BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **233,** 81–85 (1997)
ARTICLE NO. RC976399

Osteopontin Gene Expression and Protein Synthesis in Cultured Rat Mesangial Cells

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Received February 13, 1997

It is controversial whether osteopontin (OP) is expressed in glomeruli and involved in glomerular diseases. We examined whether the OP expression is present at gene and protein levels in cultured rat mesangial cells (MCs). Northern blotting revealed a 1.7 kb OP-mRNA expression in MCs. Fetal calf serum (FCS) and TNF-α increased OP gene expression in serum-starved MCs by 2.7- and 1.8-fold over 24- and 12-hour periods, respectively. PDGF, IL-1β, and TGF-β had little effect on OP gene expression. Western blotting detected the OP protein expression (69 kDa). FCS and TNF-α increased OP protein expression in serum-starved MCs over 48- and 24-hour periods, respectively. The present study clearly demonstrated the expression of OP gene and protein in cultured rat MCs. Increased OP production under serum or TNF-α stimulation suggests that intraglomerular OP may contribute to the development of glomerular diseases.

Osteopontin (OP) is a sialic acid-rich, non-collagenous bone phosphoprotein that strongly binds to the calcium phosphate-based bone matrix (hydroxylapatite). It possesses an RGD-cell attachment sequence capable of interacting with the vitronectin receptor ($\alpha_v\beta_3$ integrin) and facilitating cell attachment and spreading (1, 2, 3). As well as being a product of human bone sialoprotein I (4), osteopontin has been independently identified, as a rat 44-K bone phosphoprotein (5, 6), as a product of early T cell activation gene 1, Eta-1 (7), and as uropontin, an inhibitor of calcium oxalate crystallization (8, 9). Furthermore, OP has been reported to be expressed in various kinds of cells, such as osteoblasts (1, 10), vascular endothelial and smooth muscle cells (11, 12). OP has been reported to participate in chemotaxis (13), migration (14), pathological calcification (11, 15), and nitric oxide synthesis (16), presumably in a paracrine manner, via $\alpha_v\beta_3$ integrin (17).

It has been shown that OP mRNA is abundantly expressed in the kidney, compared to that in other tissues (18, 19). Several investigations using immunohistochemistry or *in situ* hybridizations have suggested that OP is localized within tubular epithelial cells of distal convoluted tubuli, the ascending thick limb of Henle's loop, and Bowman's capsule in normal rodents (20, 21). OP expression is known to dramatically increase under pathological conditions, such as tubulointerstitial nephritis (21, 22), in which OP is presumed to induce chemotactic migration of macrophages/monocytes (21-23). Luminal localization of OP in renal tubuli has been reported to be related to inhibition of renal stone formation (8, 9).

However, it is unclear whether OP expression is induced in glomeruli under certain pathologic conditions (21-23). Therefore, we examined whether OP gene is expressed in cultured mesangial cells (MCs) and whether important cytokines/factors acting in glomerular pathology modulate its expression.

MATERIALS AND METHODS

Materials. Fetal calf serum (FCS) and insulin-transferrin-sodium selenite (ITS mixture) were purchased from Gibco BRL(Grand Island, NY) and Sigma Chemical Co. (St. Louis, MO), respectively. Mouse tumor necrosis factor-α (TNF-α), human platelet derived growth factor-BB (PDGF-BB), human interleukin-1β (IL-1β), and human transforming growth factor-β (TGF-β) were purchased from Genzyme (Cambridge, MA). A polyclonal anti-OP antibody was raised in rabbits by immunizing with human OP which was isolated from human milk, using method similar to that described elsewhere (24). This antibody was confirmed to cross-react with rat, mouse and human OP.

