Electronegative LDL Impairs Vascular Endothelial Cell Integrity in Diabetes by Disrupting Fibroblast Growth Factor 2 (FGF2) Autoregulation

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OBJECTIVE—L5, a circulating electronegative LDL identified in patients with hypercholesterolemia or type 2 diabetes, induces endothelial cell (EC) apoptosis by suppressing fibroblast growth factor (FGF)2 expression. FGF2 plays a pivotal role in endothelial regeneration and compensatory arteriogenesis. It is likely that vasculopathy and poor collateralization in diabetes is a result of FGF2 dysregulation.

RESEARCH DESIGN AND METHODS—To investigate this mechanism, we isolated L5 from type 2 diabetic patients. In cultured bovine aortic ECs (BAECs), L5 inhibited FGF2 transcription and induced apoptosis. Because FGF2 stimulates the phosphatidylinositol 3-kinase (PI3K)-Akt pathway, we examined whether FGF2 transcription is regulated by Akt through a feedback mechanism.

RESULTS—Diabetic L5 reduced FGF2 release to the medium but enhanced caspase-3 activity, with resultant apoptosis. Inhibition of PI3K with wortmannin or suppression of Akt activation with dominant-negative Akt inhibited FGF2 expression. Transfection of BAECs with FGF2 antisense cDNA depleted endogenous FGF2 protein. In these cells, not only was Akt phosphorylation inhibited, but FGF2 transcription was also critically impaired. In contrast, transfecting BAECs with FGF2 sense cDNA augmented Akt phosphorylation. Treatment with constitutively active Akt enhanced FGF2 expression. Augmentation of either FGF2 transcription or Akt phosphorylation rendered BAECs resistant to L5.

CONCLUSIONS—These findings suggest that FGF2 is the primary initiator of its own expression, which is autoregulated through a novel FGF2-PI3K-Akt loop. Thus, by disrupting FGF2 autoregulation in vascular ECs, L5 may impair reendothelialization and collateralization in diabetes. ***Diabetes*** **57:158–166, 2008**

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Akt-CA, constitutively active Akt; Akt-DN, dominant-negative Akt; BAEC, bovine aortic endothelial cell; EC, endothelial cell; FGF, fibroblast growth factor; PAF, platelet-activating factor; oxLDL, copper-oxidized LDL; PI3K, phosphatidylinositol 3-kinase; VEGF, vascular endothelial growth factor.

Elevated plasma LDL cholesterol is a key risk factor for coronary artery disease, but diabetes is considered equivalent to presence of atherosclerotic disease regardless of whether plasma LDL cholesterol is elevated (1,2). This suggests that diabetic LDL may contain certain atherogenic components not reflected by absolute plasma LDL cholesterol concentrations. L5 is an electronegative LDL species that was originally identified in hypercholesterolemic human plasma and found to exert multiple atherogenic effects (3–5). The presence of L5 in diabetic patients with or without LDL cholesterol elevation may help explain the atherogenicity in diabetes.

Avogaro et al. (6) first reported that LDL can be divided into the electropositive [LDL(+)] and electronegative [LDL(−)] fractions by ion-exchange chromatography. Subsequently, others have described the chemical and functional properties of these dichotomized LDL subfractions (7–11). Using a modified chromatography protocol, we have been able to divide LDL into five subfractions, L1–L5, with L5 being the most electronegative (3,4). Both LDL(−) and L5 are naturally modified LDL (3,10). As reviewed by Sanchez-Quesada et al. (10), LDL(−) can induce vascular endothelial cells (ECs) and monocytes to release multiple cytokines, including interleukin-8, monocyte chemoattractant protein-1, tumor necrosis factor-α, and vascular cell adhesion molecule-1. This is compatible with our finding that L5 stimulates monocyte-EC adhesion (4). We previously showed that hypercholesterolemic L5 induces EC apoptosis (3), a critical event in the development of atherothrombosis (12,13). In both type 1 and type 2 diabetes, Rigla et al. (14) have reported the presence of LDL(−). After successfully isolating L5 from type 2 diabetic patients, we described its physical and chemical characteristics (15).

