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Activation of STAT3/Smad1 Is a Key Signaling Pathway for Progression to Glomerulosclerosis in Experimental Glomerulonephritis*

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Mesangial cell proliferation is a significant event in the development of progressive glomerular injuries. However, the issue of how cell proliferation is involved in the development of glomerulosclerosis is unclear. Recently, we showed that the overexpression of type IV collagen (Col IV), a major component of mesangial extracellular matrix, is transcriptionally regulated by Smad1 in diabetic glomerulosclerosis. In this study, we have demonstrated the effect of the administration of an anti-platelet-derived growth factor (PDGF) β-receptor antibody (APB5) blocking activation by the PDGF-B chain on rat glomerulonephritis and have examined the signaling pathways that regulate both glomerular cell proliferation and glomerulosclerosis *in vivo* and *in vitro*. Experimental mesangial proliferative glomerulonephritis (Thy1 GN) was induced by a single intravenous injection of anti-rat Thy-1.1 monoclonal antibody. In Thy1 GN, mesangial cell proliferation and the expression of Col IV peaked at day 6. Immunohistochemical staining for the expression of Smad1, phospho-Smad1 (pSmad1), and phospho-STAT3 (pSTAT3) revealed that the peak for glomerular Smad1 expression occurred at day 6, consistent with the peak for mesangial proliferation. The expression of pSmad1 was up-regulated at day 1, and the peak for glomerular pSmad1 expression occurred at day 4 of the disease. When treated with APB5, both mesangial proliferation and sclerosis were reduced significantly. The expression of Smad1, pSmad1, and pSTAT3 was also significantly reduced by the administration of APB5. PDGF induced both mesangial cell replication and Col IV synthesis in association with an increased expression of pSTAT3 and pSmad1 on cultured mesangial cells. In addition, APB5 reduced mesangial cell proliferation in association with decreased pSmad1, pSTAT3, and Col IV protein expressions *in vitro*. The introduction of dominant negative STAT3 significantly decreased the expression of Col IV in cultured mesangial cells. These data suggest that the activation of STAT3 and Smad1 participates in the developing process of glomerulosclerosis in experimental glomerulonephritis.

Both mesangial cell proliferation and glomerulosclerosis are major important pathological features in progressive glomerular damage. In many glomerular sclerosing diseases, mesangial cell proliferation is a critical process in progressive glomerular injuries (1, 2). Both events are observed simultaneously in most glomerular diseases; however, the issue of how cell proliferation contributes to the development of glomerulosclerosis remains unclear.

Platelet-derived growth factor (PDGF)[1] is known to be a critical mitogen for mesangial cells *in vitro* and *in vivo* (3, 4). Several lines of evidence indicate that PDGF plays a key role in the development of glomerulosclerosis not only in experimental models but also in human glomerular diseases (3). PDGF-BB has also been reported to be essential for mesangial cell proliferation (5), which is followed by development of glomerulosclerosis in the remnant kidney model (6). The introduction of a neutralizing anti-PDGF antibody has shown that both mesangial proliferation and glomerulosclerosis can be markedly ameliorated in a rat glomerulonephritis model (7), but little is known concerning the mechanisms of how the suppression of cell proliferation reduces glomerular sclerotic lesions.

Glomerulosclerosis is characterized by an increase in the levels of ECM. Col IV is a major component of expanded ECM in glomerular diseases, but the molecular mechanism of regulating Col IV gene transcription had not been cleared until our recent report in which we showed that Smad1 transcriptionally regulates the overexpression of Col IV in diabetic nephropathy (8). Smad1 directly transduces signals to downstream target genes that are related to renal damage such as osteopontin (9), inhibition of differentiation (10), and type I collagen (11) and is critically important for the development of kidney disease (12). These findings suggest that Smad1 is a critical transcriptional factor in the progression of glomerulosclerosis.

Signal transducer and activator of transcription (STAT) proteins have been shown to be involved in signaling by numerous cytokines and growth factors. It is well established that STAT3 activation is a key step in PDGF-induced mitogenesis (13). Nakashima *et al.* (14) report that transcriptional coactivator p300 physically interacts with STAT3 and Smad1, followed by the subsequent activation of the target gene transcription in

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[1] The abbreviations used are: PDGF, platelet-derived growth factor; PDGFβ-R, PDGFβ receptor; ECM, extracellular matrix; Col IV, type IV collagen; STAT, signal transducer and activator of transcription; pSmad1, phospho-Smad1; PAM, periodic acid-methenamine; PCNA, proliferating nuclear antigen; FCS, fetal calf serum; BrdUrd, bromodeoxyuridine; ELISA, enzyme-linked immunosorbent assay; GN, glomerulonephritis.

astrocyte differentiation. We have postulated from these findings that PDGF is able to activate the STAT3-Smad1 cross-talk pathway in mesangial proliferative glomerulonephritis and that the process is essential for the progression of mesangial cell proliferation to glomerulosclerosis. The goal of this study was to determine how the STAT3 and Smad1 signaling pathways are involved in the development of glomerulosclerosis, using anti-PDGFβ-receptor antibody in a rat glomerulonephritis model.

EXPERIMENTAL PROCEDURES

Animals

Male Wistar rats (CLEA Japan, Inc.) weighing 180–200 g were used in this study. Rats were housed under specific pathogen-free conditions. All animal experiments were performed in accordance with institutional guidelines, and the Review Board of Tokushima University granted ethical permission for this study.

Induction of Thy1 Glomerulonephritis

Experimental mesangial proliferative glomerulonephritis (Thy1 GN) was induced by a single intravenous injection of anti-rat Thy-1.1 monoclonal antibody (1 mg/kg) (Cedarlane Laboratories, Ontario, Canada) as described elsewhere (15). These rats were sacrificed at days 1, 2, 4, 6, and 12 (n = 6/group) after the administration of anti-Thy-1.1 antibody. Six age-matched rats were injected with vehicle only and were sacrificed as controls.

Protocol of Treatment with Anti-PDGFβ-R Antibody in Thy1 GN

A rat monoclonal anti-PDGFβ-receptor antibody (APB5) and its antagonistic effects on the PDGFβ-R signal transduction pathway *in vivo* and *in vitro* have been described previously (16, 17). The rats were injected intraperitoneally at daily intervals with 400 μg of APB5 or irrelevant isotype-matched control rat IgG after the administration of anti-Thy1.1 antibody from day 0. They were sacrificed at days 1, 2, 4, 6, and 12 (n = 6/group).

Histological Examination

Light Microscopy—After removal of the kidney, tissue blocks for light microscopy examination were fixed in methyl Carnoy's solution and embedded in paraffin. Sections (2 μm) were stained with hematoxylin and eosin, periodic acid-Schiff's reagent, and periodic acid-methenamine (PAM) silver.

Immunohistochemistry—Kidney sections were processed for immunohistochemistry following standard procedures. To study proliferating nuclear antigen (PCNA), Col IV, and Smad1, methyl Carnoy's solution-fixed and paraffin-embedded tissue blocks were used. Kidney sections were rehydrated and treated with 0.3% hydrogen peroxide in methanol for 30 min. To eliminate nonspecific staining, sections were incubated with the appropriate preimmune serum for 20 min at room temperature and then incubated with avidin D- and biotin-blocking solutions (Vector, Burlingham, CA) for 15 min each. Sections were incubated with the anti-PCNA antibody (1:200 dilution), anti-Col IV antibody (1:200 dilution), and anti-Smad1 antibody (1:100 dilution) (Santa Cruz Biotechnology) for 60 min at room temperature and then incubated with the appropriate biotinylated secondary antibodies followed by incubation with the avidin-biotin peroxidase complex (Vectastain Elite ABC kit, Vector). Peroxidase conjugates were subsequently localized using diaminobenzidine tetrahydrochloride. To study pSmad1 and pSTAT3, the tissues were snap-frozen in cold acetate in OCT compound (Miles Inc., Elkhart, IN), cut in 4-μm-thick sections, fixed in acetone for 5 min, and treated with 0.3% hydrogen peroxide in methanol for 30 min. Sections were treated in the same manner as PCNA with the following primary antibodies: anti-pSmad1 antibody (1:100 dilution) (Calbiochem) and anti-pSTAT3 antibody (1:100 dilution) (Santa Cruz Biotechnology). These antibodies react specifically with phosphorylated antigen. To evaluate the nuclear number, sections were counterstained with hematoxylin solution.

Quantitation of Light Microscopy—Glomerular morphometry was evaluated in PAM-stained tissues. The glomerular surface area and the PAM-positive area/glomerular area (%) were measured using an image analyzer with a microscope (IPAP, Image Processor for Analytical Pathology; Sumitomo Chemical Co., Osaka, Japan) as described (18–20). For each animal, 50 glomeruli were analyzed.

Quantitation of Immunohistochemistry—For the quantitation of proliferating cells (PCNA-positive cells), a blind test evaluated 50 glomeruli in each specimen, and the mean values/glomerulus were calculated. To quantitate the expression of pSmad1, pSmad1-positive cells/glomerular cell were counted, and the mean percentages of pSmad1-positive cells were calculated. For Col IV, Smad1, and pSTAT3, the brown area on an immunoperoxidase-stained section was selected for its color range, and the percentages of this area to the total mesangial area were quantitated using IPAP. In each animal, 50 glomeruli were evaluated.

Cell Culture Experiment

A glomerular mesangial cell line was established from glomeruli isolated from normal, 4-week-old mice (C57BL/6JxSJL/J) and identified according to a previously described method (21). Mesangial cells were maintained in B medium (a 3:1 mixture of minimal essential medium/F12 modified with trace elements) supplemented with 1 mM glutamine, penicillin at 100 units/ml, streptomycin at 100 mg/ml, and 20% fetal calf serum (FCS). The cultured cells fulfilled the generally accepted criteria for glomerular mesangial cells (22). Mesangial cells were plated in B medium/20% FCS onto 100-mm dishes. After 24 h of incubation, the cells were starved for 2 days in B medium/0.1% bovine serum albumin, cultured in B medium/2% FCS with 5 ng/ml of PDGF-B (Calbiochem), and then incubated with 100 ng/ml of APB5 or control rat IgG for 24 h.

