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TGF-β-Induced Protein βig-h3 Is Upregulated by High Glucose in Vascular Smooth Muscle Cells

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Abstract TGF-β-induced gene-h3 (βig-h3) is an adhesive molecule that interacts with integrins. Because TGF-β plays an important role in diabetic complications and βig-h3 serves as a cell substrate, we hypothesized that diabetic conditions might increase βig-h3 synthesis in vascular smooth muscle cells (VSMCs), which may subsequently contribute to the pathogenesis of diabetic angiopathy. The concentrations of βig-h3 and TGF-β were measured in conditioned media using an enzyme-linked immunosorbent assay. An immunohistochemical study showed that βig-h3 was expressed in the VSMCs and the matrix of rat aortas. TGF-β stimulated βig-h3 production, and high glucose induced βig-h3 as well as TGF-β production in the VSMCs. The high glucose-induced βig-h3 expression was almost entirely blocked by an anti-TGF-β antibody. βig-h3 protein mediated the adhesion, spreading, migration, and proliferation of rat VSMCs. These results suggest that the high glucose-induced βig-h3 in VSMCs regulates VSMC functions and may play an important role in diabetic angiopathy. J. Cell. Biochem. 88: 774–782, 2003.

Key words: βig-h3; vascular smooth muscle cells; high glucose; TGF-β; diabetic angiopathy

Hyperglycemia per se in diabetes has been suggested to be a contributing factor to the development of diabetic complications [Ziyadeh et al., 1990, 1994; Schwartz et al., 1992; Spiro et al., 1995; King and Wakasaki, 1999; Park et al., 1999]. Hyperglycemia has been suggested to affect the synthesis of extracellular matrix proteins by vascular smooth muscle cells (VSMCs) [Schwartz et al., 1992; Ross, 1993; Ha et al., 1997]. There have also been many reports demonstrating that high glucose stimulates extracellular matrix synthesis in cultured mesangial cells by stimulating TGF-β expression [Ayo et al., 1990; Studer et al., 1993; Ziyadeh et al., 1994]. Among the multiple growth factors, TGF-β is one of the key features in the development of diabetic angiopathy and atherosclerosis due to its effect on the growth of VSMCs and the accumulation of extracellular matrix proteins [Pankewycz et al., 1995; Clemmons, 1997; McCaffrey, 2000]. TGF-β-induced gene-h3 (βig-h3) is an adhesive molecule and is highly induced by TGF-β in many cell types [Brunner et al., 1991; Skonier et al., 1992]. βig-h3 is a secretion protein that contains an Arg-Gly-Asp (RGD) sequence and four internal repeat domains that are homologous with fasciclin I, a *Drosophila* neuroadhesion molecule [Skonier et al., 1992]. βig-h3 has been known to

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mediate cell adhesion through interactions with integrins and its integrin-interacting motifs have been identified [Kim et al., 2002]. In addition, βig-h3 is known to interact with other extracellular matrix proteins such as fibronectin, type I collagen, and laminin [Kim et al., 2002]. Although little is known about the biological role of βig-h3 in the vessels, O'Brien et al. reported that βig-h3 was overexpressed in atherosclerotic and restenotic human vascular lesions and suggested that βig-h3 is associated with the development of atherogenesis [O'Brien et al., 1996]. In this study, we demonstrated that high glucose induces βig-h3 expression via TGF-β in rat VSMCs and that βig-h3 mediates the adhesion, spreading, migration, and proliferation of VSMCs, thereby suggesting a potential role of βig-h3 in the pathogenesis of diabetic angiopathy.

MATERIALS AND METHODS

Cell Culture

VSMCs were isolated from the thoracic aortas of Sprague–Dawley rats, cultured as described previously [Caramelo et al., 1988], and used between passages 3 and 6. The cells were cultured in Dulbecco's modified Eagle media (DMEM, Gibco/BRL, Gaithersburg, MD) supplemented with 20% FBS, 100 U/ml of penicillin, and 100 g/ml of streptomycin; and the cells were incubated in a humidified atmosphere of 5% CO_2 at 37°C. The cells were cultivated until they reached 70% confluence, at which time recombinant human TGF-β1 ranging from 0.2 to 5 ng/ml (R&D systems, Minneapolis, MN) was added in a serum-free medium, and the cells were incubated for a further 48 h. For the study on the effects of glucose, cells were incubated for different time periods in media containing 5- or 25-mM glucose. For the last 48 h, the cells were cultured in a serum-free medium. For the study on blocking TGF-β activity, an anti-TGF-β antibody (30 μg/ml, R&D systems, Minneapolis, MN) or IgG (30 μg/ml) was added to the culture medium. The medium was then harvested and lyophilized for immunoblot and enzyme-linked immunosorbent assay (ELISA).

