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牛 대동맥 평활근 세포의 성장에 관한 shear stress의 영향

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Effect of Shear Stress on Bovine Aortic Smooth Muscle Cell Growth

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ABSTRACT

Bovine aortic smooth muscle cells cultured on the slide glass were exposed to sheared flow up to 120 hours in flow chamber to see the effect of shear stress on cell growth in wall shear stresses of 0 to 26 dyn/cm^2. From lactate dehydrogenase concentration measurement of the circulating medium, it was shown that sheared flow in the shear stress range did not remove additional smooth muscle cells from the slide glass compared with cells in stationary condition. According to smooth muscle cell counting per cm^2 of the surface, smooth muscle cells grew fastest in the stationary condition. As the wall shear stress increased, the growth of cells became slower. When the wall shear stress increased over 17 dyn/cm^2, cell growth was not observed throughout the experiment.

INTRODUCTION

When there is an injury in blood vessel, smooth muscle cells beneath the endothelial cells which contacts directly with blood flow play an important role in the cellular response. Smooth muscle cells sometimes proliferate and are accumulated in injured site, which may cause wall thickening (1, 2). It was known that a large portions of atherosclerosis lump consisted of the smooth muscle cells(3). In recent studies, atherosclerosis progression is not only affected by lipoprotein concentration such as cholesterol(4, 5), but also is assumed to be related with blood flow. Gibson *et al.*(6) indicated that below 20~30 dyn/cm^2 of wall shear stress, there was a atherosclerosis progression and above the region the arterial diameter was increased, that is, atherosclerosis was reduced. Diamond *et al.*(7), Ku *et al.* (8) also showed that low shear stress zone in arteries was easy to develop atherosclerotic lesions and enhanced thrombogenicity compared with vascular regions with high shear flow, which indicates that the shear stress of the flow may have significant effect on the growth of smooth muscle cells.

In this study, bovine aortic smooth muscle cells on the slide glass were exposed to constant shear stress of 0, 6, 11, 17 and 26 dyn/cm^2 in the rectangular flow chamber up to 120 hours to see the growth of smooth muscle cells for different shear stresses.

MATERIAL AND METHOD

Smooth Muscle Cell Culture

Bovine aortic smooth muscle cells obtained from Texas Biotechnology Co.(Houston, TX, U. S. A.) were cultured using procedures based on Campbell *et al.*(9). Smooth muscle cells were cultured in Dulbecco's modified eagle(DME) medium(Gibson, Grand Island, NY, U. S. A.) containing 10% heat-inactivated fetal bovine serum (Hyclone Laboratories, Logan, UT, U. S. A.), 1% glutamine and 1% PSN(penicillin, streptomycin and neomycin) using 25cm^2 tissue culture flasks. PH of DME medium was adjusted to 7.4 by either diluted NAOH or Hcl and smooth muscle cells were cultured in a humidified atmosphere of 5% CO_2 in air at 37 C for 3~5 days. When the smooth muscle cells were confluent in the flask, cells were trypinized with 0.25% trypsin(Gibco), centrifuged at 1,500 rpm and were seeded on the sterilized glass slide (25mm×75mm) inside the petri dish(100mm×20mm). 24 hours after initial seeding, 10 ml of DME medium was added in petri dish to flood the glass slide slightly.

Not like the endothelial cells which grow in monolayer, the smooth muscle cells only grow in monolayer under the lower concentration, but when the surface is fully covered by the cells, smooth muscle cells start to grow in multilayer. To count the number of cells properly, $10^3 \times 10^4$ cells per cm^2 of slide glass surface were seeded on the slide. For each set of flow experiment, 10 slide glasses were prepared and slide glasses with approximately same number of cells per cm^2 of the slide were selected for the experiment either in stationary condition or sheared flow conditions. Cells were exposed to the sheared flow 48 hours after the initial seeding. Fig. 1. shows typical bovine aortic smooth muscle cells on the glass slide in stationary condition for 120 hours.

