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Development of a Novel *In Vitro* Culture System : To Investigate the Effect of Hydrostatic Pressure on Renin-Angiotensin System for Monocyte

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ABSTRACT

Hemodynamic forces, which include hydrostatic pressure, stretch, and shear stress, have been shown to be important regulators of vascular structure and function. Although effects of vascular wall stretching and shear stress have been profoundly studied, very little is known about the effects of hydrostatic pressure on monocytic response. We reasoned that hydrostatic pressure may be the most significant physiological regulator of monocytic function. To examine the direct effect of hydrostatic pressure on cells, we have developed a new *in vitro* culture device : "Versatile Culture System (VCS)". VCS consists of culture chamber, a control valve, and a pressure sensor through which media pumped to a waste at a predetermined rate. We design it for a wide variety of cell culture and it has ability to load pressure ranging from 0-760 mmHg.

In this system, monocytic cell line THP1 were grown in the suspension cell chamber which was specially designed for suspension cell culture without being affected by shear stress. To clarify whether pure hydrostatic pressure itself affects the renin-angiotensin system (RAS), which has been modulated by stimulus of hemodynamic overload, we examined the expression of angiotensin -converting enzyme (ACE) and angiotensin type 1 receptor (AT1) genes of cultured monocytic cells. We subjected THP1 to 100 mmHg or 200 mmHg constant pressure for up to 12 hours. Quantitative RT-PCR analysis revealed ACE and AT1 mRNA have a tendency of alterations in a different manner by exposure to hydrostatic pressure. The present study suggest that physiological hydrostatic pressure has an ability to modulate RAS of monocytic cells at different magnitude of pressure. We conclude that VCS provides a mechanistic insight into the role of hydrostatic pressure on various cell functions. (Jikeikai Med J 2002 ; 49 : 13-21)

Key words : culture system, pressure, monocyte, angiotensin-converting enzyme, angiotensin type 1 receptor

INTRODUCTION

The hemodynamic environment to which blood vessel is exposed is known to regulate its structure during development. Recent work in cellular and molecular biology has uncovered a set of complex interactions between various components of the blood vessel including endothelium, smooth muscle, matrix and circulating blood cells such as leukocytes (monocytes, lymphocytes, and polymorphonuclear leu-

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kocytes[1]. In the blood vessel system, the hemodynamic forces induced by the passage of intravascular blood are flow and pressure. When the blood flows across the endothelial surface, frictional force produced shear stress. On the other hand, pressure is exerted radially at right angles to the axis of flow and leads to tangenital strain on the wall, causing smooth muscle/fibroblast cell and extracellular matrix constituents in the media to stretch. In addition, circulating blood cells mainly experience blood pressure, i.e., hydrostatic pressure by the surroundings.

Hypertension is characterized by structural alterations of the vasculature, depending on the size and function of the particular vessel. A large number of growth factors, cytokines, and vasoregulatory molecules have been proposed to explain the structural alterations that occur during hypertension. The renin-angiotensin system (RAS) is an important regulator of vascular tone and sodium-electrolyte balance, and its principal mediator, angiotensin II (Ang II), may also be important as a trophic factor for the maintenance of vascular structure[2]. For example, Ang II has been extensively implicated in the development of cardiac hypertrophy associated with hemodynamic overload[3], hypertension[4], and myocardial infarction[5]. The components of the RAS are present in many tissues and there is evidence for local *de novo* byosynthesis of Ang II. These tissue-based systems can be regulated independently of the endocrine RAS, and it has been suggested that the plasma RAS is predominantly important for acute regulatory mechanisms whereas the local RAS may be more involved in chronic aspects of cardiovascular regulation[6,7], and considered as an important autocrine-paracrine system for the regulation of vascular tone and structure[8,9]. Several *in vivo* studies have demonstrated that mRNA expression of ACE[10], and AT1[11,12] are upregulated in response to pressure overload or after myocardial infarction in various animal species. It has also been shown that shear stress mechanisms of ACE regulation may play an important role in the control of the local RAS for changes in vascular structure[13]. The effect of shear stress or stretch on the release of various vasoactive substances has been investigated extensively, while the effect of pressure itself is not well understood.