L5-induced EC apoptosis is associated with downregulation of fibroblast growth factor (FGF)2 (3), a pleiotropic protein conserved through evolution and ubiquitously expressed in all mammalian cells of mesodermal and neuroectodermal origin (15). By regulating expression and activity of multiple target genes, FGF2 plays an important role in cell proliferation, differentiation, survival, and angiogenesis (16–18). FGF2 plays an important role in compensatory arteriogenesis (19,20). In comparison, vascular endothelial growth factor (VEGF) is more involved in inflammatory angiogenesis as plaque neovascularization (20,21), although its participation in collateral arteriogenesis cannot be excluded. Delineating how FGF2 is dys-

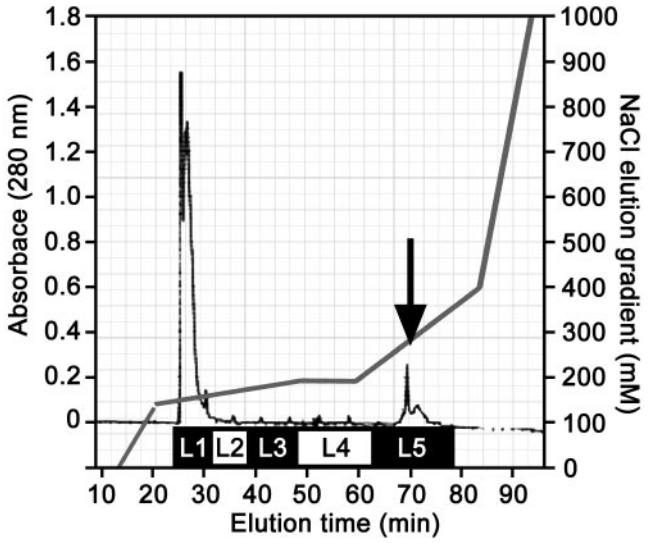

FIG. 1. L5 is a subset of diabetic LDL. A representative chromatogram of LDL subfractional zones from the plasma of a diabetic patient. The black arrow indicates the presence of L5.

regulated may yield new therapies that improve tissue perfusion in severe diabetic atherosclerosis by enhancing compensatory arteriogenesis.

FGF2 activates PI3K, which then activates Akt (22,23). Subsequently, phosphorylated Akt inactivates downstream proapoptotic proteins (24) and activates prosurvival endothelial nitric oxide synthase (25). VEGF, another mitogenic and antiapoptotic growth factor, also activates PI3K (25,26). Therefore, it is important to determine whether the FGF2 deficit can be compensated for by VEGF. Although FGF2 is able to upregulate many genes (17,27), the mechanism by which FGF2 itself is regulated remains unclear. Exogenous FGF2 can induce FGF2 expression (17), but it was unclear whether FGF2 expression depends on endogenously produced FGF2. Because FGF2 acts by activating the PI3K-Akt pathway, we hypothesized that FGF2 transcription is dependent on Akt activation through a feedback mechanism. To address this issue, we established bovine aortic EC (BAEC) cell lines in which FGF2 and Akt expression was genetically manipulated.

L5 and copper-oxidized LDL (oxLDL) differ in chemical compositions and degree of oxidation (3). However, they are both sensitive to platelet-activating factor (PAF) acetylhydrolase, which removes their proapoptotic properties by hydrolyzing PAF and PAF-like phospholipids at the *sn*-2 position (3). In this study, copper-oxLDL was used as a positive control in some experiments.

