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High pressure conditions promote the proliferation of rat cultured mesangial cells in vitro

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Abstract

Glomerular capillary pressure is involved in the development of chronic renal failure and has at least two effects on mesangial cells: transmembrane hydrostatic pressure and stretch. To clarify whether pure hydrostatic pressure itself affects the proliferation of cultured rat mesangial cells, we compared the cell number under atmospheric pressure condition with high pressure condition. At 24 and 48 h with 0.5% serum, cell number was significantly higher under high pressure condition than under atmospheric pressure condition. At 48 h, cell number under high pressure condition was increased in a pressure-dependent manner. Furthermore, flow cytometric assay indicated that pressure-load could promote DNA synthesis rate at S phase and enhance G_1/S progression induced by low concentration of serum (0.5%). These results suggest that pure hydrostatic pressure itself can promote the proliferation of cultured rat mesangial cells by advancing cell cycle progression in vitro. © 1998 Elsevier Science B.V.

Keywords: Mesangial cell; High pressure condition; Proliferation; DNA synthesis; Cell cycle

1. Introduction

Glomerular capillary hypertension could be a major factor in the development of chronic renal failure, in which progressive glomerular sclerosis is commonly observed [1–3]. Although the pathogenesis of glomerular sclerosis is not yet clear, it is known to be accompanied by expansion of the mesangium with antecedent cell proliferation [4]. Anatomically glomerular mesangial cells, which support glomerular capillaries, are in apposition to a fenestrated capillary endothelium and are exposed to glomerular capillary hydrostatic pressure necessary to sustain a normal glomerular filtration rate [5]. In glomerular capillary hypertension, mesangial cells are assumed to be exposed to high pressure [6].

Glomerular capillary pressure has at least two major effects on mesangial cells: pure hydrostatic pressure and stretch, but separating these effects in vivo is difficult. Some investigators have examined the effects of repeated stretch on cultured mesangial cells [7–9]. In addition, there are some reports, showing that pressure promotes the proliferation of rat cultured smooth muscle cells [10] and endothelial cells [11], by using pressure-loading apparatus. However, detailed information is little about the effect of pure pressure itself on mesangial cells. In this study,

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we examined the effects of hydrostatic pressure applied by a pressure-loading system without stretch on the proliferation of rat cultured mesangial cells.

2. Materials and methods

2.1. Mesangial cells

Rat mesangial cells were isolated with a method previously described by Harper et al. [12]. Briefly, 6-week-old male Wistar rats (150–210 g, Charles River, Kanagawa, Japan) were anesthetized with diethyl ether and killed with a guillotine. Kidneys were dissected and renal cortical tissues were minced with scissors under sterile condition. The minced renal cortical tissues were passed through a series of sieves of 0.3, 0.15, and 0.075 mm in pore size (Iida Seisakusho, Osaka, Japan), and trapped glomeruli were rinsed in calcium–magnesium-free Hanks' balanced salt solution, pH 7.4, and incubated with 0.2% trypsin for 20 min at 37°C. After washing twice in Hanks' balanced salt solution, glomeruli were resuspended in RPMI 1640 (Cosmo Bio, Tokyo, Japan) with 100 U/ml of penicillin, 100 μg/ml of streptomycin, and 20% bovine fetal calf serum (FCS, Upstate Biotechnology, New York, USA); plated at a density of 5×10^3 glomeruli per dish on 60 mm plastic dishes; and incubated at 37°C in a humidified atmosphere of 5% CO_2–95% air. After 2 to 3 weeks, outgrowing cells were passed to subcultures and maintained in RPMI 1640 with 20% FCS. Cells were identified as mesangial cells by their growth pattern, morphologic features, and positive staining for Thy1.1, α-actin, and myosin and negative staining for cytokeratin and factor VIII. Experiments were carried out using cells from the 7th through 15 passages. All dishes used were made by Falcon (Becton Dickinson Labware, Lincoln Park, NJ, USA).