Animals

Male Sprague–Dawley rats, each weighing 200 g, were used for the experiments according to procedures approved by the Institutional Animal Care and Use Committee. The rats were given a single intraperitoneal injection of streptozotocin (60 mg/kg body weight) (Sigma, St Louis, MO) and then examined 6 weeks later. The rats were considered diabetic when the non-fasting blood glucose level was above 300 mg/dl.

ELISA of βig-h3 and TGF-β

The βig-h3 concentrations were measured by ELISA. Ninety-six-well flat plastic microtiter plates (Coster, Cambridge, MA) were coated with 0.5 μg/ml wild-type recombinant βig-h3 protein in a 20 mM carbonate-bicarbonate buffer (pH 9.6) with 0.02% sodium azide overnight at 4°C. The plates were then rinsed three times in PBS-0.05% Tween-20 (PBST) and maintained at 4°C. Lyophilized culture media were pre-incubated with anti-βig-h3 antibodies (diluted 1:2,000 in PBS-T) in 96-well round plastic microtiter plates for 90 min at 37°C. The recombinant βig-h3 protein and anti-βig-h3 antibody have been described previously [Lee et al., 2000]. The pre-incubated samples were then transferred to pre-coated plates and incubated for 30 min at room temperature. The plates were rinsed three times in PBST and incubated for 90 min at room temperature with the peroxidase-conjugated anti-rabbit IgG antibodies (diluted 1:2,000 in PBST, Amersham, Arlington). Thereafter, the plates were rinsed again three times in PBST and incubated for 60 min at room temperature in a dark space with a substrate solution (prepared by dissolving *O*-phenylenediamine in methanol at a concentration of 10 mg/ml, diluting it 1:100 with deionized water, and adding 0.01 ml of 30% H_2O_2 per 100 ml of the solution). After stopping the reaction with 8-N H_2SO_4, the absorbance was read at 492 nm. The concentrations of total TGF-β1 in the cultured media were determined using a commercial sandwich ELISA kit (R&D System Europe Ltd., Abingdon, UK). Latent TGF-β was activated according to the instructions provided by the manufacturer. Total protein concentrations were determined using a Bio-Rad kit based on the Bradford method [Bradford, 1976].

Immunohistochemistry

The thoracic aortas from normal (n = 10) and diabetic (n = 10) Sprague–Dawley rats were fixed by perfusion via the abdominal aorta with

4% paraformaldehyde in a 0.1 M cacodylate buffer, pH 7.4. Thereafter, the rat aortas were extracted and embedded in paraffin. To reveal the antigens, sections were put in a 1 mM TRIS solution (pH 9.0) supplemented with 0.5 mM EGTA(ethyleneglycol-bis(β-amino-ethyl ether)-N,N,N′,N′-tetra acetic acid) and heated using a microwave oven for 10 min. The non-specific binding of immunoglobulin was prevented by incubating the sections in 50 mM NH_4Cl for 30 min, followed by blocking in PBS supplemented with 1% bovine serum albumin (BSA), 0.05% saponin, and 0.2% gelatin. The sections were incubated overnight at 4°C with an immune serum diluted in PBS supplemented with 0.1% BSA and 0.3% Triton-X-100 (1:3,000), and labeling was visualized using a horseradish peroxidase-conjugated secondary antibody (P448, 1:100, DAKO, Glostrup, Denmark).

Migration, Adhesion, and Spreading Assays

A cell migration assay was performed with transwell plates (8-μm pore size, Costar, Cambridge, MA). The lower surface of the membrane was coated overnight at 4°C with either βig-h3 or fibronectin (20 μg/ml) diluted in PBS and blocked with 2% BSA, or with BSA alone. Each well contained 2×10^5 rat VSMCs in 200 μl medium, and the transwell plates were seeded in the upper compartment of the filters. After 6 h of migration, cells in the upper chamber of the filter were removed, and non-migrating cells on the top of the filters were removed with a cotton swab. Cells on the lower side of the filter were fixed with 8% glutaraldehyde (Sigma) and then stained with 0.25% Crystal Violet (Sigma) in 20% methanol (w/v). Each experiment was repeated in duplicate wells, and within each well, cells were counted under nine randomly selected high power fields (HPF, 200×). The cell adhesion and spreading assays were performed as described previously [Kim et al., 2000a].