RESEARCH DESIGN AND METHODS

Cell culture, LDL preparation, and L5 isolation. Cultures of BAECs, maintained in Dulbecoo's modified Eagle's medium supplemented with 10% FBS and antibiotics, were used at 3–5 passages (28). With approval by the Baylor College of Medicine review board, blood samples were obtained from six patients with type 2 diabetes (current A1C >7%, fasting blood glucose >126 mg/dl, mean ± SEM LDL cholesterol 2.99 ± 87 mmol/l [116 ± 18 mg/dl], and age 56 ± 8 years) and six nondiabetic, normolipidemic adult subjects (LDL cholesterol 2.62 ± 0.22 mmol/l [110 ± 11 mg/dl] and age = 53 ± 4 years). LDL particles (d = 1.019–1.063 g/ml) were isolated by ultracentrifugation (28). LDL preparations were fractionated with a fast protein liquid chromatography system (Pharmacia Biotech) through a UnoQ12 anion-exchange column (BioRad). LDL subfractions were eluted with a linear gradient program at a flow rate of 2 ml/min, with concentration monitoring by absorbance at 280 nm (3). Protein concentrations were determined by the Lowry method. oxLDL was prepared by incubating LDL with 5 μmol/l $CuSO_4$ at 37°C for 24 h. Precautions were taken to prevent all LDL preparations from endotoxin contamination and further oxidation (28).

Treatments with L5, oxLDL, FGF2, VEGF, and wortmannin. BAECs were maintained in medium containing 5% serum for 6 h before incubation with various agents for 24 h. Effects of L5 (50 μg/ml), oxLDL (50 μg/ml), unfractionated normolipidemic LDL (50 μg/ml), and PBS (lipoprotein-free control) on BAEC apoptosis were determined. To evaluate their antiapoptotic effects, FGF2 (2–10 ng/ml) or VEGF (10–50 ng/ml; R&D Systems) was added to cells incubated with L5 or oxLDL. To determine the role of Akt in EC survival, cells were treated with the PI3K inhibitor wortmannin (25–200 nmol/l; Sigma-Aldrich) alone or in combination with FGF2 (10–20 ng/ml; R&D Systems) for apoptosis assessment.

Apoptosis assays. Nuclear morphology was examined in treated BAECs stained with Hoechst 33342 (Molecular Probes). Apoptotic nuclei were analyzed with MetaView software (Universal Imaging Corp) in fluorescent images (500 cells/well in three wells) through a Zeiss (Thornwood) inverted microscope (3).

Caspase-3 detection and activity assays. After treatment, activated caspase-3 in BAECs was detected by a caspase-3 detection kit (FITC-DEVD-FMK; Calbiochem) through epifluorescence microscopy. Caspase-3 activity was evaluated by EnzChek Caspase-3 Assay Kit no. 1 (E13183; Invitrogen) according to the manufacturer's instructions.

FGF2 enzyme-linked immunosorbent assay. FGF2 release from the BAECs was examined by a human FGF basic Quantikine enzyme-linked immunosorbent assay kit (DFB50; R&D systems) and analyzed after subtracting the added exogenous FGF2.

Western and Northern blot analyses of FGF2 and Akt phosphorylation assay. FGF2 protein and mRNA levels were assayed by Western and Northern blot analyses, respectively, as previously described (3,28). To assess the state of Akt activation, phosphorylated Akt levels in the cell extracts were determined by immunoblotting with murine anti–phospho-Akt antibody (Ser^{473} and Thr^{308}; Cell Signaling Technology) (24).

Transfection of constitutively active and dominant-negative Akt cDNAs in BAECs. Constitutively active Akt (Akt-CA) plasmid (plncx-HA-myr-Akt) and dominant-negative Akt (Akt-DN) plasmid (plncx-HA-myr-Akt; K179M) were provided by Dr. Marco Marcelli. The constructs were transfected by using FuGENE six (Roche Diagnostics). Briefly, BAECs were plated at a density of 1×10^5 cells/ml in six-well culture dishes and allowed to attach and grow 24 h before transfection. Each transfection mixture was prepared by diluting 3 μl of FuGENE six into 95 μl serum-free Dulbecoo's modified Eagle's medium. A 2-μl aliquot (1 μg) of DNA was gently mixed into the liposomal solution and incubated for 15 min at room temperature. The transfection mixture was slowly added to the cells, which were allowed to recover from the transfection for an additional 24 h before experimental treatments were applied. This transfection method routinely yielded a >60% efficiency.

