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Inhibitory effect of cepharanthine on fibronectin production in growth factor-stimulated rat mesangial cells

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Abstract

We examined the effect of cepharanthine, a biscoclaurine alkaloid, on extracellular matrix production in rat mesangial cells in response to platelet-derived growth factor (PDGF) or transforming growth factor-β (TGF-β). Stimulation of the cells with PDGF increased the amounts of fibronectin, one of extracellular matrix components. Pretreatment with cepharanthine (0.1–2 μM) suppressed the PDGF-stimulated increase in fibronectin in a dose-dependent manner. At a concentration of 2 μM, the alkaloid almost completely suppressed the production. Under the conditions, the alkaloid inhibited tyrosine phosphorylation of several proteins including PDGF β receptor in PDGF-stimulated cells, and also tyrosine kinase activity of the receptor prestimulated with PDGF in a cell-free assay system. Furthermore, cepharanthine suppressed TGF-β-stimulated fibronectin production at the same concentration ranges. Our results suggest that cepharanthine inhibits fibronectin production induced by growth factors, probably through suppression of receptor autophosphorylation.

Keywords: Biscoclaurine alkaloid; (PDGF) Platelet-derived growth factor; (TGF-β) Transforming growth factor-β; Mesangial cell, rat

1. Introduction

A major characteristic of glomerulosclerosis including glomerulonephritis and diabetic nephropathy is accumulation of extracellular matrix, such as collagen, fibronectin, and laminin in the glomerular mesangium (Klahr et al., 1988; Doi et al., 1991; Floege et al., 1991). The extracellular matrix levels in the glomeruli are regulated by mesangial cells contributing to both the production and degradation of extracellular matrix. It has been shown that the extracellular matrix production in rat and human mesangial cells is accelerated by platelet-derived growth factor (PDGF) or transforming growth factor-β (TGF-β) (Border et al., 1990a; Hänsch et al., 1995), which is released from platelets, macrophages, and mesangial cells themselves in an inflammation (Abboud, 1992; Sharma and Ziyadeh, 1995). In experimental animal models of glomerulonephritis and diabetic nephropathy, administration of antibodies against PDGF or TGF-β attenuates the accumulation of extracellular matrix in the glomeruli (Border et al., 1990b; Johnson et al., 1992; Sharma et al., 1996), suggesting that abnormal extracellular matrix levels under the pathological conditions are mediated by these growth factors. Furthermore, under such pathological conditions, expression of PDGF, TGF-β, and PDGF β receptor is also accelerated with concomitant accumulation of extracellular matrix (Iida et al., 1991; Yamamoto et al., 1993; Park et al., 1997). Thus, it is conceivable that PDGF and TGF-β play an important role for progressive accumulation of extracellular matrix in glomerulonephritis or diabetic nephropathy, and, thereby, that suppression of growth factor-induced extracellular matrix production might retard progression of renal diseases.

The biscoclaurine (bisbenzylisoquinoline) alkaloids including cepharanthine (Fig. 1) have been reported to suppress several cellular responses including activation of platelets (Kometani et al., 1985; Hashizume et al., 1991; Akiba et al., 1995) and of mast cells (Teh et al., 1988; Akiba et al., 1992), superoxide generation by polymorphonuclear leukocytes (Matsuno et al., 1987), and lipid peroxidation in some biomembranes (Shiraishi et al., 1980).

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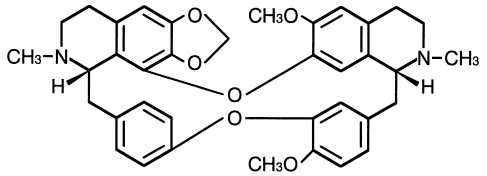

Fig. 1. Structure of cepharanthine.