Proliferation Assay

Twenty-four-well culture plates were coated with 20 μg/ml BSA, fibronectin, and wild-type βig-h3, diluted in PBS, and incubated overnight at 4°C. The plates were then rinsed three times in PBS and the uncoated surfaces were blocked with PBS containing 2% heat-inactivated BSA for 1 h at 37°C. The plates were rinsed again, and 5×10^4 rat VSMCs prepared by trypsin-EDTA treatment were added to 1 ml of culture medium in each well and grown to adherence. Next, the cells were subjected to serum starvation for 24 h in order to bring most of the cells into the G0 phase of the cell cycle. Cell proliferation was assessed by cell counting using a hematocytometer at 24-h intervals.

Statistical Analysis

The results were expressed as the mean ± SEM. One-way ANOVA and the Student–Newman–Keuls test were used to estimate the statistical significance of comparisons between groups. Differences were considered to be significant at $P < 0.05$.

RESULTS

Localization of βig-h3 in Rat Aortas

To determine whether βig-h3 is expressed in rat vascular tissues, immunohistochemistry was performed on 5-μm sections of rat aortas. Intimal endothelial cells, smooth muscle cells, and the matrix between elastic fibers stained positively for βig-h3 (Fig. 1), and diabetic rat aortas stained more strongly. Control staining with non-immune IgG was negative (data not shown).

TGF-β1 and High Glucose Induce βig-h3 Expression in Rat VSMCs

βig-h3 has been known as one of the target gene of TGF-β. To determine whether TGF-β induces βig-h3 production in VSMCs, the βig-h3 protein levels were measured by ELISA in the culture supernatants of rat VSMCs incubated with various concentrations of TGF-β1. TGF-β1 increased the βig-h3 protein levels in a dose-dependent manner (Fig. 2A). The maximum increase was observed at 1 ng/ml of TGF-β. To determine whether high glucose could induce βig-h3 expression in VSMCs, cells were exposed to 5 or 25 mM of glucose for 3, 5, and 7 days. As shown in Figure 2B, 25-mM glucose significantly increased the βig-h3 protein levels compared to 5-mM glucose after 5 days ($P < 0.01$) and 7 days ($P < 0.05$) of exposure. The high glucose-induced βig-h3 expression did not seem to be related to the effect of high osmolality per se, because the addition of D-mannitol did not have any significant effects on the βig-h3 protein levels (Fig. 2C). This result indicates that high glucose increases βig-h3 production and its increase needs to expose vascular cells to high glucose for at least 5 days.

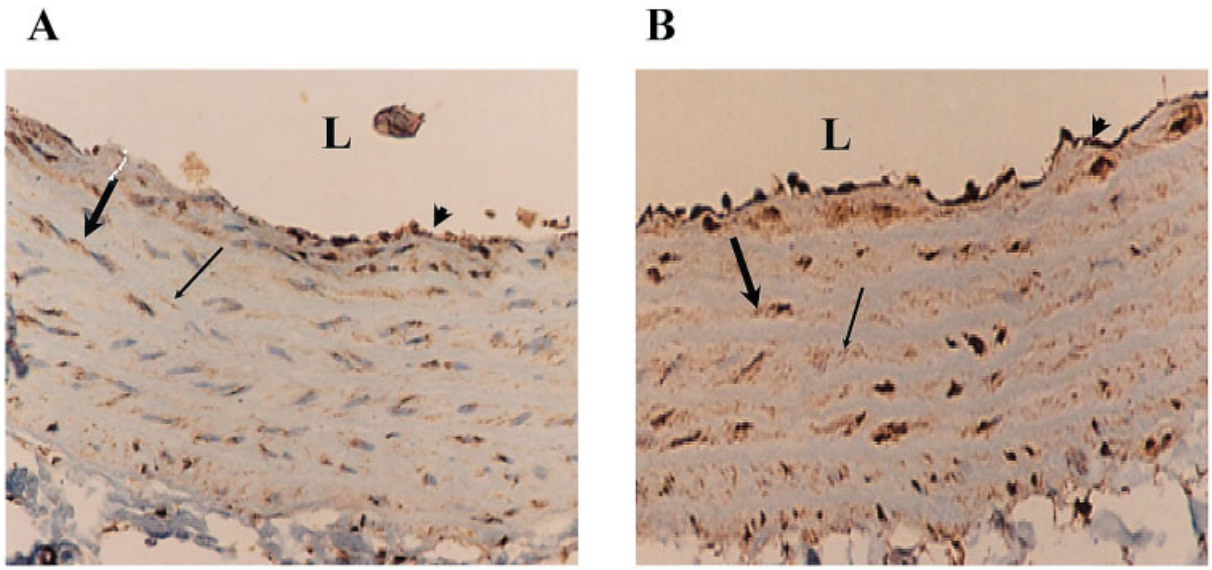

Fig. 1. Localization of βig-h3 in rat aortas by immunohistochemistry. The thoracic aortas of the control (**A**) and diabetic (**B**) rats were immunostained with an anti-βig-h3 antibody (200×). The thoracic aortas of control (A) and diabetic rats (B) have positive βig-h3 signals in endothelial cells (arrowhead), smooth muscle cells (thick arrow), and the matrix between elastic fibers (thin arrow). L, lumen of aorta.

