transmural pressure and stretch (13, 16, 29). It has been reported that application of cyclic stretch to cultured rat MC enhances cell proliferation and increases extracellular matrix (13, 18, 19, 29) and proteoncogene c-fos (3), through activation of both protein kinase C and S6 kinase (16). These findings suggest that stretching of MC is important in the pathogenesis of mesangial expansion and the development of glomerulosclerosis. Recently, Kawata et al. (20) have reported that a high pressure also enhances MC proliferation through activation of mitogen-activated protein kinases, although the detailed signaling mechanism through which this pressure promotes cell proliferation remains unknown at present.

Growth factors such as platelet-derived growth factor (PDGF) and basic fibroblast growth factor (bFGF) can potentially mediate many biological responses of MC (1, 2, 9, 10), and they are most potent mitogens of MC in vivo (1, 9, 10). PDGF and bFGF are released not only from activated platelets, macrophages, and endothelial cells but also from MC (9, 30) and have also been reported to stimulate MC proliferation in vitro (1, 2, 30). These results suggest that PDGF and bFGF possibly contribute to the proliferation of MC via an autocrine and/or paracrine manner (1, 2, 9). In a rat remnant kidney used as a model of advanced renal failure (4, 6, 9), MC proliferation precedes the development of glomerulosclerosis and is associated with an increased expression of PDGF mRNA and its protein in the glomeruli (9). Recent studies have demonstrated that in cultured rat MC exposed to stretch, the production of extracellular matrix components was increased in parallel with the increment of PDGF mRNA expression, thus suggesting the possible role of PDGF in the pathogenesis of glomerulosclerosis associated with glomerular hypertension (18, 19). However, it remains to be determined whether pressure directly induces such gene expression.

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.

GLOMERULAR CAPILLARY HYPERTENSION is one of the most important factors that cause glomerular sclerosis (6, 8). Increased intraglomerular pressure is directly transmitted to the capillary wall. The increased physical forces that are transmitted to glomerular cells, including endothelial and mesangial cells (MC), are likely to participate in the pathogenesis of glomerulosclerosis (13, 21, 29). Since the mesangium occupies a central location and is surrounded by glomerular capillaries, MC are believed to perceive at least two physical forces: transmural pressure and stretch (13, 16, 29). It has been reported that application of cyclic stretch to cultured rat MC enhances cell proliferation and increases extracellular matrix (13, 18, 19, 29) and proteoncogene c-fos (3), through activation of both protein kinase C and S6 kinase (16). These findings suggest that stretching of MC is important in the pathogenesis of mesangial expansion and the development of glomerulosclerosis. Recently, Kawata et al. (20) have reported that a high pressure also enhances MC proliferation through activation of mitogen-activated protein kinases, although the detailed signaling mechanism through which this pressure promotes cell proliferation remains unknown at present.

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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 (Germantown, Germany). [3H]thymidine and an inositol 1,4,5-trisphosphate (IP3) 3H radioreceptor assay kit were from Amersham Japan (Tokyo). 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 synthesis, 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 (4 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- octylamine (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, 80 µl of the resulting supernatants was assayed for IP3 with 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 lysate was 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'-CagATCCACAGCATCCgggA-3', antisense 5'-CTCCTCTAACCCTCACCTgggA-3'; PDGF-B, sense 5'-CTCCTCTCtGtCTCTGttG-3', antisense 5'-gCCACTgTCTCACACTTgCA-3'; bFGF, sense 5'-NCCAGTCCTGACTGTC-3'.
RESULTS

Effects of duration and level of pressure on DNA synthesis. Application of pressure at 80 mmHg to MC significantly promoted the incorporation of [3H]thymidine in a time-dependent manner (Fig. 1A). At room air pressure, [3H]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 [3H]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 [3H]thymidine incorporation compared with pressurization at 80 mmHg (Fig. 1B). Treatment of cells with 1% or 10% FCS for 24 h increased [3H]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.

Effects of inhibitors of phospholipase C and protein kinases or GdCl3 on pressure-induced DNA synthesis. Treatment of cells with NCDC (20 µM) significantly decreased DNA synthesis induced by pressure alone to 62 ± 3%. H-7 (10 µM) and chelerythrine (5 µM) inhibited pressure-induced DNA synthesis to 40 ± 17 and 56 ± 10%, respectively. Genistein (5 µM) also significantly inhibited pressure-induced DNA synthesis to 52 ± 10% (Fig. 3). These inhibitors had no effect on the basal level of [3H]thymidine incorporation at each experiment (data not shown). The stretch-activated mechanosensitive inhibitor GdCl3 at a concentration of 10 µM failed to inhibit the pressure-induced DNA synthesis (Fig. 3).
Effect of pressure on IP₃ production. Pressure at 80 mmHg significantly increased IP₃ 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 IP₃ production without affecting the basal level (Fig. 4 and data not shown). Angiotensin II (100 nM), a well-known compound that increases IP₃ levels in MC (28), also stimulated the rapid formation of IP₃ 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.

Fig. 2. Effects of duration (A) and intensity (B) of pressure on MC proliferation. A: MC in serum-free DMEM were incubated in absence (○) or presence (●) of pressure at 80 mmHg for indicated time intervals, and cell number was counted 48 h after cessation of pressure. Data are means ± SD (n = 8). *P < 0.05 compared with values at 0 min, 0 mmHg (control, 77,100 ± 260 cells/well). B: MC in serum-free DMEM were subjected to a high pressure for 1 h at indicated pressure levels, and cell number was determined 48 h after pressure. Data are means ± SD (n = 8). *P < 0.05 compared with values at 0 min, 0 mmHg (control, 57,800 ± 300 cells/well).

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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 ± 6% 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. 7. Effects of neutralizing antibodies against PDGF or bFGF on pressure-induced \[^{3}H\]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 \[^{3}H\]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.

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⁺ 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-
increased 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 is probably increased in MC in such pathologic 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 (JNK) 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 JNK. Even if JNK is activated in response to pressure in our study, it is not clear why state-induced JNK activates mRNA expression for PDGF, which in turn, enhances cell proliferation. There is no report as to whether activations of stress-induced JNK/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.

We thank A. Sugimoto for technical assistance.

This work was supported by Ministry of Education, Science and Culture of Japan Grant 09707861 and by a grant from Renal Anemia Foundation, Tokyo, Japan (to A. Osajima).

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Received 20 October 1998; accepted in final form 30 March 1999.

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