Cells and cell culture. Glomeruli of the kidneys from Sprague-Dawley rats were isolated by the sieving method (25). MCs, obtained from the outgrowths of isolated glomeruli (25), were cultured in plastic flasks or plates in Dulbecco's Modified Eagle Medium (DMEM,

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0006-291X/97 $25.00

GIBCO) supplemented with 10% FCS (GIBCO), penicillin (100 units/ml), and streptomycin (100 mg/ml) at 37 °C in a humidified atmosphere containing 5% CO_2. MCs of the 4th to 7th passages were used for the experiments. MCs were serum-starved for 12 hours by culturing them in DMEM with ITS mixture (5 μg/ml insulin, 5 μg/ml transferrin, 5 ng/ml sodium selenite, at final concentration). After 12-hour serum-starvation, MCs were treated with the various reagents and further incubated for the indicated periods to harvest total RNAs or cellular proteins.

Preparation of cDNA probes. A rat OP cDNA probe containing a 411-base pair fragment (corresponding to 332 to 742) in the coding region was obtained by reverse-transcription (RT) of an mRNA from rat primary vascular smooth muscle cells (1), followed by polymerase chain reaction (PCR) and subcloning into a TA cloning vector (Invitrogen, San Diego, CA). Sequences of the obtained cDNA were confirmed by the didoxy sequencing method (26).

Northern blotting. Total RNA from MCs was extracted by using acid guanidinum thiocyanate-phenol-chloroform method (27). For Northern blot analysis, total RNA was denatured with 6% formaldehyde, fractionated by 1% agarose gel electrophoresis, and transferred to a nylon membrane (Hybond-N, Amersham, Buckinghamshire, UK). The membranes were hybridized in a buffer containing 50% formamide, 3 × SSC (1 × SSC; 0.15 M NaCl and 0.015 M sodium citrate, pH 7.4), 50 mM Tris-HCl, 20 mg/ml tRNA, 20 mg/ml boiled salmon sperm DNA, 1 mM EDTA, 0.02% bovine serum albumin, 0.2% polyvinylpyrolidone, and 0.02% Ficoll, with ^{32}P-labeled rat OP, or rat glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) (28) cDNA as a probe at 37 °C for 48 hours. After washing, filter membranes were exposed to X-ray film at − 70 °C for detection of hybridized RNA. The DNA probes used were electrophoretically purified from agarose gels and labeled with [α-^{32}P]dCTP by using a random priming method (Megaprime DNA labeling system, Amersham). For quantification of the changes in OP mRNA expression, OP/GAPDH mRNA ratio was used after densitometric estimation of each band.

Western blotting. After washing culture plates with ice-chilled phosphate-buffered saline (PBS) three times, MCs were scraped off from the culture plates on ice, and dissolved in a lysing buffer (150 mM NaCl, 1.0% Nonidet P-40, 0.5% deoxycholic acid, 0.1% sodium diphosphate, 50mM Tris-HCl) which contains phenylmethylsulfonyl fluoride (PMSF) (1 mM) and aprotinin (2 mg/ml). Protein contents were measured by using a BCA protein assay kit (Pierce, IL). The protein extracts were then electrophoresed under reducing conditions on 11% polyacrylamide gels and transferred to nitrocellulose filters. Membranes were stained for OP with specific rabbit anti-OP antibody as a primary antibody at a dilution of 1:3,000, then a horseradish peroxidase-conjugated goat anti-rabbit antibody as a secondary at a dilution of 1:100. The immune complexes were detected using a chemiluminescence kit (ECL, Amersham).

Statistical analysis. All results are expressed as the mean ± SE and statistical significance was tested using one-way analysis of variance (ANOVA) and multiple comparisons (Scheffe's *F* type).