Previously, we demonstrated that the inhibitory effect of cepharanthine on platelet aggregation is reversible upon removal of the alkaloid (Kometani et al., 1985), indicating that the alkaloid inhibits the response probably through alteration of membrane properties. Therefore, it is likely that cepharanthine may affect extracellular matrix production induced by PDGF and TGF-β through modification of physicochemical properties of plasma membranes. In this study, to evaluate the efficacy of the biscoclaurine alkaloids on cellular responses of mesangial cells, we examined effects of cepharanthine on the production of fibronectin, one of extracellular matrix ingredients, in PDGF- and TGF-β-stimulated rat mesangial cells.

2. Materials and methods

2.1. Materials

Cepharanthine was obtained from Kaken Shoyaku (Tokyo, Japan). Recombinant human PDGF-BB was obtained from Pepro Tech EC (London, UK), TGF-β (human platelets) from Biomedical Technologies (Stoughton, MA, USA), rabbit anti-human PDGF β receptor antibody from Santa Cruz Biotechnology (Santa Cruz, CA, USA), rabbit anti-rat fibronectin antibody from Biogenesis (Poole, UK), and peroxidase-conjugated goat anti-rabbit antibody from Southern Biotechnology Associates (Birmingham, AL, USA). Peroxidase-conjugated anti-phosphotyrosine antibody and enhanced chemiluminescence Western blotting detection kit were from Amersham Pharmacia Biotech (Buckinghamshire, UK).

2.2. Cell culture

Rat mesangial cells were prepared as described previously (Hayama et al., 1997). Briefly, mesangial cells were obtained from a culture of glomeruli isolated from Sprague–Dawley rats (100–150 g) by sieving, and grown in RPMI 1640 supplemented with 20% heat-inactivated fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 5 μg/ml insulin, 5 μg/ml transferrin, and 5 ng/ml selenious acid. The cells, between the third and sixth passages, were made quiescent by incubating with serum-free medium containing 0.1 mg/ml fatty acid-free bovine serum albumin for 24 h, and subjected to the following experiments.

2.3. Immunoblot studies for fibronectin and tyrosine-phosphorylated proteins

The quiescent cells were pretreated with various concentrations of cepharanthine for 1 h, and stimulated with PDGF-BB or TGF-β as described in the figure legends. The extracellular medium was removed and centrifuged. The supernatant obtained and the remaining cells were solubilized with a buffer (2% sodium dodecyl sulfate, 1 mM EDTA, 2 mM EGTA, 2% β-mercaptoethanol, 10% glycerol, 0.01% bromophenol blue, and 100 mM Tris–HCl, pH 6.8), and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis on a 7.5% gel. The separated proteins were transferred onto a nitrocellulose membrane. For the detection of fibronectin, anti-fibronectin primary antibodies (1/1000 dilution) and peroxidase-conjugated secondary antibody (1/20 000 dilution) were applied. For the detection of proteins with phosphotyrosine residues, peroxidase-conjugated anti-phosphotyrosine antibodies (1/100 dilution) were applied. The bound antibodies were visualized using enhanced chemiluminescence reagents.

2.4. Autophosphorylation of PDGF β receptor

The quiescent cells were pretreated with 2 μM cepharanthine for 1 h, and stimulated with 50 ng/ml PDGF-BB for 10 min. The cells were lysed in an ice-cold lysis buffer (1% Triton X-100, 150 mM NaCl, 5 mM EGTA, 50 mM NaF, 1 mM $NaVO_4$, 10 μg/ml leupeptin, 1 mM *p*-(amidinophenyl)methanesulfonyl fluoride, and 20 mM Tris–HCl, pH 7.4). The lysate was incubated with protein A-agarose for 30 min, as a pre-clearing step, and centrifuged at $12{,}000 \times g$ for 5 s. The supernatant obtained was incubated with anti-PDGF β receptor antibodies overnight at 4°C, and further with protein A-agarose for 2 h at 4°C. After centrifugation, the pellet obtained was washed three times with the lysis buffer and solubilized. The sample was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis on a 7.5% gel. Tyrosine-phosphorylated PDGF β receptors were detected with peroxidase-conjugated anti-phosphotyrosine antibodies as described above.