2.2. Incubator and pressure-loading apparatus

The small chamber (Fig. 1) which was resealable acrylic chamber, was designed to fit inside a standard incubator. This chamber had three openings: entrance and exit ports for compressing air and a hole for passing wires to a pCO_2/pO_2 sensor (Microgas 7640, Kontron Instruments, Tokyo, Japan) and a pH sensor

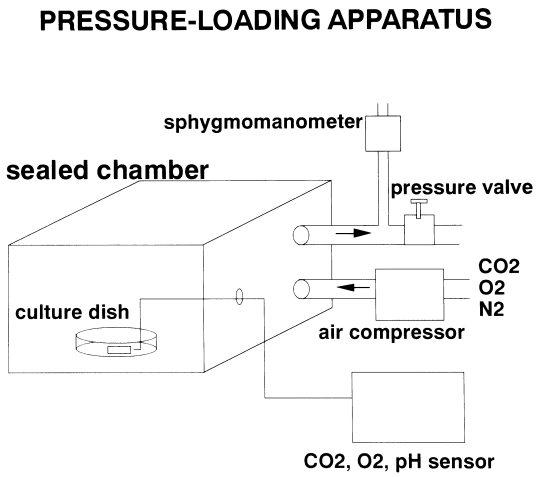

Fig. 1. Schematic diagram of the high pressure chamber in the pressure-loading apparatus. This high pressure chamber was set up in a standard CO_2 incubator, and the air in the incubator was compressed into the high-pressure chamber.

(Digitrapper Mk III, Synectics Medical, Stockholm, Sweden). The entrance port was connected with a tube to an air compressor (Astec, Fukuoka, Japan), and the exit port was connected with a tube to a sphygmomanometer (Handy Manometer, Copal Electronics, Tokyo, Japan) and an air-release valve with controller (Astec). Constant pressure ranging from 0 to 120 hectopascal (hPa) (0–90 mmHg) could be achieved by compressing air and allowing air to fill the chamber to the appropriate level. When the air pressure in the small chamber was increased, the values of pO_2 and pCO_2 in the chamber were slightly changed. By changing the composition of the compressed air in advance (such as $O_2 = 20.8\%$, $CO_2 = 5.0\%$ in atmospheric pressure, $O_2 = 20.0\%$, $CO_2 = 4.8\%$ in 40 hPa, $O_2 = 19.5\%$, $CO_2 = 4.7\%$ in 67 hPa, $O_2 = 19.0\%$, $CO_2 = 4.6\%$ in 93 hPa, $O_2 = 18.6\%$, $CO_2 = 4.5\%$ in 120 hPa), we could keep pO_2 and pCO_2 concentrations of the air in the small chamber almost equal to those of the atmospheric air ($pO_2 = 205 \pm 0.7$ hPa, $pCO_2 = 49 \pm 0.7$ hPa). In this study, rat mesangial cells were exposed to five conditions at 37°C: atmospheric pressure condition (APC) and four high pressure conditions (HPCs) by 40, 67, 93, and 120 hPa (30, 50, 70, and 90 mmHg) in addition to atmospheric pressure (0 hPa). Measurements confirmed that pH was identical ($pH = 7.4 \pm 0.05$) in each medium of this regulated condition.

2.3. Preparation of cells

Mesangial cells in growing were plated at a density of 5×10^4 cells per dish in 35 mm culture dishes and at a density of 5×10^5 cells per dish in 100 mm culture dishes. After cells were incubated in the serum-free RPMI 1640 medium for 24 h, the medium was changed to the one containing 0.5% FCS and the cells were incubated for 24 and 48 h in APC and HPCs at 37°C. Cells in 35 mm dishes were used for direct cell counting and those in 100 mm dishes were used for flow cytometry.

2.4. Cell counting

After medium in dishes was removed, 0.3 ml of 0.25% EDTA and 2.7 ml of 0.2% trypsin were added to each dish for several minutes. After confirming removal of all cells, samples were mildly agitated to obtain a homogeneous suspension. One ml of an aliquot was used to count the cell number with Coulter Counter (Coulter Electronics, Hialeah, FL, USA).

