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Evidence for low-density lipoprotein–induced expression of connective tissue growth factor in mesangial cells

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Evidence for low-density lipoprotein–induced expression of connective tissue growth factor in mesangial cells.

Background. Although hyperlipidemia is a risk factor for the progression of renal damage, the relationship between increased plasma lipoproteins and glomerular injury is poorly defined. Connective tissue growth factor (CTGF) is emerging as a key determinant of progressive fibrotic diseases and its expression is up-regulated by diabetes. To define the mechanisms through which low-density lipoproteins (LDLs) promote glomerular injury, we evaluated whether LDL can modulate the expression of CTGF and collagen I.

Methods. The effects of LDL on CTGF and collagen I expression were carried out in rat mesangial cells.

Results. Treatment of mesangial cells with LDL for 24 hours produced a significant increase in the protein levels of CTGF and collagen I compared to unstimulated controls. To explore if CTGF and collagen I are downstream targets for regulation by transforming growth factor-β (TGF-β), mesangial cells were treated with various concentration of TGF-β for 24 hours. TGF-β produced a concentration-dependent increase in the protein levels of CTGF and collagen I. The increase in CTGF and collagen I induced by LDL was significantly inhibited by neutralizing anti-TGF-β antibodies. Inhibition of $p38^{mapk}$ or $p42/44^{mapk}$ activities did not affect LDL-induced TGF-β1, CTGF, and collagen I expression, whereas inhibition of c-Jun NH2-terminal kinase (JNK) suppressed LDL-induced TGF-β, CTGF, and collagen I expression.

Conclusion. These findings implicate JNK pathway and TGF-β1 as key players in LDL signaling leading to CTGF and collagen I expression in mesangial cells. The data also point to a potential mechanistic pathway through which lipoproteins may promote glomerular injury.

Diabetic nephropathy is the leading cause of end-stage renal failure, and is clinically manifested by albuminuria, hypertension, and a progressive decline in glomerular filtration rate (GFR) [1–3]. About 30% to 40% of patients with type 1 diabetes develop progressive nephropathy [3]. A very characteristic and initial event of the development of diabetic nephropathy is glomerulosclerosis, which is featured by increased thickness of the glomerular basement membrane (GBM), and a widening of the mesangium with accumulation of extracellular matrix (ECM). The development of glomerulosclerosis is clearly dependent on hyperglycemia since intensive control of glycemia in type 1 diabetic patients was associated with a reduction of glomerular lesions [4]. Although the association of chronic hyperglycemia and diabetic nephropathy is well established, the risk factors and cellular signaling mechanisms that promote glomerulosclerosis in diabetes are still undefined. In this regard, the cytokine transforming growth factor-β (TGF-β) has been shown to play a pivotal role in mesangial cell expansion and matrix deposition [5]. Recent evidence indicates that the profibrotic signals initiated by TGF-β are mediated via activation of connective tissue growth factor (CTGF) [6].

CTGF was originally identified as a product of human umbilical vein endothelial cells that was both chemotatic and mitogenic for fibroblasts [7]. It is now known that CTGF belongs to a new gene family, CCN (named after prototype members of this family CTGF, cyr61, and nov) [8]. The molecular weight of CTGF-like factors varies between 35 and 40 kD, and the structure of these molecules consists of four modules: an N-terminal insulin-like growth factor binding protein (IGFBP)-like domain, a von Willebrand factor type C repeat domain, a thrombospondin type 1 repeat domain, and a C-terminal dimerization domain [8].

The biologic actions of CTGF are pleiotropic and seem to be cell specific, but the cellular mechanisms of its actions are still undefined. An emerging role of CTGF is that of a prosclerotic factor. Renal expression of CTGF is up-regulated by the diabetic state and by other progressive renal diseases [9]. In addition, CTGF has been shown to increase collagen I expression in human mesangial cells [10].

Poorly controlled type 1 diabetes is usually associated with elevated levels of plasma LDL, intermediate-density lipoproteins and very low-density lipoprotein levels

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[11–14]. Besides quantitative abnormalities, patients with type 1 diabetes are known to have significant qualitative lipoprotein abnormalities. Abnormalities in lipid and lipoprotein metabolism are commonly associated with end-stage renal disease (ESRD) [15–17]. Specifically, hyperlipidemia and the glomerular deposition of atherogenic lipoproteins (LDL and oxidized LDL) are implicated in key pathologic processes involved in the development of glomerular disease, including stimulation of monocyte infiltration into the mesangial space, mesangial cell hypercellularity, and ECM deposition [17, 18].

Although hyperlipidemia is now considered a risk factor for the progression of renal damage, the relationship between increased plasma lipoproteins and glomerular injury is poorly defined. Hyperlipidemia can directly or indirectly stimulate the synthesis and release of factors from resident renal cells which in turn can stimulate mesangial cell growth, as well as ECM production in an autocrine or paracrine manner [19, 20]. Therefore, the present study was designed to explore the potential role of LDL in modulating the expression of CTGF in mesangial cells and to delineate the cellular signaling mechanisms through which this regulation may occur.

METHODS

Mesangial cell culture

Rat glomerular mesangial cells were prepared by a modification of the method of Lovett et al [21]. Glomerular cells collected as described above were incubated in phosphate-buffered saline (PBS) plus 0.1% gentamysin solution and 1% antibiotic antimycotic, pH 7.4, containing collagenase (5mg/mL), at 37°C for half an hour to remove epithelial cells, leaving the glomerular cores containing mesangial and endothelial cells, vortexed every 10 minutes during the incubation. The cores were diluted in 1.5 mL RPMI 1640 medium with Hepes and L-glutamine (Invitrogen Corporation, Carlsbad, CA, USA) per kidney, containing 0.1% gentamycin solution, 1% antibiotic antimycotic, 0.5% insulin transferring solution, and 20% fetal bovine serum (FBS), conditions which favor growth of mesangial cells. Cells were incubated at 37°C in a humidified atmosphere of 95% air/5%CO_2. Cell viability was assessed by standard dye exclusion techniques, using 0.1% Trypan blue. Mesangial cells were identified by the following criteria. Mesangial cells stained positive for intracellular cytoskeletal fibrils of actin and smooth muscle cell (SMC)-specific myosin (indicative of contractile cells), desmin, and vimentin and negative for cytokeratin and factor VIII antigens. Morphologically, mesangial cell had an elongated and stellate or spindle-shaped morphology. Mesangial cells isolated by this procedure were homogenous and used in all studies between passages 3 and 8.