2.5. Cell-free assay for tyrosine kinase of PDGF β receptor

Tyrosine kinase activity of PDGF β receptor was measured according to the method of Yagi et al. (1997). The quiescent cells were lysed in an ice-cold lysis buffer (1% Triton X-100, 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 10% glycerol, and 50 mM HEPES, pH 7.5). The lysate was incubated with 50 ng/ml PDGF for 30 min at 4°C, and subjected to immunoprecipitation as described in Section 2.4. The PDGF β receptors immunoprecipitated were washed and resuspended in a buffer (10 mM $MnCl_2$ and 50 mM Tris–HCl, pH 7.5). The suspension was incubated with cepharanthine for 30 min at 4°C. Tyrosine

kinase activity of the receptor was estimated by receptor autophosphorylation, which was initiated by incubation of the suspension with 40 μM ATP for 10 min. Tyrosine-phosphorylated PDGF β receptors were detected as described in Section 2.3.

3. Results

3.1. Effect of cepharanthine on PDGF-stimulated fibronectin production

As shown in Fig. 2A, stimulation of rat mesangial cells with PDGF-BB (10–50 ng/ml) for 48 h increased dose-dependently the amounts of fibronectin in the extracellular medium and in the residual cells, indicating that the growth factor accelerated fibronectin production in rat mesangial cells. To examine the effect of cepharanthine on the PDGF-stimulated fibronectin production, the cells were pretreated with various concentrations of cepharanthine (0.1–2 μM) and stimulated with 50 ng/ml PDGF-BB. The results shown in Fig. 2B revealed that the alkaloid dose-dependently inhibited PDGF-stimulated fibronectin production in the extracellular medium and in the residual cells. The quantitative results demonstrated almost complete suppression of PDGF-stimulated fibronectin production at 2 μM of cepharanthine (Fig. 2C). Under the conditions, cepharanthine (up to 2 μM) did not affect cell viability, as estimated by trypan blue dye exclusion (data not shown).

