pattern (**Figure 14a, c**).

Formation of capillary tubes: compared with the control group, the formation of capillary tubes was suppressed. Also, it appeared to have led to damage to the migratory cells (**Figure 15a, c**). **Figure 16** indicates the results of statistical analysis.

Figure 9. After 10 days of cultivation of soleus muscles. (a) Mouse soleus muscle, (b) rat soleus muscle. Phase-contrast microphotographs. Arrows indicate numerous and long capillary tubes. Scale bar: 90 μm . (c) Rat soleus muscle, tomato lectin staining. Arrows indicate numerous and long capillary tubes (cf. **Figure 7**). Scale bar: 50 μm . Asterisks show a muscle tissue explant.

3.6. ELISA

Compared with the culture medium, significant difference ($p < 0.01$) was observed only in the concentration of FGF-2 in the culture medium of the mouse soleus muscle. In the rat, no significant difference was observed. No significant difference in the concentration of eight kinds of angiogenic factor was observed between the soleus and tibialis anterior muscles from the rat and mouse (data not shown). In the α -isoproterenol administration group, increase in leptin was observed in the rat soleus muscle. However, no significant difference was observed. In the electrical stimulation group, increase in angiogenic factors except for the TNF- α was observed. Significant difference ($p < 0.01$) was seen in the concentration of the VEGF, FGF-2 and EGF (**Table 1**).

Figure 10. Rat soleus muscle. (a) Electron micrograph of capillary tube from rat soleus muscle. In a cross section of capillary tube, the lumen is surrounded by two to three cells. Scale bar: 1 μm . (b) Higher magnification of a part of (a). Arrowheads indicate a focal adhesion or an adherens junction. Arrows indicate plasmalemmal vesicles or caveolae. Scale bar: 200 nm.

Figure 11. Rat soleus muscle. Electron micrograph of a large capillary tube. The wall is made up by five cells. Scale bar: 1 μm .

Figure 12. Capillary density of rat soleus and tibialis anterior muscles. (a) Rat soleus muscle, (b) rat tibialis anterior muscle. Capillary tubes were stained by Giemsa solution. Scale bar: 50 μm .

Figure 13. Statistical analysis of length of capillary tubes. Capillary density of soleus muscles had significantly greater than tibialis anterior muscles at $p < 0.01$.

Figure 14. Electron micrographs of tissue explants of rat soleus muscle. (a) Control, (b) α -isoproterenol administration, (c) electrical stimulation. Both α -isoproterenol and electrical stimulation reduced the degeneration of skeletal muscles. Banding pattern of skeletal muscles is maintained when compared to the control. Scale bar: 1 μ m.

Figure 15. Rat soleus muscle. Effects of α -isoproterenol administration and electrical stimulation. Numerous capillary tubes were observed both control and α -isoproterenol administration groups. No capillary tubes were observed in the electrical stimulation group. (a) Control, rat soleus muscle. (b) α -Isoproterenol administration. (c) Electrical stimulation. Capillary tubes were stained by Giemsa solution. Numerous capillary tubes were observed both control and α -isoproterenol administration groups. No capillary tubes were observed in the electrical stimulation group. Scale bar: 100 μ m.

Figure 16. Statistical analysis of length of capillary tubes. Control: rat soleus muscle. The formation of capillary tubes was promoted by α -isoproterenol administration. At low-frequency electrical stimulation, the formation of capillary tubes was inhibited. Capillary density of α -isoproterenol administration group had significantly greater than control group at $p < 0.01$.

	Control	α -Isoproterenol	Electric stimulation
TNF- α	1	1.040	1.016
VEGF	1	0.843	1.542*
IL-6	1	0.980	1.350
FGF-2	1	0.912	1.335*
IFN- γ	1	0.969	1.286
Leptin	1	1.232	1.446
IGF-1	1	0.913	1.003
EGF	1	1.027	1.313*

Data were shown as fold change relative control. In the α -isoproterenol administration group, no significant difference was observed. In the electrical stimulation group, significant difference ($*p < 0.01$) was seen in the concentration of the VEGF, FGF-2 and EGF.

Table 1. ELISA of eight angiogenic factors.

