that are transmitted to glomerular cells, including to the capillary wall. The increased physical forces are important factors that cause glomerular sclerosis (6, 8). Increased intraglomerular pressure is directly transmitted to at least two physical forces: mechanical stretch and high transmural pressure. Increased transmural pressure, as well as mechanical stretch, promotes MC proliferation, which may enhance glomerulosclerosis. The exact mechanism of this effect is not fully understood. We examined the effects of transmural pressure alone on cell proliferation and DNA synthesis and investigated the role of platelet-derived growth factor (PDGF) and basic fibroblast growth factor (bFGF), candidates for mediation of glomerular diseases, in the pressure-induced events. Pressure was applied to cultured MC placed in a sealed chamber using compressed helium gas. Application of pressure resulted in a time-dependent (–2 h) and pressure level-dependent (–80 mmHg) increase in cell number (1.4-fold) and [3H]thymidine incorporation (2.7-fold).

Pressure-induced DNA synthesis was significantly suppressed by inhibitors of phospholipase C (2-nitro-4-carboxy-isoquinolinylsulfonyl)-2-methylpiperazine and chelerythrine), transient formation of inositol 1,4,5-trisphosphate, which was blocked by the phospholipase C inhibitor. Pressure also promoted a rapid increase in tyrosine kinase activity. Pressure increased mRNA levels of PDGF-B, with a peak at 6 h, but not those of PDGF-A or bFGF. Pressure-induced DNA synthesis was partially inhibited by a neutralizing antibody to PDGF but not by an antibody against bFGF or nonimmune IgG. Our results indicated that pressure by itself increases DNA synthesis and proliferation of cultured rat MC possibly through activation of protein kinase C and tyrosine kinases, and PDGF-B could be partially involved in these pathways.

In the present study, we examined the effects of pressure alone on cell proliferation and DNA synthesis and the mechanism of intracellular signal transduction using cultured rat MC subjected to a high pressure. We also investigated the role of PDGF or bFGF in the pressure-induced cell proliferation.
PRESSURE PROMOTES MESANGIAL CELL PROLIFERATION

MATERIALS AND METHODS

Materials. Multiwell plates (6 and 24 wells) and 150-mm cell culture flasks were purchased from Iwaki (Tokyo, J apan). 1-(5-Isoquinolinylsulfonyl)-2-methylpiperazine (H-7), genistein, and GdCl₃ were obtained from Sigma (St. Louis, MO). Chelerythrine was from Funakoshi (Tokyo). Cellulose acetate filters (0.45 mm thick) were from Whatman (Gettmgen, Germany). [³H]Thymidine and an inositol 1,4,5-triphosphate (IP₃) kit were from Amersham Japan (Tokyo), and [¹²⁵I]cAMP and [¹²⁵I]cGMP radioimmunoadsay kits were from Yamasa (Chiba, J apan). A tyrosine kinase assay ELISA kit was from Takara Biomedicals (Kyoto, J apan). DMEM and 0.25% trypsin-EDTA were from GIBCO (Life Technologies, Rockville, MD). FCS was from Sanko J unyaku (Tokyo). Neutralizing antibodies against PDGF (polyoncal mouse anti-human PDGF-AB) and bFGF type-1 (monoclonal mouse anti-bovine bFGF) were obtained from Upstate Biotechnology (Lake Placid, NY). 2-Nitro-4-carboxyphenyl-N,N-diphenylcarbamate (NCDC), 1,1,2-trichloro-1,2,2-trifluoroethane (TCTFE), and other chemicals were from Nacalai Tesque (Kyoto, J apan).

MC cultures. Rat MC were obtained from the intact glomeruli of 4-wk-old Wistar rats prepared by using the sieving method previously described by Osajima et al. (27) from our laboratory. The correct cell type was confirmed by the contraction reaction with angiotensin II (5). In brief, MC were grown in DMEM supplemented with 20% FCS, 200 µg/ml penicillin, and 100 µg/ml streptomycin at 37°C in a humidified atmosphere of 95% air-5% CO₂. The cells were detached by using 0.25% trypsin-EDTA and seeded on plates. To set the cells in the quiescent stage, cells were cultured with DMEM containing 20% FCS for 24 h, changed to DMEM containing 0.5% FCS, and cultured for additional 48 h, then changed to fresh serum-free DMEM before the experiments. Cells were used between passages 5 and 10.