Fig. 1. Typical bovine aortic smooth muscle cells on the glass slide in stationary condition for 120 hours.

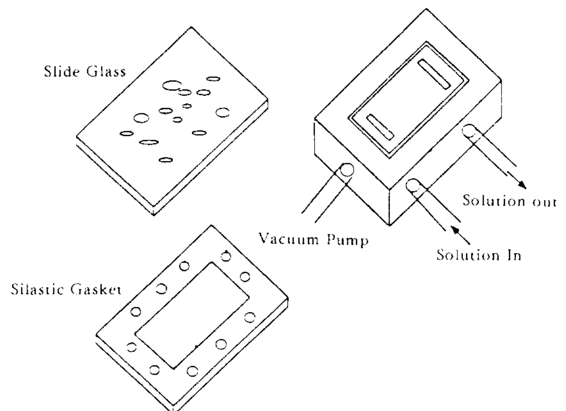


Fig. 2. Flow chamber.

Flow System

Smooth muscle cells were exposed to sheared flow of DME medium in a parallel plate flow chamber as shown in Fig. 2. The flow chamber consisted of a polycarbonate surface, a silastic gasket(Dow Corning, Midland, MI, U. S. A.) and the glass slide on which cells were attached. To prevent the leakage of the medium in the flow chamber, glass slide was attached firmly to the polycarbonate surface by connecting to vacuum pump through the hole outside the flow chamber. The flow chamber was put on the microscope table in reverse to prevent a damage that might cause by an air bubble in the chamber. During the experiment, smooth muscle cells on slide glass was checked time to time through the microscope to identify cell contamination. Surface area of the slide exposed to the flow was 15cm^2.

The flow unit consisted of an upper channel,

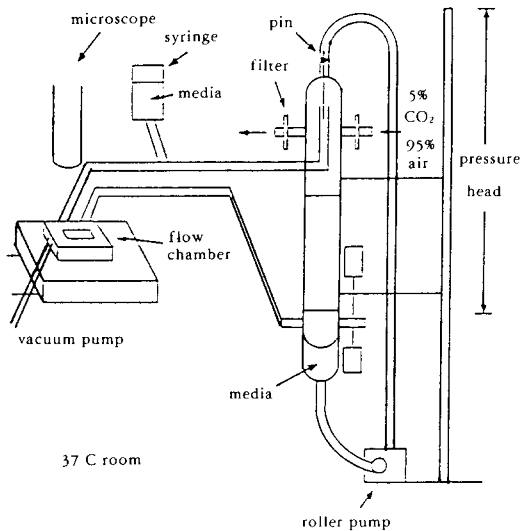


Fig. 3. Folw unit.

lower channel, and middle section with various length which could be added or removed depending on the pressure head as shown in Fig. 3. All three units were connect by rubber tubes. The medium was transported to the flow chamber through Tygon tube(Fisher Scientific, St.Louis, MO. U. S. A.) connected to the upper channel. The flow rate of the medium was decided by a constant hydrostatic pressure head created by the vertical distance between the top of the flow unit and flow chamber. Outlet DME medium from the flow chamber was transported to the lower channel and was recirculated from the lower channel to the upper channel by a peristaltic pump (Fisher Scientific, St.Louis, MO. U. S. A.). Small outlet port was installed in the lower channel to draw the circulating medium by the cylinze. After the flow unit was assembled, it was autoclaved with polycarbonate surface and silastic gasket. After cooling, glass slide on which smooth muscle cells are cultured is assembled with polycarbonate surface and silastic gasket to make a flow chamber. The flow chamber was connected to the flow unit, DME medium was inserted by the cylinze and circulated for 5 minutes to check the stability of the flow unit. The flow unit was set in 37 C room and a humidified mixture of 95% air and

Table 1. Volumetric flow rates, Reynolds numbers and wall shear stresses applied in the experiment.