FGF2 expression vector construction and transfection. The primer pair used to amplify a 1,754 base pairs of human FGF2 cDNA consisted of 5′-CAGAAAACCCGAGCGAGTAG-3′ (forward strand) and 5′-ACACAGCGGTTCGAGAAGTT-3′ (reverse strand) custom sequences. The amplified full-length FGF2 cDNA was inserted into the expression vector pcDNA 3.1 (Invitrogen) in the sense and antisense orientations, generating pFGF2(+) and pFGF2(−) expression vectors, respectively. For transfection, 10^6 BAECs were seeded per 100-mm dish and incubated with 100 ng pFGF2(+) or pFGF2(−) plasmid DNA, using the calcium phosphate method (Roche). Stably transfected cells were selected with 800 μg/ml G418 for 14 days. G418-resistant BAECs that harbored pFGF2(+) and pFGF2(−) DNAs were designated BAEC[FGF2(+)] and BAEC[FGF2(−)], respectively. To detect FGF2 expression in transfected cells, G418-resistant BAECs were fixed in 4% paraformaldehyde and incubated with 6% BSA for 20 min, followed by incubation with anti-FGF2 antibody (R&D Systems; 1:100 dilution) for 24 h at 4°C. After incubation in PBS containing 0.2% Triton X-100, cells were treated with AlexaFluor 594 goat anti-rabbit IgG. Immunocytochemical reactions of the samples were analyzed by fluorescence microscopy.

Nuclear run-on analysis. To assess the rate of FGF2 gene transcription, EC nuclei were isolated with NP-40 lysis buffer and stored at −80°C. After thawing, nuclei from 5×10^7 cells were added to 100 μl 2 × reaction buffer containing 100 μCi [α-^{32}P]UTP at 30°C for 30 min. To remove template DNA after transcription, RNase-free DNase I and 1 mol/l $CaCl_2$ were added to the mixture and incubated at 26°C for 30 min. To enhance protein digestion, proteinase K and tRNA (Roche) were added to the mixture for an additional incubation period of 30 min at 37°C. The labeled nascent RNA transcripts were extracted with Tri-Reagent (Molecular Research Center) plus chloroform and precipitated with isopropanol. The RNA pellet were then washed with 70% ice-cold ethanol and dissolved in 0.2% SDS. To detect the newly transcribed RNAs, 1 μg each of FGF2 and β-actin cDNA were immobilized