LDL preparation and characterization

LDL was prepared as previously described [22]. Briefly, blood was taken from fasting healthy nondiabetic volunteers into a lipoprotein preservative/antioxidant cocktail (LPPC) containing ethylenediaminetetraacetic acid (EDTA) (0.1% wt/vol), chloramphenicol (20 μg/mL), gentamycin sulfate (50 μg/mL), and ε-amino-n-caproic acid (0.13%, wt/vol). Phenylmethylsulfonyl fluoride (PMSF), 20μg/mL final concentration, was added to plasma to retard proteolysis. All samples were processed at low temperature and in the absence of white light to minimize oxidation. All density solutions were supplemented with LPPC, degassed, and purged with N_2. Plasma density was increased to d = 1.21 g/mL using dried KBr and 11 mL layered under d = 1.019 g/mL saline/LPPC. After ultracentrifugation (Beckman VTi50 rotor), $2\frac{1}{2}$ hours 50,000 rpm 7°C with slow acceleration and deceleration, the LDL band was harvested by piercing the tube and aspirating into a syringe. LDL isolated by this procedure was free from contamination with apolipoprotein A (ApoA-I) and albumin.

Each LDL preparation was characterized for purity by electrophoresis on 1% agarose gels (Paragon gels) (Beckman, Brea, CA, USA). The LDL pools were tested for endotoxin contamination by the Limulus Amebocyte Lysate (BioWhittaker, Walkersville, MD, USA) according to the manufacturer's suggestion.

Cell extracts

Mesangial cells treated with LDL were washed twice in ice-cold PBS, scraped in PBS containing 2 mmol/L sodium vanadate, and centrifuged at 3000*g* for 5 minutes. Pellets were resuspended in 100 mL of lysis buffer (20 mmol/L Tris, 130 mmol/L NaCl, 10% glycerol, 10 mmol/L CHAPS, 1 mmol/L PMSF, 2 mmol/L sodium vanadate, 100 mU/mL aprotinin, and 0.156 mg/mL benzamidin, pH 8.0), incubated on ice for 30 minutes and centrifuged at maximum speed for 5 minutes. The supernatant was used as the protein source and its concentration was determined by a BCA Protein Assay Kit (Pierce, Rockford, IL, USA) using bovine serum albumin (BSA) as a standard protein.

Western blotting of CTGF and collagen I

Mesangial cells were cultured in 6-well plates (9.6 cm^2/well). At 80% confluence, cells were serum starved by the changing of serum-free media (RPMI medium1640 with 25 mmol/L Hepes buffer, 11.11 mmol/L glucose, and L-glutamine) within 24 hours. Quiescent mesangial cells were stimulated with LDL (50 μg/mL) for 24 hours in the presence and absence of either a p42/$p44^{mapk}$ inhibitor (PD98059), 40μmol/L (Calbiochem, La Jolla, CA, USA), a $p38^{mapk}$ inhibitor (SB203580), 10 μmol/L (Calbiochem), and/or the c-Jun NH2-terminal kinase (JNK)

inhibitor (SP600125), 30 μmol/L (A.G. Scientific, San Diego, CA, USA). Soluble protein (20 to 25 μg) obtained as described above was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (12% CTGF and 7.5% collagen I), under reducing conditions and transferred onto nitrocellulose membrane (Bio-Rad, Hercules, CA, USA) with semitransfer method (Bio-Rad) at 20 volts for 50 minutes. The membranes were immunoblotted with anti-CTGF polyclonal antibody (1:1000 dilution), anticollagen I polyclonal antibody (1:1000 dilution) (Southern Biothechnology, Birmingham, AL, USA), and antiactin antibody (1:1000 dilution) (Sigma Chemical Co., St. Louis, MO, USA) overnight at 4°C followed by incubating the membranes in a secondary antibody conjugated to horseradish peroxidase (HRP). The immunoreactive bands were visualized using the chemiluminiscence reagent Renaissance (NEN Life Science Products, Boston, MA, USA), according to the procedure described by the supplier. Membranes were exposed to Kodak LS film and bands were measured by densitometry and quantified by Scion Image program (Scion Corporation, Frederick, MA, USA).

Mitogen-activated protein kinase (MAPK) assays

Quiescent mesangial cells stimulated with LDL (50 μg/mL) for 5 minutes. Twenty-five to thirty micrograms of protein was analyzed by SDS-PAGE and the separated proteins were transferred to nitrocellulose membrane with semitransfer method and immunoblotted with antiphospho-p42/p44^{mapk} polyclonal antibody that detects p42 and p44 MAPK only when activated by phosphorylation at Thr202 and Tyr204 (1:4000 dilution) (Cell Signaling Technology, Inc., Beverly, MA, USA), antiphospho-p38^{mapk} antibody that detects p38 only when activated by phosphorylation at Thr180 and Tyr182 (1:1000 dilution) (Cell Signaling Technology, Inc.) and anti-JNK antibody that detects JNK only when activated by phosphorylation at Thr183 and Tyr185 (1:1000 dilution) (Cell Signaling Technology, Inc.). Immunoreactive bands were visualized using the chemiluminiscence reagent Renaissance, according to the procedure described by the supplier. Membranes were exposed to Kodak LS film and bands were measured by densitometry and quantified by Scion Image program.

TGF-β1 protein level determination

Mesangial cells were cultured in 6-well plates (9.6 cm^2/well). At 80% confluence, cells were serum starved by the changing of serum-free media within 24 hours. Cells were then stimulated for 24 hours with LDL (50 μg/mL), in presence or absence of the p42/p44^{mapk} inhibitor (PD98059), 40 μmol/L, the p38^{mapk} inhibitor (SB203580), 10 μmol/L, and/or the JNK inhibitor (SP600125), 30 μmol/L, in exactly 1.5 mL of medium. TGF-β1 protein levels were determined by colorimetric enzyme-linked immunosorbent assay kit (ELISA) (R& D System, Minneapolis, MN, USA) in the conditioned media that were activated by 1 N HCl (to measure both active and latent TGF-β1) according to the manufacturer's instructions, and expressed as picograms per milliliter.

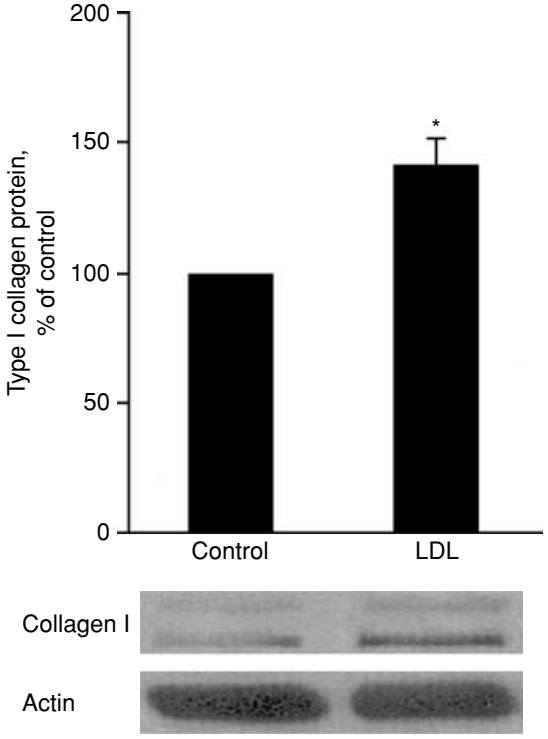

Fig. 1. Low-density lipoprotein (LDL) stimulates the regulation of collagen I protein in mesangial cells. Quiescent mesangial cells were stimulated with 50 μg/mL of LDL for 24 hours. Cell proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotted with antibodies against collagen I (1:1000) and actin (1:1000). Bar graph represents intensities of collagen I bands relative to actin and expressed as percent above control. Blots shown are representative of 22 experiments. *$P < 0.0005$ vs. control.