RESULTS

Expression of OP gene in MCs. As preliminarily exploring gene expression of OP in MCs, we performed RT/PCR of OP gene fragment by using the primers as described in Methods. A fragment of the predicted length was amplified in MCs as compared with that in rat primary vascular smooth muscle cells as a positive control (Data not shown). To further confirm the expression of OP gene, Northern blot analysis was performed by using rat OP cDNA prepared as described. A 1.7 kb OP mRNA was detected in MCs. To examine

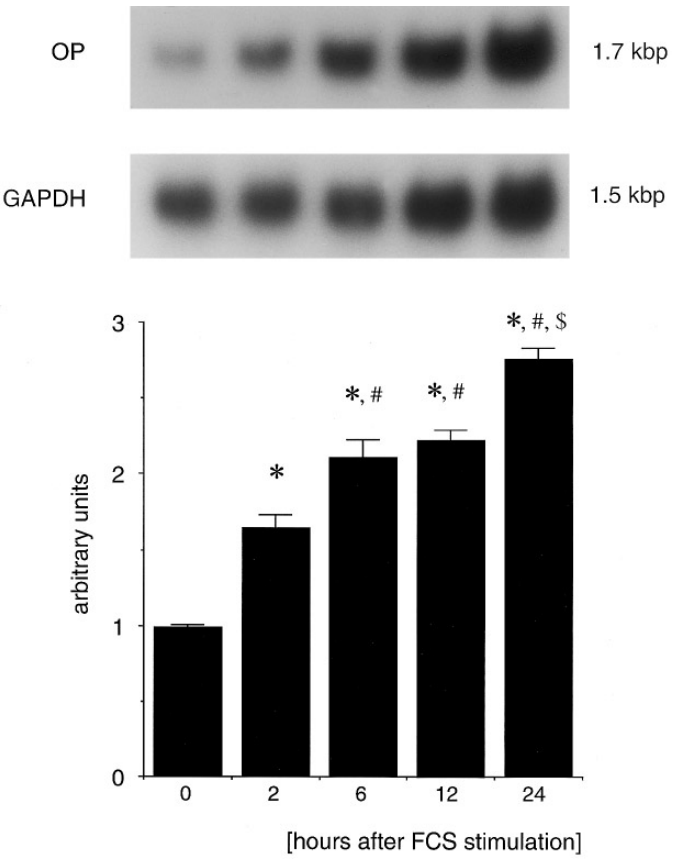

FIG. 1. Effect of FCS on OP mRNA expression. Northern blotting (upper panel) and its densitometric analysis (lower panel) were carried out as described in *Methods*. After MCs were cultured for 12 hours in serum free medium, medium containing 10% FCS was added, and RNA was extracted before (0) and 2, 6, 12, 24 hours after stimulation. The autoradiogram shown in the upper panel is representative of three separate experiments. Densitometric analysis (lower panel) showed a significant increase in OP/GAPDH mRNA ratio at 24 hours. *; $p < 0.01$ vs 0 hour. #; $p < 0.01$ vs 2 hour. $; $p < 0.01$ vs 6 hour.

the effects of various stimuli on OP gene expression, Northern blotting was conducted as follows; after MCs were cultured for 12 hours in serum free DMEM with ITS mixture, FCS (10%) or a cytokine, such as TNF-α, PDGF, IL-1β, and TGF-β at 10 ng/ml concentration, were added. Then RNA was extracted before and 2, 4, 6, 12, 24 hours after stimulation. As shown in Fig. 1, FCS markedly increased the band of 1.7 kb OP mRNA over a 24-hour period after stimulation, and 2.7-fold increase compared with the control was observed at 24 hours. TNF-α also increased OP gene expression over a 12-hour period (Fig. 2) and the maximal induction (1.8-fold increase) was detected at 12 hours. On the other hand, IL-1β (Fig. 3), PDGF (not shown), and TGF-β (not shown) had little effect on OP gene expression over a 24-hour period after stimulation.