Cell Proliferation Test by BrdUrd ELISA

The proliferation of mesangial cells was also determined using a colorimetric immunoassay, based on the measurement of BrdUrd incorporation during DNA synthesis (Amersham Biosciences). The BrdUrd ELISA was performed according to the manufacturer's instructions. Briefly, mesangial cells were plated out at a low density in 96-well flat-bottomed microtiter plates in B medium/10% FCS and allowed to adhere overnight. The subconfluent cells were then starved for 2 days in B medium/0.1% bovine serum albumin. 100 ng/ml of APB5 was added to cells in B medium/2% FCS with 5 ng/ml PDGF-B and 10 mM BrdUrd. After 6 h of culture, the plates were centrifuged and the cells denatured with a fixative solution and then incubated for 30 min with 1:100 diluted anti-BrdUrd monoclonal antibodies conjugated to peroxidase. After removing the antibody conjugate, substrate solutions were added for 15 min, and the reaction was terminated by adding 1 M sulfuric acid. The absorbance was measured within 5 min at 450 nm with a reference wavelength at 690 nm using an ELISA plate reader (Model550; Bio-Rad Laboratories). The blank corresponded to 100 μl of culture medium with or without BrdUrd.

Western Blot Analysis

Cultured mesangial cells were starved for 24 h in B medium/0.1% bovine serum albumin. The cells were stimulated with 5 ng/ml PDGF-BB with 100 ng/ml APB5 or control IgG for 120 min. Cells were suspended in lysis buffer, resolved by SDS-polyacrylamide gel electrophoresis, and transferred to a nitrocellulose membrane. They were subjected to Western blot using a 1:1000 dilution of antibodies for pSTAT3, 1:1000 dilution of antibodies for pSmad1, and 1:2000 dilution of antibodies for Col IV and detected using an enhanced chemiluminescence detection system (Amersham Biosciences).

Treatment of AG490 in Cultured Mesangial Cells

Cultured mesangial cells were starved for 24 h in B medium/0.1% bovine serum albumin. The cells were stimulated with 5 ng/ml PDGF-BB with 50 μM AG490 (Calbiochem), a well characterized inhibitor of STAT3 phosphorylation (23, 24), or control vehicle for 8 h. Cell lysis and Western blot analysis were performed as previously described. The proliferation of mesangial cells was also determined using a BrdUrd ELISA system.

Cell Transfection

Plasmid constructs of expression vectors of wild type STAT3 and dominant negative STAT3 were kindly provided by Jackie Bromberg (Rockefeller University) (25). Mesangial cells (60-mm dish) were transfected with an expression vector encoding wild type STAT3 (8 mg) or dominant negative STAT3 (8 mg) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. After 6 h of transfection, the medium was changed to growth medium (60% Dulbecco's modified Eagle's medium, 20% F12, 20% fetal calf serum). After 48 h, cells were suspended in lysis buffer, and Western blot analysis was performed as previously described. The proliferation of transfected mesangial cells was also determined using a BrdUrd ELISA system.

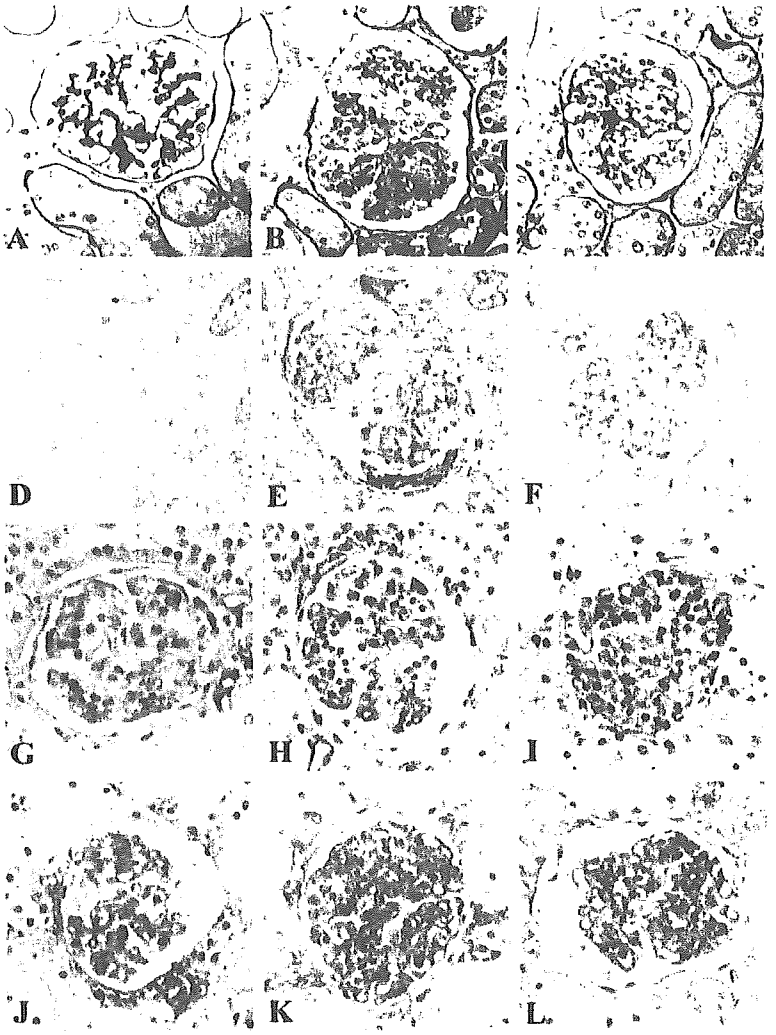

FIG. 1. **Morphological changes and effect of administration of APB5 in Thy1 GN glomeruli.** The microscopic lesions in Thy1 GN rats took the form of the diffuse proliferation of mesangial matrix and an expansion of the mesangial area. Immunohistochemical staining with anti-Col IV antibodies revealed the overexpression of Col IV in the expanded mesangial area of glomeruli from kidneys of Thy1 GN. APB5 reduced the mesangial proliferation and expression of Col IV. Thy1 GN also showed significantly positive expression of PDGF-B and PDGFβ-receptor in the glomeruli, and APB5 could reduce these overexpressions. *A–C*, PAM. *D–F*, Col IV. *G* and *H*, PDGF-B chain. *J* and *K*, PDGFβ-receptor. *A*, *D*, *G*, *J*, normal control rats. *B*, *E*, *H*, *J*, disease control rats on day 6. *C*, *F*, *I*, *L*, APB5-treated rats on day 6.

Statistical Analysis

All values were expressed as the mean ± S.E. and analyzed by Mann-Whitney nonparametric analysis or one-way analysis of variance with a modified *t* test. Statistical significance was defined as $p < 0.05$. Statistical analysis of the cell proliferation test and expression of Smad1 mRNA in cultured mesangial cells were done by *t* test. Quantitation of immunohistochemistry and expression of Smad1 mRNA in glomeruli were analyzed by one-way analysis of variance followed by the *post hoc* test. *p* values <0.05 were considered significant. Data are expressed as mean ± S.D.

RESULTS

Morphological Changes in Thy1 GN—We examined the *in vivo* role of Smad1 phosphorylation in glomerulonephritis. We utilized an acute model of mesangial proliferative glomerulonephritis known as Thy1 glomerulonephritis. In Thy1 GN, the proliferation of mesangial cells begins at day 2, peaks at day 6, and subsides in 12 days after the injection. Fig. 1 shows a representative light microscopic picture at day 6 for each group. The Thy1 GN group showed the increase of mesangial matrix, which peaked at day 6 (Fig. 1*B*). Increased replication of glomerular cells was assessed by immunostaining of PCNA. PCNA-positive cells were markedly increased in the Thy1 GN group and peaked at day 6 (data not shown). Col IV is a main component of the ECM in the glomerulosclerosis. Col IV was weakly visible along the glomerular basement membrane and insignificant in the mesangial area in the normal control group (Fig. 1*D*). On the other hand, the Thy1 GN group showed a strong positive reaction for Col IV in the expanded mesangial area (Fig. 1*F*).

Thy1 GN also showed overexpression of the PDGF-B and PDGFβ-receptor in the glomeruli (Fig. 1, *H* and *K*). These findings indicate that the excessive proliferation of mesangial cells, glomerular hypertrophy, and glomerular sclerotic changes occur simultaneously in glomerulonephritis induced by the anti-Thy1 antibody.

Anti-PDGFβ-receptor Antibody Inhibits Both Glomerular Cell Proliferation and Glomerulosclerosis in Vivo—APB5 inhibited the objective PDGFβ-R-mediated signaling pathway as described previously. Treatment with APB5 resulted in significant reductions of both glomerular cell numbers and glomerular PCNA-positive cells in Thy1 GN at each point studied (Figs. 1*C* and 2, *A* and *B*). Overexpression of the PDGF-B chain and PDGFβ-R were significantly reduced after the administration of APB5 (Fig. 1, *I* and *L*). APB5 treatment also reduced the increase in mesangial matrix in Thy1 GN, as assessed by the PAM-positive area/glomerular area using an image analyzer with a microscope (Fig. 2*C*). The mesangial expression of Col IV in Thy1 GN was suppressed by treatment with APB5 (Fig. 2*D*). These data indicate that APB5 is able to inhibit mesangial cell proliferation and the mesangial matrix expansion of Thy1 GN.