2.5. Cell culture with conditioned medium

To examine the effects of conditioned medium alone on cell proliferation, we tried experiments as below. After we incubated mesangial cells in the serum-free medium under APC and an HPC (93 hPa) for 24 h, each conditioned medium was applied to another quiescent cultured mesangial cells for 24 h with 5% CO_2–95% air at 37°C. Cell counting was done as shown above.

2.6. Flow cytometry

2.6.1. DNA histogram

After washing with phosphate-buffered saline (PBS), cells were trypsinized and resuspended in 1 ml of PBS and fixed in 4 ml of 100% ethanol at 4°C. DNA staining was achieved with propidium iodide (50 μg/ml) after treatment with 0.1% RNase (Sigma, St. Louis, MO, USA).

2.6.2. Bivariate DNA / BrdU distribution

Cells were incubated with 10 μM of bromodeoxyuridine (BrdU, Sigma) for 30 min at each time point for pulse-label experiments, or incubated for 12 h for continuous label experiments. After washing with PBS, cells were trypsinized and resuspended in 1 ml of PBS and fixed in 4 ml of 100% ethanol at 4°C. BrdU incorporation was detected with a modification of the method previously described by Dolbeare et al. [13]. DNA denaturation was done with 4 N HCl at room temperature for 30 min. Between all following steps, cells were washed in 7 ml of Borax–Borate buffer (pH 8.9) and centrifuged at 1200 rpm. BrdU and DNA staining was done with a standard two-step procedure as follows. Both the primary antibody (anti-BrdU monoclonal antibody, Becton Dickinson, San Jose, CA, USA) and fluorescein isothiocyanate (FITC)-conjugated goat $F(ab')^2$ anti-mouse IgG (Caltag Laboratories, CA, USA) as a secondary antibody were used at a dilution of 1:50 in 1 ml of PBS containing 0.5% Tween 20, 0.5% bovine serum albumin, and 10% normal goat serum for 30 min at room temperature. DNA staining was achieved with 0.5 ml of propidium iodide (30 μg/ml) after treatment with 0.1% RNase to remove double-strand RNA.

Samples were filtered with nylon mesh and were used for the flow cytometric analysis with a flow cytometer (FACScan, Becton Dickinson, Sunnyvale, CA, USA). Fluorescence intensity was measured at a wavelength of 488 nm. At least 1×10^4 events were acquired for each sample.

To analyze bivariate DNA/BrdU distribution for pulse-labelling experiment, mean FITC-fluorescence intensity of cells at mid S phase, showing BrdU incorporation, was calculated. To analyze DNA/BrdU distribution for continuous labelling experiment, BrdU-positive cells at S phase, showing G_1/S progression, were counted.

2.7. Statistical analysis

The results are expressed as means ± S.E. and statistical significance was assessed by analysis of variance post hoc Scheffe's F test. Values of $P < 0.05$ were considered significant.

3. Results

3.1. Morphological appearances

There were no significant differences in growing pattern, cell size, and cell shape of mesangial cells

between APC and HPC (93 hPa)with phase-contrast light microscope (data not shown). There were also no significant differences in staining of cells for Thy1.1, α-actin, and myosin between APC and 93 hPa.

3.2. Proliferation assay

To synchronize mesangial cells at G_0/G_1 phase of cell cycle, they were incubated in serum-free medium for 24 h before the start of the experiments. To promote a slow, sustained rate of growth, mesangial cells were grown in RPMI 1640 with 0.5% FCS and pressurized for 24 and 48 h. In APC, there were no significant changes in cell number at 0, 24, and 48 h (Fig. 2). However, cell numbers in HPCs (40, 67, 93, and 120 hPa) at 24 h were significantly higher than in APC. Furthermore, cell numbers at 48 h were even higher in a pressure-dependent manner (Fig. 2). The trypan blue exclusion test showed that more than 90% of mesangial cells were viable throughout the experiments.