Pressure loading apparatus. We used the pressure-loading apparatus previously described by Oishi et al. (26), with slight modifications. The apparatus consisted of a resalable steel chamber with inlet and outlet ports (Miwa, Osaka, J apan). The inlet port was connected through a tube to a reservoir of compressed helium, whereas the exit port was connected through a tube to a sphygmonanometer and an air-release valve. Compressed helium gas was pumped in the chamber to raise the internal pressure. During the delivery of helium gas into the apparatus, no prepacked room air was released, so that the partial pressures of the gases originally contained in the chamber, such as oxygen, nitrogen, and carbon dioxide, were kept constant (15), consistent with Boyle-Charle's law, as described previously (26). The plates (24-well) used for assays of cell proliferation and DNA syntthesis were placed on a warm plate (37°C) inside the chamber. The partial pressure of oxygen, temperature, and pH of the incubation medium in the plates remained constant throughout the experiments. Cell viability was assessed by staining with trypan blue and light microscopy and was always >90% throughout experiments. The cells appeared morphologically intact, and the number of detached cells was negligible during experiments.

DNA synthesis rate. [³H]Thymidine incorporation assay was performed as described previously (26). MC were seeded at a density of 5 × 10⁴ cells/well in 24-well plates, rendered quiescent, and subjected to a pressure loading from 40 to 120 mmHg for 30–120 min. After 22 h following pressurization, 1 µCi/ml [³H]thymidine was added to each well, and the cells were further incubated for an additional 2 h. Cells were washed twice with ice-cold PBS, once with 5% (wt/wt) trichlo-roacetic acid, and once with ethyl alcohol/diethyl ether (3:1, vol/vol), and cells were then harvested with 0.3 M NaOH. After neutralization with 0.6 M HCl, the suspension was passed through a cellulose acetate filter, and the retained radioactivity was determined with a liquid scintillation spectrometer (LS5000; Beckman, Fullerton, CA).

Cell proliferation. Cells were seeded at a density of 5 × 10⁴ cells/well in 24-well plates and rendered quiescent, after which they were pressurized with helium at pressures of 40 to 80 mmHg for 30–120 min. The cells were then incubated in an incubator (95% air-5% CO₂) at 37°C for 48 h, detached with trypsin, and the cell number was counted with the Coulter Counter (Coulter Electronics, Luton, UK) (32).

Effects of inhibitors for a phospholipase C and protein kinases or GdCl₃ on pressure-induced DNA synthesis. To investigate the mechanism of pressure-induced DNA syntthesis, cultured MC in 24-well plates were treated with inhibitors for phospholipase C (20 µM NCDC), protein kinase C (10 µM H-7 and 5 µM chelerythrine), or tyrosine kinases (5 µM genistein) for 30 min before and during the pressurization. To examine the role of stretch-activated mechanosensitive channels in pressure-induced DNA synthesis, GdCl₃ at a concentration (10 µM) that inhibits the channel activity (12) was treated for 30 min before and during pressurization.

Measurement of IP₃ production. After cells were subjected to 80 mmHg pressure loading for 30, 60, 90, 120, 180, or 300 s, ice-cold 70% (wt/wt) perchloric acid was rapidly injected into each well (24-well plates) with a syringe and needle to give a final concentration of 10% (vol/vol). The cells were maintained on ice for 15 min, scraped, sonicated, and centrifuged at 10,000 g for 5 min at 4°C as previously described (26). The resulting supernatant (800 µl) was transferred to a polypropylene tube containing 200 µl of 10 mM EDTA, and then 1.4 ml of TCTFE/tri-n-octyamine (1:1, vol/vol) was added to each tube. The tubes were capped, shaken vigorously, and maintained at room temperature for 3 min. After centrifugation at 10,000 g for 5 min, 800 µl of the resulting supernatants was assayed for IP₃ by a [³H]IP₃ assay kit (Amersham).