Statistical analysis

Data is expressed as mean ± SE and analyzed by Student t test for unpaired analysis. Differences were considered significant if $P < 0.05$.

RESULTS

Induction of collagen I by LDL

To investigate whether LDL induces matrix formation, we measured collagen I expression. Type I collagen is not usually expressed under normal physiologic conditions in mesangial cells, but can be induced in response to injury or to growth factors. Mesangial cells were stimulated with LDL (50 μg/mL) for 24 hours, and the protein levels of collagen I were measured by Western blots. The results shown in Figure 1 demonstrate that LDL stimulation resulted in a significant increase in the production of

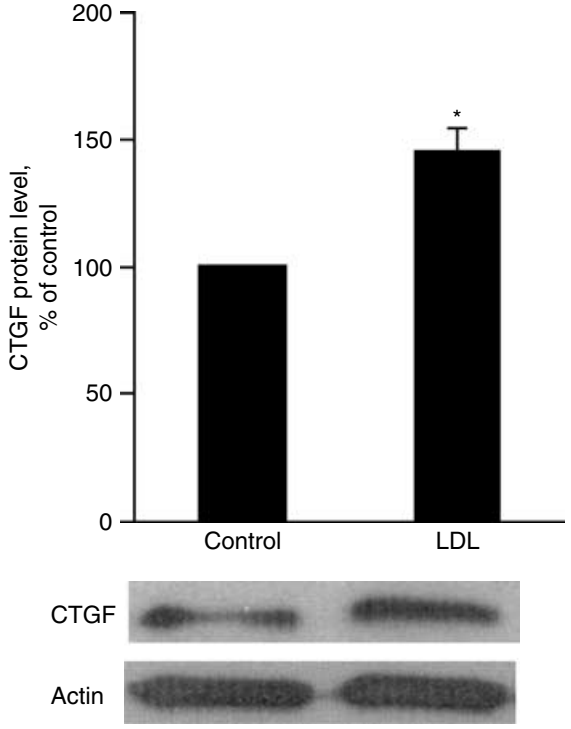

Fig. 2. Low-density lipoprotein (LDL) stimulates the regulation of connective tissue growth factor (CTGF) in mesangial cells. Quiescent mesangial cells were stimulated with 50 μg/mL of LDL for 24 hours. Cell proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (12%) and immunoblotted with an antibody against CTGF (1:1000) and actin (1:1000). Bar graph represents intensities of CTGF bands relative to actin expressed as percentage above control. Blots shown are representative of 23 experiments. $^*P <$ 0.0001 vs. control.

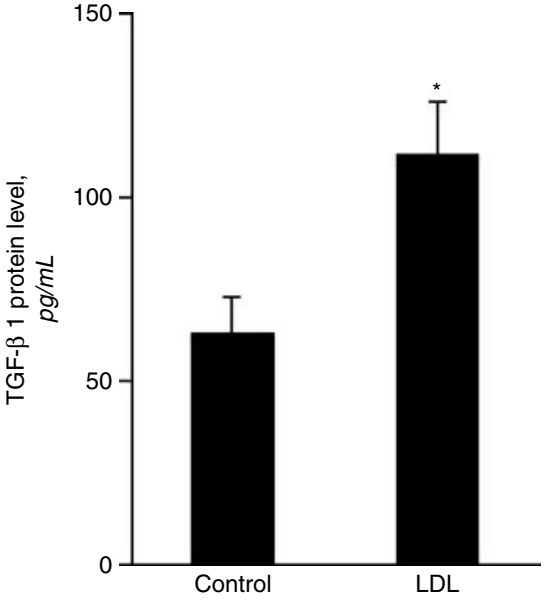

Fig. 3. Induction of transforming growth factor-β1 (TGF-β1) protein secretion by low-density lipoprotein (LDL) in mesangial cells. Quiescent mesangial cells were stimulated by LDL (50 μg/mL) for 24 hours. Conditioned media were collected and activated to determine the total level of TGF-β1 protein, using colorimetric enzyme-linked immunosorbent assay (ELISA) kit assay. Values are means ± SE of TGF-β1 levels expressed in pg/mL ($N = 26$). $^*P < 0.0001$ vs. control.

collagen I compared to unstimulated cells (2346 ± 289 vs. 1589 ± 232 densitometric units; LDL vs. control, respectively) ($P < 0.0005$) ($N = 22$). The levels of the structural protein actin protein levels measured in the same cell extracts as control were not different between control and LDL-treated cells.

Induction of CTGF by LDL

To begin to understand the cellular mechanisms through which LDL promotes renal injury, we evaluated whether LDL can modulate the expression of CTGF in renal cells prone to injury. Mesangial cells were stimulated with LDL (50 μg/mL) for 24 hours. The expression of CTGF was measured by Western blots using specific anti-CTGF antibodies. Figure 2 represents the intensities of the CTGF bands expressed as percent increase above control. The results indicate that LDL produced a significant increase in the expression of CTGF (1384 ± 144 vs. 897 ± 129 densitometric units; LDL vs. control, respectively) ($P < 0.0001$) ($N = 23$). The levels of the structural protein actin were also measured in the same cell extracts as control, to ensure equal loading of the samples into the gel, and were not different between control and LDL-treated cells.

This finding provides the first evidence that LDL can directly modulate the expression of CTGF in mesangial cells.

LDL stimulates TGF-β production in mesangial cells

We next assessed whether LDL will stimulate the production of TGF-β protein levels. Mesangial cells were treated with LDL (50 μg/mL) for 24 hours, and TGF-β protein levels were measured in the conditioned media by ELISA. The results shown in Figure 3 demonstrate that LDL treatment resulted in a significant increase in the production of TGF-β levels compared to unstimulated cells (111.8 ± 14.2 pg/mL vs. 63.2 ± 9.7 pg/mL; LDL vs. control, respectively) ($P < 0.0001$) ($N = 26$).