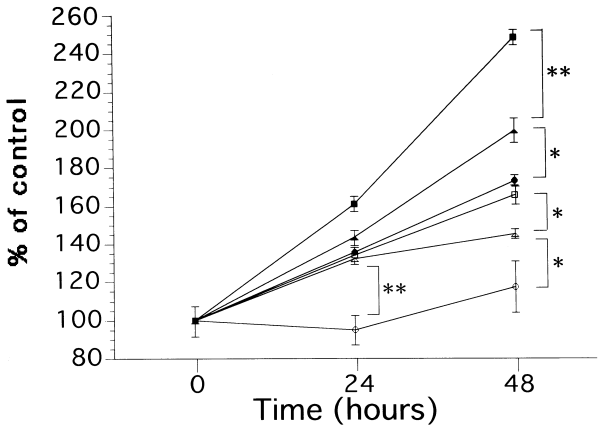

Fig. 2. Percent of control in mesangial cell proliferation at 24 and 48 h. This experiment was started after incubating with serum-free medium for 24 h and the cell number at that time was used as control. Cells were incubated for 48 h with 0.5% FCS medium. Cell counting was performed at 24 and 48 h under APC (○) and HPCs of 40 (△), 67 (□), 93 (●), and 120 (▲) hPa. Cells incubated with 10% FCS medium (■) under APC are shown for comparison. Data are shown as means ± S.E. $* P < 0.05$, $** P < 0.01$, $n = 6$.

Table 1
The cell numbers after incubation for 24 h both in APC-derived and HPC-derived conditioned media

	cell number ($\times 10^4$) ($n = 6$)
control	9.1 ± 0.3
APC-derived conditioned medium (at 24 h)	10.0 ± 0.1
HPC-derived conditioned medium (at 24 h)	9.6 ± 0.2

The cell number before incubation in conditioned medium is shown as control.

3.3. Cell culture with conditioned medium

To investigate the contribution of humoral factors that might be secondarily secreted in response to pressure-load, experiments using conditioned medium were done. After synchronization at G_0/G_1 phase, mesangial cells were incubated at atmospheric pressure for 24 h in APC-derived and HPC-derived conditioned media that were obtained from dishes in which cells had been exposed to APC or an HPC (93 hPa) for 24 h. There were no significant differences in the percent increase in cell number between APC and HPC (Table 1).

3.4. Flow cytometric assay

3.4.1. DNA histogram

To elucidate the mechanisms by which pressure-load promotes cell proliferation, we measured DNA contents of mesangial cells incubated in an HPC (93 hPa) and APC. After serum-starvation for 24 h, mesangial cells were incubated with RPMI 1640 supplemented with 0.5% FCS for 24 h, then cells were collected. There were no significant differences in DNA histograms between these two groups (Fig. 3). In addition, each proportion of G_1, S and G_2/M phase did not significantly differ between APC and 93 hPa HPC (Table 2).

3.4.2. BrdU incorporation rate at S phase

For further analysis of the effect of pressure, BrdU incorporation rate and DNA content of mesangial cells were measured simultaneously with flow cytometric analysis (Fig. 4). The incorporation of pulse-labelled BrdU into a cell, reflecting DNA synthesis rate, was shown as a value of FITC fluorescence intensity, while DNA content of a cell was deter-

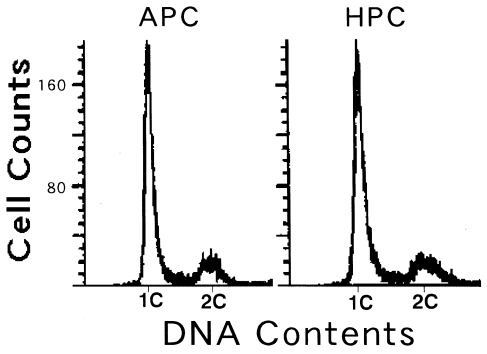

Fig. 3. DNA histograms of mesangial cells incubated for 24 h under APC and HPC. After mesangial cells were incubated with serum-free medium for 24 h, they were stimulated for 24 h with 0.5% serum under APC and 93 hPa HPC. There were no significant differences in the DNA histogram between APC and HPC.

mined by measuring propidium iodide fluorescence intensity. We compared mean FITC-fluorescence intensity of cells at mid S phase in 93 hPa HPC with that in APC. To synchronize cells at G_1 phase, they were incubated with serum-free medium for 24 h before the experiment. Stimulation with pressure-load and low concentration of serum significantly increased BrdU incorporation rate at mid S phase in 93 hPa HPC in comparison with APC at 24 h and 48 h (Fig. 4B). These findings indicate that pressure-load can increase DNA synthesis rate stimulated by low concentration of serum.