Measurement of tyrosine kinase activity. After cells were subjected to 80 mmHg pressure loading for 1, 2 or 10 min, cells were washed twice with ice-cold PBS, then scraped and homogenized. The cell lysates were centrifuged at 10,000 g for 10 min, and 100 µl of supernatant was assayed for tyrosine kinase activity with a tyrosine kinase assay ELISA kit according to the manufacturer (Takara, Kyoto, J apan).

Measurement of cAMP and cGMP. For assay of cyclic nucleotides, cells containing 0.5 mM IBMX in DMEM were subjected to a pressure of 80 mmHg for up to 60 min, as previously described (27). After reaction, cells were washed twice with ice-cold PBS, added to 0.1 N HCl (1 ml), scraped, transferred to tubes, and boiled for 5 min. The cell lysates were centrifuged at 3,000 g for 5 min, and 100 µl of the supernatant was assayed for cAMP and cGMP with [¹²⁵I]cAMP and [¹²⁵I]cGMP assay kits, respectively (Yamasa).

Semiquantitation of RT-PCR. MC, rendered quiescent, were homogenized, and total RNA was extracted with a RNeasy kit (Qiagen, Hilden, Germany). RT was performed using a first-strand cDNA synthesis kit (Pharmacia Biotech, Tokyo) with a random primer supplied with the kit. The reaction mixture was incubated for 60 min at 37°C and heated for 5 min at 90°C in a thermal cycler (Takara Biomedicals, Kyoto, J apan). For the PCR, the primers used were based on the sequences of rat cDNAs in the GenBank database, and they were as follows: PDGF-A, sense 5'-CTCTTCACTCCATCGGA-3', antisense 5'-CTCCTTCTACACTGTC-3'; PDGF-B, sense 5'-CTCCTTCTGTTCTCTGTCG-3', antisense 5'-GCCACTGCTTCACTGGCA-3'; bFGF, sense 5'-CAGATCCACGCCAGCATCGG-3', antisense 5'-CTCTTCAGTCCGACGACG-3'; and for RT-PCR, MC, rendered quiescent, were homogenized, and total RNA was extracted with a RNeasy kit (Qiagen, Hilden, Germany). RT was performed using a first-strand cDNA synthesis kit (Pharmacia Biotech, Tokyo) with a random primer supplied with the kit. The reaction mixture was incubated for 60 min at 37°C and heated for 5 min at 90°C in a thermal cycler (Takara Biomedicals, Kyoto, J apan). For the PCR, the primers used were based on the sequences of rat cDNAs in the GenBank database, and they were as follows: PDGF-A, sense 5'-CTCTTCACTCCATCGGA-3', antisense 5'-CTCCTTCTACACTGTC-3'; PDGF-B, sense 5'-CTCCTTCTGTTCTCTGTCG-3', antisense 5'-GCCACTGCTTCACTGGCA-3'; bFGF, sense.
Effects of duration and level of pressure on DNA synthesis. Application of pressure at 80 mmHg to MC significantly promoted the incorporation of \([^{3}H]\)thymidine in a time-dependent manner (Fig. 1A). At room air pressure, \([^{3}H]\)thymidine incorporation did not change throughout the observation period. We also examined the dose-response to step changes in the applied pressure. Application of pressure for 60 min resulted in a significant increase in \([^{3}H]\)thymidine incorporation, which was proportional to the level of applied pressure (between 40 and 80 mmHg, Fig. 1B). However, a higher pressure of 120 mmHg tended to decrease \([^{3}H]\)thymidine incorporation compared with pressurization at 80 mmHg (Fig. 1B). Treatment of cells with 1% or 10% FCS for 24 h increased \([^{3}H]\)thymidine incorporation to 190 ± 22% (n = 8, P < 0.001) or 726 ± 23% (n = 8, P < 0.001) of the control level, respectively. Hence, the increased DNA synthesis rate by 80 mmHg pressure seems to be almost comparable to that caused by 1% FCS in our study.