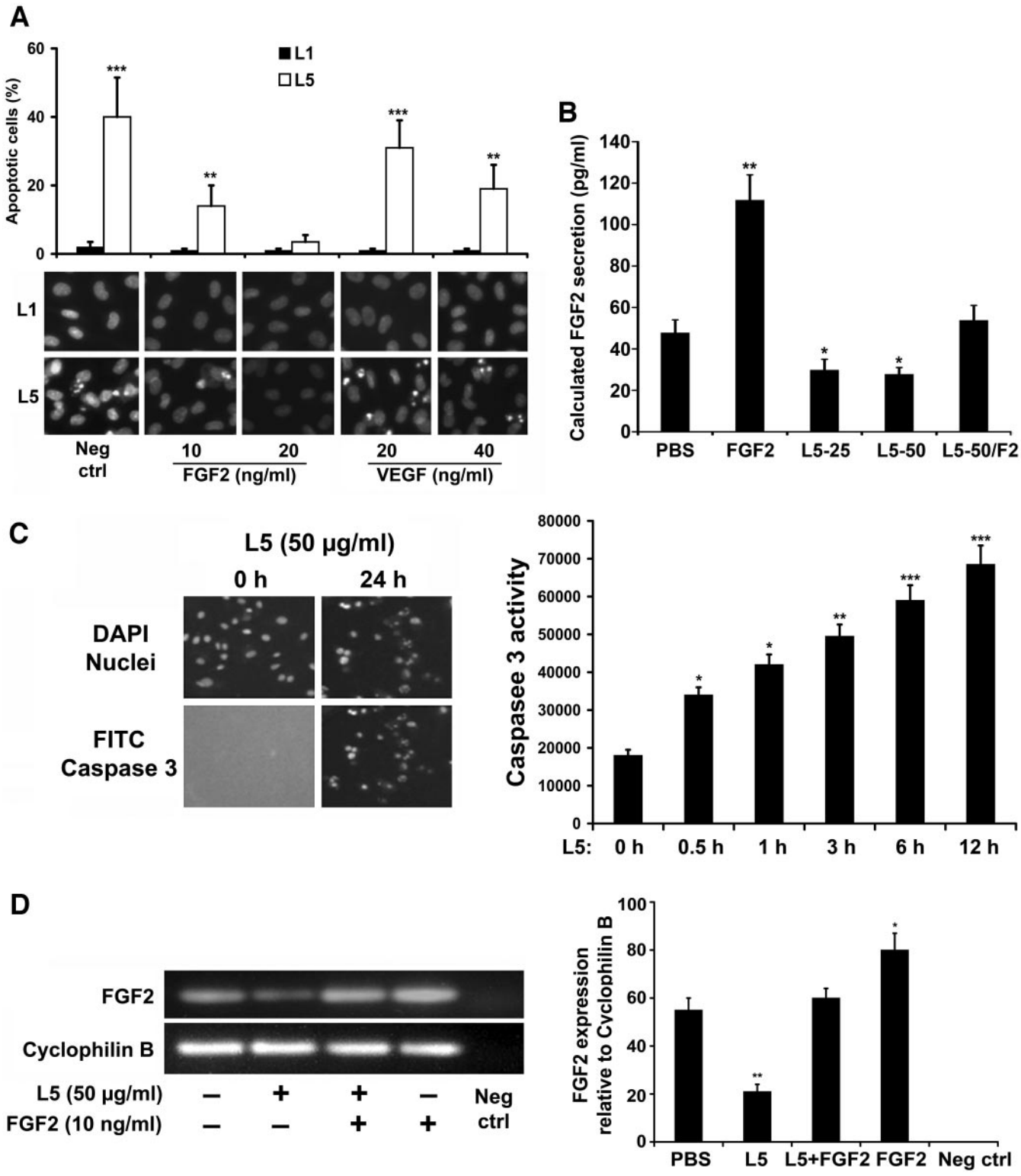

FIG. 2. Differential effects of FGF2 and VEGF. ***A*: Apoptosis evidenced by condensed nuclear staining in BAECs treated for 24 h with diabetic L1 and L5 alone, or together with FGF2 or VEGF, was examined by epifluorescence microscopy. **$P < 0.01$, ***$P < 0.001$ vs. LDL alone ($n = 6$). *B*: Enzyme-linked immunosorbent assay–determined FGF2 concentrations in the culture media of BEACs treated with PBS, FGF2, L5, or a combination of L5 and FGF2. *$P < 0.05$, **$P < 0.01$ vs. PBS ($n = 6$). *C*: Detection of caspase-3 at 24 h (*left*) and time course of caspase-3 activity (*right*) challenged by 50 µg/ml L5. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs. 0 h ($n = 4$). *D*: RT-PCR analysis of FGF2 mRNA from BAECs treated with 50 µg/ml L5 or 10 ng/ml FGF2. *$P < 0.05$, **$P < 0.01$ vs. PBS ($n = 3$).**

onto nitrocellulose, prehybridized in hybridization buffer, and hybridized at 65°C for 24 h with ^{32}P-labeled nascent RNA transcripts. The membranes were washed twice with 2 × sodium chloride–sodium citrate at 65°C for 20 min and exposed to X-ray film for 24 h.