Time Course for the Expression of Smad1, Phospho-Smad1 (pSmad1), and Phospho-STAT3 (pSTAT3) in Thy1 GN—We examined the expression of Smad1 in a Thy1 GN rat kidney by

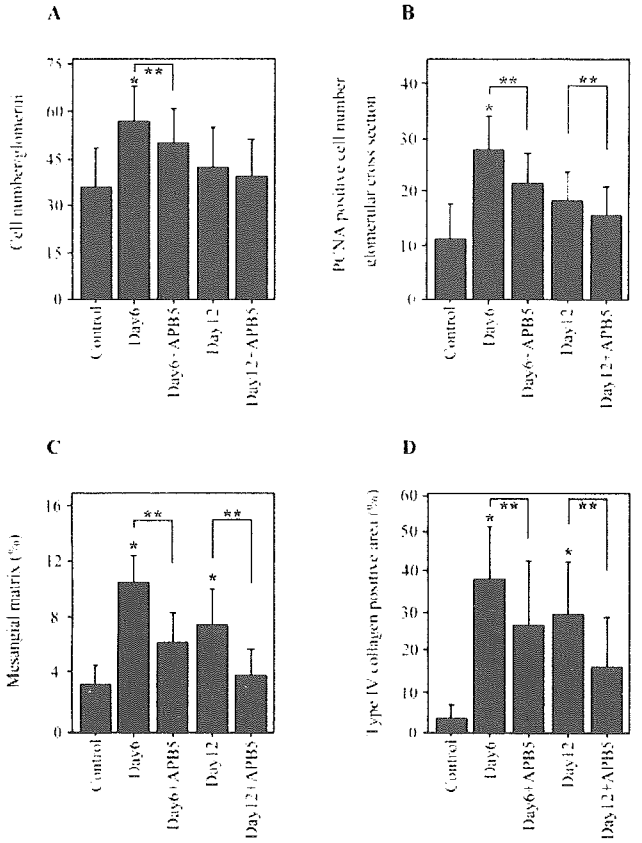

FIG. 2. **Quantitation of mesangial cell proliferation and glomerulosclerosis in Thy1 GN.** To quantitate histological changes in Thy1 GN and the effect of administration of APB5, glomerular cell number and PCNA-positive cell number were determined, and an IPAP system was used to quantitate the mesangial matrix and Col IV-positive areas. *A*, glomerular cell number. Increase in glomerular cell number was observed in Thy1 GN groups. *B*, PCNA-positive cell number of Thy1 GN. The number of PCNA-positive cells in the glomeruli of rats treated with APB5 was significantly reduced at each point studied. *C*, mesangial matrix proliferation. Mesangial matrix proliferation was observed at day 6 in Thy1 GN rats. APB5 significantly reduced this proliferation on each point studied. *D*, expression of type IV collagen. In the control group, Col IV was strongly positive in the expanded mesangial area. APB5 significantly reduced this expression. *, $p < 0.001$ *versus* Control. **, $p < 0.001$ *versus* non-treated disease control.

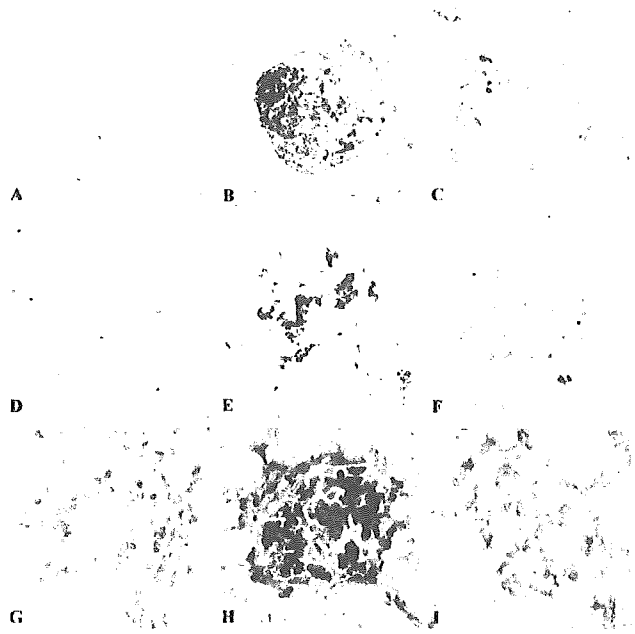

FIG. 3. **Immunohistochemical staining of Smad1, pSmad1, and pSTAT3 in Thy1 GN.** A remarkable increase in the expression of Smad1, pSmad1, and pSTAT3 was noted by immunohistochemical staining in the Thy1 rat glomeruli. pSmad1 was markedly observed with a nuclear pattern in Thy1 GN. APB5 treatment led to a significant decrease. *A–C*, Smad1. *D–F*, pSmad1. *G–I*, pSTAT3. *A*, *D*, *G*, normal control rat. *B*, *E*, *H*, untreated Thy1 rat glomeruli at day 6. *C*, *F*, *I*, treated with APB5 at day 6.

immunostaining. Smad1 was barely detectable in control glomeruli (Fig. 3*A*). However, in the glomerulus of Thy1 GN at day 6, Smad1 was extensively expressed with a typically expanded mesangial pattern (Fig. 3*B*). An IPAP system was used to quantitate the expression of Smad1. Glomerular Smad1 expression peaked at day 6 (Fig. 4*A*), consistent with the peak for mesangial proliferation. We next examined whether the phosphorylation and translocation of Smad1 are affected in Thy1 GN. By immunohistochemistry, pSmad1 was barely observed in the control group (Fig. 3*D*). However, in the Thy1 GN group, pSmad1 was extensive with a nuclear pattern (Fig. 3*E*). To quantitate the expression of pSmad1, the number of pSmad1-positive cells/glomerular cell was determined (Fig. 4*B*). The expression of pSmad1 was up-regulated at day 1 of Thy1 GN and glomerular pSmad1 expression peaked at day 4 of the disease, which also corresponds to the early phase of mesangial cell proliferation. Because PDGF-B and PDGFβ-R were up-regulated in Thy1 GN and APB5 reduced the overexpression, we performed immunostaining for phospho-STAT3, a transcription factor of the PDGF signaling pathway (26). The expression of pSTAT3 was extensively increased in Thy1 GN (Fig. 3, *G* and *H*) and peaked at day 6 (Fig. 4*C*).

The APB5-treated groups had significantly reduced expression of Smad1 and pSmad1 proteins in glomeruli in Thy1 GN (Figs. 3, *C* and *F*, and 5, *A* and *B*). In addition, the overexpression of pSTAT3 was significantly inhibited by the administration of ABP5 at each point studied (Figs. 3*I* and 5*C*).

Effect of Anti-PDGFβ-R Antibody in Vitro—To determine whether APB5 inhibits the proliferation of mesangial cells, we examined the proliferation of mesangial cells in the absence and the presence of APB5 by using the BrdUrd ELISA system. As shown in Fig. 6*A*, the addition of APB5 suppressed PDGF-induced DNA synthesis in cultured mesangial cells. Next we examined whether the presence of phosphorylation inhibitors can prevent the actions of STAT3 in mesangial cells. AG490, an inhibitor of STAT3 phosphorylation, significantly inhibited STAT3 phosphorylation in cultured mesangial cells and mesangial proliferation (Fig. 6*B*). We also examined by Western blot analysis whether APB5 reduces the expression of pSTAT3, pSmad1, and Col IV in mesangial cells after stimulation with PDGF-B. We found that APB5 decreased phosphorylation of STAT3 and Smad1 and the expression of Col IV (Fig. 6*C*). These data suggest that STAT3 and Smad1 are involved to a significant extent in the expression of Col IV induced by stimulation of the PDGF-B signaling pathway.

Interaction between STAT3 and Smad1—To elucidate the role of the interaction between STAT3 and Smad1 for the increased expression of Col IV, transfection with a vector encoding dominant negative STAT3 was performed in cultured mesangial cells. Transfection with the dominant negative STAT3 clearly decreased the expression of pSmad1 and Col IV compared with wild type STAT3 (Fig. 6*D*). Using the BrdUrd ELISA system, we further examined whether transfection with dominant negative STAT3 reduces mesangial proliferation. The dominant negative STAT3 suppressed PDGF-induced DNA synthesis in cultured mesangial cells.

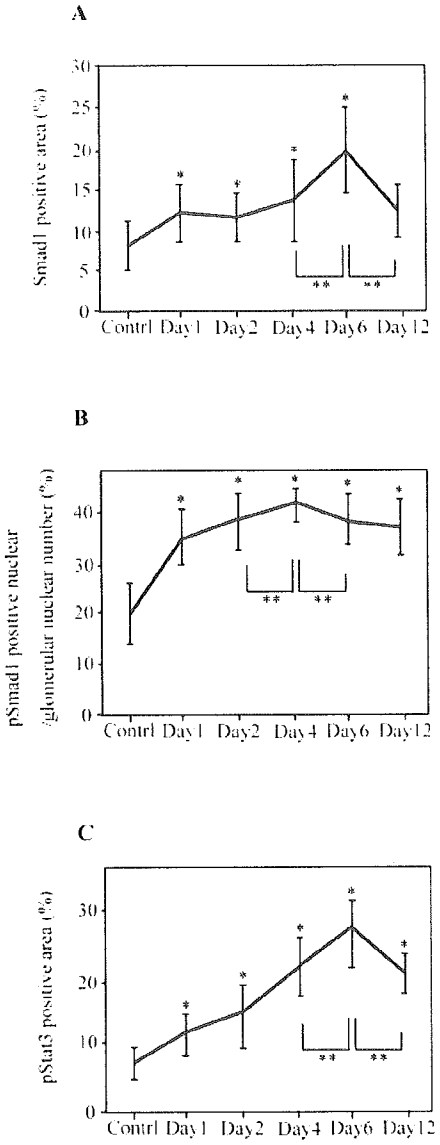

FIG. 4. **Time course of expression of Smad1, pSmad1, and pSTAT3.** Kidney sections from Thy1 GN rats on days 0, 1, 2, 4, 6, and 12 were subjected to immunohistochemical staining with antibodies against Smad1, pSmad1, and pSTAT3. *A*, expression of Smad1 in Thy1 GN. Expression of Smad1 peaked at day 6 and subsided on day 12. *B*, the time course for the ratio of pSmad1-positive cells to total glomerular cell number. Expression of pSmad1 peaked at day 4. *C*, time course of expression of pSTAT3. The ratio of pSTAT3-positive area to mesangial area increased by day 6 and subsided on day 12. *, $p < 0.001$ *versus* Control. **, $p < 0.001$ *versus* each day.