Effects of duration and level of pressure on cell proliferation. Application of pressure at 80 mmHg to the cells significantly increased cell numbers in a time-dependent manner (Fig. 2A). At 0 mmHg, the cell number remained stable throughout the observation period (data not shown). Application of 0 to 80 mmHg pressure on the cells for 60 min significantly increased cell number in a pressure-dependent manner (Fig. 2B). Incubation of cells with 10% FCS for 24 h increased cell number to 152 ± 23% (n = 8, P < 0.001) of the control level. Based on the results shown in Figs. 1 and 2, application of pressure at 80 mmHg for 60 min was selected in the remaining experiments.
Effect of pressure on IP$_3$ production. Pressure at 80 mmHg significantly increased IP$_3$ level at 30 s (2.4-fold rise from the basal level), but the rise was transient and soon decreased to basal levels (within 90 s, Fig. 4). NCDC (20 µM) inhibited the pressure-induced IP$_3$ production without affecting the basal level (Fig. 4 and data not shown). Angiotensin II (100 nM), a well-known compound that increases IP$_3$ levels in MC (28), also stimulated the rapid formation of IP$_3$ at 30 s (2.2-fold rise from basal level), and its level returned to the basal level within 90 s (Fig. 4).

Effect of pressure on tyrosine kinase activity. After application of pressure at 80 mmHg to MC, the level of tyrosine kinase activity was significantly increased at 1 min to 122 ± 4% of the control, and the level remained constant at least at 10 min. In contrast, the levels of tyrosine kinase activity in nonpressurized MC remained unchanged for up to 10 min (Fig. 5).

Effect of pressure on cAMP and cGMP production. Application of pressure at 80 mmHg for 10, 30, or 60 min had no effect on intracellular cAMP and cGMP levels in MC (data not shown). On the other hand, treatment of cells for 10 min with adrenomedullin (100 nM) and atrial natriuretic peptide (100 nM), compounds known to increase cAMP and cGMP levels in MC, respectively (27), increased the nucleotide levels approximately 5- to 6-fold and 150- to 165-fold, respectively.
Effects of pressure on expression of mRNA levels of PDGF-A, PDGF-B, or bFGF in MC. We examined the effects of 80 mmHg pressure applied for 60 min on mRNA expression of PDGF-A, PDGF-B, or bFGF 24 h later. PDGF-B mRNA increased significantly at 3 h with a peak at 6 h but progressively decreased thereafter to basal levels at 24 h (Fig. 6, A and B). In contrast, the mRNA levels of PDGF-A and bFGF remained unchanged throughout the experiment (Fig. 6, A and B).

Effect of neutralizing antibodies against PDGF or bFGF on pressure-induced DNA synthesis in MC. Addition of a neutralizing antibody against PDGF at a concentration of 100 or 1,000 ng/ml to the incubation medium significantly reduced pressure-induced DNA synthesis to 42 ± 16% and 26 ± 14%, respectively. However, at 10 ng/ml, the antibody had no effect on the pressure-induced events (Fig. 7). In contrast, an antibody against bFGF, even at a high concentration (1,000 ng/ml), had no effect on pressure-induced DNA synthesis. Neutralizing antibodies against PDGF (1,000 ng/ml) or bFGF (1,000 ng/ml) did not change DNA synthesis in nonpressurized MC (Fig. 7). In addition, a nonimmune IgG did not affect the mitogenic response of pressure (data not shown).

DISCUSSION

Glomerular hypertension results in at least two major effects: a high transmural pressure and stretch on MC (13, 16, 29). Previous studies have shown that application of stretch to MC increases cell proliferation and synthesis of extracellular matrix, both of which consequently resulted in glomerulosclerosis (13, 29). In the present study, we have developed an experimental setup that allows examination of the effect of pressure on MC proliferation without stretching, and clearly demonstrated that cell proliferation and DNA synthesis increased proportionally with the magnitude of applied pressure. Our results showed that the maximal increase in DNA synthesis and cell proliferation occurred at a pressure level of 80 mmHg. The level is

Fig. 5. Effects of pressure on tyrosine kinase activity. MC in serum-free DMEM were incubated in absence (○) or presence (●) of pressure at 80 mmHg for indicated periods, and tyrosine kinase activity was determined with a tyrosine kinase assay ELISA kit. Data are means ± SD (n = 4–6). *P < 0.05 compared with values at 0 min, 0 mmHg (control, 2.696 ± 0.226 U/well).