Q(ml/s)	N_{RE}	τ_w(dyn/cm^2)
0.12	9.7	6
0.22	18	11
0.34	27	17
0.52	42	26

5% CO_2 was gassed to maintain pH of the medium to 7.4 through the small port connected by 0. 45μm filter(Millipore, MA. U. S. A) in the upper channel. Total medium volume circulated in the flow unit was 25ml.

Shear stress at the surface in laminar flow region was calculated by the following equation (10):

$$\tau_w = 6\ \mu\ Q/(B^2\ W) \qquad [1]$$

where τ_w is the shear stress at the surface of slide glass, Q is the volumetric flow rate(ml/s), μ is the viscosity of the medium(1 cp), W is the width of the flow chamber(2.5cm) and B is the gap of the flow chamber(220μm). Shear stress was adjusted by the volumetric flow rate(Q) which depended on the hydrostatic pressure head in the flow unit. In turn, hydrostatic pressure head can be adjusted by changing the length of middle section of the flow unit. Volumetric flow rates, Reynolds numbers(N_{RE}) that are based on the equivalent diameter(11) and wall shear stresses based on eq.1 are summarized in Table 1. The sheared flow in the experiment was clearly in the laminar flow region. In human body, wall shear stress is 16 dyn/cm^2 in large veins, 56dyn/cm^2 in large arteries, 64dyn/cm^2 in capillaries and 15 dyn/cm^2 in ascending aorta, whereas Reynolds number is 110~850 in large arteries, 210~570 in large veins and 0.0007~0.003 in capillaries(12).

3 slides on which smooth muscle cells were cultured were put inside the petri dish and were flooded with 25ml of fresh DME medium. Then, slides were put in the incubator for the same conditions as that in the flow experiment to observe the growth of cells in the stationary condition.

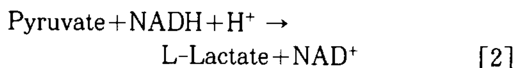
The flow experiment was done up to 120 hours to see the difference on the cell growth in between the stationary condition and the sheared flow conditions. 1ml of each medium was taken every 24 hours, stored at -4 C for the lactate dehydrogenase concentration measurement and fresh medium of the same volume was added in the lower channel by the cylinze through the port.

Smooth Muscle Cell Counting

To measure cell growth, pictures of smooth muscle cells in both stationary and sheared flow conditions were taken for every 24 hours by camera that was mounted to the microscope (Nikon TMS, Tokyo, Japan). For each slide, 3 pictures of smooth muscle cells in the center region of the slide glass were taken, the number of cells in each picture was counted and average number of cells was calculated. A picture of slide glass with scale was also taken in the same magnification and average number of cells per cm^2 of slide glass was obtained.

Lactate Dehydrogenase Concentration Measurement

Even though smooth muscle cells are known to adhere glass surface strongly(9), the possibility of cell washout from the surface due to the sheared flow needs to be investigated. In the smooth muscle cells, following biological reactions takes place in anaerobic and aerobic conditions (13);



$$\text{Pyruvate} + \text{NADH} + H^+ \rightarrow \text{L-Lactate} + \text{NAD}^+ \quad [2]$$

Lactate dehydrogenase catalyzes this reaction and the amount of L-lactate generated is proportional to the lactate dehydrogenase concentration in the solution. Lactate dehydrogenase is known to be released when cells are ruptured. Viable cells in the medium can be measured by breaking the cell membrane using triton (x-100, Sigma Chemical Co., St.Louis, MO, U. S. A.). The number of smooth muscle cells which are off from the surface can be counted indirectly by measuring

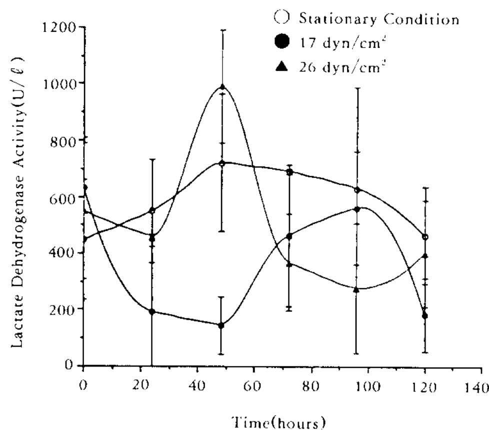


Fig. 4. Activity of lactate dehydrogenase in the circulating medium at the wall shear stress of 0, 17 and 26 dyn/cm^2.