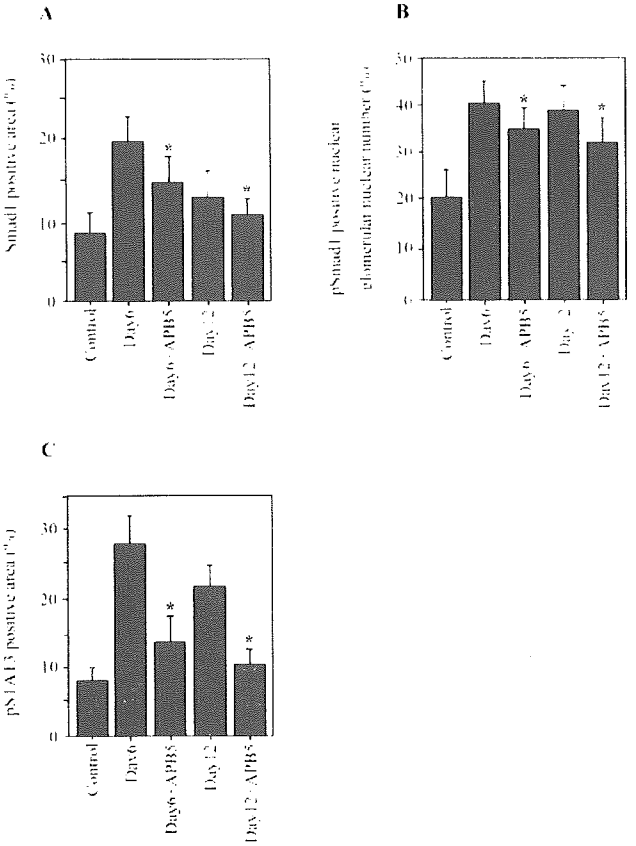

FIG. 5. **Effects of APB5 treatment on Smad1, pSmad1, and pSTAT3 expression.** Immunohistochemical staining and quantitation of Smad1, pSmad1, and pSTAT3 expression revealed treatment with APB5 reduced the expression of these proteins similar to mesangial matrix expansion and Col IV expression in glomeruli. *A*, expression of Smad1. *B*, expression of pSmad1. *C*, expression of pSTAT3. *, $p < 0.001$ *versus* non-treated disease control.

DISCUSSION

Many glomerular diseases are characterized by both mesangial cell proliferation and progressive glomerulosclerosis, but the common mechanisms related to both of these important pathological findings remain unresolved. The findings herein have shown that the activation of STAT3 and Smad1 plays a key role in regulating both of these critical events of progressive glomerular damage. Based on these findings, we have proposed a new direction of research concerning the pathogenesis and a therapeutic approach for chronic glomerulonephritis and diabetic nephropathy, which are major problems in the 21st century.

Glomerulosclerosis is characterized by an increased amount of ECM mainly in the mesangium. Col IV is one of the major components of ECM and is overproduced in glomerulosclerosis (27). We recently reported that Smad1 is a key transcriptional factor in the regulation of Col IV expression in diabetic nephropathy *in vitro* and *in vivo* (8). In Thy1 GN, Col IV is strongly expressed in the sclerotic lesions of glomeruli as previously described (19). We showed here that phosphorylated Smad1 is strongly expressed in parallel with the up-regulated expression of Col IV and the expanded ECM in this glomerulonephritis. Moreover, the area in which Smad1 is strongly expressed is consistent with the Col IV-positive area. These findings suggest that Smad1 is a critical factor in the development of glomerulosclerosis not only in diabetic nephropathy but also in glomerulonephritis as well.

Glomerulosclerosis has the pathological features of progressive glomerular injuries, including chronic glomerulonephritis, IgA nephropathy, and diabetic nephropathy. Glomerular cell proliferation at an early stage in a number of glomerular diseases progresses to the subsequent development of glomerulosclerosis, which eventually progresses to end stage glomerular damages (1, 2). This process is seen in IgA nephropathy, membranoproliferative glomerulonephritis, diabetic nephropathy, and light chain systemic diseases in humans as well as in animals such as the anti-thy1 glomerulonephritis and the rat renal ablation models (2, 28). Blocking glomerular cell proliferation with an anti-PDGF antibody (7), anti-coagulant heparin (30), or vitamin D analogue (19) has been demonstrated to abolish the subsequent development of progressive glomerulosclerosis, but responsibility for this remains unclear. In the

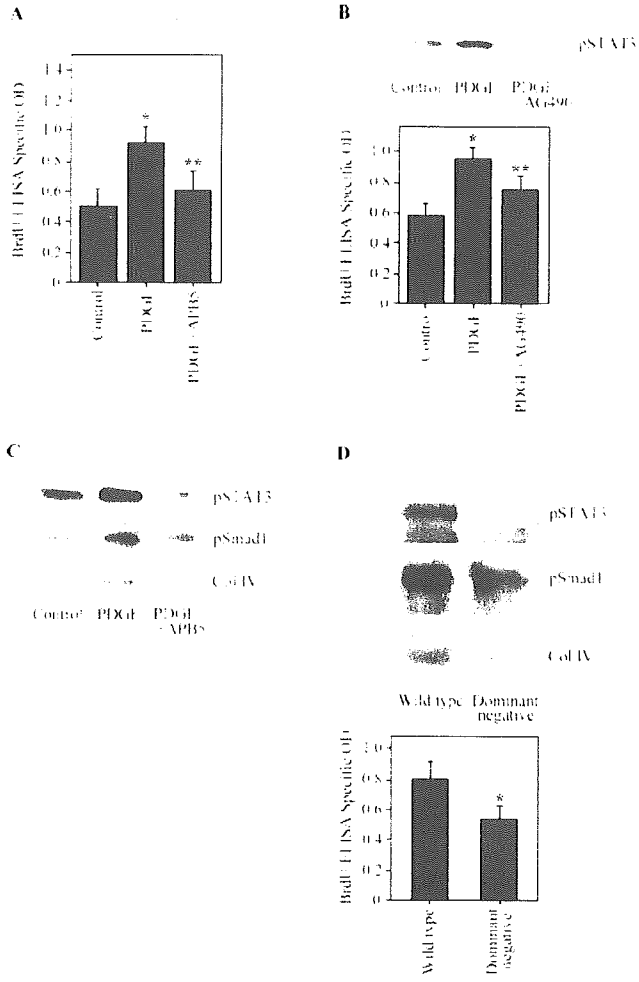

FIG. 6. **Effect of APB5 *in vivo* and Western blot analysis of transfected mesangial cells.** *A*, inhibitory effects of APB5 on mesangial cell proliferation. Addition of PDGF-B increased the proliferation of mesangial cell, and APB5 significantly inhibited this proliferation. *, $p < 0.05$ *versus* control. **, $p < 0.05$ *versus* PDGF-B-stimulated control. *B*, AG490 significantly inhibited STAT3 phosphorylation in PDGF-B-stimulated mesangial cells and mesangial proliferation. *, $p < 0.05$ *versus* Control. **, $p < 0.05$ *versus* PDGF-B-stimulated Control. *C*, Western blot analysis revealed that expression of pSTAT3, pSmad1, and Col IV protein was reduced by addition of APB5. One of three independent experiments is shown. *D*, expression of pSmad1 and Col IV protein was reduced by dominant negative STAT3. One of three independent experiments is shown. Mesangial proliferation was reduced by dominant negative STAT3. *, $p < 0.05$ *versus* wild type.

current study, we demonstrated a possible mechanism for regulating the interaction between mesangial cell proliferation and glomerulosclerosis for these pathological processes.

In this study, we showed that pSTAT3 is strongly expressed in mesangial proliferative lesions in Thy1 GN. Thy1 GN is a well characterized model in rats that leads to an acute phase of complement-dependent mesangial cell lysis, followed by a phase of intense mesangial cell proliferation and expansion of the mesangial matrix resembling the morphological features of human mesangial proliferative glomerulonephritis. A previous study reported that STAT3 serves as a signaling molecule in the development of glomerulonephritis (31). The current study suggests that the activation of Smad1 and STAT3 is involved in the development of glomerulosclerosis from mesangial proliferation.

A receptor for PDGF has been identified in murine and human mesangial cells (32). PDGF is a key potent mitogen for mesangial cells and is constitutively synthesized as an autocrine growth factor by these cells *in vitro* (32, 33). PDGF plays an important role in the development of pathological conditions, including glomerulonephritis, diabetic nephropathy, and progressive glomerulosclerosis *in vitro* and *in vivo* (3, 4). It has been previously reported that the activation of PDGF receptor tyrosine kinase induces the tyrosine phosphorylation of STAT3 proteins (13, 34). This activation is associated with growth regulation and differentiation (28, 29). The findings herein have demonstrated that the overexpression of phosphorylated STAT3 occurs simultaneously with the increased expressions of both PDGF and its β-receptor in this experimental glomerulonephritis and that APB5 ameliorates glomerulonephritis in association with reduced expression of PDGF, its β-receptor, and STAT3 *in vivo*. We have also shown that treatment with APB5 reduces the expression of Smad1 in Thy1 GN, indicating that the PDGF pathway can affect Smad1 production *in vivo*.

We confirmed the interaction of STAT3 and Smad1 in regulating the critical gene of glomerulosclerosis. The introduction of dominant negative STAT3 significantly decreased the expression of Col IV in cultured mesangial cells. The activation of STAT3 and Smad1 appears to be independent, although both factors are activated by PDGF. Furthermore, the activation of Smad1 appears to be involved in the activation of STAT3, based on the findings of a partial reduction of phosphorylated Smad1 by the introduction of dominant negative STAT3. These findings suggest that STAT3 activation by PDGF interacts with the overexpression of Smad1, followed by the subsequent activation of Col IV in experimental glomerulonephritis. Nakashima *et al.* (14) report that the transcriptional coactivator p300 physically interacts with STAT3 and Smad1 and that the formation of a complex between STAT3 and Smad1, bridged by p300, is involved in the cooperative signaling of the pathway. Thus, we concluded that the blocking of PDGF could affect the signaling of Smad1 and reduce the overproduction of Col IV *in vitro* and *in vivo*. The clear elucidation of both signaling pathways is essential for developing a complete understanding of the pathological process for development of progressive glomerular injury.

Therapeutic approaches for sclerosis in diverse organs are currently limited to supportive therapy to slow the loss of function of these organs. Our findings offer insights into the nature of the proliferative diseases that lead to sclerosis. Because both Smad1 and STAT3 are nearly absent in normal glomeruli, blocking Smad1 and/or STAT3 signaling may be beneficial for inhibiting the progression of various renal diseases leading to sclerosis by suppressing the pathologically activated proliferation and production of ECM.