High Glucose Increases TGF-β Production and an Anti-TGF-β Antibody Abrogates the High Glucose-Induced βig-h3 Protein Production

Because TGF-β is known to be induced by high glucose in mesangial cells and renal tubular epithelial cells [Wolf et al., 1992; Ziyadeh et al., 1994], we assumed that high glucose may increase TGF-β in VSMCs, which in turn, induces βig-h3 expression. After 5 days of exposure to 25-mM glucose, the TGF-β protein levels were measured by ELISA in the culture supernatants of rat VSMCs. The TGF-β protein levels increased threefold in cells treated with 25-mM glucose compared to those treated with 5-mM glucose (Fig. 3A). To test whether high glucose-induced TGF-β could mediate the high glucose-induced βig-h3 production, experiments were performed with a neutralizing anti-TGF-β antibody. Incubation with the anti-TGF-β antibody almost completely blocked the high glucose-induced βig-h3 production, whereas incubation with the control IgG did not (Fig. 3B). The result indicates that upregulation of βig-h3 production by high glucose is mediated through the TGF-β production.

βig-h3 Supports the Adhesion, Spreading, Migration, and Proliferation of Rat VSMCs

Since βig-h3 is known to mediate the adhesion of several cell types [Skonier et al., 1992, 1994; Kim et al., 2000a], the ability of βig-h3 to mediate the adhesion of rat VSMCs was investigated by using a recombinant βig-h3 protein. In addition, the effects of βig-h3 on the spreading, migration, and proliferation of rat VSMCs were also examined. For the cell adhesion and spreading assay, a cell culture plate with recombinant βig-h3 protein (20 μg/ml) was used. As shown in Figure 4, βig-h3 mediated both cell adhesion and spreading in a dose-dependent manner. Next, the ability of βig-h3 to mediate VSMC migration and proliferation was tested. For the migration assay, a transwell plate coated with the indicated protein was used. βig-h3 significantly increased the VSMC migration compared to BSA (Fig. 5A,B). Cells seeded on βig-h3-coated culture plates showed a marked increase in number compared to those on plates coated with BSA alone (Fig. 5C). The βig-h3 activities on VSMC were comparable with those of fibronectin, although the activity of βig-h3 was somewhat lower than that of fibronectin.

DISCUSSION

In this study, we demonstrated that βig-h3 is expressed in smooth muscle cells, endothelial cells, and the matrix between elastic fibers in the rat thoracic aorta. A similar result has been reported in a study using the bovine aorta, showing that βig-h3 most strongly stained