lactate dehydrogenase concentration in the medium. The concentration of lactate dehydrogenase in the circulating medium was measured by Gilford LD-P essay kid (Oberlin, OH, USA). 1ml of 10% triton was added to 0.4ml of stored medium from either stationary condition or sheared flow conditions and left for 24 hours. Then $20\mu\ell$ from the mixture was added to 1 ml of LD-P reagent which contained pyruvate, NADH and mixed rapidly. Absorbance of whole solution at 340 nm was measured at 15 and 75 seconds after the mixing by the spectrophotometer(System 2600, Gilford Instrument Laboratories, Oberlin, OH, U. S. A.). Lactate dehydrogenase activity for each condition was calculated by the equation presented in the LD-P essay kid.

RESULTS AND DISCUSSION

Possibility of Smooth Muscle Cell Washout from the Surface due to the Sheared Flow

As shown in Fig. 4. lactate dehydrogenase activity of the medium did not show a significant difference for 120 hours in between the stationary condition and the shear flow conditions of 17 and 26 dyn/cm^2, which means that sheared flow in this range does not remove additional cells from the surface compared with cells in the sta-

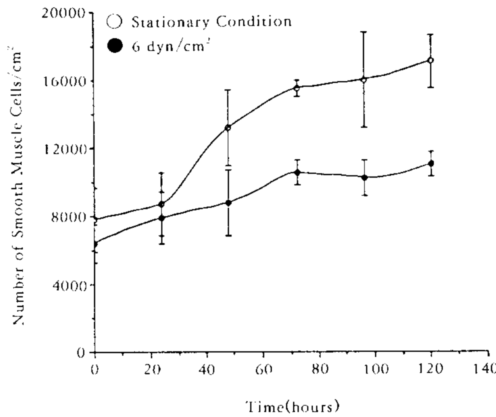


Fig. 5. Number of smooth muscle cells per cm^2 of glass slide at the wall shear stress of 0 and 6 dyn/cm^2 up to 120 hours.

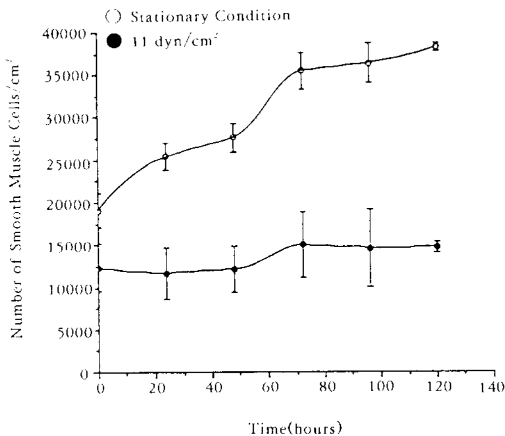


Fig. 6. Number of smooth muscle cells per cm^2 of glass slide at the wall shear stress of 0 and 11 dyn/cm^2 up to 120 hours.

tionary condition. Error bars in Fig. 4. represent a standard deviation. For the lower shear stress of 6 and 11 dyn/cm^2, lactate dehydrogenase concentration was the same as that in the stationary condition. Therefore possibility of smooth muscle cell washout from the slide due to the sheared flow was neglected.