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CXCL16 is a novel angiogenic factor for human umbilical vein endothelial cells

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Abstract

CXCL16 is a unique chemokine with characteristics as a receptor for phosphatidylserine and oxidized low density lipoproteins in macrophages, and is involved in the accumulation of cellular cholesterol during atherosclerotic lesion development. In this study, we report a new function of CXCL16 as a novel angiogenic factor in human umbilical vein endothelial cells (HUVEC). CXCL16 stimulated proliferation and chemotaxis of HUVEC in a dose-dependent manner, reaching a maximum at 1 nM. CXCL16 also significantly induced tube formation of HUVEC on Matrigel. Further, exposure of HUVEC to CXCL16 led to a time- and dose-dependent activation of mitogen-activated protein kinase (ERK1/2), which was completely inhibited by a mitogen-activated protein kinase kinase inhibitor, PD98059. Proliferation and tube formation in response to CXCL16 were also blocked by the pretreatment with PD98059, but not CXCL16-induced chemotaxis. Thus, our data indicate that CXCL16 may act as a novel angiogenic factor for HUVEC and that ERK is involved as an important signaling molecule to mediate its angiogenic effects.

Keywords: CXCL16; Angiogenesis; Endothelial cell

Angiogenesis, the formation of new blood vessels from pre-existing endothelium, is a fundamental step in a variety of physiological and pathological conditions including wound healing, embryonic development, chronic inflammation, and tumor growth [1–3]. The process of angiogenesis is complex and involves several discrete steps, such as extracellular matrix degradation, proliferation, migration, and morphological differentiation of endothelial cells to form tubes [4]. The angiogenic process is tightly controlled by a wide variety of positive or negative regulators, and many of these factors are initially characterized in other biological activities.

CXCL16 was identified as a chemokine produced from dendritic cells, which attracts T cells in lymphoid organ [5,6]. We independently cloned this molecule as a scavenger receptor that binds phosphatidylserine and oxidized lipoprotein (SR-PSOX) [7], and showed that this molecule is expressed by macrophages in vitro and in atherosclerotic lesions in vivo [7,8]. It is also involved in the massive accumulation of cellular cholesterol during the generation of macrophage foam cells associated

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with atherosclerotic lesion development [9]. In addition to macrophages, Hofnagel et al. [10] have demonstrated CXCL16 expression in cultured aortic smooth muscle cells (ASMC) by RT-PCR. More recently, it has been reported that human ASMC express CXCR6, and that CXCL16 increases cell–cell adhesion and ASMC proliferation [11]. We recently found that CXCL16 was strongly expressed in valvular and neocapillary endothelial cells in patients with infective endocarditis, rheumatic, and atherosclerotic valvular disease [12]. These data indicate that CXCL16 can also be involved in proliferation and migration of endothelial cells. Accordingly, we asked whether CXCL16 might act as a novel angiogenic stimulus directly involved in the processes of angiogenesis.

In the present study, we investigated the angiogenic activity of CXCL16 for endothelial cells. We also examined the signaling cascades involved in CXCL16-mediated angiogenic effects in human umbilical vein endothelial cells (HUVEC) in vitro.

Materials and methods

Materials. A mitogen-activated protein kinase kinase (MEK) inhibitor, PD98059, was obtained from Calbiochem (San Diego, CA). Recombinant human CXCL16 was from R&D Systems (Minneapolis, MN). bFGF was from Kurabo (Osaka, Japan).

Cell culture. HUVEC were purchased from Kurabo and grown in HuMedia EG2 (Kurabo) culture medium. Cells were maintained at 37 °C in 5% CO_2 and 95% ambient air. Experiments were performed with cells in 3–6 passages.

Cell proliferation assay. Cell proliferation was examined using WST-1. WST-1 assay is based on formazon dye formation, which directly correlates with the number of metabolically active cells in the culture. 6×10^3 cells grown in a 96-well plate for 24 h were partially starved in Humedia EB2 supplemented with 1% FBS for 24 h and then stimulated by CXCL16 for another 48 h. In some cases, cells were pretreated with PD98059 (25 μM) for 30 min before stimulation. Ten microliters of WST-1 solution (Takara, Japan) was added to each well and incubated for 3 h, and absorbencies at 450 nm of the medium were measured using a microplate reader (Bio-Rad, Model 550, Tokyo, Japan).

Chemotaxis assay. The chemotactic motility was assessed using a modified Boyden chamber with a 5 μm-pore polycarbonate filter (Neuro Probe, Gaithersburg, MD). Cells were starved in Humedia EB2 with 1% FBS for overnight before assays. Fresh 0.5% BSA Humedia EB2 containing CXCL16 was placed in the lower wells. Trypsin-harvested cells were suspended at a final concentration of 5×10^5/ml in Humedia EB2 containing 0.5% BSA. PD98059 was given to the cells for 30 min at room temperature before seeding. Fifty microliters of the cell suspension was loaded into each upper well and the chamber was then incubated at 37 °C for 4 h. Non-migrating cells on the upper surface of the filter were removed by rubber swab. Filters were fixed and stained with Diff-Quik solution (International Reagents, Kobe, Japan). Cell motility was quantified by counting cells that migrated across the filter toward the lower surface in three fields per filter under optical microscope at 100× magnification.

Tube formation assay. The tube formation assay was performed as previously described [13]. Briefly, 250 μl of growth factor-reduced Matrigel (BD Biosciences Discovery Labware, Bedford, MA) was added per well of a 24-well plate and allowed to polymerize at 37 °C for at least 30 min. Trypsin-harvested cells (5×10^4) suspended in 250 μl of Humedia EB2 containing CXCL16 or bFGF were seeded onto Matrigel. PD98059 was given to the cells for 30 min at room temperature before seeding. After incubation for 24 h at 37 °C, capillary-like structures within the Matrigel layer were stained with calcein (Molecular Probes, Eugene, OR) and photographed under fluorescent microscope. Total tube length was quantified using image analysis software of Image-Pro Plus (version 3.0; Media Cybernetics, Baltimore, MD).

Immunoprecipitation and Western blotting. HUVEC were incubated in Humedia EB2 with 1% FBS overnight before stimulated with CXCL16. In some cases, cells were pretreated with PD98059 (25 or 50 μM) for 30 min before stimulated by CXCL16. ERK activation was measured by p44/42 MAP Kinase Assay Kit (Cell Signaling Technology, Beverly, MA). Briefly, the cell lysate was collected by ice-cold cell lysis buffer provided by the kit. Immunoprecipitation was done by using immobilized phospho-p44/42 MAP kinase (Thr202/Tyr204) monoclonal antibody. The precipitate is then incubated with Elk-1 fusion protein to active ERK to phosphorylate Elk-1. Subsequently, protein from each sample was loaded onto a sodium dodecyl sulfate 12% polyacrylamide gel and transferred onto a PVDF membrane. The blocked membranes were then incubated with rabbit anti-phospho-Elk-1 antibody (1:1000). Immunoreactive bands were visualized using detection reagents.

Statistical analysis. Data are expressed as means ± SD. Statistical analyses were performed by using one-way ANOVA followed by Dunnett's test to evaluate statistical significance between two groups. p values less than 0.05 were considered statistically significant.

Results and discussion

CXCL16 induces proliferation, migration, and tube formation of HUVEC

To determine whether CXCL16 can induce angiogenesis in endothelial cells, the ability of CXCL16 as an angiogenic stimulus has been assessed in in vitro angiogenesis models. We first examined the effect of CXCL16 on HUVEC proliferation by WST-1 assay. Incubation of the cells with CXCL16 for 48 h resulted in an increase of HUVEC in a dose-dependent manner significantly from 0.1 to 10 nM and the cell number reached a plateau at 1 nM (Fig. 1A). CXCL16 (1 nM) increased the cell number by approximately 35% over the control. We then compared the effect of CXCL16 with that of basic fibroblast growth factor (bFGF) (10 ng/ml), which is known to stimulate HUVEC proliferation (Fig. 1B), and found that the effect of CXCL16 on endothelial proliferation was less potent than that of bFGF. CXCL16 is also shown to induce ASMC proliferation at 5 nM [11]. Therefore, the effect of CXCL16 on endothelial proliferation is consistent with the effect on other cell type. We next determined the effect of CXCL16 on chemotactic motility of HUVEC by modified Boyden chamber assay (Figs. 1C and D). CXCL16 stimulated the chemotactic motility of HUVEC in a dose-dependent manner. The chemotactic motility at 1 nM CXCL16 was 1.4-fold over the control. This effect of CXCL16 was also less potent than that of bFGF.