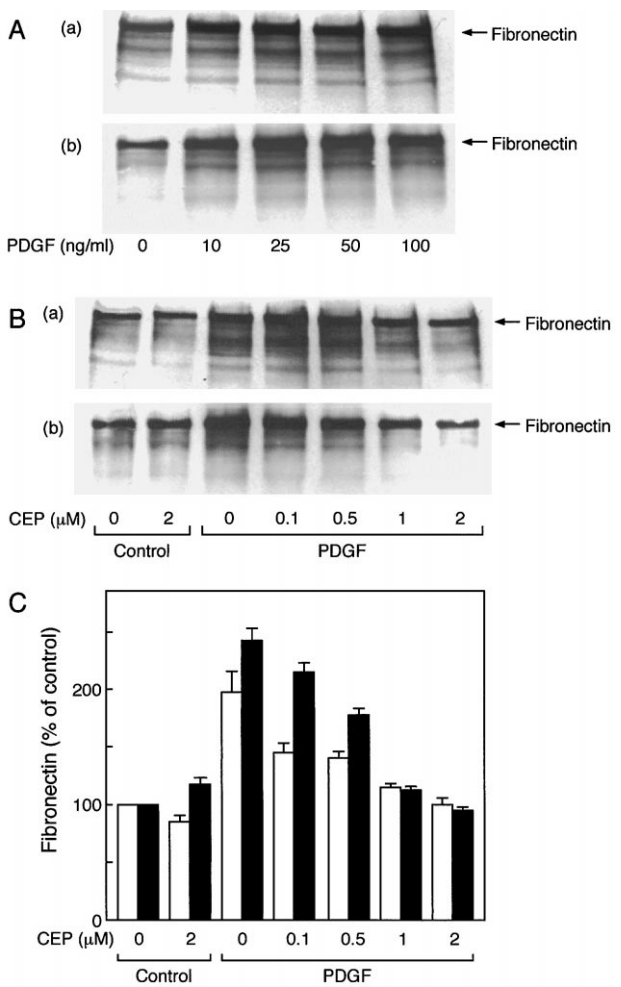

Fig. 2. Effect of cepharanthine on PDGF-stimulated fibronectin production. (A) Rat mesangial cells were stimulated with various concentrations of PDGF-BB for 48 h. Fibronectin in the extracellular medium (a) and in the cells (b) was detected as described in Section 2. (B) Cells were treated with various concentrations of cepharanthine for 1 h, and stimulated with (PDGF) or without (control) 50 ng/ml PDGF-BB for 48 h. Fibronectin was detected as in (A). (C) Amounts of fibronectin in the extracellular medium (open columns) and in the cells (hatched columns) were estimated by measuring the density of fibronectin band shown in (B) and expressed as the percent of control (untreated cells). Data represent the mean ± S.E.M. of three separate experiments.

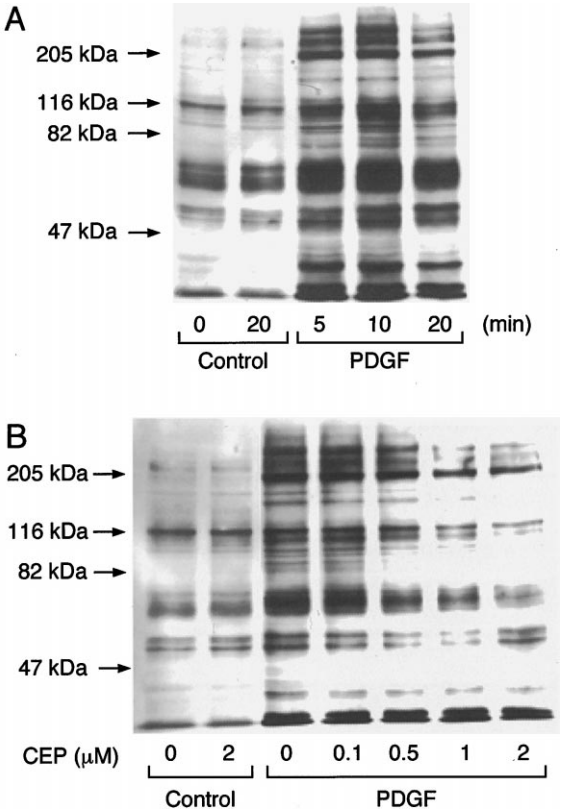

Fig. 3. Effect of cepharanthine on PDGF-induced protein tyrosine phosphorylation. (A) Rat mesangial cells were stimulated with (PDGF) or without (control) 50 ng/ml PDGF-BB for indicated periods. (B) Cells were treated with various concentrations of cepharanthine for 1 h, and stimulated with (PDGF) or without (control) 50 ng/ml PDGF-BB for 10 min. Tyrosine-phosphorylated proteins were detected as described in Section 2. The results are representative of three experiments.

3.2. Effect of cepharanthine on PDGF-induced protein tyrosine phosphorylation

In rat mesangial cells, PDGF-BB induces several cellular responses including extracellular matrix production,