Monocyte is a type of white blood cell normally flowing in the blood stream. In response to recruiting signals, monocyte adheres to the vessel wall and migrate across the endothelium in to the sub-endothelial space and surrounding tissue. It has been reported that these enhanced monocytes may recognize hemodynamic forces and mediate any necessary response in the regulation of vascular structure and functions[14]. In this study, we develop a new pressure-loading apparatus and reported that whether hydrostatic pressure modulates local RAS genes, including ACE and AT1 gene expression in human monocytic cells.

MATERIAL AND METHODS

Pressure-loading apparatus

A new *in vitro* culture device ; "Versatile Culture System (VCS)", have developed to clarify the effects of hemodynamic force on both vascular wall and circulating cells. This enabled us to culture a wide variety of cell with the change of cell chamber and to load pressure ranging from 0-760 mmHg. Fig. 1 shows schematic diagram of the VCS designed to suspension cell culture with constant pressure. Culture medium was pumped to a waste through culture chamber, pressure sensor and a control valve at a predetermined flow. All system was set up in a standard CO_2 incubator (Astec, Fukuoka, Japan) and the entire period of perfusion was recorded on chart using a printer (M & S, Tokyo, Japan ; LC-1001). We have modified a disposable fuser (Terumo, Tokyo, Japan ; L060K01) as suspension cell chamber and used syringe pump (Terumo, Tokyo, Japan ; TE-311) and Swagelok valve (Nupro, Ohio, U.S.A. ; SS-SS2) to regulate pressures. The other disposable components such as connecting tube, pressure sensor (PR-AS123S) and syringe were purchased from Terumo (Tokyo, Japan).

Cell culture

THP1 (American Type Culture Collection), a

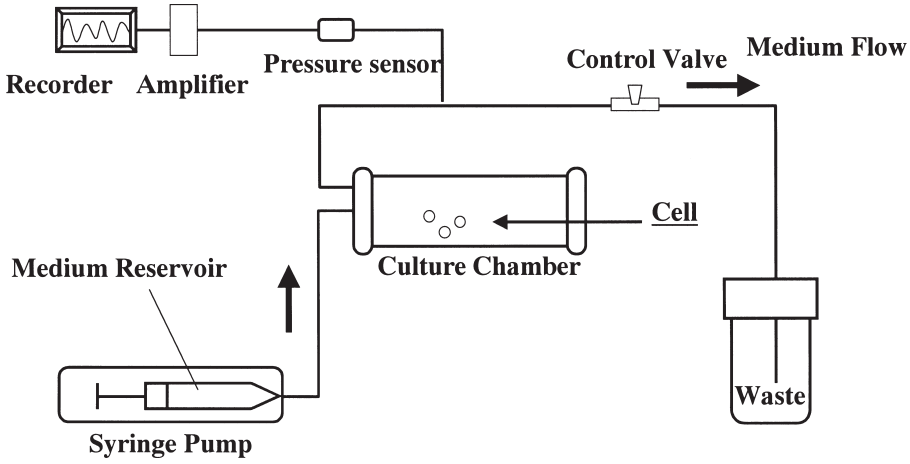

Fig. 1. Versatile Culture System (VCS)
Unit for constant pressure/suspension cell culture

human monocytic cell line, was grown in culture using RPMI1640 (GIBCO-BRL), supplemented with 10% fetal bovine serum. Cells were resuspended in 175 cm^2 culture flask (Falcon) and passaged at 1 : 10 in a 95% humidified air/5% CO_2 environment at 37°C. Initially, 30 ml of cell suspension at density of 1×10^5 cells/ml were incubated for 12 hours to eliminate possible effects due to the addition of fresh medium. Then, cells were transferred to a chamber at 5×10^4 cells/ml (chamber volume 66 ml). All cells used were between passages 6 and 12 from the initial purchase. To note, extremely care was taken to avoid the loading of additional mechanical stress to cells (*e.g.*, conventional centrifugation for subculture).