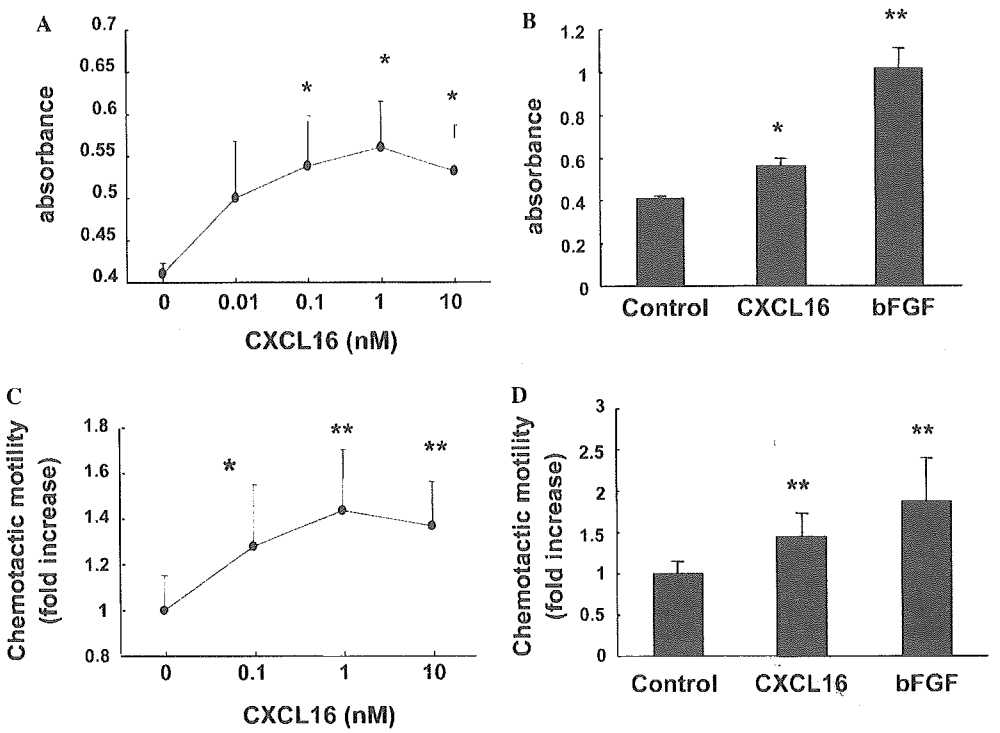

Fig. 1. CXCL16-induced proliferation and migration in HUVEC. (A) After HUVEC were cultured with the indicated concentrations of CXCL16 for 48 h, cell proliferation was determined by WST-1 assay ($n = 7$). (B) Effects of CXCL16 and bFGF on HUVEC proliferation. Cells were stimulated with 1 nM CXCL16 or 10 ng/ml bFGF for 48 h and cell proliferation was determined by WST-1 assay ($n = 7$). (C) CXCL16-induced chemotaxis in HUVEC. HUVEC were placed in the upper chamber, and the various concentrations of CXCL16 were added in the lower chamber. Cell migration was assayed by modified Boyden chamber assay ($n = 9$). (D) Effects of CXCL16 and bFGF on chemotaxis of HUVEC ($n = 9$). EB2 (0.5% BSA) with 1 nM CXCL16 or 20 ng/ml bFGF was placed in the lower wells. After 4 h, chemotactic motility of HUVEC was measured as described under Materials and methods. Data A and B represent means ± SD, and similar results were obtained in three independent experiments. Data C and D are expressed as fold increase over control ± SD from three independent experiments with triplicate determinations. $^{*}p < 0.05$; $^{**}p < 0.01$ vs. control.

The effect of CXCL16 on the morphological differentiation of HUVEC into vascular structures in vitro was investigated using Matrigel tube formation assay. When placed on growth factor-reduced Matrigel in the absence of angiogenic factors, HUVEC did not form complete tube-like structures. However, by adding 1 nM of CXCL16, we could observe the networks similar to those formed by bFGF, which consisted of a large number of cells (Fig. 2A). By measuring the length of tube in the tube network using an image analysis program, CXCL16 (1 nM) stimulated tube formation by 1.5-fold over the control (Fig. 2B). In addition, CXCL16 stimulated the tube formation in a dose-dependent manner with a significant increase from 0.01 to 10 nM (data not shown). These results indicate that CXCL16 has a novel angiogenic activity in in vitro human endothelial cell culture system. However, these effects of CXCL16 were weaker than those of bFGF.

CXCL16 activates ERKs in HUVEC

Recent studies have shown that activation of ERK is closely involved in proliferation, migration, or morphogenesis of endothelial cells induced by various angiogenic factors [13–17]. Therefore, we examined whether CXCL16 can stimulate ERK in HUVEC. Subconfluent HUVEC were exposed to various concentrations of CXCL16, and the activation of ERK was analyzed by immunoprecipitation and Western blot analysis as described under Materials and methods. As shown in Figs. 3A and B, CXCL16 induced the phosphorylation of Elk-1 in a dose- and time-dependent manner. The phosphorylation peaked at 10 min after stimulation with CXCL16 and declined thereafter. We then checked the effect of a specific MEK inhibitor, PD98059, on ERK activation by CXCL16 by adding the inhibitor 30 min before stimulation with CXCL16. As expected, pretreatment with PD98059 abolished CXCL16-induced ERK activation both at 25 and 50 μM (Fig. 3C).

ERK activation is involved in CXCL16-mediated angiogenesis in HUVEC

The activation of the ERK pathway by CXCL16 prompted us to determine whether this biochemical

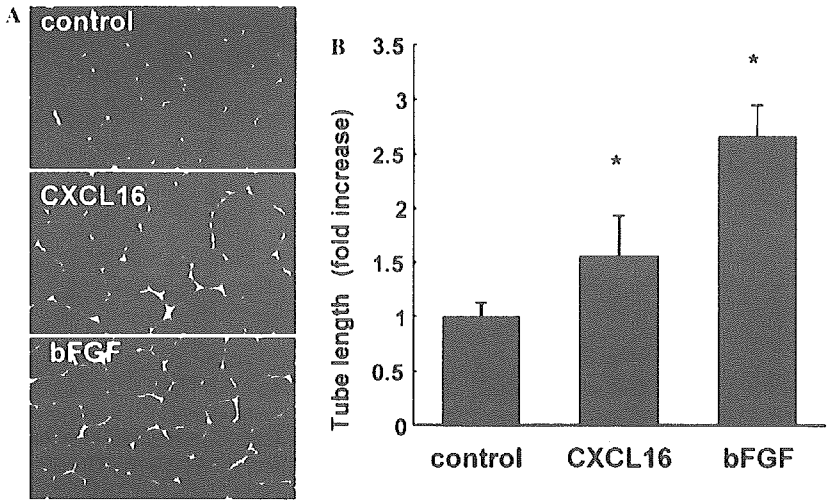

Fig. 2. CXCL16-induced tube formation of HUVEC. HUVEC were plated on Matrigel-coated wells with media alone, 1 nM CXCL16, or 20 ng/ml bFGF (A). After 20 h, the tubes were visualized using calcein staining and photographs were taken under microscope (40×). Shown is a representative picture from three independent experiments. (B) The length of tube was quantitated using Image-Pro Plus software. Data are expressed as fold increase over control and means ± SD from five independent experiments with triplicate determinations. $^{*}p < 0.01$ vs. control.

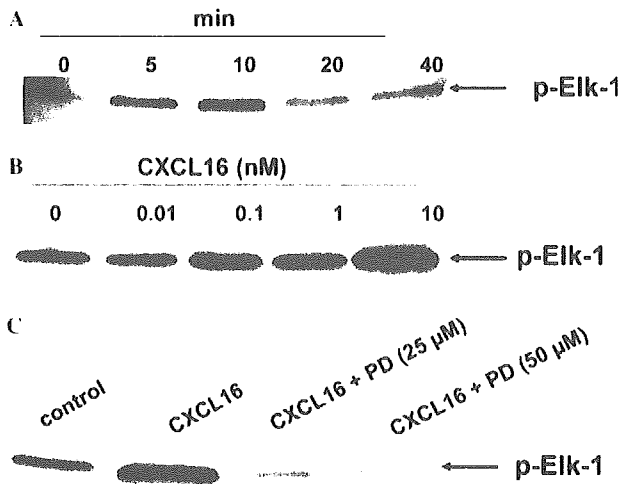

Fig. 3. CXCL16-induced ERK activation was blocked by PD98059 in HUVEC. (A) Time course of CXCL16-induced ERK activation. HUVEC were stimulated with 1 nM CXCL16 for the indicated times. Activation of ERK was determined as described under Materials and methods. Shown is a representative Western blot from three independent experiments. (B) Dose-dependent ERK activation. HUVEC were stimulated with various concentrations of CXCL16 for 10 min. Shown is a representative Western blot from three independent experiments. (C) HUVEC were pretreated with PD98059 (25 and 50 μM) for 30 min and stimulated with 1 nM CXCL16 for 10 min. Shown is a representative Western blot from three independent experiments.

event is important for angiogenic activities of CXCL16 in HUVEC. First, we tested the effect of PD98059 on CXCL16-induced HUVEC proliferation. As shown in Fig. 4A, pretreatment of the cells with PD98059 (25 μM) completely inhibited CXCL16-induced HUVEC proliferation, suggesting that ERK is closely involved in CXCL16-induced endothelial cell proliferation.

It has been reported that ERK is required for endothelial cell chemotaxis stimulated by bFGF and the placental angiogenic hormone proliferin [17,18]. In contrast, VEGF-induced HUVEC migration is reported to be independent of ERK [19]. Therefore, we next examined the effect of PD98059 on CXCL16-induced chemotaxis in HUVEC. As shown in Fig. 4B, PD98059 had no significant effect on CXCL16-induced cell motility, even though the same concentrations of PD98059 blocked CXCL16-induced ERK activation (Fig. 3C). These results do not indicate that CXCL16-induced chemotaxis is mediated by ERK. Although the present study could not identify the signaling mechanisms of CXCL16-induced chemotaxis, our data did not show that activation of ERK by CXCL16 is required for the chemotactic effect in HUVEC.

Finally, we examined the signaling mechanism of CXCL16-induced tube formation in HUVEC. PD98059 (25 μM) preteatment markedly inhibited tube formation induced by CXCL16 in HUVEC (Fig. 5), suggesting that G protein-dependent activation of the ERK pathway is critically involved in promoting morphological differentiation of endothelial cells on Matrigel.

In this report, we demonstrate that CXCL16 has a novel function as an angiogenic factor for endothelial cells. In in vitro angiogenesis models, we have revealed that the stimulation of HUVEC with CXCL16 leads to an increase in cell proliferation as well as chemotactic motility, and strongly induces the formation of tube network. Our data also showed that ERK signaling is involved in cell proliferation and tube formation induced by CXCL16 in HUVEC.