Induction of CTGF and collagen I by TGF-β

To explore the cellular mechanisms through which LDL regulates the expression of CTGF and collagen I in mesangial cells, we determined first if CTGF and collagen I are downstream targets for regulation by TGF-β. Mesangial cells were treated with various concentration of TGF-β (0, 0.2, 0.5, 1, 5, and 10 ng/mL) for 24 hours. The protein levels of CTGF and collagen I were measured by Western blots. The levels of the structural protein actin were also measured in the same cell extracts as control, to

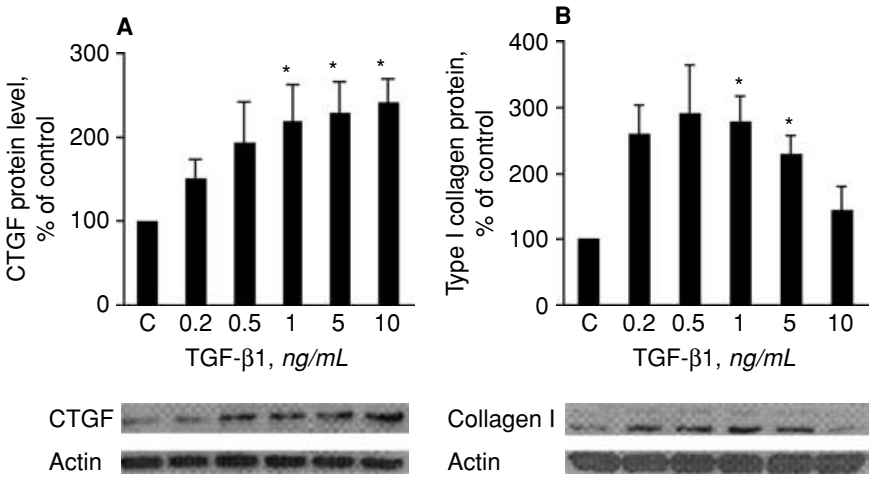

Fig. 4. Induction of connective tissue growth factor (CTGF) and collagen I by transforming growth factor-β (TGF-β). Mesangial cells were treated with various concentration of TGF-β 1 (0, 0.2, 0.5, 1, 5, and 10 ng/mL) for 24 hours. (*A*) Cell proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (12%) and immunoblotted with an antibody against CTGF (1:1000) and actin (1:1000). Bar graph represents intensities of CTGF bands relative to actin expressed as percentage above control. Blots shown are representative of five separate experiments. $^{*}P < 0.05$ vs. control. (*B*) Cell proteins were separate by SDS-PAGE (7.5%) and immunoblotted with an antibody against collagen I (1:1000) and actin (1:1000). Bar graph represents intensities of collagen I bands relative to actin expressed as percentage above control. Blots shown are representative of three separate experiments. $^{*}P < 0.05$ vs. control.

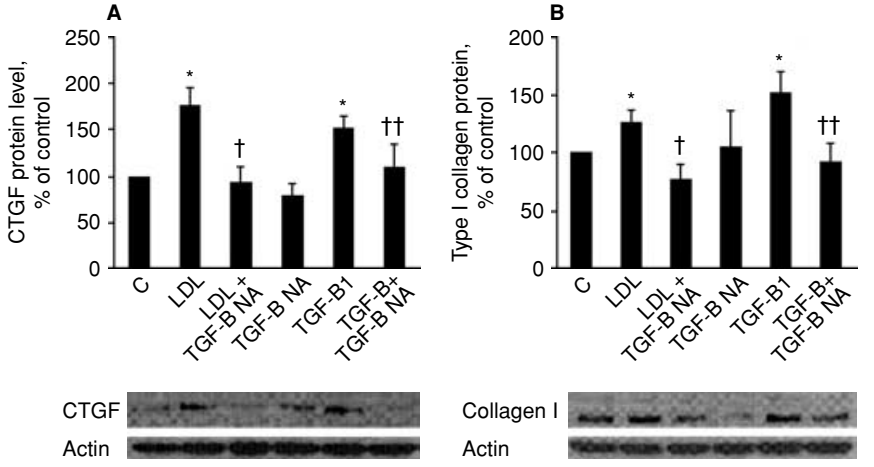

Fig. 5. Low-density lipoprotein (LDL) induces connective tissue growth factor (CTGF) and collagen I expression via autocrine activation of transforming growth factor-β (TGF-β). Mesangial cells were stimulated with 50 μg/mL of LDL or 5 ng/mL of TGF-β1 for 24 hours in presence or absence of 5 μg/mL of neutralizing anti-TGF-β antibody (TGF-β NA). (*A*) Equal amounts of proteins were resolved on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (12%) and immunoblotted with an antibody against CTGF (1:1000) and actin (1:1000). Bar graph represents intensities of CTGF bands relative to actin expressed as percentage above control. Blots shown are representative of nine experiments. $^{*}P < 0.01$ vs. control; $^{\dagger}P < 0.05$ vs. LDL; $^{\dagger\dagger}P < 0.05$ vs. TGF-β1. (*B*) Equal amounts of proteins were resolved on SDS-PAGE (7.5%) and immunoblotted with an antibody against collagen I (1:1000) and actin (1:1000). Bar graph represent intensities of collagen I bands relative to actin expressed as percentage above control. Blots shown are representative of eight experiments. $^{*}P < 0.05$ vs. control; $^{\dagger}P < 0.05$ vs. LDL; $^{\dagger\dagger}P < 0.01$ vs. TGF-β1.

ensure equal loading of the samples into the gel. The results shown in Figure 4 demonstrate that TGF-β resulted in a concentration-dependent increase in the expression of CTGF ($P < 0.05$) ($N = 5$) and collagen I ($P < 0.05$) ($N = 3$) in mesangial cells. Actin protein levels were not different between control and TGF-β–treated cells.

These findings indicate that TGF-β is upstream of CTGF and collagen I and could play a key role in modulating the expression of the prosclerotic cytokine CTGF and of matrix protein collagen I.

Role of TGF-β in LDL-induced CTGF and collagen I expression

To assess whether the induction of CTGF and collagen I we observed in response to LDL is mediated via autocrine activation of TGF-β, mesangial cells were treated with LDL (50 μg/mL) for 24 hours in the presence and absence of neutralizing anti-TGF-β (TGF-β NA) antibodies (5 μg/mL) (R&D System), a concentration shown to neutralize TGF-β action [23]. As a positive control, mesangial cells were also treated with TGF-β (5 ng/mL) for 24 hours in the presence and absence of neutralizing anti-TGF-β antibodies. The protein levels of CTGF, collagen I, and actin were measured by Western blots.