Mechanical stimulation

In this study, culture medium, which was preincubated 6 hours in CO_2 incubator, was pumped at 4 ml/hr and either 100 ± 2 or 200 ± 2 mmHg of constant pressure, achieved by adjusting the control valve. The cells were exposed to pressure for 6 and 12 hours and compared to the control specimens from the same set of replicate cultures under 0 mmHg These parallel sets of experiments in each time course were performed simultaneously. The partial pressure of oxygen and pH of the medium showed no significant changes for 24 hours after the application of pressure and the viability of cells was ascertained by a trypan blue exclusion assay.

RNA isolation and quantitative PCR

Total cellular RNA in monocytic cell lines were isolated by RNeasy Mini Kit (Qiagen) at 0, 6, 12 hours of pressure-loading, and total RNA was prepared according to the manufacturer's recommendations. The RNA was dissolved in 30 μl of H_2O and the concentration was determined by spectrophotometer (A 260/280 nm). Reverse transcription of RNA was carried out in 20 μl of a reaction mixture by using Omniscript (Qiagen), containing 1 μg of total RNA. The reaction master mix was prepared according to the manufacturer's protocol to give final concentrations of 1×reaction buffer, 0.5 mM each dNTPs, 0.5 units/μl RNase inhibitor, 0.2 units/μl reverse transcriptase, 0.5 μM random hexamers. To monitor gene expression we used real time PCR analysis, which has been described previously[15]. Briefly, 50 ng of total RT product was added to a 50 μl of a reaction mixture. PCR amplification was carried out in the presence of 1×reaction buffer, 0.2 mM each dNTPs, 3.5 mM $MgCl_2$, 0.02 units/μl AmpliTaq Gold DNA polymerase, a 300 nM concentration of the primers, and a 200 nM concentration of the corresponding probe. Primers and probes of ACE, AT1, GAPDH genes were designed by the Primer Express Program according to Heid et al[16]. For sequence information of all oligonucleotides and probes, see Table 1. All primers were synthesized at Lifetech Oriental (Tokyo, Japan) using conventional nucleic acid synthesis chem-

Table 1. Oligonucleotide primers and probes used for real time PCR

Gene	Oligonucleotide	Sequence	Annealing temp.
ACE	forward primer	5′-ggataaaggcgatcttgtcaagg-3′	55°C
	reverse primer	5′-aagacttacctgtggccttgagg-3′	
	probe	5′-Fam-tcagcaggttgagactgtgcaggtgctt-Tamra-3′	
AT1	forward primer	5′-ggccctttggcaattacctat-3′	60°C
	reverse primer	5′-gtgagtagaaacacactagcgtacagg-3′	
	probe	5′-Fam-agattgcttcagccagcgtcagtttca-Tamra-3′	
GAPDH	forward primer	5′-gaaggtgaaggtcggagtc-3′	58°C
	reverse primer	5′-gaagatggtgatgggatttc-3′	
	probe	5′-Fam-caagcttcccgttctcagcc-Tamra-3′	

istry. Fluorescent probes and reagents were purchased from PE Applied Biosystems.

PCR and resulting relative increase in reporter fluorescent dye emission were monitored in real time by the 7,700 sequence detector (PE Applied Biosystems). Signals were analyzed by sequence detector 1.6 program (PE Applied Biosystems). All quantitative PCR analyses were performed with identical lots of reagents and stock solutions. The PCR amplification profile for each of genes examined involved an initial denaturation step at 95°C for 10 min. Each temperature cycle consisted of 94°C for 30 sec., annealing step for 30 sec. and 72°C for 30 sec. The primer annealing step was as follows: ACE 55°C, AT1 60°C, glyceraldehyde phosphate dehydrogenase (GAPDH) 58°C (Table 1). Expression of GAPDH mRNA served as internal standard and mRNA amounts of target gene were normalized for each sample with respect to the corresponding GAPDH amounts. Then, the score in the pressurized state was expressed relative to a control condition, and 0 hrs sample was used as standard. To ensure that RT-PCR products were not amplified from genomic DNA, all RNA samples were additionally amplified without reverse trasncriptase. In all the cases, PCR of RNA samples was negative in the absence of reverse transcriptase (data not shown).

