Effect of Smooth Muscle Cells on the Mechanical Response of Rabbit Carotid Arteries in Culture*

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The effects of smooth muscle cells on the mechanical response of arterial walls were investigated for the common carotid arteries in culture. Tubular segments obtained from male Japanese White rabbits were cultured for 6 days in the unloaded state in DMEM+20% FBS. Prior to culture, half of the specimens were soaked in liquid nitrogen (liq-N₂) for 20 - 40 seconds to kill cells in their walls. The mechanical response of the two groups following culture was examined using the pressure-diameter test and morphometry of the cultured segments. The arterial segments cultured without liq-N₂ treatment had significantly smaller outer diameter than their uncultured counterparts over 80 mmHg ($P < 0.02$), and also their wall thickness was significantly reduced ($P < 0.005$). These changes were not significant for the segments treated with liq-N₂. These results suggest that mechanical response observed in the cultured arteries are attributable to active remodelling of arterial wall caused by smooth muscle cells.

Key Words: Biomechanics, Material Testing, Elasticity, Mechanical Adaptation, Blood Vessel Wall, Smooth Muscle, Tissue Culture

1. Introduction

It has been pointed out that soft biological tissues change their morphology and mechanical properties in response to change in the mechanical environment to which they are exposed *in vivo*. Many of these responses can be considered to be adaptation processes to increased load. For example, wall thickening occurs in arterial^{(1),(2)} and ventricular⁽³⁾ walls of hypertensive subjects so as to maintain circumferential stress at a normal level. Incremental elastic modulus of arterial wall in hypertensive subjects is much lower than that in their normotensive counterparts especially at higher blood pressure^{(2),(4),(5)}. This may suggest the restoration of elastic properties at physiological conditions. These changes are, however, also attributable to changes in other factors such as hormonal environment and nervous stimuli. In fact, several papers including Liu et al.⁽⁶⁾ have reported that

hormonal change alone can cause wall thickening and other morphological changes.

To evaluate the effects of mechanical and chemical stimulations separately, we developed a perfusion system to culture blood vessels under various controlled mechanical conditions while maintaining their chemical and other non-mechanical conditions constant. We have cultured rabbit carotid arteries with this system for 6 days, and found that an appropriate pressure is necessary to maintain their normal pressure-diameter relationship and their wall thickness increased and decreased in response to intraluminal pressure elevation and reduction, respectively⁽⁷⁾. Most of these changes may be caused by cells in the arterial wall, especially by medial smooth muscle cells. Smooth muscle cells are one of the major components of blood vessel walls and their contraction and relaxation will lead to a change in the blood vessel diameter. In addition, smooth muscle cells produce proteins such as elastin and collagen, and various biochemical agents in response to various mechanical and chemical signals.

The changes during culture may, however, also be attributed to passive deformation such as creep and

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swelling of the tissue. To examine this possibility, we cultured arterial segments after killing their cells by rapid freezing and compared the change in their mechanical properties and dimensions during culture with that of intact segments.

2. Materials and Methods

2.1 Specimen

Right and left common carotid arteries of adult male Japanese white rabbits weighing 3.0 - 3.5 kg were used as the test model. Arteries taken from one side were cultured for 6 days and the contralateral arteries were used to establish the mechanical properties and dimensions before the culture. The choice of artery used for culture was made in a random manner.

Tubular segments of the arteries were excised at both sides from the animals under sterilized condition after they were killed by an overdose of sodium pentobarbital, and immediately transferred into a Petri dish filled with a physiological saline at room temperature. Longitudinal lengths of each segment were measured with the accuracy of 0.1 mm in the Petri dish within 5 minutes after excision. Right and left specimens obtained from six animals were then soaked in liquid nitrogen (liq-N₂) for 20 - 40 seconds to kill cells in their walls⁽⁸⁾.