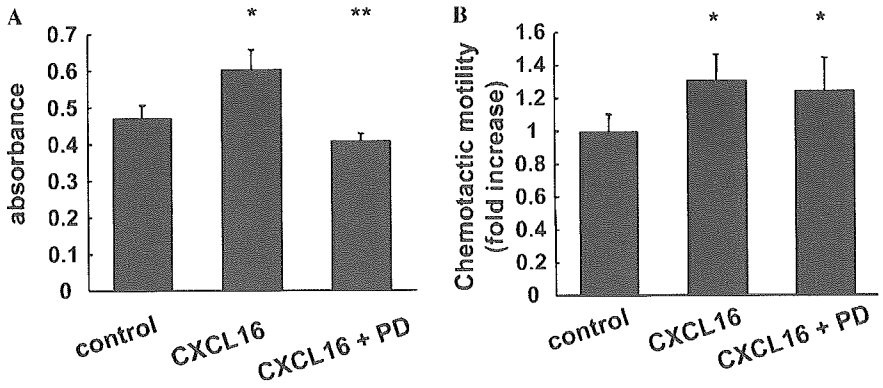

Fig. 4. MEK inhibitor suppressed CXCL16-induced proliferation, but not chemotaxis in HUVEC. (A) Cells were pretreated with or without PD98059 (25 μM) for 30 min and stimulated with 1 nM CXCL16 for 48 h. Cell proliferation was then determined by WST-1 assay. (B) CXCL16-induced chemotactic mobility of HUVEC was determined in the absence or presence of MEK inhibitor PD98059. Cells were preincubated for 30 min with or without PD98059 (25 μM) prior to treating with 1 nM CXCL16. Data A represent means ± SD, and similar results were obtained in two different experiments. Data B are expressed as fold increase over control ± SD from two different experiments with triplicate. *$p < 0.05$ vs. control; **$p < 0.05$ vs. CXCL16 (1 nM).

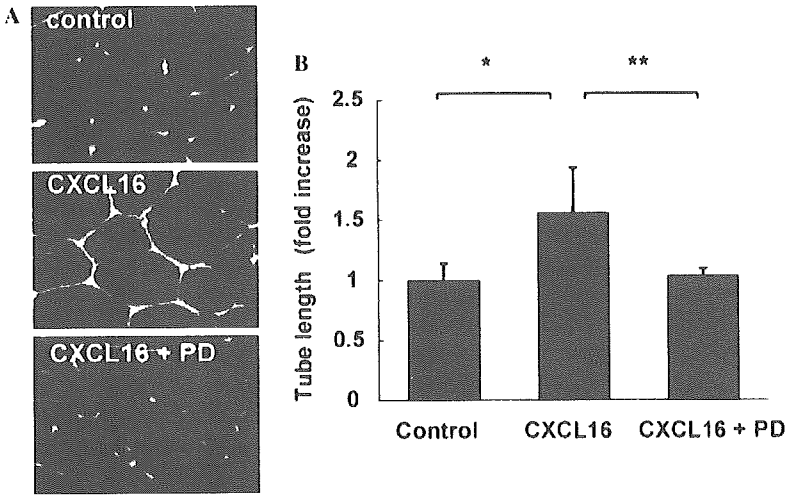

Fig. 5. MEK inhibitor inhibited CXCL16-mediated tube formation in HUVEC. (A) Cells (5×10^4/well) were pretreated with or without PD98059 (25 μM) for 30 min before stimulation with CXCL16 (1 nM). After incubation on Matrigel-coated wells for 20 h, photographs were taken under microscope (40×). Representative pictures are shown. (B) The length of tube was quantitated using Image-Pro Plus software. Data are expressed as fold increase over control ± SD from two independent experiments with triplicate determinations. *$p < 0.05$ vs. control; **$p < 0.05$ vs. CXCL16 (1 nM).

Acknowledgments

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Depression, activities of daily living, and quality of life of community-dwelling elderly in three Asian countries: Indonesia, Vietnam, and Japan

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Abstract

The purpose of this study was to examine the prevalence of screening-based depression and the association of depression with activities of daily living (ADL) and quality of life (QOL) of community-dwelling elderly in the developing and developed countries. A total of 2695 community-dwelling elderly subjects aged 60 years or older living in five rural Asian towns (Indonesia: 411, Vietnam: 379, Japan: 1905) participated in this cross-sectional study. Depressive symptoms were assessed using a 15-item geriatric depression scale (GDS-15). ADL, higher daily activities, and medical and social history were assessed by interviews or self-report questionnaires. For the assessment of subjective QOL, a 100 mm visual analogue scale was used. Using a cut-point of 5/6 for the GDS-15, 782 participants (29.0%) appeared to have depression (Indonesia: 33.8%, Vietnam: 17.2%, Japan: 30.3%). Subjects with depression had significantly lower scores for both

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ADL and QOL than those without depression in all the three countries. In all the three countries, 17.2–33.8% of community-dwelling elderly subjects had screening-based depression, which was commonly associated with both lower quantitative ADL and lower QOL.

Keywords: Community-dwelling elderly; Prevalence of depression; Activities of daily living; Quality of life; Geriatric depression scale short form

1. Introduction

Depressive illness is not only a major threat to the mental health and well-being of elderly patients but also a matter of concern to any primary physician and caregiver treating such patients. Depressive illness is projected to be the second-leading cause of disability worldwide by 2020 (Murray and Lopez, 1997). Thirty-four studies have reported prevalences of depression in the community-dwelling elderly, but with widely varying findings (0.4–35%) (Beekman et al., 1999). Furthermore, most of these studies were performed in developed countries. Methodological differences among studies preclude firm conclusions concerning cross-cultural and geographical variations in the prevalence of depression. In the community, the prevalence of major depression ranges from 1 to 2% among the elderly (Unutzer et al., 1997; Penninx et al., 1998), but other disabling depressive illnesses include dysthymia (a chronic low-grade depression) and minor depression (an episodic, less severe illness), which are common in the elderly.

Pursuit of successful aging is now emphasized, and comprehensive geriatric assessment is considered very important for the achievement of this (Rowe and Kahn, 1987). The health status of the elderly should be assessed comprehensively with regard to their not only physical condition but also emotional condition, social activity, and quality of life (QOL). In the community-based setting, depression in the elderly is difficult to define clearly, and the significance of screening for depression in comprehensive determination of the health status of the elderly has remained unclear. To our knowledge, few studies have examined the relationship of depression to both QOL and activities of daily living (ADL), especially in less developed countries. Xavier et al. (2002) reported that subjects with minor depression had significantly lower life satisfaction and worse indices of life quality in Brazil. However, the size of their random sample was only 77. The purpose of the present study was to examine the prevalence of depression as defined by the 15-item geriatric depression scale (GDS-15) score in two developing countries in southeast Asia and in a developed country, Japan, and to clarify the relationship of depression with quantitative ADL and QOL scores in community-dwelling elderly persons.

2. Methods

2.1. Sample size

A total of 2695 Asian subjects aged 60 years or older living in three Asian countries (two towns in Indonesia, two towns in Vietnam, and one town in Japan) were studied between

June 2002 and November 2003. All five towns were rural. Standardized questionnaires were used throughout. In Indonesia and Vietnam, the questionnaires were translated into the native languages, and then translated back to check for consistency in meaning and content, and standardized interviews were also carried out. Those elderly subjects who agreed to reply and could answer the GDS-15, self-rating questionnaire for ADL impairment, and quantitative subjective QOL examination were included. They were also assessed for living condition, lifestyle, and social and medical history (hypertension, current use of anti-hypertensives, and history of stroke, heart disease, and osteoarthropathies).

2.1.1. Indonesia

A total of 411 subjects (female 59.9%, mean (±S.D) age: 72.3 (±7.3), range 62–105 years, 44.8% of eligible subjects) from two towns in Java Island were studied in February 2003. One is S town, near Bandun city, and the other is K town, about 50 km east of the capital Jakarta. They had populations of 6965 and 11,505, respectively, and their proportions of individuals aged 65 years or older were 5.5% and 4.7%. All the elderly persons living in these towns were informed that they could undergo a health check-up including questionnaire, blood test, and blood pressure measurement as well as consultation with a physician. Finally, 204 and 207 subjects (53.7% and 38.5% of eligible subjects, respectively) participated in this survey and local physicians implemented the questionnaire.

2.1.2. Vietnam

Two rural towns were selected for this study. N town is about 150 km northwest of the capital Hanoi. In this town, four communities were selected for inclusion in this study. All the elderly aged 60 years or older living in these communities were informed that they could have a health check-up including questionnaire, blood test, and blood pressure measurement, as well as consultation with a physician, and 191 subjects participated (coverage rate, range, 60–90%). V town is about 80 km northwest of Hanoi. One area of this town was selected for this study, and 262 individuals (26.9% of the elderly living in the area) were randomly selected based on a local government list. However, 62 subjects rejected the inclusion and 12 were excluded because they were under 59 years of age. A total of 379 subjects (female 55.1%, mean age: 70.7 ± 8.0, range 60–98 years, 28.3% of those in the study area) from these two towns were studied in November 2003.

2.1.3. Japan

S town is situated in the midportion of Japan, and has a population of 16,700. The proportion of those aged 65 years or older was 20.0%. All the elderly living in this town were given a self-rating questionnaire. A total of 1450 subjects did not complete the survey for the following reasons: away from the area, in a hospital, in a nursing home, subject could not answer because of severe disability or cognitive impairment, or subject refusal. Finally, 1905 subjects (female 58.0%, mean age; 74.0 ± 6.6, range 65–100 years, 57.0% of eligible subjects) participated in this survey in June 2002.

2.2. Depression screening

The GDS-15 (Yesavage et al., 1982; Sheikh and Yesavage, 1986; Yesavage, 1988) was used for depression screening, and required approximately 4 min to complete and score. Using a cut-point of 6 or more, the GDS-15 has a sensitivity of 88–92% and specificity of 62–81%, compared with results of structured clinical interviews for the determination of depression in Western countries (Gerety et al., 1994; Herrmann et al., 1996; Lyness et al., 1997). In Japan, Schreiner et al. (2003) reported that the cut-point for the GDS-15 for Japanese subjects was the same as that reported for Western subjects. In Indonesia and Vietnam, however, no validation study of the GDS-15 has been reported yet. Thus, the definition of depression was unified with a cut-point for the GDS-15 of 6 or more in these three countries, and a cut-point of 10 or more was used for reference. In Japan, medical histories were self-reported and history of hypertension was defined as self-reported high blood pressure and counter-checked with another item of the questionnaire on the use of anti-hypertensives. In Indonesia and Vietnam, all subjects had blood pressure measured in the sitting position, and hypertension was defined as 140 mmHg or higher systolic blood pressure. In cases in which elderly individuals were not able to directly answer the questions (e.g., unable to read or write clearly), a proxy helped them complete the questionnaire. However, in such cases, questions related to QOL and the GDS-15 were left unanswered if the individuals were unable to indicate the answers themselves.