RESULTS

Several features of VCS

The three main module that constitute VCS are the control valve, pump unit and culture chamber. Furthermore, this system also use universal joint of connecting tube and disposable parts, we could easily assemble modules and modify entire circuit. With the change of culture chamber, it provide a wide variety of cell culture. For example, we have developed suspension cell chamber for monocytic cells and adhisive cell chamber for human umbilical vein endothelial cells. This system also offers constant or pulsatile pressure ranging from 0-760 mmHg. We already have a pulsatile pressure system, in which peristaltic pump and solenoid valve are used. In this study, constant magnitude of pressure is regulated by both medium flow rate and adjusting the control valve. We did not recirculate the medium because the conditioned medium obtained from the cells may contain humoral factors which can be secondarily secreted in response to pressure. Medium flow rate of 4 ml/hr seems to have enough amount of nutrients compared with medium change of routine cell culture. Inner wall of chamber was coated with silicon oil and therefore cell could not attach the wall, which completely exlude the possibility of flow induced shear stress. THP1 exposed to elevated hydrostatic pressure demonstrated no detectable decrement in cell viability as assessed by Trypan blue exclusion and there were no significant differences in growing pattern, cell size, and cell shape compared to control (unpublished observations), which means pressure does not promote differentiation of THP1. These results implied VCS was not detrimental to the cells.

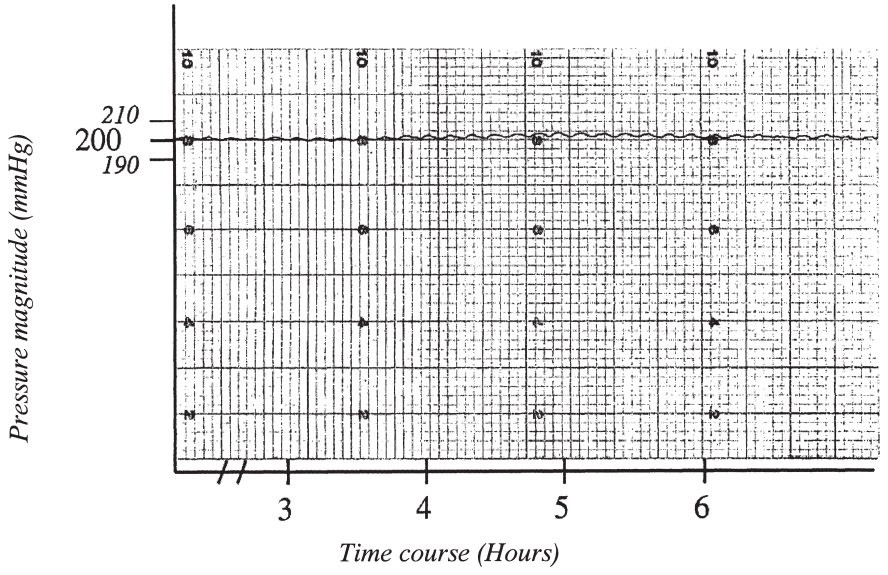

Fig. 2. Representative data of 200 mmHg constant pressure monitoring to which monocytic cells were exposed