DNA synthesis, and proliferation, which are initiated by autophosphorylation of tyrosine residues of PDGF β receptors (Abboud, 1992; Heldin et al., 1998). The receptor autophosphorylation is followed by protein tyrosine phosphorylation of several intracellular proteins. The present study confirmed that 50 ng/ml PDGF-BB increased tyrosine phosphorylation of various proteins, which were estimated by immunoblot analysis with anti-phosphotyrosine antibodies (Fig. 3A). The maximal response of PDGF-induced tyrosine phosphorylation was observed 10 min after the stimulation. To characterize the mechanism underlying the inhibition by cepharanthine of PDGF-stimulated fibronectin production, we examined the effect of cepharanthine on the protein tyrosine phosphorylation in response to PDGF-BB. As shown in Fig. 3B, pretreatment of the cells with cepharanthine (0.1–2 μM) resulted in a dose-dependent attenuation of tyrosine phosphorylation of several proteins including approximately 40, 140 and 170 kDa proteins in 50 ng/ml PDGF-stimulated rat mesangial cells. These results led us to examine the possibility that cepharanthine may inhibit autophosphorylation of tyrosine residues of PDGF β receptor. To verify this possibility, we examined the effect of cepharanthine on PDGF-induced receptor autophosphorylation, which was estimated by immunoprecipitation with an antibody against PDGF β receptor, followed by detection of phosphotyrosine residues. The result shown in Fig. 4A indicates that tyrosine phosphorylation of PDGF β receptor induced by 50 ng/ml PDGF-BB was attenuated when rat mesangial cells were pretreated with 2 μM cepharanthine. Furthermore, the effect of cepharanthine on tyrosine kinase activity of PDGF β receptor in a cell-free assay system was examined. As shown in Fig. 4B, when cell lysate was incubated with 50 ng/ml PDGF-BB, followed by immunoprecipitation of PDGF β receptor, the receptor tyrosine phosphorylation was stimulated by adding 40 μM ATP into the immunoprecipitates. Pretreatment of the receptors with 2 μM cepharanthine prior to addition of ATP resulted in a decrease in tyrosine phosphorylation of the PDGF-preactivated receptors.

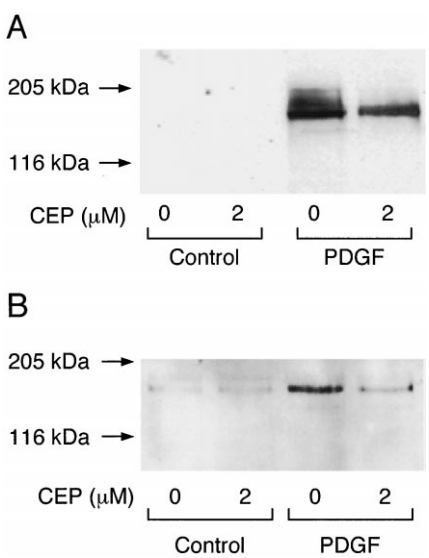

Fig. 4. Effect of cepharanthine on tyrosine phosphorylation of PDGF β receptor in PDGF-stimulated cells (A) and in a cell-free assay system (B). (A) Rat mesangial cells were treated with or without 2 μM cepharanthine for 1 h, and stimulated with (PDGF) or without (control) 50 ng/ml PDGF-BB for 10 min. (B) Immunoprecipitates, which were obtained with antibodies against PDGF β receptor from cell lysate preincubated with (PDGF) or without (control) 50 ng/ml PDGF-BB, were treated with or without 2 μM cepharanthine for 30 min, and further incubated with 40 μM ATP for 10 min. Tyrosine phosphorylation of PDGF β receptors was detected as described in Section 2. The results are representative of three experiments.