The results shown in Figure 5 demonstrate that both LDL and TGF-β produced a significant increase in CTGF and collagen I production ($P < 0.01$ vs. control) ($N = 9$). This increase in CTGF and collagen I in response to LDL or TGF-β was significantly inhibited by the anti-TGF-β NA ($P < 0.05$ vs. LDL and vs. TGF-β). These findings are the first to implicate TGF-β as a key player in LDL

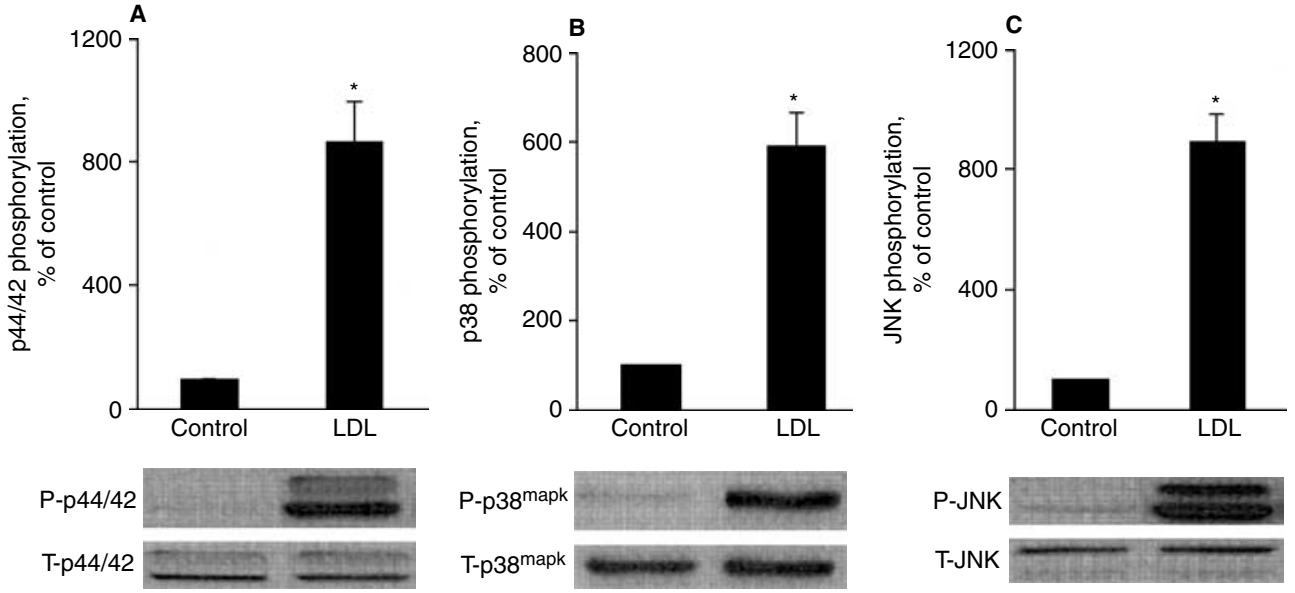

Fig. 6. Phosphorylation of mitogen-activated protein kinase (MAPK) pathway by low-density lipoprotein (LDL). Quiescent mesangial cells were stimulated with 50 μg/mL of LDL for 5 minutes. Cell proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (12%) and immunoblotted with a polyclonal antibody against phospho-p44/42 (1:4000) and total-p44/42 (1:4000) (*A*), phospho-p38 (1:1000) and total-p38 (1:4000) (*B*), and phospho-c-Jun NH2-terminal kinase (JNK) (1:1000) and total-JNK (1:4000) (*C*). Blots shown are representative of 12 experiments. Bar graph represents intensities of phospho-MAPK relative to total-MAPK expressed as percent phosphorylation above control. Bars represent means ± SE of 12 experiments. $^*P < 0.0001$ vs. control.

signaling leading to CTGF and collagen I expression in mesangial cells.

Phosphorylation of MAPK pathway by LDL

The MAPK signal transduction pathway represents an important mechanism by which growth factors regulate cell proliferation. In mammalian cells the MAPK family include the extracellular signal-regulated kinases 1 and 2 (Erk 1/2), or p42, and $p44^{mapk}$, the JNK/or stress-activated protein kinase (SAPK) and $p38^{mapk}$. We have previously reported that LDL can active the $p42/p44^{mapk}$ in mesangial cells [22]. To further explore if LDL will activate other members of the MAPK family, mesangial cells were treated with LDL (50 μg/mL) for 5 minutes. Cytosolic proteins were analyzed by SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted with either antiphospho-$p42/p44^{mapk}$ antibodies (1:4000 dilution), or antiphospho-$p38^{mapk}$ (1:1000 dilution), and/or antiphospho-JNK antibodies (1:1000 dilution). The immunoreactive bands were visualized using enhanced chemiluminescence (ECL) reagent. The membranes were also immunoblotted with total nonphosphorylated $p42/p44^{mapk}$, $p38^{mapk}$, and JNK antibodies to ensure equal amounts of MAPK loaded into the gels.

The results shown in Figure 6 demonstrate that LDL resulted in a significant increase in the phosphorylation of $p42/p44^{mapk}$ ($P < 0.02$) ($N = 12$), $p38^{mapk}$ ($P < 0.02$) ($N = 12$), and of JNK ($P < 0.001$) ($N = 12$). Total nonphosphorylated $p42/p44^{mapk}$, $p38^{mapk}$, and JNK were not different between control and LDL-treated cells.

Role of JNK in LDL-induced TGF-β production

To explore whether the MAPK pathway modulates the production of TGF-β in response to LDL, we measured TGF-β protein levels in mesangial cells treated with LDL (50 μg/mL) for 24 hours, in the presence and absence of membrane permeable inhibitors of $p42/p44^{mapk}$ (PD98059), 40 μmol/L, $p38^{mapk}$ (SB 203580), 10 μmol/L, and JNK (SP600125), 30 μmol/L. The results shown in Figure 7 demonstrate that LDL once again produced a significant increase in TGF-β protein levels compared to unstimulated cells. This increase in TGF-β protein levels was significantly reduced by the JNK inhibitor SP600125 (155.6 ± 21.7 pg/mL vs. 54.7 ± 6.9 pg/mL LDL vs. LDL+SP600125) ($P < 0.0001$) ($N = 10$). Treatment of mesangial cells with the JNK inhibitor SP600125 significantly reduced the basal production of TGF-β protein levels (29.3 ± 3.0 pg/mL vs. 84.1 ± 14.6 pg/mL; SP600125 vs. C) ($P < 0.001$) ($N = 10$). On the other hand, selective inhibition of $p42/p44^{mapk}$ by PD98059 and of $p38^{mapk}$ by SB203580 did not significantly alter the increased production of TGF-β protein levels in response to LDL stimulation, nor did they significantly influence the basal levels of TGF-β. These findings demonstrate that LDL utilizes the JNK pathway to modulate the production of TGF-β in mesangial cells.