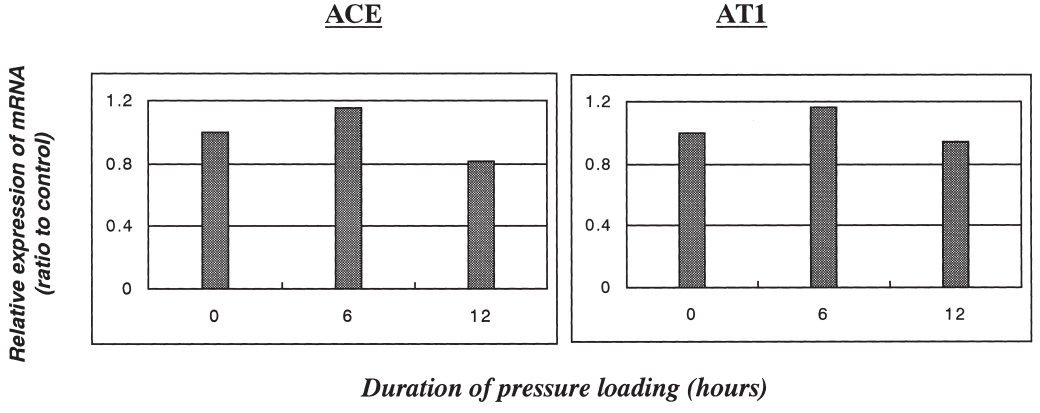

Fig. 3. The ratio of ACE and AT1 mRNA expression in THP-1 under 100 mmHg versus control pressure conditions The ratio of both ACE and AT1 mRNA level was elevated (ACE ; 1.16 fold, AT1 ; 1.16 fold) at 6 hours after the initiation of pressure loading. Data shown are the mean for triplicate analysis of the three independent experiments.

Effects of hydrostatic pressure on gene expression of ACE and AT1

Expression of GAPDH mRNA was found not to be affected by hydrostatic pressure and was thus used to normalize other target mRNA for validation in PCR. Fig. 3 shows a ratio of ACE and AT1 mRNA expression in THP1 under 100 mmHg versus control pressure condition. The ACE mRNA was elevated at 6 hours and then declined. At the 6 hrs, we observed 1.16-fold increase and at the 12 hrs, a 0.81-fold decrease of the ACE mRNA levels. The AT1 mRNA also demonstrated similar pattern. At the 6 hrs, we observed 1.16-fold increase and at the 12 hrs, a 0.94-fold decrease of the AT1 mRNA levels. Fig. 4 shows a ratio of ACE and AT1 mRNA expression in THP1 under 200 mmHg versus control pressure condition. The ACE mRNA was dropped at 6 hours and then sustained. At the 6 hrs, we observed 0.52-fold induction and at the 12 hrs, a 0.64-fold induction of the ACE

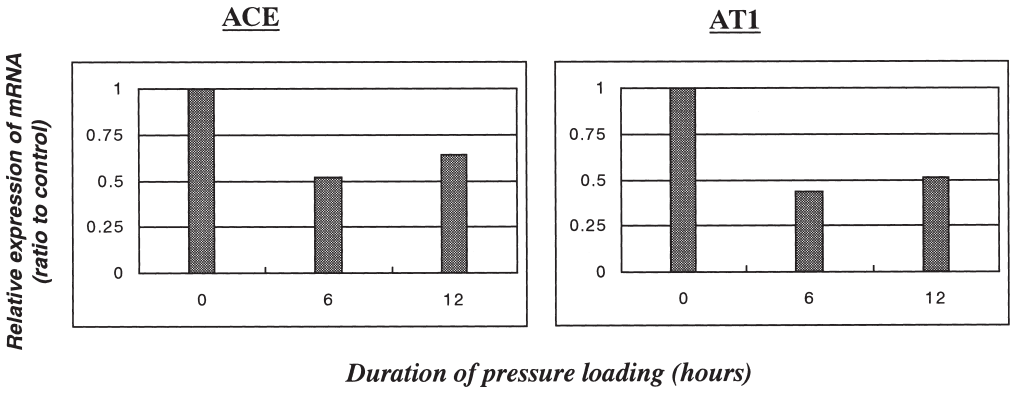

Fig. 4. The ratio of ACE and AT1 mRNA expression in THP-1 under 200 mmHg versus control pressure conditions The ratio of both ACE and AT1 mRNA level was dropped (ACE ; 0.52 fold, AT1 ; 0.44 fold) at 6 hours after the initiation of pressure loading. Data shown are the mean for triplicate analysis of the three independent experiments.

mRNA levels. The AT1 mRNA also demonstrated similar pattern. At the 6 hrs, we observed 0.44-fold induction and at 12 hrs, a 0.51-fold induction of the AT1 mRNA levels. Both ACE and AT1 mRNA expression show some tendency of alteration at different magnitude of pressure, although the difference was not significant for each time point.