Effects of smooth muscle viability on the mechanical response of a cultured artery were mainly studied in the segments incubated in an unloaded state. Frozen and non-frozen specimens for culture were incubated for 6 days in a Petri dish filled with an aerated Dulbecco's Modified Eagle Medium (DMEM) supplemented with 20% fetal bovine serum (FBS) and penicillin-streptomycin (1 unit/ml) at 37°C and then subjected to a mechanical test. They were denoted as 'Freeze+NoLoad' and 'NoLoad', respectively. Uncultured frozen and non-frozen specimens, denoted as 'Freeze' and 'Normal' respectively, were immediately subjected to the mechanical test.

Mechanical changes of a cultured artery were also studied under a loaded condition in one animal. Two arterial segments were taken from a rabbit in an identical manner to that described previously. One segment was treated with liq-N₂ and then cultured for 6 days in the blood vessel culture apparatus mimicking mechanical environment *in vivo*⁽⁷⁾ in the aerated DMEM±20% FBS. Flow rate and pressure were quasi-static. Flow rate of the medium was set at 80 ml/min to apply physiological shear stress of 1 Pa to the specimens. Intraluminal pressure was set at 80 mmHg⁽⁹⁾. Longitudinal stretch ratio of the specimen, the ratio of cultured length to unloaded length, was set at 1.4.

2.2 Mechanical test

Quasi-static pressure-diameter relationships for each specimen was obtained with the experimental apparatus used in the previous study⁽⁷⁾. The specimen was transferred to a tissue bath filled with an aerated Krebs-Henseleit solution at 37°C and stretched longitudinally to 1.4 times of its unloaded length measured just after excision. It was then inflated and deflated with the intraluminal pressure of the solution varying between 0 and 200 mmHg at a rate of about 3 mmHg/sec. The external diameter D_0 was monitored with a TV system and the intraluminal pressure P_i with a pressure transducer. The inflation and deflation were repeated 5 to 8 times until the pressure-diameter (P - D) loop became reproducible. The ascending limb of the last stable loop was used for the control P - D curve. Subsequently, 100 mM of KCl was added to the bath while keeping $P_i=30$ mmHg to check smooth muscle viability through potassium-induced contraction. After diametrical change of vessel ceased, P_i was decreased to 0 mmHg and the vessel was inflated once again to 200 mmHg to obtain the P - D curve under smooth muscle contraction.

2.3 Data analyses

After these pressure-diameter tests, each specimen was removed from the apparatus to measure its unloaded length after culture. A normalized longitudinal length λ_{NL} , which is the ratio of the unloaded length of the specimen immediately after mechanical test to that after excision, was calculated to establish the change in longitudinal dimension. A ring-like segment was then obtained from the specimen and soaked in a weak gentian violet solution (saturated gentian violet aqueous solution diluted >100 times with the saline solution) for 5 - 10 minutes to stain medial tissue pale blue. The segment was then transferred into the saline and its inner, medial external, and outer diameters in the unloaded state (d_i , d_m , and d_o , respectively) were measured under a measuring microscope. Inner diameter of a pressurized specimen D_i was calculated assuming the incompressibility of the wall material, as follows:

$$D_i = \sqrt{D_0^2 - \frac{1}{\lambda_z^*}(d_o^2 - d_i^2)} \quad (1)$$

where λ_z^* is the *in vitro* stretch ratio (= [length during testing]/[unloaded length after culture]).

Mechanical properties of the specimens were evaluated with an incremental elastic modulus $H_{\theta\theta}$ ⁽¹⁰⁾ obtained from the unloaded diameters and the slope of P - D curve at each pressure:

$$H_{\theta\theta}(P_i) = 2 \left(\frac{\Delta P_i}{\Delta D_0} \frac{D_0 D_i^2}{D_0^2 - D_i^2} + \frac{P_i D_0^2}{D_0^2 - D_i^2} \right) \quad (2)$$

where ΔD_0 is the increment of outer diameter with the pressure increase ΔP_i . The incremental elastic

modulus represents the inherent elastic properties of wall material.