4. Discussion

4.1. Formation of capillary tube from tissue explant of skeletal muscles

In this study, we tested tissue explants of skeletal muscles, brain, kidney, liver and spleen. In the light microscopic level, tubular structures are newly formed from all tissue explants tested. By electron microscopic observation, tubular structures with a lumen were observed only from the tissue explant of skeletal muscles and kidney cortex. By further detail electron microscopic observation, tubular structures from the kidney cortex have microvilli. The

capillary tubes had quite similar architecture observed in the collagen gel culture of aortic explant [12]. Plasmalemmal vesicles or caveolae were also observed. Plasmalemmal vesicles are plasma membrane invaginations. They are particularly numerous in the continuous endothelium of microvascular beds such as skeletal muscles in which they have been identified as transcytotic vesicular carriers [20, 21].

In the present study, we demonstrated that capillary tubes have architecture of capillary by tomato lectin, as we have previously shown [22]. Cell adhesion molecules are a family of closely related cell surface glycoproteins involved in cell-cell interactions during growth and are thought to play an important role in embryogenesis and development. PECAM-1, also referred to as CD31, is a glycoprotein expressed on the cell surfaces of endothelial cells, as well as platelets and mononuclear cells. PECAM-1 positive cells were clearly demonstrated as a capillary-like structure. ICAM-1, also referred to as CD54, is an integral membrane protein of the immunoglobulin superfamily and recognizes the $\beta 2\alpha 1$ and $\beta 2\alpha M$ integrins. In the present study, ICAM-1 positive cells were sparse. It may accord with $\beta 2$ integrin which was not detected. We have reported that pericyte-like cells were observed in the aortic explant culture (for a detailed review see Ref. [12]). Pericyte-like cells were positive for actin [12, 23]. In this study, pericyte-like cells were also observed around the capillary tubes, as shown in the aortic explant culture. Pericytes were reported to stimulate angiogenesis through the secretion of growth factors such as fibroblast growth factor (FGF) [24], VEGF and placenta growth factor [25]. In addition, pericytes appear to promote endothelial cell (EC) survival [26] and affect EC behavior such as sprouting [27]. It is strongly suggested that pericytes play an important role in angiogenesis. VEGF is a key promoter of angiogenesis. VEGF acts as a chemoattractant and directs capillary growth. VEGF concentration gradients are important for activation and chemotactic guidance of capillary sprouting [28, 29]. Zhang et al. [30] reported that a variety of cells in the body, including myocyte (skeletal muscle fibers), secrete VEGF at different rates. Ji et al. [31] also reported that VEGF is secreted by myocytes and binds VEGF receptors and neuropilin-1 on endothelial cell surface. Further studies are needed to understand the angiogenic factors.

It should be noted that, when compared with mice and rats, capillary tubes formation was predominant in rats than mice. We could not demonstrate the difference in angiogenic factors between rats and mice. It is widely accepted that endothelial cells derived from different species display different morphological, biochemical and phenotypical heterogeneity [32, 33]. For instance, FGF1 or collagen-coated dish is not required for culturing endothelial cells from the bovine and pig, unlike the rabbit and rat [34]. Difference in the properties of endothelial cells by species is considered in angiogenesis. It should be also noted that there is difference between red and white muscles. The number of capillaries in the soleus muscle of rat is 2.8/muscle fiber. The corresponding value for the tibialis anterior muscle is 1.2–2.0 [35]. The density of capillaries in the soleus muscle is greater than the tibialis anterior muscle. The difference in the capillary density may relate the formation of capillary tubes.

4.2. Effects of α -isoproterenol

Isoproterenol (β -adrenergic receptor agonist) promotes skeletal muscle hypertrophy in several animals, including rats and mice [36, 37]. The hypertrophy by isoproterenol induces through the stimulation of β_2 -adrenergic receptor [38], and β -adrenergic receptor is involved

in skeletal muscle growth and regeneration [39]. Expression of β -adrenergic receptor and its coupling to cAMP are important components of the signaling mechanism that controls atrophy and hypertrophy of skeletal muscle [40]. We have reported that α -isoproterenol reduced the degeneration of muscle after the facial nerve crush [41]. In this study, the direct effect of α -isoproterenol was confirmed even in vitro.