DISCUSSION

The mammalian circulation generates a number of complex physical forces within the vascular system. Shear stress, stretch, and hydrostatic pressure are the major hemodynamic forces. In addition, the role of hemodynamic forces in the pathogenesis of vascular disease is receiving increased attention. The participation of shear stress and stretch in vascular structure and functions has been widely and intensely investigated by well-recognized system[17]. Although a few number of *in vitro* models have been used to examine the biology of hydrostatic pressure, there are several requirements for previous pressure-loading apparatus will be widely used. Tokunaga and Watanabe reported that the behavior of human umbilical vein endothelial cells (HUVEC) in culture was affected by pressure[18]. Utilizing a sealed T-300 culture flask to which a syringe needle, connected to a manometer, was inserted, they were able to subject endothelial cells (EC) or smooth muscle cells (SMC) to constant pressure up to 160 mmHg. They noted a transient increase in ^{3}H-thymidine incorporation in EC by day 2 when exposed to 80 mmHg pressure, which declined to lower levels on continued exposure. Acevedo and colleagues have described a model for subjecting bovine pulmonary artery EC in culture to pressure[19]. EC are grown on glass coverslips and placed at the base of a long enclosed culture well. By altering the height of the media (1.5-15 cm), they altered the hydrostatic pressure on the EC in culture. These investigators noted time and pressure-dependent morphological changes in endothelial cells, with a more elongated shape and locally coaligned cell clusters. They also observed EC with fibroblast-like microfilament patterns growing in multiple layers, as opposed to typical monolayers, that could be easily disturbed and noted that cell proliferation was enhanced at increasing hydrostatic pressure. Transfer of conditioned media to freshly plated EC or fibroblasts caused similar proliferative and morphological changes. A number of potential problems exist with these hydrostatic models. The different levels of fluid can produce differences in pO_2 and pCO_2 with different levels of hydrostatic pressure. There is also a diffusion/transport issue, due to the increase in volume of media overlying the cells which may result in artifact the conditioned media studies. Lastly, the physiologic significance of the level of hydrostatic pressure in unclear since 15 cm H_2O corresponds to 11 mmHg. In addition, there are some reports showing that pressure promotes the proliferation of rat cul-

tured SMC[20], EC[21], and rat mesangial cells[22], by using other pressure-loading apparatus, which were able to subject adhesive cells to constant pressure up to 120 mmHg. In these system, compressed air or helium gas was pumped into the sealed chamber, in which culture dish was set. These pressure-loading apparatus has an advantage to enable culture cells at the same condition of pO_2, pCO_2, and pH at under atmosphreic pressure. However, it was difficult to exchange the culture medium without unsealing the chamber and inevitably causing elevated pressures of the plates to fall to atmospheric pressure. Based upon these points, we developed a new pressure-loading apparatus VCS, which has several advantages over previous systems. In the present study, this VCS model (constant pressure/suspension cell chamber) has been developed specifically for monocytic cells and medium replenishment was attained by flow of the system. VCS also enabled us to culture cells at the same conditions of pO_2, pCO_2, and pH as under atmospheric pressure, even if the pressure was high. Furthermore, this VCS is easy to assemble, reproducible, and provide diverse utility.