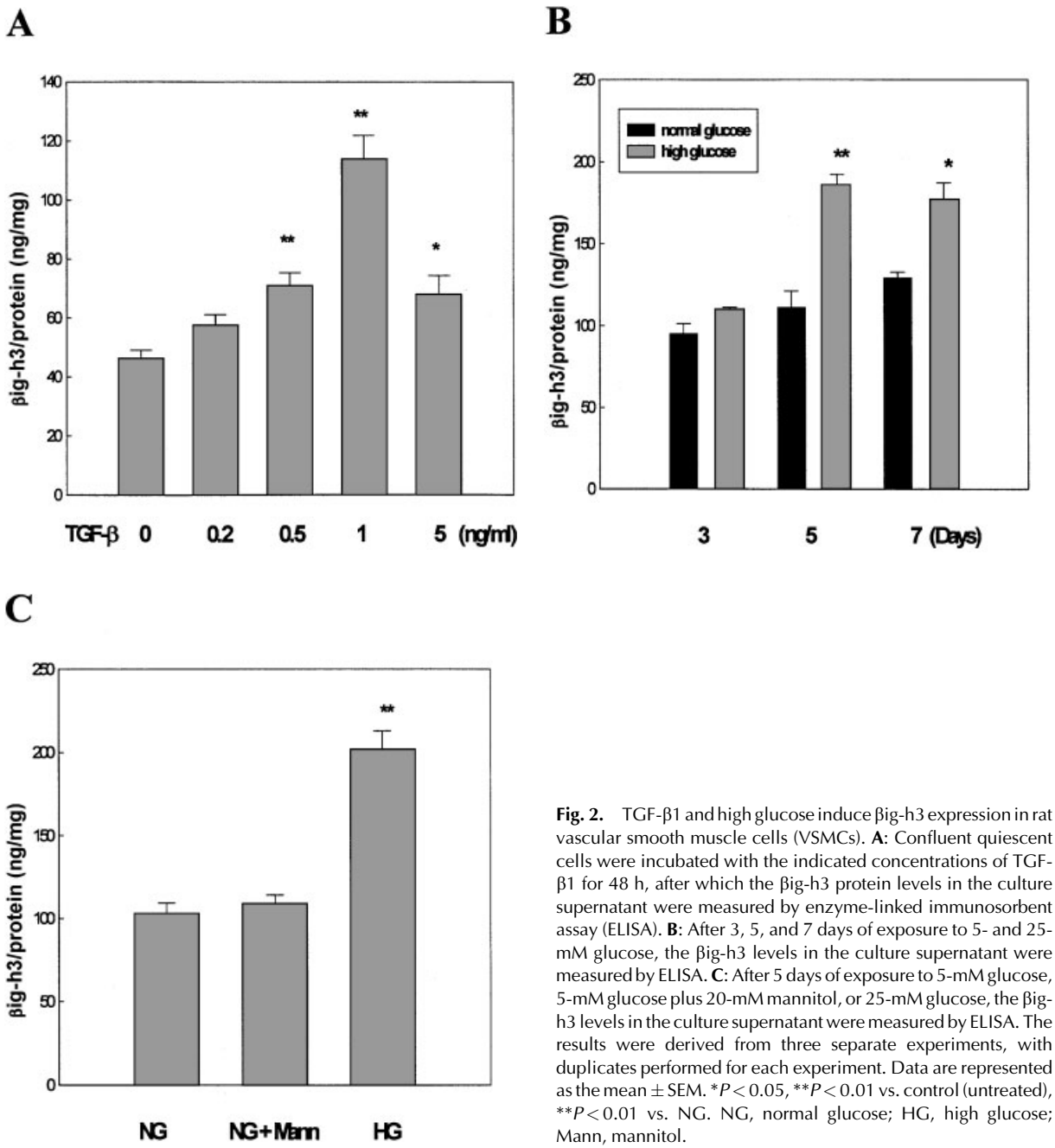

Fig. 2. TGF-β1 and high glucose induce βig-h3 expression in rat vascular smooth muscle cells (VSMCs). **A**: Confluent quiescent cells were incubated with the indicated concentrations of TGF-β1 for 48 h, after which the βig-h3 protein levels in the culture supernatant were measured by enzyme-linked immunosorbent assay (ELISA). **B**: After 3, 5, and 7 days of exposure to 5- and 25-mM glucose, the βig-h3 levels in the culture supernatant were measured by ELISA. **C**: After 5 days of exposure to 5-mM glucose, 5-mM glucose plus 20-mM mannitol, or 25-mM glucose, the βig-h3 levels in the culture supernatant were measured by ELISA. The results were derived from three separate experiments, with duplicates performed for each experiment. Data are represented as the mean ± SEM. $*P < 0.05$, $**P < 0.01$ vs. control (untreated), $**P < 0.01$ vs. NG. NG, normal glucose; HG, high glucose; Mann, mannitol.

the matrix between the elastic fibers and also localized to smooth muscle cells [Gibson et al., 1997]. However, in that report it was not clear whether the immunostaining of βig-h3 was evident in the endothelial cells of the bovine aorta. In contrast, O'Brien et al. [1996] reported that both βig-h3 proteins and mRNAs were detected in endothelial cells, whereas neither proteins nor mRNAs were detected in smooth muscle cells of the human normal internal mammary artery. However, βig-h3 is expressed in the subendothelial smooth muscle cells of some arteries with modest intimal thickening and smooth muscle cells in restenotic lesions and atherectomy tissues [O'Brien et al., 1996]. The discrepancy in βig-h3 expression in the normal artery could be due to using different antibodies, different species, or different arteries, but further studies are needed to clarify this. In spite of this discrepancy, it has been