Effects of pressure on expression of mRNA levels of PDGF-A, PDGF-B, or bFGF in MC. We examined the effects of 80 mmHg pressure applied for 60 min on mRNA expression of PDGF-A, PDGF-B, or bFGF 24 h later. PDGF-B mRNA increased significantly at 3 h with a peak at 6 h but progressively decreased thereafter to basal levels at 24 h (Fig. 6, A and B). In contrast, the mRNA levels of PDGF-A and bFGF remained unchanged throughout the experiment (Fig. 6, A and B).

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Fig. 7. Effects of neutralizing antibodies against PDGF or bFGF on pressure-induced [³H]thymidine incorporation. Quiescent MC were subjected to a pressure of 80 mmHg for 1 h in absence or presence of indicated concentration of a neutralizing antibody against PDGF (10, 100, and 1,000 ng) (left) or bFGF (10, 100, and 1,000 ng) (right). Twenty-two hours later, cells were incubated for 2 h with [³H]thymidine, then DNA synthesis was determined. Data are means ± SD (n = 6). *P < 0.05 compared with 0 mmHg (1,717 ± 91 cpm/well). +P < 0.05 compared with 80 mmHg.

almost identical to that transmitted to the glomerular capillary wall of five-sixths renal ablation rat, a model of advanced renal failure (4). Based on these results, we suggest that the continuously elevated transmural pressure, in addition to cyclical stretching (13, 16, 18, 19, 29), enhances the proliferation of MC and may lead to sclerosis of glomeruli in advanced renal failure (9).

In MC, as well as vascular smooth muscle cells (23), stretch has been reported to induce the expression of c-fos through an activation of phospholipase C/protein kinase C pathway (3, 13, 16). In our study, pressure-induced DNA synthesis was significantly inhibited not only by NCDC, H-7, or chelerythrine but also by genistein, indicating that pressure-induced DNA synthesis is mediated by a phospholipase C/protein kinase C and a tyrosine kinase pathway. Our tyrosine kinase assay result also supports the involvement of tyrosine kinase pathway in the pressure-induced events. In rat vascular smooth muscles or intestinal epithelial cells, such dual kinase involvement in cell proliferation was proposed in the signaling mechanism through which pressure promoted cell proliferation (14, 15). Our present results are different from those recently reported by Kawata et al. (20). These investigators showed that pressure-induced proliferation of MC was significantly inhibited by genistein but not by protein kinase C inhibitors, chelerythrine and GF-109203X. The reason for this discrepancy is not known at present, but it may be due to differences in the experimental setup such as the pressure-loading apparatus (air vs. helium) or cell culture conditions.

Our results also showed that the applied pressure resulted in a rapid but transient increase in IP₃ level, which was inhibited by NCDC. This finding suggests the involvement of phospholipase C activation and presumably an increase in intracellular Ca²⁺ from the IP₃-sensitive Ca²⁺ stores (24) in pressure-induced DNA synthesis. The present results are essentially similar to those reported by Hishikawa et al. (15), who showed that application of pressure to cultured rat vascular smooth muscle cells increased IP₃ levels, intracellular Ca²⁺ concentrations, and DNA synthesis in these cells. However, the time-course studies indicated that pressure-induced changes in IP₃ production were transient, whereas those obtained in vascular smooth muscle cells were sustained (15). The exact mechanisms of the difference in IP₃ pressor response between MC and smooth muscle cells are not clear at present; however, the differences may be due to experimental conditions or cell types employed.