3.4.3. Analysis of G_1/S progression by continuous labelling method

To examine the effect of pressure-load on cell cycle progression, we measured continuous labelling index of BrdU which was percentage of cells labelled with BrdU continuously for various times. After synchronization at G_1 phase, BrdU was added at 0 h, and samples were fixed at each time point after stimulation with either 93 hPa pressure-load plus 0.5% serum or only 0.5% serum. Bivariate DNA/BrdU distribution shows that cells enter into S phase and BrdU incorporation occurs prosperously (Fig. 5A). Histogram shows the percentage of BrdU-positive cells at each time (Fig. 5B). The labelling indices increased in proportion to time after addition of BrdU.

Table 2
The proportion of each phase in the cell cycle of mesangial cells at 24 h under APC and HPC

	G_1 (%)	S (%)	G_2/M (%)
APC ($n=6$)	74.4 ± 1.6	17.5 ± 2.3	8.1 ± 1.6
HPC (70 mmHg) ($n=6$)	76.6 ± 2.0	14.1 ± 1.5	9.3 ± 1.3

There were no significant differences in the proportion of each phase between APC and HPC.

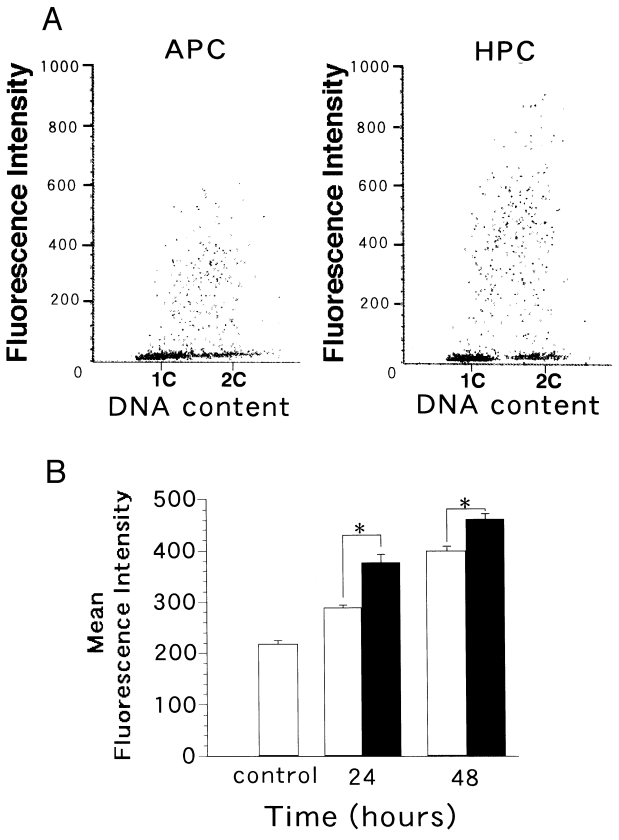

Fig. 4. (A) Effects of pressure-load on bivariate DNA/BrdU distributions measured for cultured rat mesangial cells after pulse-labelling with 10 μM BrdU for 30 min at 24 h. Dot plots showing BrdU content (y axis) and DNA content (x axis). After serum-starvation for 24 h, mesangial cells were stimulated with 0.5% serum under APC and 93 hPa HPC for 24 h. Mean fluorescence intensity at mid-S phase, which was proportional to DNA synthesis rate, was significantly higher in HPC than in APC. (B) Mean fluorescence intensities at mid S phase of mesangial cells incubated under APC and HPC (93 hPa) at 24 and 48 h. The values after serum-starvation for 24 h are shown as control. The mean fluorescence intensity under HPC is significantly higher than that under APC at 24 and 48 h. (□): APC, (■): HPC. $* P < 0.01$, $n = 6$.