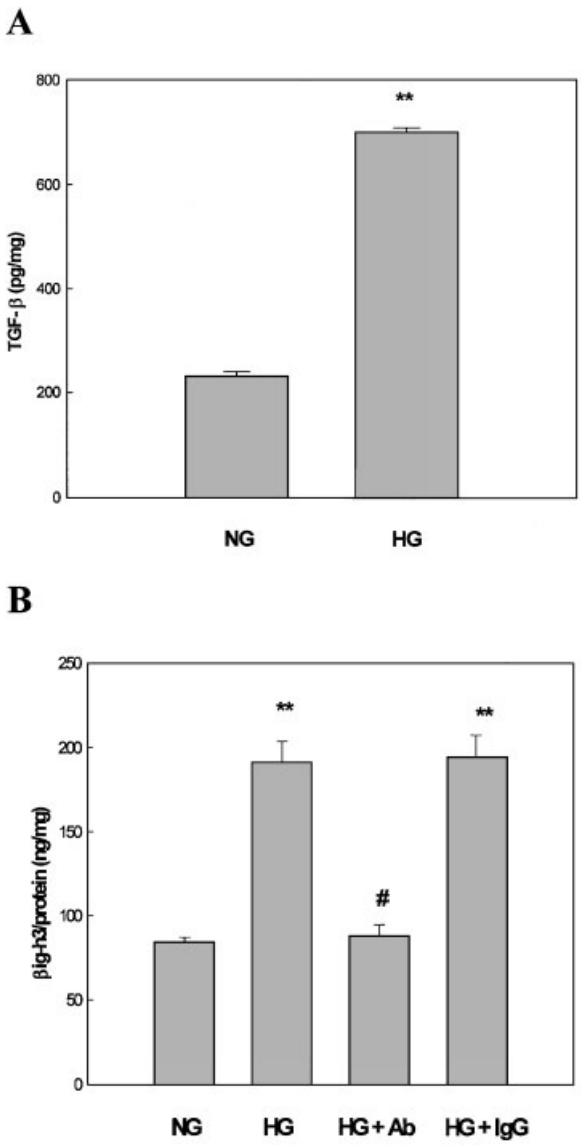

Fig. 3. High glucose induces TGF-β protein expression and a TGF-β antibody blocks high glucose-induced expression of βig-h3 in rat vascular smooth muscle cells (VSMCs). **A**: After 5 days of exposure to 5- or 25-mM glucose, the supernatants were harvested and TGF-β protein levels were measured by ELISA. **B**: The cells were incubated for 5 days in media with 5- or 25-mM glucose plus 30 μg/ml anti-TGF-β antibody or 30 μg/ml nonspecific IgG. The results were derived from three separate experiments, with duplicates performed for each experiment. Data represented is the mean ± SEM. $^{**}P < 0.01$ vs. NG, # $P < 0.01$ vs. HG. NG, normal glucose; HG, high glucose; Ab, antibody.

suggested that βig-h3 plays an important role in vascular diseases because it is expressed in vascular tissues and is highly induced by TGF-β [O'Brien et al., 1996; Gibson et al., 1997]. Although our immunohistochemical staining study of diabetic and normal rat aortas showed that they have the same pattern of βig-h3 distribution, the immunostaining intensity was stronger in the diabetic aortas than in the normal aortas.

Our in vitro studies supported the hypothesis that βig-h3 expression is increased in the diabetic aorta. Exposure of VSMCs to high glucose (25 mM) for 5 days led to a significant increase of βig-h3 production. In addition, the high glucose-induced βig-h3 production appears to be mediated by TGF-β because TGF-β production is also increased by high glucose and an anti-TGF-β antibody almost completely blocks the βig-h3 production induced by high glucose. There have been a variety of studies reporting that high glucose per se would be an important contributing factor to the development of diabetic complications, such as diabetic nephropathy and atherosclerosis [Ayo et al., 1990; Ziyadeh et al., 1994; Spiro et al., 1995; Ha et al., 1997; King and Wakasaki, 1999]. It is well known that high glucose can stimulate TGF-β expression in mesangial cells and proximal tubular epithelial cells, which, in turn, stimulates the production of extracellular matrix [Ziyadeh et al., 1990, 1994]. Recently, it was reported that high glucose did not increase the amount of active and total TGF-β in the conditioned media of VSMCs [Yasuda et al., 2001], although it was reported that the proliferation of VSMCs was significantly accelerated under high glucose conditions and that the anti-TGF-β antibody inhibited this increase. Although they could not detect increased TGF-β production, they suggested that high glucose-induced cell proliferation could be mediated by TGF-β. They explained these results by speculating that it might be due either to using fetal bovine serum (FBS), which is supposed to have large amounts of TGF-β, or to the short half-life of TGF-β. In our study, we cultured cells in the presence of high glucose for 5 or 7 days rather than 3 weeks and omitted FBS in the culture media for the last 48-h incubation and were able to detect an increased amount of TGF-β in the conditioned media of cells exposed to high glucose.