Viability of the specimens was determined by smooth muscle contraction induced with KCl. Diameter change due to smooth muscle contraction $\% \Delta D(P_i)$ was calculated for every 10 mmHg as follows:

$$\% \Delta D(P_i) = \frac{D_0(P_i) - D_{KCl}(P_i)}{D_0(P_i)} \times 100 \quad (3)$$

where $D_0(P_i)$ and $D_{KCl}(P_i)$ are the outer diameters at P_i before and after KCl administration, respectively.

2.4 Histological analysis

Ring-like segments were fixed with formalin solution in the unloaded condition, cut into $\sim 2.5 \mu\text{m}$ -thick slices to obtain cross sections, and stained with hematoxylin-eosin for light microscopy. Number of lamellar units⁽¹¹⁾ and number of smooth muscle cell nuclei per unit area were measured in enlarged ($\times 350$) photographs.

2.5 Statistical analysis

Data are expressed as mean $\pm$ SEM. Differences were analyzed by the Student's paired and unpaired t -test and were considered significant when $P < 0.05$.

3. Results and Discussion

Figure 1 shows the change in diameter induced with KCl. In case of Normal specimens, the reduction in diameter has its peak generally within the physiological pressure range. Frozen specimens showed almost no contraction, suggesting that freezing deprived the specimens of their viability. NoLoad specimens contracted up to approximately 45%. The peak response was almost identical to that of the normal segments. However, the NoLoad segments exhibited almost no contraction above 100 mmHg.

Fig. 1 Contraction responses of rabbit common carotid arteries. Normal, segments tested just after excision; Freeze, segments frozen with liq-N₂ and thawed; NoLoad, segments without freezing cultured for 6 days in the unloaded condition; Freeze+NoLoad, segments treated with liq-N₂ and cultured for 6 days in the unloaded condition.

Smooth muscle viability of the NoLoad segments might have been affected during culture.

The effects of freezing and thawing on the unloaded diameters are summarized in Fig. 2. Six ring-like segments were obtained in series from an arterial segment and their unloaded dimensions were measured before and after the liq-N₂ treatment. Freezing and thawing of the arterial segments did not change either outer or medial external diameters, but increased inner diameter significantly (Fig. 2(a)). In accordance with these changes, both total and medial wall thicknesses decreased significantly. These results indicate that freezing itself has a considerable effect on artery wall dimensions by decreasing the medial volume, possibly the volume of the smooth muscle cells.

The change in the dimensions of cultured segments are shown in Figs. 3 and 4. With regard to diameters, there was no significant change between Normal and NoLoad (Fig. 3(a)). However, the inner diameter of Freeze was significantly greater than Normal, as shown in Fig. 2, and this increase was maintained after 6-day culture period (Freeze+NoLoad). The segments cultured under the unloaded condition without the liq-N₂ treatment exhibited significantly thinner medial walls than the normal ones (Fig. 3(b)), which may be attributable to atrophy of the tissue. The effect of the freezing treatment was to reduce the thickness of both the total and

(a) Diameters

(b) Wall thicknesses

Fig. 2 Effect of the liquid nitrogen treatment on unloaded dimensions of ring-like segments of arterial tissue.

Fig. 3 Change in diameters (a) and wall thicknesses (b) of arterial segments following unloaded culture for 6 days. See Fig. 1 for legends.

Fig. 4 Change in longitudinal length of arterial segments following unloaded culture for 6 days. Longitudinal length measured after the mechanical test was normalized by the length just after excision. See Fig. 1 for legends.