Effect of Shear Stress on the Smooth Muscle Cell Growth

Fig. 5. shows smooth muscle cell growth up to

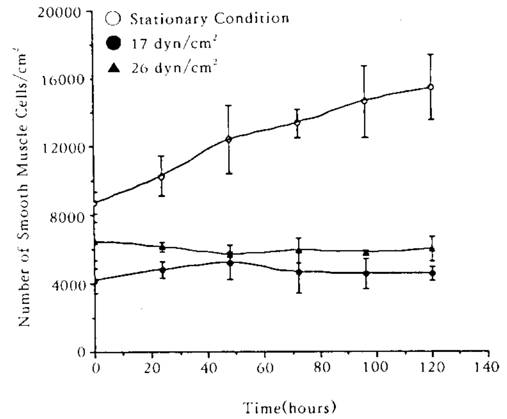


Fig. 7. Number of smooth muscle cells per cm^2 of glass slide at the wall shear stress of 0, 17 and 26 dyn/cm^2 up to 120 hours.

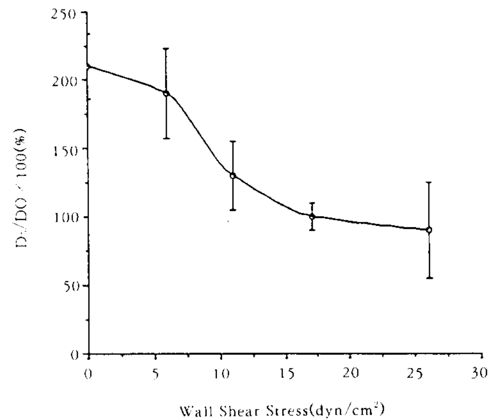


Fig. 8. Number of smooth muscle cell on glass slide at 120 hours (D_5) to that in the beginning (D_0) for the wall shear stress between 0 and 26 dyn/cm^2.

120 hours in wall shear stress of 6 dyn/cm^2 compared with that in the stationary condition and error bars indicate a standard deviation. Smooth muscle cell concentration on slide was increased 120% from 7,800 cells/cm^2 to 17,000 cells/cm^2 for 120 hours in the stationary condition, whereas cell concentration was increased 72% from 6,400 cells/cm^2 to 11,000 cells/cm^2 in the 6 dyn/cm^2. Fig. 6. shows that cell concentration was increased 100% for the stationary condition even

though initial cell concentration was 19,000 cells/cm^2. Cell concentraion was increased 25% from 12,000 cells/cm^2 to 15,000 cells/cm^2 in the wall shear stress of 11 dyn/cm^2 for 120 hours. Fig. 7. shows that cell concentration was also increased 100% in the stationary condition, whereas cell growth was negligible in shear stresses of both 17 dyn/cm^2 and 26 dyn/cm^2.

From the cell growth data, smooth muscle cell growth was fastest in the stationary condition and as the shear stress increased, cell growth became slower. Smooth muscle cell growth was not observed in wall shear stress of 17 dyn/cm^2 or higher. Fig. 8. shows relationship of smooth muscle cell growth for 120 hours with the wall shear stress applied. According to the Fig. 8, smooth muscle cell growth is inversely proportional to the wall shear stress and smooth muscle cells do not grow in the wall shear stress higher than about 20 dyn/cm^2. Even though the research was based on the smooth muscle cells, the data obtained in the study agreed well with Gibson et al(6) which suggested that increased shear stress reduced atherosclerosis progression.

요 약

평활근 세포에 대한 shear stress의 영향을 알아보기 위해 슬라이드 글라스 위에 배양된 牛 대동맥의 평활근 세포를 120시간 동안 0~26 dyn/cm^2의 각기 다른 일정한 shear stress의 유체에 노출하였다. 실험 장치를 순환하는 배지의 lactate dehydrogenase의 농도측정 결과, 본 연구에서 적용된 shear stress 범위에서는 유체의 흐름으로 인하여 세포가 슬라이드 글라스 표면으로부터 이탈되는 현상은 없었다. 표면 cm^2 당 존재하는 세포의 수를 측정한 결과, 평활근 세포는 정체배양시 가장 빨리 성장하였다. 표면에서의 shear stress가 증가할수록 평활근 세포의 성장은 늦었으며, shear stress가 17 dyn/cm^2 이상에서는 세포의 성장이 관찰되지 않았다.

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