There is evidence that hydrostatic pressure plays a considerable role in vascular alterations associated with hypertension. Changes in the hydrostatic pressure associated with altered blood flow has especially direct effects on circulating blood cells (ie, monocytes). A growing number of recent reports have clarified the effects of mechanical stress on vascular wall cells, but its role on monocyte has been less precisely defined. The present study was an attempt to clarify the effects of hydrostatic pressure alone on RAS alteration from human monocytic cells. The local RAS is considered an important autocrine -paracrine system for the regulation of vascular tone and structure[8,9]. It is well known that systemic as well as glomerular hypertension is a trigger of the remodeling process in target organ of Ang II including heart and kidney[23]. Previous studies have demonstrated that mechanical stretch of cultured rat cardiac myocytes cause rapid secretion of Ang II and that this autocrine production of Ang II plays a critical role in many, if not all, stretch-induced hypertrophic responses[24,25]. Furthermore, Malhotra and colleagues also indicate that mechanical stretch of rat cardiac myocytes upregulate expression of ACE and AT1 and suggest the possibility of further amplification of Ang II[26].

Altered expression of RAS genes under pressure condition at 6 hrs could be supported by the study of shear stress. Recent evidence indicates that steady fluid shear stress (6 hrs duration) regulates the expression of a number of endothelial products in a specific fashion. Endothelin 1 (ET1) and platelet-derived growth factor B (PDGF-B), factor which cause both vasoconstriction and increase smooth muscle and fibroblast growth, show decreased expression under physiological steady shear stress in a magnitude-dependence fashion[27]. In contrast, elevated shear increases production of basic fibroblast growth factor (bFGF), which induce growth of both EC and SMC[28]. Previously, Hishikawa et al have demonstrated that pressure from 40 to 120 mmHg promote DNA synthesis of SMC in a pressure-dependent manner[20]. ET1 production also revealed similar results[29]. So, it may be possible that around 200 mmHg, which is the upper limit of physiological pressure, saturation behavior of vasoregulatory molecules would be observed. Although the precise reason of sustained decrease expression of RAS genes under 200 mmHg are not clear, it would be due to the deactivation of pressure-sensitive mechnoreception, or minor cell injury.

Unfortunately, the present *in vitro* study failed to show the significant difference between the control and pressurized conditions. The reason for these nonsteady-state control during this time period may be a small number ($n=3$) and sample to sample variability. In addition, it may reflect differences in levels of cell growth state and underscore the difficulty of modeling *in vitro* fluid mechanical environments. However, these results may show coordinate tendency of ACE and AT1 mRNA expression at each magnitude of pressure. This imply the possibility of RAS genes showing reverse behavior at magnitudes between 100 mmHg and 200 mmHg.

ACE is localized mainly on endothelial cells and angiotensinogen is synthesized in the media and adventitial layers[30]. Indeed, Riedar et al have suggested that elevation in the level of shear stress alter

endothelial cell function by suppressing ACE gene and protein expression *in vitro* and *in vivo*[13]. Thus, it is likely that hydrostatic pressure has the similar potential to alter ACE and AT1 mRNA of monocytic cells and it may participate in the local activation of RAS in autocrine manner. The concept of the local control of vascular function by locally synthesized compounds has been recently described. We also have hypothesized that locally synthesized RAS genes from monocytic cells may play a definite role in the regulation of both vascular tone and structure. It should be noted that mRNA measurements alone do not provide insight into a functional role of the local RAS, although they are an important precondition for the investigation of such a role.

In conclusion, circulating blood cells *in vivo* undergo dynamic and static mechanical force from superimposed cardiac loads, and this hydrostatic pressure may be important regulator of vascular wall remodeling. We demonstrated that hydrostatic pressure has a possibility of regulatory factor of monocytic cells by influencing autocrine RAS genes. Although our model does not perfectly recreate the flow conditions that occur *in vivo*, it goes significantly beyond previous models. Finally, it is tempting to speculate that perhaps one or more of these local mechanisms of growth factors, cytokines, and vasoregulatory molecules become activated when hydrostatic pressure is high. The characterization of these mechanisms will be a subject for future studies.

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