Shear stress as well as stretch is reported to regulate cell functions through activation of a phospholipase C/protein kinase C pathway in various cell types (3, 13, 15, 16, 22, 23). Both mechanical forces also activate adenylate cyclases and guanylate cyclases in some cells (22, 25, 32). Our results showed that a phospholipase C/protein kinase C but not cAMP or cGMP pathway was involved in the transmural pressure-induced MC proliferation. Kawata et al. (20) showed that increased pressure activated tyrosine kinases and mitogen-activated protein kinases, which resulted in increase in cell proliferation. Nonetheless, the mechanism by which mechanical forces activate these protein kinases is not well defined so far. It has been reported that a stretch-activated ion channel or a mechanosensitive ion channel could be a candidate to transduce signals from mechanical forces to cell membranes (12). Such channel is reported to be present in MC (7). However, our results suggest that the channel is not involved in pressure-induced DNA synthesis since GdCl₃, a stretch-activated mechanosensitive inhibitor, failed to inhibit the pressure-induced events. At present, in our study, we do not have clear explanation how the cell senses increased pressure. Taken together, the present findings suggest an important role for phospholipase C/protein kinase C- and tyrosine kinase-dependent mechanisms (but not mechanosensitive- or cyclic nucleotide-mediated mechanisms) in pressure-induced DNA synthesis in cultured MC.

MC are known to express mRNAs for PDGF-A, PDGF-B, and bFGF and to secrete high amounts of PDGF and bFGF in response to several growth factors (1, 2, 9, 30). The present study clearly demonstrated that pressure alone significantly and specifically in-
creased the level of PDGF-B mRNA expression, but it did not change the mRNA levels of PDGF-A and bFGF. This finding is consistent with previous in vivo results showing that the expression of PDGF-B mRNA in glomeruli was markedly increased in five-sixths nephrectomized rats with glomerular hypertension (9). The results of experiments using antibodies further confirmed the above findings; the neutralizing antibody against PDGF partially inhibited pressure-induced DNA synthesis, whereas antibody against bFGF and a nonimmune IgG had no effect. This finding is similar to the immunohistochemical results reported by Floege et al. (9), who demonstrated increased expression of glomerular PDGF-B protein in proliferating MC. The fact that PDGF-B had more potent mitogenic effects than PDGF-A and bFGF in cultured MC (1, 9, 10) may support our interpretation that PDGF-B but not PDGF-A and bFGF is involved, at least in part, in the pressure-induced proliferation of MC. Since MC are exposed to a continuously high level of transmural pressure in glomerular hypertension, the mRNA level of PDGF-B is probably increased in MC in such pathological conditions. Recent reports have shown that mechanical stretch of MC enhanced the expression of PDGF mRNA and that stretch-induced cell proliferation was inhibited by neutralizing antibodies against PDGF (18, 19). Based on these results, it is likely that PDGF plays an important role in the pathogenesis of MC proliferation induced not only by mechanical stretch but also by transmural pressure in glomerular hypertension.

Shear stress and stretch are reported to increase mRNA expression for PDGF through activation of a phospholipase C/protein kinase C pathway in various cell types including MC (17–19, 33). Since we demonstrated that a phospholipase C/protein kinase C pathway was responsible for the pressure-induced cell proliferation, this pathway may be involved in the pressure-induced PDGF gene expression. We showed that genistein inhibited the pressure-induced DNA synthesis, suggesting the involvement of tyrosine kinase pathways in the pressure-induced events. However, it is not certain whether genistein inhibits tyrosine kinases responsible for induction of PDGF gene expression, whether it inhibits tyrosine kinase-coupled PDGF receptor signaling pathways activated by PDGF, which is synthesized by the pressure, or whether it inhibits both pathways. On the other hand, increased pressure may be perceived as a stressor by MC, resulting in stress responses mediated by stress-related protein kinases such as c-jun NH2-terminal kinases (J NK) or stress-activated protein kinases (SAPK) (11). However, a recent study by Kawata et al. (20) showed that applied pressure to cultured MC promoted the activation of mitogen-activated protein kinases but not J NK. Even if J NK is activated in response to pressure in our study, it is not clear whether J NK activation changes mRNA expression for PDGF, which in turn, enhances cell proliferation. There is no report as to whether activations of stress-induced J NK/SAPK increase PDGF gene expression in MC. Therefore, it is not clear at present why increased pressure upregulates mRNA for PDGF. Further studies are required to clarify the detailed mechanism.

In conclusion, our results showed that pressure by itself promotes DNA synthesis in cultured rat MC through activations of protein kinase C and tyrosine kinase pathways. PDGF-B, presumably secreted from MC in response to increased levels of transmural pressure, could be partially involved in these pathways. Our results point to the importance of lowering blood pressure in glomerular hypertension to modulate the proliferation of MC.

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