Fig. 5 Change in pressure-diameter curves of rabbit common carotid arteries following unloaded culture for 6 days. Difference in diameter among the groups was evaluated at 20, 80, and 160 mmHg. See Fig. 1 for legends.

medial walls as shown in Fig. 2. However, the change in the wall thickness following the culture was not significant. Figure 4 illustrates the change in the normalized longitudinal length following culture and/or freezing. The value was below unity even in the control segments because arterial specimens shrink to a small degree after excision, possibly due to their viscoelastic response. When cultured in the unloaded condition, the specimen shortened significantly by greater than 10%. The liq-N₂ treatment did not change the longitudinal length significantly either before or following culture. These results indicate that the unloaded culture produces a reduction in the thickness and the length of the arterial segments when smooth muscle cells are alive, but such dimensional changes do not occur when smooth muscle cells were killed. Dimensional changes observed during culture must therefore be mainly caused by the action of the smooth muscle cells. The mechanism of such dimensional changes is, however, unknown. Remodelling of the arterial tissue accompanying atrophy caused by the smooth muscle cells may be one of the most probable causes. Biochemical analysis such as a DNA/RNA assay would be necessary.

Pressure-diameter curves obtained from each group are shown in Fig. 5. The segments cultured in the unloaded condition without the liq-N₂ treatment (NoLoad) had significantly smaller diameter than the normal ones above 30 mmHg, as observed in the segments cultured in the unloaded condition while being stretched to 1.4 times its no-load length (a hypotensive condition)⁽⁷⁾. The liq-N₂ treatment itself had a significant effect on the pressure-diameter relationship, i.e., outer diameter of the treated specimens (Freeze) was significantly smaller than the normal segments at both 80 and 160 mmHg. However, the

Fig. 6 Summary of the incremental elastic moduli of all experimental groups used in the present study. See Fig. 1 and Table 1 for legends.

effect of culture on diameter was minimal over the whole range of pressures.

Figure 6 summarizes the incremental elastic modulus at 20, 80, and 160 mmHg obtained from the frozen and non-frozen vessels cultured in both the unloaded condition and the *in vivo* condition. In response to the unloaded culture, the non-linearity in the pressure-diameter curve of the untreated specimens decreased: the elastic modulus at 20 and 80 mmHg was higher in NoLoad than in Normal and that at 160 mmHg was lower in NoLoad, and thus the ratio of elastic modulus at 160 mmHg to that at 20 mmHg decreased from 207 to 47. The freezing treatment had a similar effect: it increased the elastic modulus at 20 and 80 mmHg, and decreased it at 160 mmHg. Subsequent culture under the unloaded condition decreased the modulus at 80 mmHg, while the elastic modulus at 80 mmHg increased when the untreated specimens were cultured under the unloaded condition. The change in the elastic modulus of the specimens with intact smooth muscle is opposite to the passive change observed in the frozen vessels. These results obtained from Figs. 5 and 6 indicate that change in mechanical properties observed during culture is mainly caused by the smooth muscle cells. The reason for the increase and decrease in the non-linear-

Fig. 7 Change in pressure-diameter curves of frozen segments of rabbit common carotid arteries following perfusion culture. Difference in diameter among the groups was evaluated at 20, 80, and 160 mmHg. See Fig. 1 and Table 1 for legends.

ity in the pressure-diameter curve is uncertain at this stage. However, microstructural changes may have a close correlation with the mechanical parameters. For example, increase in the variance of the slackness (or waviness) of collagen fibers and their deterioration may decrease the non-linearity, and *vice versa*.

Fig. 7 shows the pressure-diameter curves obtained from the frozen specimen cultured under the *in vivo* condition for 6 days and from the contralateral specimen immediately after excision. The averaged curves of Normal and Freeze in Fig. 5, and that of specimens cultured under the *in vivo* condition without liq-N₂ treatment (Normo)⁽⁷⁾ are also included for comparison. As could be seen by comparing the curves of Normal and Freeze, frozen vessels have smaller diameter than its controls over 50 mmHg. When cultured under the *in vivo* condition, the outer diameter of the frozen specimen was restored. The pressure-diameter curve of Freeze+Normo was almost similar to that of the contralateral counterpart (Normal). We regard this restoration as a direct result of creep of the wall material caused by the intraluminal pressure applied for 6 days during culture. The incremental elastic moduli were smaller at 20, 80, and 160 mmHg than those measured for Freeze (Fig. 6). This may also be the passive change of the arterial wall during culture. On the other hand, the modulus of cultured specimens increased significantly at 80 mmHg when smooth muscles were alive. This also suggests that the changes in the mechanical properties observed in the untreated specimens are caused by an active response of smooth muscle cells.