2.3. Disability

For basic-ADL assessment, each subject rated his/her independence in seven items (walking, ascending and descending stairs, feeding, dressing, going to the toilet, bathing, and grooming). Each basic-ADL item was evaluated using four levels: 3, completely independent; 2, needing some help; 1, needing much help; 0, completely dependent. The seven basic-ADL scores were summed to a total score (0–21). For higher-level daily activities, each subject rated his/her independence on the Tokyo Metropolitan Institute of Gerontology index of competence (TMIG-IC) (Koyano et al., 1991, 1993; Ishizaki et al., 2000). This is a 13-item index including three sublevels of competence: (1) instrumental self-maintenance (five items: ability to use public transport, buy daily necessities, prepare a meal, pay bills, and handle banking matters, rated on a yes/no basis); (2) intellectual activities (four items: ability to fill out forms, read newspapers, read books or magazines, and interest in television programs or news articles on health-related matters, rated on a yes/no basis); and (3) social role (four items: ability to visit own friends, give advice to relatives and friends who confide in one, visit someone at the hospital, and initiate conversation with younger people, rated on a yes/no basis).

2.4. Quality of life

QOL was assessed using a 100 mm visual analogue scale (VAS) (Morrison, 1983; Matsubayashi et al., 1997) (worst QOL on the left end of the scale, best on the right) for the following five items: subjective sense of health, relationship with family, relationship with friends, financial status, and subjective happiness. We have already confirmed the

inter-rater reliability ($R = 0.74$) and test-retest reliability ($R = 0.82$) of the VAS (Matsubayashi et al., 1994).

2.5. Statistics

Statistical analysis was performed using StatView ver. 5 for Macintosh (SAS Institute Inc., Cary, NC). Student's t-test was used for continuous variables. P-values less than 0.05 were considered significant.

3. Results

The characteristics of the 2695 subjects of three different populations are shown in Table 1. In Indonesia, subjects living alone numbered more than those in the other two countries, and prevalence of hypertension was highest among the subjects there. The majority of subjects from Indonesia were Muslims and did not drink alcohol. Mean GDS-15 score was lowest in Vietnam. The lowest TMIG-IC scores were found in Indonesia, but since the study areas there were relatively remote, one of the TMIG-IC questions, "Can you handle your own banking?", was probably biased. Figs. 1 and 2 show proportions of depressed subjects among the three populations using as cut-points 5/6 and 9/10 on the GDS-15. Using the cut-point 5/6, subjects with depression comprised 33.8% of those in Indonesia, 17.2% of those in Vietnam, and 30.3% of those in Japan, while with a cut-point of 9/10 the corresponding percentages were 11.7%, 4.7%, and 10.7%. Although the proportion of subjects with basic ADL independence was lowest in Vietnam, the prevalence of depression was also lowest there. The prevalence of depression was higher in women than in men in all three countries. On linear regression analysis, GDS-15 score exhibited weak but significant inverse correlations with basic ADL score, self-maintenance score, intellectual activity score, social role score, TMIG-IC score, and each item of QOL score (data not shown).

The elderly subjects with depression had significantly lower scores for each item of the ADL and QOL than those without depression, as assessed by GDS-15 using a cutoff of 5/6 even after adjustment for the effect of age (Table 2), except self-maintenance in Vietnam.

4. Discussion

The prevalences of depression as determined using a cut-point of 5/6 for the GDS-15 were 33.8% in Indonesia, 17.2% in Vietnam, and 30.3% in Japan. The reported prevalences of screening-based depression using the GDS-15 in other countries have varied (14% in US study, with the cut-point of 5/6 (Whooley et al., 2000); 40.2% in Estonia, with the cut-point of 5/6 (Saks et al., 2002); and 8.8% in Taiwan, with the cut-point of 4/5 (Tsai et al., 2003)). In Korea, Cho et al. (1999) suggested a score of 8 or more as the optimal cut-point for the GDS-15 for screening DSM-III-R major depression among clinical subjects, and also concluded that relatively high cut-points require further evaluation in the viewpoint of culturally determined style of response for the depression questionnaire in Korea. The

Table 1
Baseline characteristics of 3255 elderly subjects in three Asian countries

	K and S towns, Indonesia ($N = 411$)	N and V towns, Vietnam ($N = 379$)	S town, Japan ($N = 1905$)
Eligible subjects (%)	44.8	28.3	57.0
Age, mean ± S.D.	72.3 ± 7.3	70.7 ± 8.0	74.0 ± 6.6
Female (%)	59.9	55.1	58.0
Lifestyle			
Partner alive (%)	53.5	70.5	66.6
Living alone (%)	31.3	6.3	10.0
Alcohol consumption (%)	0.7	43.9	35.9
Non-smoker (%)	55.0	77.6	78.8
Working everyday (%)	53.9	85.0	65.5
Average monthly income[a], mean (USD)	Not available	29.0	1244.0
GDP/capita (International $)	3121	2459	25901
Total health expenditure/capita	84	129	2009
Life expectancy at birth, male/female	64.4/67.4	66.9/71.8	77.9/84.7
Medical			
Hypertension[b] (%)	76.1	58.8	41.2
Current use of antihypertensives (%)	Not available	15.9	44.5
Past medical history of heart disease (%)	4.4	16.4	20.3
History of stroke (%)	1.2	5.6	5.0
History of osteoarthropathy (%)	22.5	52.1	42.7
History of fall (%)	Not available	16.5	18.9
ADL			
Basic ADL score (range, 0–21)	20.0 ± 12.6	20.6 ± 2.0	19.9 ± 3.2
Tokyo Metropolitan Institute of Gerontology index (range 0–13)	6.7 ± 3.2	10.1 ± 3.6	10.7 ± 3.3
GDS-15, mean ± S.D.	4.9 ± 3.3	3.4 ± 2.7	4.1 ± 3.6

Four hundred eleven elderly from K and S towns, Indonesia, and 379 elderly from N and V towns, Vietnam, were measured for blood pressure in sitting position, with hypertension defined as 140 mmHg or higher casual systolic blood pressure. S.D.: standard deviation; USD: US dollar; GDS-15: 15-item Geriatric Depression Scale.

[a] A total of 991 and 40 elderly individuals from S town of Japan and N town of Vietnam, respectively, volunteered the information.

[b] A total of 1905 elderly individuals from S town, Japan, reported awareness of their own hypertension.

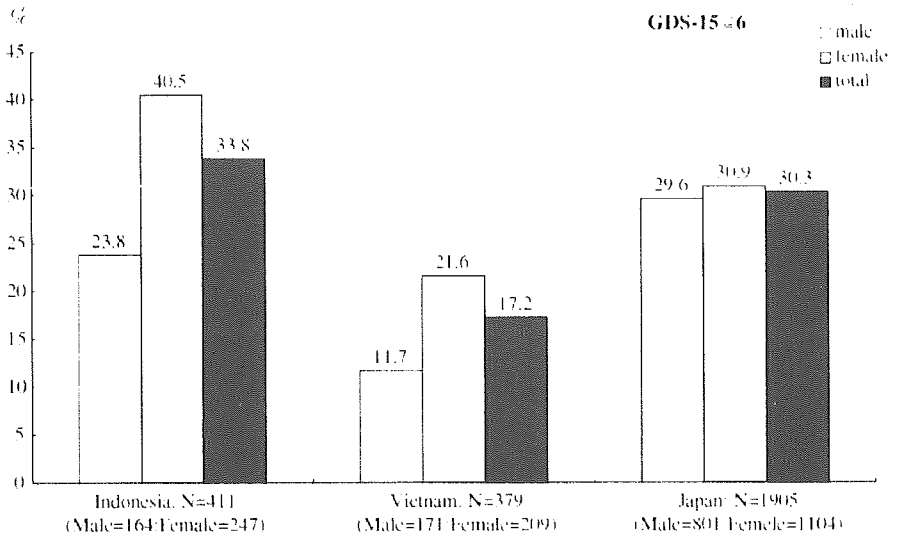

Fig. 1. Prevalence of depression defined as a 15-item GDS score of 6 or more.

GDS-15 (usual cut-point, 5/6) is limited by low specificity in detecting depression (Mulrow et al., 1995; Whooley et al., 1997). A higher cut-point might thus be useful for screening in these Asian countries. In the present study, the lowest prevalence of depression among the elderly was found in Vietnam using both 5/6 and 9/10 as cut-points. Since translation from the English version of GDS-15 to a native language may preclude firm conclusions regarding interpretations of results, further evaluation by a native psychiatrist in each of these countries is needed to confirm our results. Differences in socio-economic factors, social support, ecological environment, and even cultural conditions probably affected the

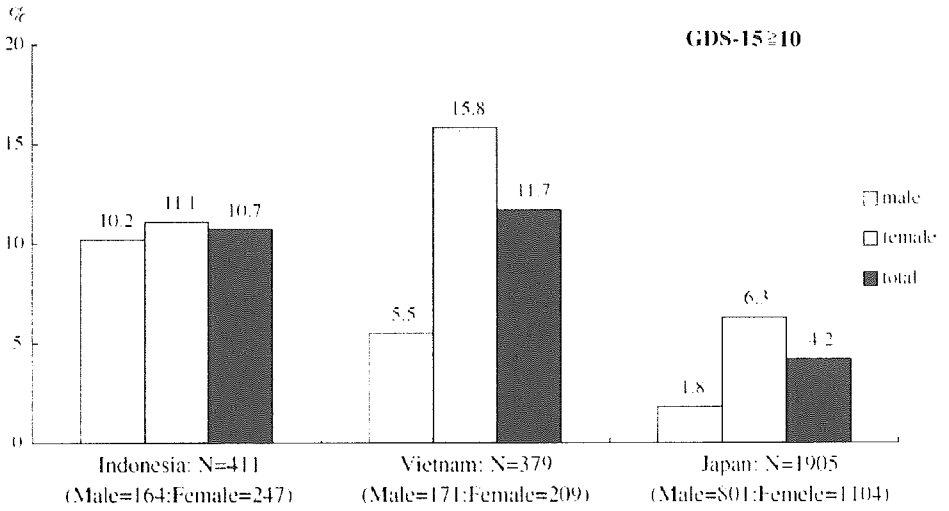

Fig. 2. Prevalence of depression defined as a 15-item GDS score of 10 or more.