Expression of OP protein in MCs. Since we demonstrated that FCS and TNF-α induce the expression of OP

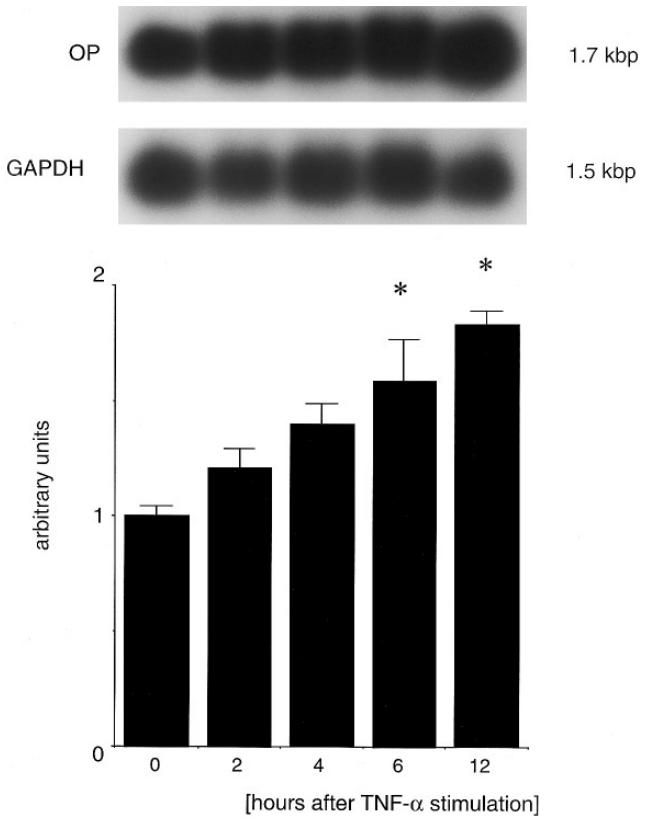

FIG. 2. Effect of TNF-α (10 ng/ml) on OP mRNA expression. Northern blotting (upper panel) and its densitometric analysis (lower panel) were carried out as described in *Methods*. After MCs were cultured for 12 hours in serum free medium, TNF-α was added, and RNA was extracted before (0) and 2, 4, 6, 12 hours after stimulation. The autoradiogram shown in the upper panel is representative of three separate experiments. Densitometric analysis (lower panel) showed a significant increase in OP/GAPDH mRNA ratio at 12 hours. *; $p < 0.05$ vs 0 hour.

gene in a time-dependent manner, we next examined the effects of these factors on OP protein production by MCs by Western blotting using a polyclonal anti-OP antibody. Twelve-hour serum-starved MCs were stimulated by FCS (10%) or TNF-α (10 ng/ml), and were harvested before and 12, 24, 48 hours after the stimulation. As shown in Fig. 4, a single band at 69 kDa, which corresponded well to OP molecular size reported previously (25, 32), was detected in MCs. The intensities of the bands of either FCS- or TNF-α-treated cells were increased over 24-hour and 48-hour periods, respectively. In addition to the 69 kDa band, a 37 kDa band was also seen (not shown), probably corresponding to the thrombin-digested OP (25, 29). The further experiments regarding the regulation of this small fragment of OP by these reagents is now in progress.

DISCUSSION

The present study clearly demonstrated the presence of both gene expression and protein synthesis by cultured rat MCs by Northern blotting and Western blotting. Furthermore, we investigated factors affecting OP expression. Among several factors examined, FCS and TNF-α increased both OP gene expression and protein synthesis.

Cytokines that are expressed and participate in glomerular diseases, in either an autocrine or paracrine manner, such as TNF-α, PDGF, TGF-β and IL-1 (30), are also known to induce OP expression in other cell types (16, 31-33). In our experiments, TNF-α, one of these cytokines, markedly increased OP gene expression and protein synthesis in cultured MCs. Since TNF-α is known to stimulate production of complement (34), monocyte adhesion (35), and nitric oxide synthesis (36) in *in vitro* studies on MCs, it is possible that TNF-α-induced OP production may be involved in the development of glomerulonephritis *in vivo*. On the other hand, in the present study, a pro-inflammatory cytokine IL-1β, which has been reported to affect prostaglandin synthesis and tyrosine phosphorylation (37), had little effect on OP gene expression at 10 ng/ml, a concentration usually used in *in vitro* MC studies. TGF-β and PDGF, pleiotropic cytokines which are involved in extracellular matrix production, chemoattraction and MC proliferation (30,38), did not affect gene expression of OP at 10 ng/ml. This suggests that a unique regulatory