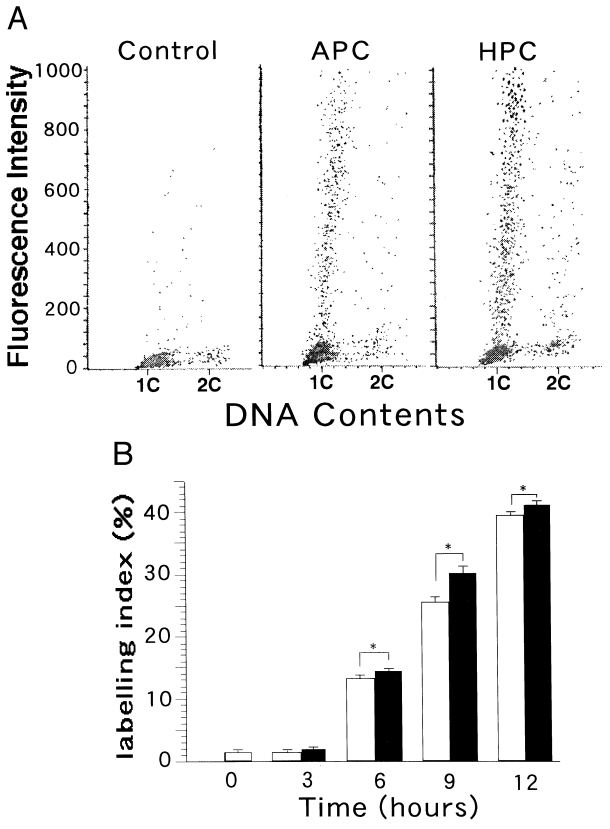

Fig. 5. (A) Bivariate DNA/BrdU distributions measured for mesangial cells after continuous labelling with 10 μM BrdU for 30 min (Control), for 9 h under APC (APC), and for 9 h under 93 hPa HPC (HPC). Dot plots showing BrdU content (y axis) and DNA content (x axis). After serum-starvation for 48 h, mesangial cells were stimulated with 0.5% serum under APC and 93 hPa HPC, and cells were collected at each time point. The proportion of BrdU-positive cells at S phase in total were significantly higher in HPC than in APC. (B) Time course of the proportion of BrdU-positive cells at S phase. The proportion of BrdU-positive cells at S phase are higher in HPC than in APC at each time point. (□): APC, (■): HPC. $* p < 0.05$, $n = 6$.

It is known that the rise of labelling indices are in accord with the rate of entry into S phase [14,15]. By time course of our bivariate DNA/BrdU distributions, cells do not yet enter into S phase before the time point of 3 h, and cells begin to move out of S phase and through G_2/M phase after 12 h (data not shown). It was therefore thought that the increase of labelling indices between 3 h and 9 h could reflect the rate of G_1/S progression in this experiment. Significant increase of labelling indices was recognized during the second 3 h (3–6 h) and the third 3 h (6–9 h) in HPC as compared to APC. These results indicate that pressure-load can enhance G_1/S progression induced by low concentration of serum.

3.4.4. Time course of the S phase in serum-free medium under HPC

In mammalian cells, growth factors are usually necessary for G_1/S progression [16]. To examine whether pure pressure-load itself without serum could progress G_1 phase cells into S phase, we compared the time course of S phase on DNA histogram in HPC with that in APC while cells were incubated with serum-free medium. The proportion of S phase was time-dependently decreased both in HPC and APC (Fig. 6). This finding indicated that cells in G_1 phase were inhibited entering S phase by serum-starvation. It was suggested that the effect of just pressure itself without serum was insufficient for G_1/S progression, although pressure-load could enhance cell proliferation induced by low concentration of serum. The proportion of S phase cells in HPC was decreased more rapidly than that in APC under such a condition that G_1/S progression was inhibited by serum starvation (Fig. 6). This result suggests that DNA synthesis rate in S phase cells was increased under HPC as compared with APC. Comparison of these findings with the results of DNA synthesis rate