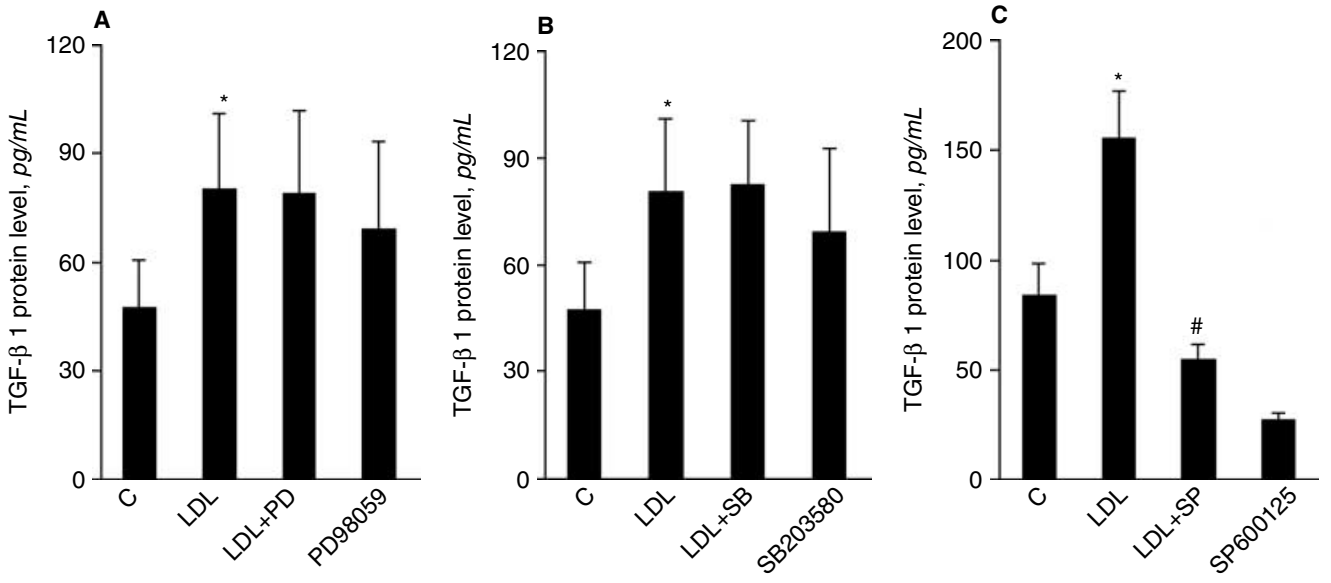

Fig. 7. Role of c-Jun NH2-terminal kinase (JNK) in low-density lipoprotein (LDL)-induced transforming growth factor-β (TGF-β) production. Quiescent mesangial cells were stimulated with LDL (50 μg/mL) for 24 hours in presence or absence of p44/42^{mapk} inhibitor, PD98059 (40 μmol/L) (*A*), p38^{mapk} inhibitor, SB203580 (10 μmol/L) (*B*), and JNK inhibitor, SP600125 (30 μmol/L) (*C*). Conditioned media were collected and activated to determine the total level of TGF-β1 protein using colorimetric enzyme-linked immunosorbent assay (ELISA) kit assay. Values are means ± SE of TGF-β1 levels expressed in pg/mL. $^{*}P < 0.05$ vs. control; $^{\#}P < 0.0001$ vs. LDL ($N = 4$ to 10 experiments).

Role of MAPK in LDL-induced CTGF production

To explore whether the MAPK pathway modulates the production of CTGF in response to LDL, we measured CTGF protein levels in mesangial cells treated with LDL (50 μg/mL) for 24 hours, in the presence and absence of membrane permeable inhibitors of p42/p44^{mapk} (PD98059), 40 μmol/L, p38^{mapk} (SB 203580), 10 μmol/L, and JNK (SP600125), 30 μmol/L. CTGF levels were measured by Western blots. The results shown in the Figure 8 demonstrate that LDL once again produced a significant increase in CTGF protein levels compared to unstimulated cells. However, in the presence of the p44/42^{mapk} inhibitor PD 98059 and/or the JNK inhibitor SP600125, the increase in CTGF protein levels in response to LDL was significantly suppressed ($P < 0.005$) ($N = 8$). On the other hand, inhibition of the p38^{mapk} by SB203580 did not alter the expression of CTGF in response to LDL. Actin protein levels measured at the same time and in the same cell extracts were not significantly different among the groups.

This is the first demonstration that the induction of CTGF by LDL is mediated via phosphorylation of the MAPK pathway.

LDL increases collagen I via JNK-dependent pathway

To evaluate whether activation of the MAPK pathway modulates the increase in collagen I in response to LDL, mesangial cells were pretreated with the p42/p44^{mapk} (PD98059), 40 μmol/L, p38^{mapk} (SB 203580), 10 μmol/L, and JNK (SP600125), 30μmol/L inhibitors for 45 minutes, followed by LDL stimulation for 24 hours. The results shown in Figure 9 demonstrate that LDL once again produced a significant increase in collagen I protein levels compared to unstimulated cells. This increase in collagen I protein levels was significantly reduced by the JNK inhibitor SP600125 ($P < 0.001$) ($N = 8$). However, selective inhibition of p42/p44^{mapk} by PD98059 and of p38^{mapk} by SB203580 did not significantly alter the increased production of collagen I protein levels in response to LDL stimulation. These findings demonstrate that LDL utilizes the JNK pathway to modulate the production of collagen I in mesangial cells. Actin protein levels measured at the same time and in the same cell extracts were not significantly different among the groups.

DISCUSSION

In the present study we have demonstrated that LDL exerts a significant effect on the expression of CTGF and collagen I in mesangial cells. We have shown that LDL increases the protein levels of CTGF, TGF-β, and collagen I in mesangial cells. This effect of LDL was mediated via phosphorylation of the MAPK pathway.