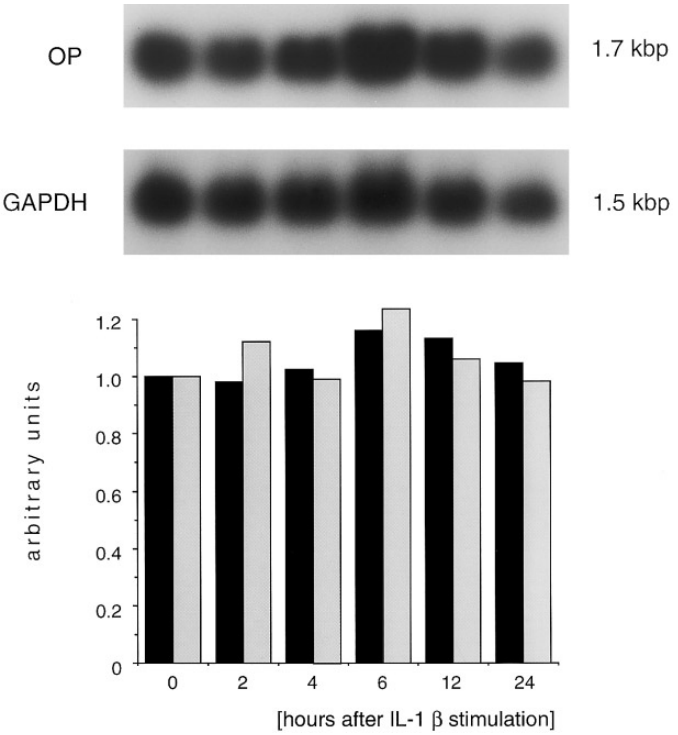

FIG. 3. Effect of IL-1β (10 ng/ml) on OP mRNA expression. After MCs were cultured for 12 hours in serum free medium, IL-1β was added, and RNA was extracted before (0) and 2, 4, 6, 12, 24 hours after stimulation. ■; experiment 1 ▨; experiment 2. Little effect of IL-1β on OP mRNA expression was observed.