Table 1 summarizes the changes in dimensions of the frozen artery following the *in vivo* culture. Each value is normalized to that of its contralateral whose

Table 1 Change in dimensions (in mm) of arterial segments.

(mean $\pm$ SEM)				
Group	n	Outer diameter	Inner diameter	Total wall thickness
Freeze+Normo	1	1.00	1.03	0.91
Freeze	6	0.98 $\pm$ 0.03	1.02 $\pm$ 0.03**	0.87 $\pm$ 0.04*
Normo	8	1.04 $\pm$ 0.01*	1.01 $\pm$ 0.01	1.11 $\pm$ 0.05*

Dimensions of frozen and/or cultured segments normalized by those of their freshly isolated contralateral counterparts. Values are at intraluminal pressure of 10 mmHg and longitudinal stretch ratio of 1.4. Freeze, arterial segments frozen with liquid nitrogen; Normo, segments perfusion-cultured under the intraluminal pressure of 80 mmHg, shear stress of 1 Pa, and longitudinal stretch ratio of 1.4 for 6 days; Freeze+Normo, frozen segment perfusion-cultured for 6 days. * $P < 0.05$; ** $P < 0.005$.

Table 2 Summary of histological observation.

(mean $\pm$ SEM)			
Group	Number of lamellar units	Lamellar unit thickness (μ m)	Cell nuclei count (/mm ²)
Normal	8.1 $\pm$ 0.3	21.8 $\pm$ 0.7	1342 $\pm$ 11
NoLoad	7.5 $\pm$ 0.3	19.6 $\pm$ 0.7*	1308 $\pm$ 237
Freeze	7.4 $\pm$ 0.3	19.1 $\pm$ 0.9*	902 $\pm$ 68**
Freeze+NoLoad	7.5 $\pm$ 0.5	18.7 $\pm$ 1.0*	979 $\pm$ 86**

Data of each group were obtained from 12 positions in 2 slices. See Fig. 1 for legends. * $P < 0.05$; ** $P < 0.005$.

dimensions were measured without both freezing and culture. Normalized values of frozen non-cultured artery (Freeze) and non-frozen cultured artery (Normo) are also included. When cultured under the *in vivo* condition, untreated arteries (Normo) exhibited an increased outer diameter and total wall thickness, as shown by each normalized value exceeding unity. In contrast, the frozen vessel did not change its dimensions during culture and, as a result, the normalized values of Freeze+Normo were very close to the corresponding values for Freeze. The wall thickening observed in Normo may be attributable to an active response of smooth muscle cells rather than simple tissue swelling.

The results obtained from the histological observation are summarized in Table 2. Lamellar unit thickness was calculated by dividing medial wall thickness by the number of lamellar units. The number of lamellar units was not significantly different

between any of the four test groups. Thus, the change in the thickness of the lamellar unit followed closely that of the medial thickness (Fig. 3(b)). The number of smooth muscle nuclei per unit area was significantly smaller in frozen specimens. Difference between Freeze and Freeze+NoLoad was not significant. Because the freezing treatment decreases medial volume, the number of nuclei should increase in the treated specimens. The reason for this decrease is not clear at this stage. The freezing treatment might destroy cell nuclei and deprive nuclei of their affinity to hematoxylin.

The effects of smooth muscle cells on mechanical response of arterial walls were investigated for the common carotid arteries in culture. Although the freezing itself has remarkable effects on the pressure-diameter relationship and wall dimensions, the changes observed in the cultured specimens disappeared in the frozen vessels. These results suggest that mechanical response observed in the cultured arteries are attributable to active remodelling of arterial wall caused by smooth muscle cells.

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