Statistical analysis. The significance of the differences between group means was assessed by a modified *t* test (Bonferroni test) for multiple comparisons. A value of $P < 0.05$ was considered significant. Results are expressed as means ± SEM.

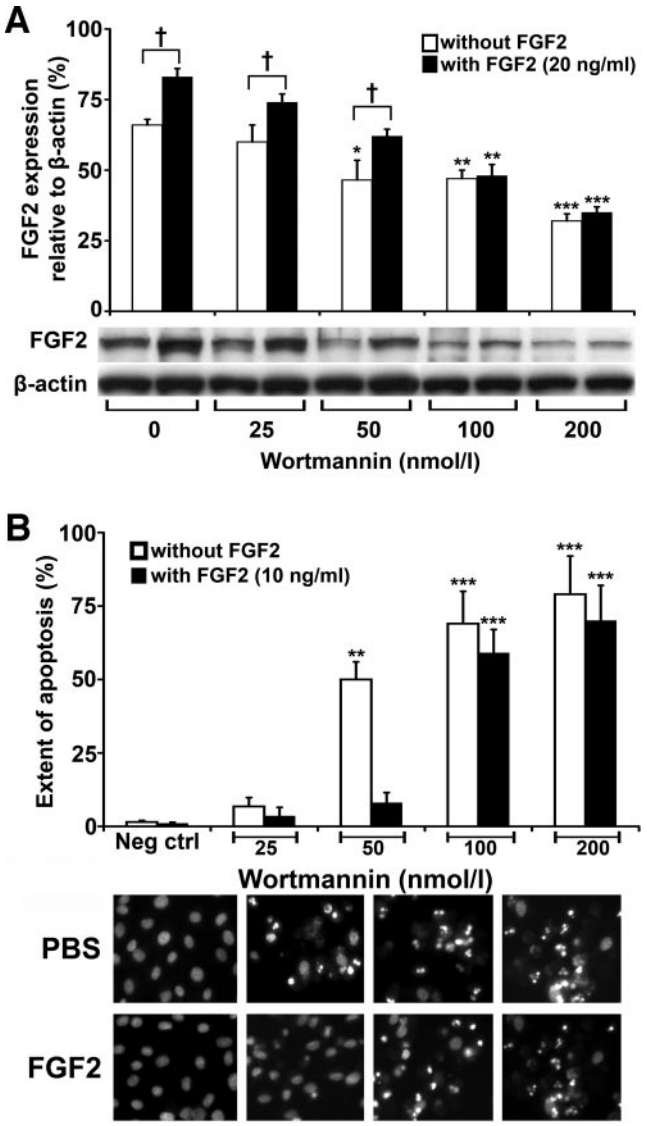

FIG. 3. Effects of wortmannin on FGF2 expression and apoptosis. *A*: Representative Western blot analysis of FGF2 protein levels in BAECs treated with FGF2 or wortmannin. †$P < 0.05$ between each other ($n = 3$), **$P < 0.01$, ***$P < 0.001$ vs. no wortmannin/no FGF2 ($n = 3$). *B*: Apoptosis in BAECs treated with PBS, wortmannin, or FGF2 was examined by epifluorescence microscopy. **$P < 0.01$, ***$P < 0.001$ vs. no treatment ($n = 3$).

RESULTS

Appearance of L5 in plasma. L5 was present in each of the six diabetic patients but not in any of the nondiabetic, normolipidemic subjects, although the plasma LDL cholesterol concentrations in the six diabetic patients were as low as those in the control group. An example of the elution profile of diabetic L5 resolved by anion-exchange chromatography is shown (Fig. 1).