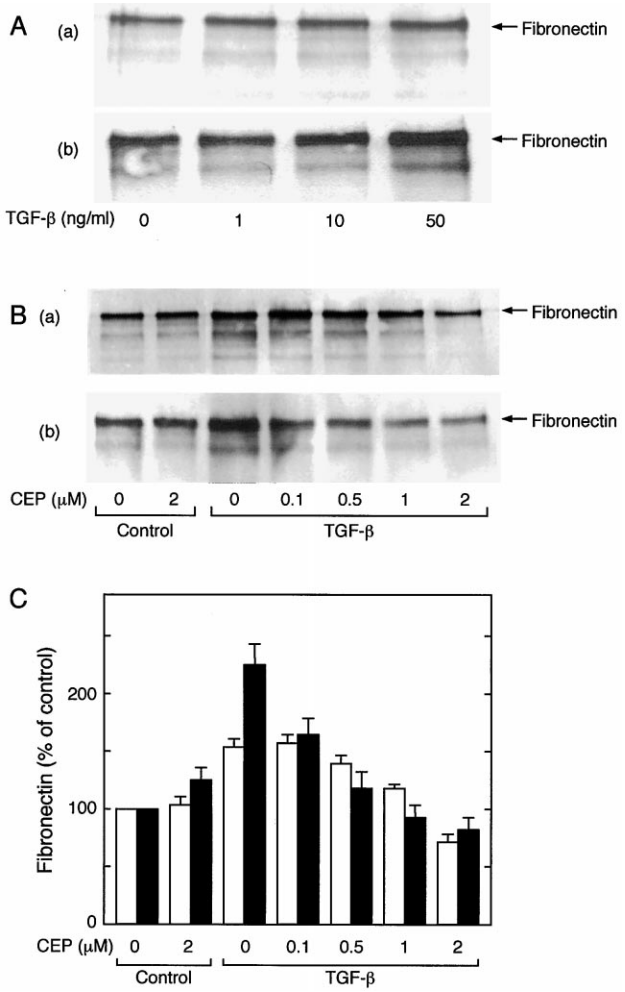

Fig. 5. Effect of cepharanthine on TGF-β-stimulated fibronectin production. (A) Rat mesangial cells were stimulated with various concentrations of TGF-β for 48 h. Fibronectin in the extracellular medium (a) and in the cells (b) was detected as described in Section 2. (B) Cells were treated with various concentrations of cepharanthine for 1 h, and stimulated with (TGF-β) or without (control) 50 ng/ml TGF-β for 48 h. Fibronectin was detected as in (A). (C) Amounts of fibronectin in the extracellular medium (open columns) and in the cells (hatched columns) were estimated by measuring the density of fibronectin band shown in (B) and expressed as the percent of control (untreated cells). Data represent the mean ± S.E.M. of three separate experiments.

3.3. Effect of cepharanthine on TGF-β-stimulated fibronectin production

In addition to PDGF, TGF-β also stimulates extracellular matrix production in rat mesangial cells. We confirmed that stimulation of the cells with TGF-β (1–50 ng/ml) for 48 h increased dose-dependently the amounts of fibronectin in the extracellular medium and in the residual cells (Fig. 5A). Under the condition, when cepharanthine (0.1–2 μM) was added to the medium prior to stimulation with 50 ng/ml TGF-β, the alkaloid suppressed TGF-β-stimulated fibronectin production in a dose-dependent manner (Fig. 5B). The quantitative results shown in Fig. 5C also demonstrated significant suppression of the production by cepharanthine.

4. Discussion

The biscoclaurine alkaloids including cepharanthine have been shown to suppress several cellular responses in inflammatory cells, such as platelets (Kometani et al., 1985; Hashizume et al., 1991; Akiba et al., 1995), mast cells (Teh et al., 1988; Akiba et al., 1992), and polymorphonuclear leukocytes (Matsuno et al., 1987). In this study, we demonstrated that cepharanthine suppressed fibronectin production in rat mesangial cells stimulated with PDGF-BB and TGF-β. The extracellular matrix components including fibronectin, collagen, and laminin are accumulated in the mesangium in glomerulonephritis and diabetic nephropathy (Klahr et al., 1988; Doi et al., 1991; Floege et al., 1991). The accumulation of extracellular matrix in these renal diseases has been suggested to be mediated by PDGF and TGF-β, because antibodies against these growth factors attenuate the extracellular matrix accumulation in the glomeruli in experimental animal models of glomerulonephritis and diabetic nephropathy (Border et al., 1990b; Johnson et al., 1992; Sharma et al., 1996). Furthermore, expression of PDGF and TGF-β is also upregulated in the glomeruli in experimental glomerulosclerosis (Iida et al., 1991; Yamamoto et al., 1993; Park et al., 1997). Thus, these growth factors play a pivotal role for progression of glomerulosclerosis. Therefore, our results suggest that cepharanthine might interfere with the progressive accumulation of extracellular matrix in the renal disease.