Poorly controlled type 1 diabetes is usually associated with elevated levels of plasma LDL, intermediate density lipoproteins, and very low-density lipoprotein levels [11–14]. In contrast, HDL cholesterol levels are low during poor glycemic control and increase to normal or above normal when adequate control is attained [24, 25]. In the Pittsburgh Epidemiology of Diabetes Complications Cohort of type 1 diabetic patients, Orchard et al [26]

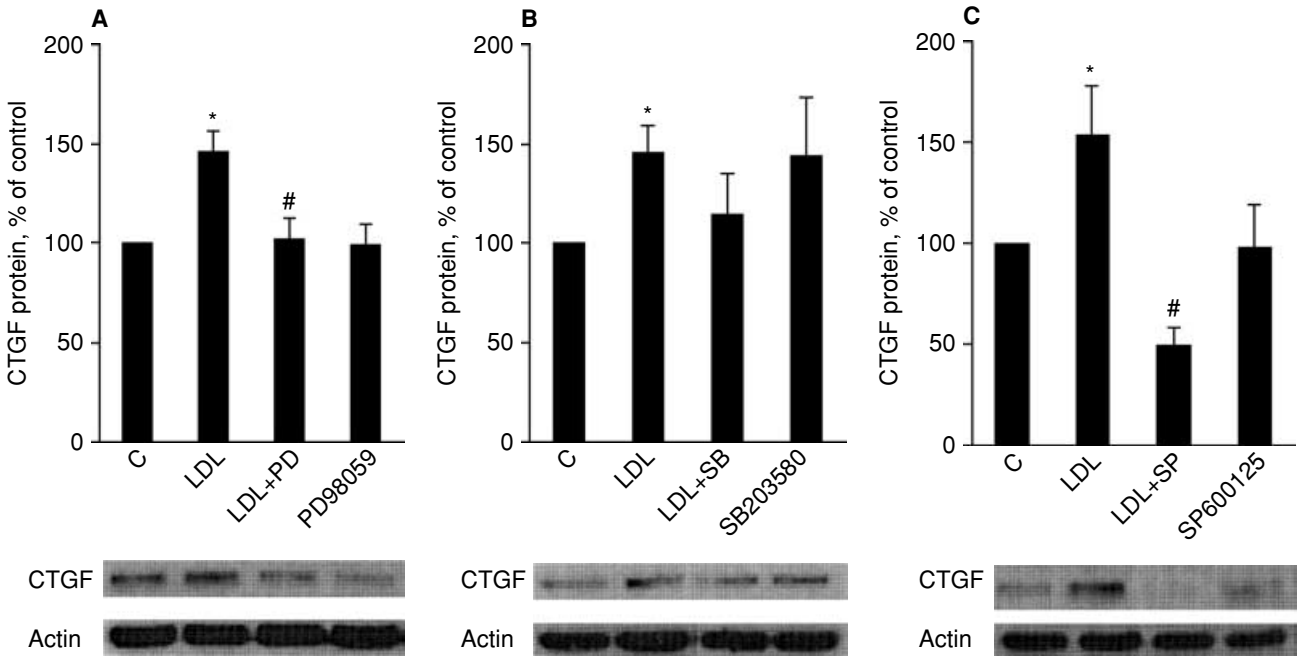

Fig. 8. Effect of mitogen-activated protein kinase (MAPK) inhibitors on low-density lipoprotein (LDL)-induced regulation of connective tissue growth factor (CTGF). (*A*) Mesangial cells were stimulated with LDL (50 μg/mL) for 24 hours in presence or absence of $p44/42^{mapk}$ inhibitor, PD98059 (40 μmol/L) for 45minutes. Equal amounts of proteins were resolved on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (12%) and immunoblotted with an antibody against CTGF (1:1000) and actin (1:1000). Bar graph represents intensities of CTGF bands relative to actin expressed as percent above control. Blots shown are representative of eight experiments. $^{*}P < 0.05$ vs. control; $^{\#}P < 0.005$ vs. LDL. (*B*) Quiescent mesangial cells were stimulated by LDL (50 μg/mL) for 24 hours in presence or absence of $p38^{mapk}$ inhibitor, SB203580 (10 μmol/L) for 45minutes. Blots shown are representative of four experiments. $^{*}P < 0.05$ vs. control. (*C*) Quiescent mesangial cells were stimulated by LDL (50 μg/mL) for 24 hours in presence or absence of c-Jun NH2-terminal kinase (JNK) inhibitor, SP600125 (30 μmol/L) for 45minutes. Blots shown are representative of eight experiments. $^{*}P < 0.05$ vs. control; $^{\#}P < 0.001$ vs. LDL.

related baseline lipids to nephropathy and showed that LDL cholesterol and triglycerides and hypertension were predictive of nephropathy onset within 5 years but not 6 to 10 years. Hemoglobin A_{1c} (HbA_{1c}) levels predicted the later onset of nephropathy [26]. Abnormalities in the distribution of lipoprotein subclasses in type 1 diabetes have been recently described [27]. Using nuclear magnetic resonance (NMR), LDL particle concentration was positively associated with albumin excretion rate (AER) and LDL diameter was inversely associated with AER [27]. Interestingly, a recent study showed that the regression of microalbuminuria in type 1 diabetic patients is associated with low levels of total serum cholesterol and triglycerides [28]. Furthermore, type 1 diabetic patients with proteinuria have a tenfold risk of macrovascular disease relative to type 1 patients without proteinuria. The relation of microalbuminuria to vascular disease complications such as carotid intima-medial thickness was recently illustrated in the DCCT/EDIC cohort of type 1 diabetic patients [29]. Diabetic renal disease is associated with elevations of blood pressure and dyslipidemia, conditions that predict and accelerate the progression of vascular disease in diabetic patients [28].

Although hyperlipidemia is now considered a risk factor for the progression of renal damage, the initiating and sustaining signals that link hyperlipidemia to glomerular sclerosis are not fully realized. Accumulation of LDL within the mesangium may activate glomerular cells to produce various cytokines and growth factors that may alter mesangial cell behavior by inducing cell proliferation, hypertrophy and increasing ECM accumulation [19, 20]. In this regard our findings demonstrate that the protein levels of TGF-β, CTGF and collagen I were induced in mesangial cells in response to LDL challenge. Inhibition of TGF-β activity with neutralizing antibodies prevented the rise in CTGF and collagen I, thus demonstrating that the increase in CTGF and collagen I protein levels in response to LDL were mediated via autocrine activation of TGF-β. These data support other findings and lends support to the notion that CTGF and collagen I is downstream targets of TGF-β.

Even though TGF-β has long been regarded as a major driving force in many progressive fibrotic diseases, attention has recently focused on the role of CTGF as a profibrotic factor [30]. CTGF, a newly described factor that promotes ECM deposition and fibrosis in many tissue, appears to act downstream of TGF-β to induce ECM production [31]. Several studies demonstrated CTGF is an important mediator in the pathogenesis of glomerulosclerosis in diabetic nephropathy and other forms of