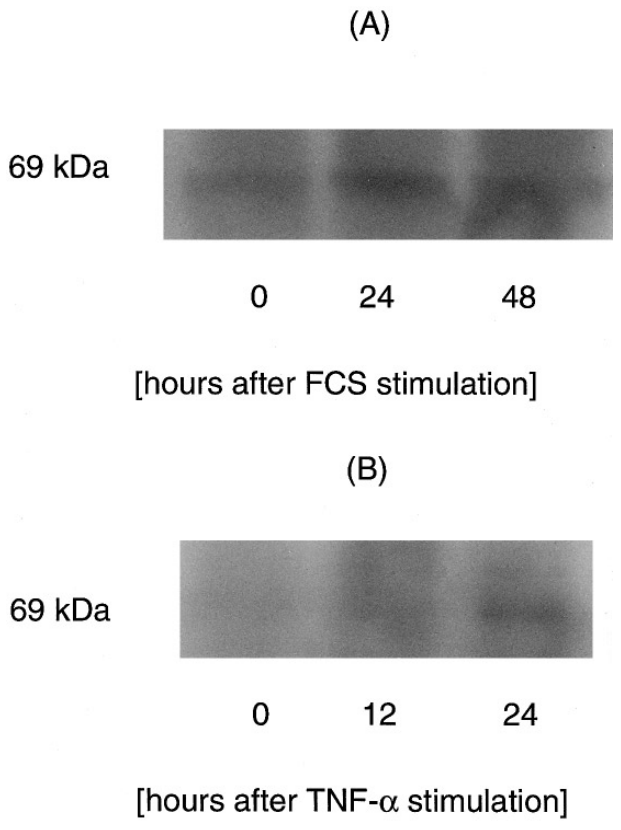

FIG. 4. Effect of 10% FCS (upper panel) and TNF-α (10 ng/ml) (lower panel) on OP protein synthesis in MCs. After MCs were cultured for 12 hours in serum free medium with ITS mixture (5 μg/ml insulin, 5 μg/ml transferrin, 5 ng/ml sodium selenite, at final concentration), FCS or TNF-α was added and extracts of MC layers were obtained before (0) and 12, 24, 48 hours after the stimulation. Fifty μg of the samples were electrophoresed and analyzed by Western blotting using a polyclonal anti-OP antibody as described in *Materials and Methods.* OP protein synthesis (69 kDa) was demonstrated, and the intensity of bands was increased after stimulation by either FCS or TNF-α. The result is representative of three separate experiments.

mechanism of OP production is present, different from that of other extracellular matrix proteins, such as collagen and laminin which is strongly affected by TGF-β (39). It is also suggested that OP is not related to be a product associated with MC proliferation induced by IL-1 or PDGF.

Several reports by utilizing immunohistochemistry and/or *in situ* hybridization have demonstrated that OP expression was restricted to tubular epithelial cells of distal convoluted tubuli and the ascending thick limb of Henle's loop, and Bowman's capsule in normal rodents (20, 21). Moreover, in various pathological conditions, such as angiotensin II-induced tubulointerstitial nephritis (21), the antithymocyte serum model of mesangial proliferative glomeluronephritis, passive Heymann nephritis model of membranous nephropathy, and aminonucleoside model of focal segmental glomerulosclerosis (23), a dramatic increase in OP expression was documented only in renal tubuli, and not in glomeruli (21-23). However, Proels *et al* recently reported that OP mRNA is detectable in glomeruli isolated from rats of anti-Thy 1 glomerulonephritis (40). They emphasized the role of OP in maintaining and restoring the structural integrity of the mesangium, since OP mRNA was dramatically upregulated in the later stages of the repair process in anti-Thy 1 glomerulonephritis. Therefore, it is possible that OP may play some roles in the pathophysiology in glomerular lesions. However, exact role of OP in glomerular pathology including glomerulonephritis remains to be clarified. One of the reasons OP was not detected in previous studies may be related to the technical limits of OP detectability by immunohistochemistry and/or *in situ* hybridization, since a far greater OP expression in tubuli than in glomeruli might have stopped the antibody-antigen and *in situ* hybridization reaction before reaction becomes detectable in glomeruli.

It is known that OP is cleaved into a small fragment (20-38 kDa) by thrombin digestion (25, 32), and both the native and digested form are found in normal human milk (24), although which form is physiologically active *in vivo* is unknown. Ulrich *et al* demonstrated the presence of a small fragment of OP in normal rat kidney and indicated the possibility of an intracellular, proteolytic maturation step in MDCK cells, a canine kidney-derived cell line close to the distal tubular epithelial cells (30). Our results in which a small molecular band at 37 kDa was present in cultured MCs corresponds to previous reports (24, 30) supposing that the diverse functions of OP in various tissues might be attributed to specific processing of distinct polypeptides (32).

In conclusion, this study clearly demonstrated the presence of OP gene expression and protein synthesis in cultured rat MCs. Increased OP production under the stimulation of serum or TNF-α suggests that intraglomerular OP may contribute to the development of various glomerular diseases.

ACKNOWLEDGMENT

We thank Dr Minehiko Yokoyama (Meiji Nyugyo Central Laboratory, 540 Narita, Odawara, Kanagawa 250, Japan) for generously providing the anti-OP antibody.

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