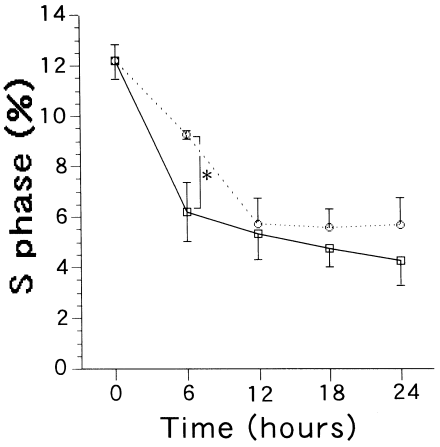

Fig. 6. Time course of S phase under APC and HPC (93 hPa) in serum-free medium. After serum-starvation for 24 h, mesangial cells were incubated in serum-free RPMI 1640 medium and cells were collected at each time point. Results are expressed as the percent population of S phase on a DNA histogram. (○): APC, (□): HPC. $* P < 0.05$, $n = 5$.

in Fig. 4 showed good agreement. Our data indicate that HPC can promote DNA synthesis rate in S phase as well as can enhance G_1/S progression induced by low concentration of serum.

4. Discussion

In this study we compared effects of hydrostatic pressure in HPCs from 40 to 120 hPa applied by means of a pressure-loading apparatus with that in APC on the proliferation of cultured mesangial cells as a model of glomerular capillary hypertension. Our results showed that pressure-load significantly increased both of cell number and DNA synthesis rate. In addition, our data indicated that pressure-load could enhance G_1/S progression induced by 0.5% serum.

We developed a pressure-loading apparatus with which pressure within a chamber can easily be regulated. As pO_2, pCO_2, and pH of the medium were measured within the sealed chamber, we could easily correct changes in the values due to high pressure by altering the composition of the air. Therefore, our pressure-loading apparatus 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.

Glomerular capillary hydraulic pressure averages 44 mmHg (59 hPa) in Munich-Wistar rats that have normal renal function, while it averages 67 mmHg (89 hPa) in uninephrectomized spontaneously hypertensive rats in which glomerulosclerosis develops at an accelerated rate [17,18]. We therefore compared cell proliferation in the pressure ranging from 0 (APC) to 120 hPa with our pressure-loading system.

In the present study, we found that cell proliferation in HPCs ranging from 40 to 120 hPa was increased in a pressure-dependent manner at 48 h. To examine the involvement of humoral factors, mesangial cells were incubated in APC- and HPC (93 hPa)-derived conditioned media. Cell number did not differ significantly between these conditions. Thus, these results suggest that pressure itself promotes cell proliferation with minimal participation of humoral factors. Cheng et al. reported that a single transient mechanical stimulus increased DNA synthesis in human vascular smooth muscle cells by autocrine or paracrine release of fibroblast growth factor-2 [19]. As their experiment was done with three-dimensional collagen gel cultures using smooth muscle cells, which were subjected to compression, those differences between their findings and ours could be attributed to the differences of magnitude and mechanism in force and the type of cell.

Morphologically, there were no significant differences in mesangial cells between under APC and under HPC with light microscope. In yeast and bacteria, no alterations in the subcellular structure were observed electron microscopically below 100 MPa (0.1 MPa = 750 mmHg), and the nuclear membrane was more susceptible to pressure (at > 100 MPa) than cell membrane [20,21]. The possibility exists that hydrostatic pressure can transmit the force to the nucleus without any deformities of cell membrane as well as with deformities of cell membrane, although electron microscopic changes of mesangial cells by hydrostatic pressure are not yet examined and the pressure used in our study is below 120 hPa.

Flow cytometric assay with BrdU-labelled cells demonstrated that pressure-load could promote DNA synthesis rate in the S phase. Besides, it indicated that pressure-load could enhance G_1/S progression induced by low concentration of serum. These findings suggest that pressure-load can enhance the cell cycle progression in each phase, in other words, it can transmit the signal for proliferation to the mitotic system in the nucleus.

In conclusion, we demonstrated that pressure-load itself could promote cell proliferation by enhancing cell cycle progression, especially by enhancing G_1/S progression induced by low concentration of serum and promoting DNA synthesis rate at S phase. What type of stress occur to cell by pressure-load and what type of signal transduction pathway is activated by stimuli derived from pressure-load are not yet known, so further examination will be needed.

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