There are a number of reports about the increase in skeletal muscle capillary density with exercise. Exercise such as endurance training increases the capillary network to adapt to oxygen demand, particularly arteriolar portion of capillaries to favor the oxygen supply [42, 43]. When the endurance training was loaded in normal rats, angiogenesis of the soleus muscle is promoted, and arteriolar portion of capillaries is increased significantly [44]. Increase in arteriolar portion of capillaries is believed to be caused by "arteriolarization of capillaries" promoted by an increase in wall tension [44–46]. From the fact that circulating catecholamines (adrenalin and noradrenalin) are concerned with contraction/expansion of the blood vessels, catecholamines are expected to be associated with an increase in capillary density. Circulating catecholamines, which are adrenergic receptor agonist, are the main hormones whose concentrations increase markedly during exercise [47]. Many researchers have worked on the effect of exercise on these catecholamines and reported 1.5 to >20 times basal concentrations depending on exercise characteristics (e.g., duration and intensity) [48]. The increase in circulating catecholamines results in stimulating of β -adrenergic receptor activity and, consequently, increased intercellular concentration of cyclic AMP [49]. However, we have no direct effect that catecholamines associate with an increase in capillary density. Although an experiment in culture, in the brown fat precursor cells, noradrenalin encourages the growth of capillaries [50]. In the soleus muscle in the present study, α -isoproterenol, which is also an adrenergic receptor agonist, encourages the growth of capillary tubes. Although we could not detect the angiogenic factors, it has become possible to study the direct effect of α -isoproterenol on the skeletal muscle and formation of capillary tubes.

4.3. Effects of low-frequency electrical stimulation

From the results of the electrical stimulation, the effect of suppressing the denaturation of the muscle was observed. Young et al. [39] reported that electrical stimulation increased the number of β -receptors and promoted the synthesis of cAMP.

In this study, we could demonstrate that electrical stimulation also reduced the degeneration of skeletal muscles.

For angiogenesis in skeletal muscle in vivo, Cotter et al. [51] showed an increase in capillary density by the low-frequency electrical stimulation. After that, angiogenesis by skeletal muscle has been made under the various conditions of electrical stimulation [52–54].

For electrical stimulation in vitro, studies using myoblast cell line have been reported [55–57]. However, in vitro study of skeletal muscles capillaries is very few. Endothelial cells isolated from skeletal muscle capillaries are studied to make physical and chemical stress to the cells [58]. A number of angiogenic factors involve in angiogenesis by electrical stimulation [59, 60]. VEGF can be mentioned as the most important proteins. VEGF encourages the growth of vascular endothelial cells. VEGF played a central role in angiogenesis [59, 60].

As the mechanism, skeletal muscle contraction due to electrical stimulation has been considered to induce hypoxia [61, 62] and shear stress [63]. However, Kanno et al. [19] applied the electrical stimulation (50 Hz) without muscle contraction to the skeletal muscle and showed the increase in VEGF protein in vitro, and they showed the increased in capillary density in the rat model of hindlimb ischemia. In this study, the condition (50 Hz) according to the report of Kanno et al. [19] was adopted.

Electrical stimulation upregulated FGF-2 and EGF protein levels in the brains of stroke rats [64]. In this study, VEGF, FGF-2 and EGF protein levels are increased. However, under the condition used in this study, it was harmful to the migrating cells. The effect of promoting angiogenesis was not observed. On the contrary, angiogenesis was inhibited. Further studies including the setting of the condition are necessary.

4.4. The usefulness of this in vitro model

Endothelial progenitor cells derived from bone marrow are present in the peripheral blood. These cells reach the ischemic site, and angiogenesis occurs by proliferation and differentiation. However, it is difficult to collect a large amount of bone marrow stem cells for treatment of ischemia. Transplantation of CD133-positive endothelial precursor cells to the damaged muscle tissue has been studied [65]. In this report, angiogenesis is promoted, and the damaged muscle tissue is expected to recover. Recently, cardiac tissue sheets from human iPS cells have been shown to be effective in engraftment and transplantation in the rat model of myocardial infarction [66]. This cardiac tissue sheets include vascular cells (vascular endothelial cells and pericytes), in addition to the cardiomyocytes. Higher survival rate than the sheet of only cardiomyocytes has been shown. Transplantation including vascular cells may become research to increase the possibility of therapeutic angiogenesis.

In this study, we propose the possibility of autologous transplantation using tissue explants of skeletal muscle in cardiovascular disease including critical hind limb ischemia.

4.5. The drawback of this in vitro model

Clearly, those capillary tubes that emanate from the muscle explants are very similar to capillaries in vivo. However, the capillary tubes are not filled with flowing blood. Although the structure as capillary tubes is maintained at least 2 weeks, further studies are needed for long-term culture.

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