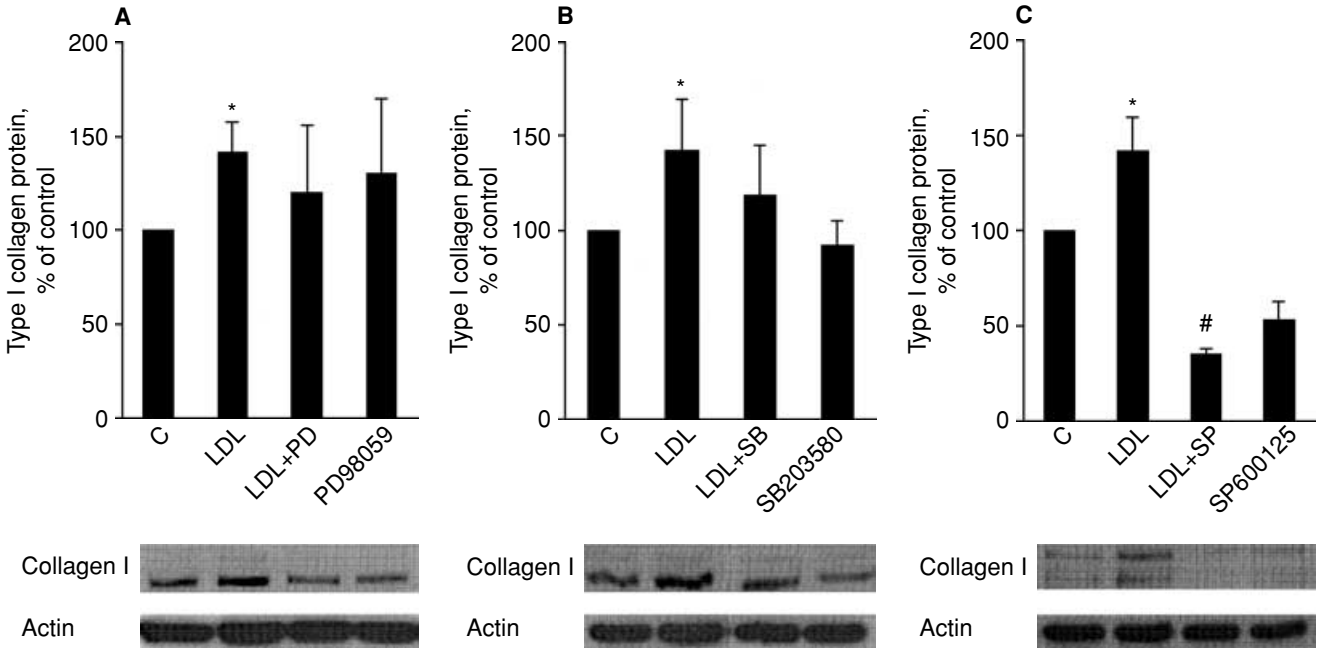

Fig. 9. Role of mitogen-activated protein kinase (MAPK) pathway in low-density lipoprotein (LDL)-induced collagen I expression. (*A*) Quiescent mesangial cells were stimulated with LDL (50 μg/mL) for 24 hours in presence or absence of p44/42^{mapk} inhibitor, PD98059 (40 μmol/L) for 45minutes. Equal amounts of proteins were resolved on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (12%) and immunoblotted with an antibody against collagen I (1:1000) and actin (1:1000). Bar graph represents intensities of collagen I bands relative to actin expressed as percentage above control. Blots shown are representative of seven experiments. $^{*}P < 0.05$ vs. control. (*B*) Quiescent mesangial cells were stimulated by LDL (50 μg/mL) for 24 hours in presence or absence of p38^{mapk} inhibitor, SB203580 (10 μmol/L) for 45minutes. Blots shown are representative of four experiments. $^{*}P < 0.05$ vs. control. (*C*) Quiescent mesangial cells were stimulated by LDL (50 μg/mL) for 24 hours in presence or absence of c-Jun NH2-terminal kinase (JNK) inhibitor, SP600125 (30 μmol/L) for 45minutes. Blots shown are representative of eight experiments. $^{*}P < 0.05$ vs. control; $^{\#}P < 0.0001$ vs. LDL.

renal disease [32–35]. CTGF in mesangial cells is up-regulated by high extracellular glucose, TGF-β and angiotensin II and is down-regulated by nitric oxide [31, 36–38]. However, in the present study we demonstrate for the first time that LDL can up-regulate CTGF in mesangial cells.

It is widely accepted that LDL stimulation results in the activation of members of the MAPK family, and activation of this pathway is known to be important in regulating gene expression and mesangial cell growth and function [22, 39]. The MAPK are a family of serine-threonine protein kinases that are activated in response to a variety of extracellular stimuli. ERK (p42/44^{mapk}), p38^{mapk}, and JNK constitute three major subfamilies of MAPK that appear to mediate cellular responses, including proliferation, differentiation, and apoptosis [40]. ERK plays a major role in cell proliferation and differentiation, as well as in survival mediated by various growth factors [40]. On the other hand, p38^{mapk} and JNK are activated by various inflammatory cytokines and environmental stressors and they play important roles in apoptosis and cytokine production [41]. To elucidate which member of the MAPK family may be responsible for the LDL-induced increase in CTGF and collagen I, we elected to study the role of p42/44^{mapk}, p38^{mapk}, and JNK in mesangial cells. Our findings indicate that inhibition of JNK by SP600125 suppressed the expression of TGF-β, CTGF, and collagen I levels in response to LDL stimulation, whereas inhibition of the p42/44^{mapk} by PD98059 resulted in suppression of LDL-induced expression of CTGF. On the other hand, blockade of the p38^{mapk} pathway by SB203580 did not significantly alter the expression of TGF-β, CTGF, or collagen I in response to LDL. This finding implicates JNK as a key player in modulating the signals through which LDL promotes TGF-β, CTGF, and collagen I expression in mesangial cells. Furthermore, it is of interest to note that inhibition of basal JNK activity also reduced the basal production of TGF-β, thus implicating a key role for JNK kinase in modulating the production of TGF-β in mesangial cells. Other studies have implicated JNK in TGF-β1–induced CTGF mRNA expression in human lung fibroblasts, whereas p38^{mapk} and p42/44^{mapk} pathways were implicated in TGF-β–induced ECM deposition [42–44].

Although the initiating and sustaining signals that link LDL to CTGF expression are not yet fully defined, several possibilities may exist. Our data demonstrate that LDL stimulates JNK activation and JNK has been shown to bind and phosphorylate the DNA binding protein c-Jun and increase its transcriptional activity [41]. c-Jun is a

component of the activating protein-1 (AP-1) transcription complex which is an important regulator of gene expression [45]. In this regard, the autoinduction of TGF-β has been shown to be mediated via activation of the AP-1 complex [46]. Once activated, TGF-β can induce CTGF expression via the SMAD pathway [47]. In fact, a SMAD response element is present on the promoter of the CTGF gene [48].

CONCLUSION

We demonstrate that the expression of CTGF and collagen I in mesangial cells are up-regulated by LDL and that this regulation is mediated via autocrine activation of TGF-β. The results also implicate JNK as a key player in modulating the expression of TGF-β, CTGF, and collagen I in response to LDL stimulation. The data also point to a potential novel mechanistic pathway through which lipoproteins may promote diabetic glomerular injury.

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