PDGF exists as disulphide-bonded dimer of A- and B-chains termed PDGF-AA, PDGF-AB or PDGF-BB (Heldin et al., 1998). The receptors for PDGF consist of two structurally related molecules, α and β. PDGF-AA binds to α receptor only, whereas PDGF-AB and PDGF-BB bind to both α and β receptors. Binding of PDGF to the receptors induces receptor dimerization to activate intrinsic tyrosine kinase, leading to autophosphorylation of tyrosine residues within the intracellular domains of the receptor. Rat mesangial cells possess PDGF β receptor only or predominantly (Huwiler et al., 1995; Plüss et al., 1995), and, thereby, PDGF-BB causes numerous cellular responses including DNA synthesis, extracellular matrix production, and proliferation. It was shown that inhibition of β receptor autophosphorylation by a quinoline derivative results in suppression of DNA synthesis and proliferation in rat mesangial cells (Yagi et al., 1997). Thus, the receptor autophosphorylation is an initial and essential step for PDGF-elicited signal transduction. The present work showed that cepharanthine prevented autophosphorylation of β receptor as well as tyrosine phosphorylation of several proteins upon stimulation with PDGF-BB, suggesting that the suppression by cepharanthine of PDGF-stimulated fibronectin production may be due to termination of intracellular signaling through impairment of receptor autophosphorylation. Furthermore, we demonstrated that cepharanthine also exhibited inhibitory effect on tyrosine kinase activity of PDGF-preactivated β receptor in a cell-free assay system. Therefore, it seems likely that cepharanthine suppresses autophosphorylation of β receptor, probably through inhibition of intrinsic tyrosine kinase activity of the receptor.

TGF-β is also one of potent agonists for mesangial cells to stimulate extracellular matrix production (Hänsch et al., 1995). This growth factor transmits its signals through a heteromeric complex consisting of types I and II receptors, which have serine/threonine kinase activities in their cytoplasmic domains (Wrana et al., 1994). Binding of TGF-β to type II receptor causes association with type I receptor, and the type I receptor is phosphorylated by the type II receptor on both serine and threonine residues. Thus, TGF-β-mediated signaling is also initiated by receptor autophosphorylation. In this study, we showed that TGF-β-stimulated fibronectin production was suppressed by cepharanthine. Considering the results with PDGF, we speculate that the suppression of fibronectin production by cepharanthine might be due to inhibition of TGF-β receptor activation including receptor autophosphorylation. Previously, TGF-β-stimulated collagen production was shown to be attenuated in the presence of antibodies against PDGF in human mesangial cells (Hänsch et al., 1995), suggesting that expression of PDGF is involved, at least in part, in the collagen production. Therefore, the inhibition by cepharanthine of TGF-β-stimulated fibronectin production might be partially explained by suppression of the response mediated by PDGF expressed in response to TGF-β.

It has been suggested that inflammatory cells including platelets and polymorphonuclear leukocytes are involved in pathogenesis of glomerulonephritis (Couser, 1998). The activated inflammatory cells release diverse mediators, such as cytokines, growth factors, eicosanoids, and reactive oxygen species, which stimulate proliferation of resident glomerular cells or induce inflammation in glomerular lesions. These inflammatory cells and proinflammatory mediators have been identified as therapeutic targets (Nassar and Badr, 1998). We and other investigators have

shown that biscoclaurine alkaloids including cepharanthine have inhibitory effects on aggregation and arachidonic acid liberation in platelets (Kometani et al., 1985; Hashizume et al., 1991; Akiba et al., 1995), and on superoxide generation in polymorphonuclear leukocytes (Matsuno et al., 1987). Considering these observations together with the findings obtained here, cepharanthine seems to exert its suppressive effects on inflammatory and glomerular cells, which are involved in pathogenesis or progression of glomerulonephritis. We suppose, therefore, that cepharanthine might be therapeutically useful. In particular, administration of the alkaloid, as anti-inflammatory drugs, might contribute to use of low doses of steroid.

In summary, the present study demonstrated that cepharanthine suppressed fibronectin production in response to PDGF in rat mesangial cells, probably through inhibition of intrinsic tyrosine kinase activity of PDGF β receptor, suggesting that cepharanthine may retard progression of glomerulosclerosis mediated by growth factor-stimulated mesangial cells.

Acknowledgements

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