Our results suggest that βig-h3 may contribute to the high glucose-induced accelerated proliferation of VSMCs. Although various mechanisms might be involved, high glucose-induced TGF-β has been suggested to be a factor in the accelerated proliferation of VSMCs in

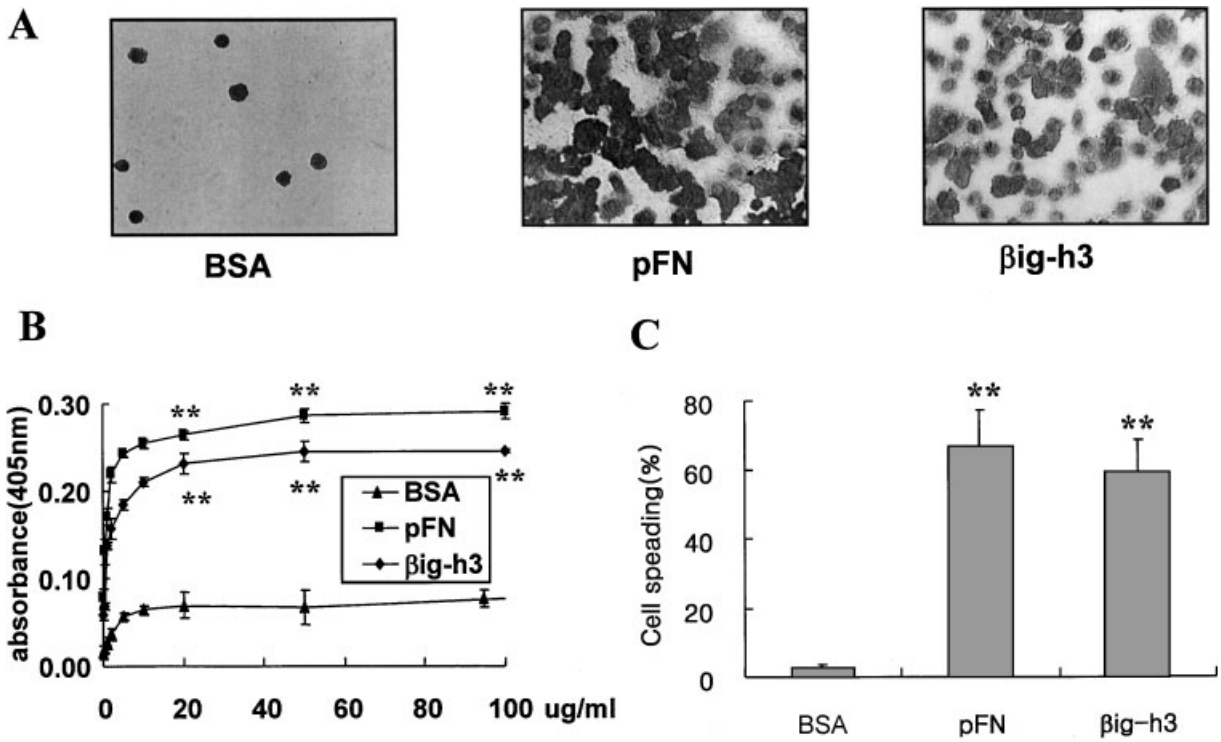

Fig. 4. βig-h3 mediates cell adhesion (**A**,**B**) and spreading (**C**) of rat vascular smooth muscle cells (VSMCs). Ninety-six-well culture plates were coated with BSA, fibronectin, and wild-type βig-h3. Cells were seeded to each well and incubated for 1 h, then the attached cells were analyzed for cell adhesion and spreading. The results were derived from three separate experiments, with duplicates performed for each experiment. Data are represented as the mean ± SEM. $*P < 0.01$ vs. BSA. BSA, bovine serum albumin; pFN, purified fibronectin.

diabetes [Wolf et al., 1992]. High glucose-induced TGF-β could stimulate VSMCs to produce βig-h3, which would serve as a substrate for mediating not only VSMC adhesion and migration but also proliferation.

Although the important roles of TGF-β in diabetic complications are well known, the biological roles of βig-h3 in diabetic complications are not well understood. While studies on the biological functions of βig-h3 have only recently begun, initial studies have suggested that this molecule is a matrix protein involved in cell adhesion and growth [Skonier et al., 1992, 1994]. VSMC adhesion, migration, and proliferation are critical processes in neointima formation and atherosclerosis [Dodge and D'Amore, 1992; Raines and Ross, 1993; Ross, 1993; O'Brien and Chait, 1994; Faries et al., 2001]. In our studies, we demonstrated that βig-h3 mediates VSMC adhesion, migration, and proliferation. Recently, we have identified two cell adhesion motifs within the 2nd and 4th fasciclin domains of βig-h3, which interact with the α3β1 integrin to mediate cell adhesion [Kim et al., 2000a]. These two motifs, however, do not seem to be responsible for the βig-h3-mediated adhesion of VSMCs, because we found that βig-h3 has other motifs that mediate VSMC adhesion via interactions with integrins consisting of the αv integrin subunit rather than α3β1 integrin (data not shown). Recently, it has been found that αvβ5 and αvβ3 integrins mediate VSMC adhesion and migration, suggesting that they participate in the regulation of neointima formation [Kappert et al., 2001].

Although there has been much evidence showing that high glucose can stimulate the production of extracellular matrix proteins via TGF-β in kidney mesangial and tubular cells [Ayo et al., 1990; Ziyadeh et al., 1990, 1994; Wolf et al., 1992], there have been only a few reports of the roles high glucose plays in VSMCs. This study presents the first evidence that high glucose, by stimulating TGF-β production in VSMCs, results in the increased production of βig-h3, which subsequently mediates VSMC

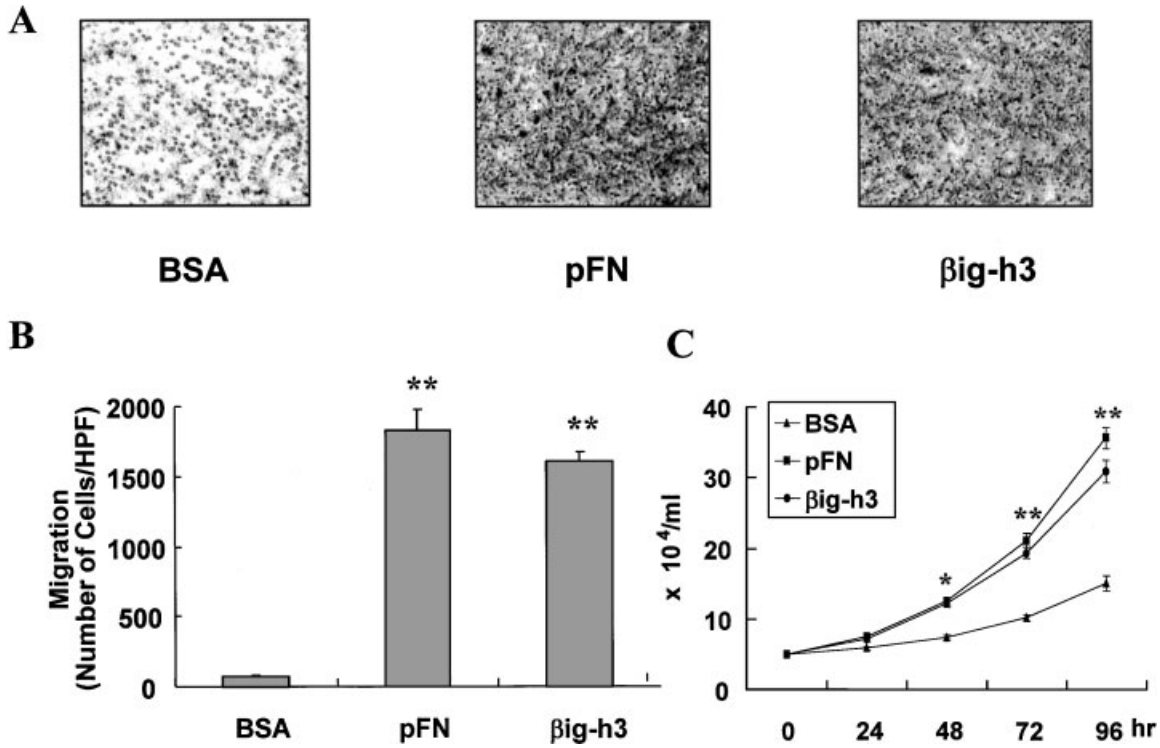

Fig. 5. βig-h3 mediates cell migration and proliferation. **A** and **B**: A cell migration assay was performed with transwell plates. The membrane surface was coated with BSA, fibronectin and wild-type βig-h3, then cells were seeded in the upper compartment of the filter. Cells that migrated to the lower side of the filter were fixed, stained, and counted. **C**: The cells were seeded on plates coated with different proteins and then the cell growth was measured at different time points. The results were derived from three separate experiments, with duplicates performed for each experiment. Data are represented as the mean ± SEM. $*P < 0.01$ vs. BSA. BSA, bovine serum albumin; pFN, purified fibronectin.

adhesion, migration, and proliferation. βig-h3 may thus play an important role in the development of diabetic vascular complications.

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