Differential protection of FGF2 and VEGF against L5-induced apoptosis. L5 was potent in inducing EC apoptosis, while L1 has no effect. Both PI3K stimulators, FGF2 and VEGF, were tested to ascertain whether they are equally protective against L5 (50 μg/ml). Unlike FGF2, which completely prevented apoptosis at a concentration of 20 ng/ml, VEGF up to 40 ng/ml only resulted in a mild attenuation (Fig. 2*A*). After subtracting the added amount, exogenous FGF2 induced FGF2 secretion threefold in the absence of L5. It also preserved FGF2 secretion at the baseline level, which would otherwise have been reduced in the presence of L5 (Fig. 2*B*). Enhanced caspase-3 detectability and activities in L5-treated BAECs confirmed the apoptotic nature of L5 (Fig. 2*C*).

The sharp difference between the effects of FGF2 and VEGF prompted us to focus on the role of endogenous FGF2 in EC protection. Further investigations showed that L5 downregulated FGF2 at the transcriptional level, as determined by RT-PCR. This was, again, reversed by the addition of 10 ng/ml FGF2 (Fig. 2*D*).

Effects of wortmannin on FGF2 expression and apoptosis. Wortmannin, a PI3K inhibitor, decreased FGF2 protein levels in a concentration-dependent (25–200 nmol/l) manner. At 200 nmol/l, it reduced FGF2 protein by more than one-half in comparison with that reduced by β-actin (Fig. 3*A*). Supplementation with 20 ng/ml FGF2 increased FGF2 expression in cells treated with 0–50 nmol/l wortmannin. It was able to preserve FGF2 expression to the baseline level, where no exogenous FGF2 or wortmannin was given, in the presence of 50 nmol/l wortmannin. However, FGF2 failed to effectively counteract 100–200 nmol/l wortmannin. Because wortmannin blocks and FGF2 stimulates Akt phosphorylation, the competition between them suggests that FGF2 expression depends on Akt activity and that when PI3K is adequately suppressed, FGF2 is unable to stimulate its own expression.

Since FGF2 protects EC from spontaneous apoptosis, we examined whether wortmannin alone can induce EC apoptosis. Incubation of EC with wortmannin resulted in spontaneous apoptosis within 24 h in a concentration-dependent manner (Fig. 3*B*). At lower wortmannin concentrations (25 and 50 nmol/l), wortmannin-induced apoptosis could be completely prevented by 10 ng/ml FGF2. At 100 nmol/l, wortmannin induced apoptosis in 75% of cells. FGF2 expression began to succumb to 100 nmol/l wortmannin and failed to attenuate wortmannin-associated apoptosis. At 200 nmol/l of wortmannin, nearly all cells underwent apoptosis, and FGF2 was unable to overcome the effect of wortmannin.

Effects of constitutively active and dominant-negative Akt on Akt phosphorylation, FGF2 expression, and apoptosis in BAECs. To confirm the findings made with wortmannin, we transiently transfected BAECs with an Akt-CA or Akt-DN expression vector to establish BAEC(Akt-CA) and BAEC(Akt-DN) cells. Compared with empty vector transfected BAEC(ev), the baseline phosphorylated Akt levels were unchanged in BAEC(Akt-CA) and significantly decreased in BAEC(Akt-DN) (Fig. 4*A*). L5 suppressed Akt phosphorylation in BAEC(ev) but had no significant effect on BAEC(Akt-DN) or BAEC(Akt-CA) transfectants. When 10 ng/ml FGF2 was added simultaneously with 50 μg/ml L5, it completely prevented L5-mediated phosphorylated Akt downregulation in BAECs. In BAEC(Akt-DN) and BAEC(Akt-CA), the phosphorylated Akt levels remained unchanged with or without FGF2 supplementation.

Expression of Akt-CA or Akt-DN markedly altered FGF2 expression in opposite directions. To determine whether elevated FGF2 mRNA levels in BAEC(Akt-CA) may be negatively regulated by L5, cells were treated with L5 for 24 h and FGF2 mRNA levels determined by Northern blotting. As shown in Fig. 4*B*, L5 treatment significantly decreased FGF2 mRNA in BAEC(ev), while it had no effect on FGF2 mRNA levels in BAEC(Akt-CA) or BAEC(Akt-DN) transfectants.

The above findings indicate that endogenous FGF2 is

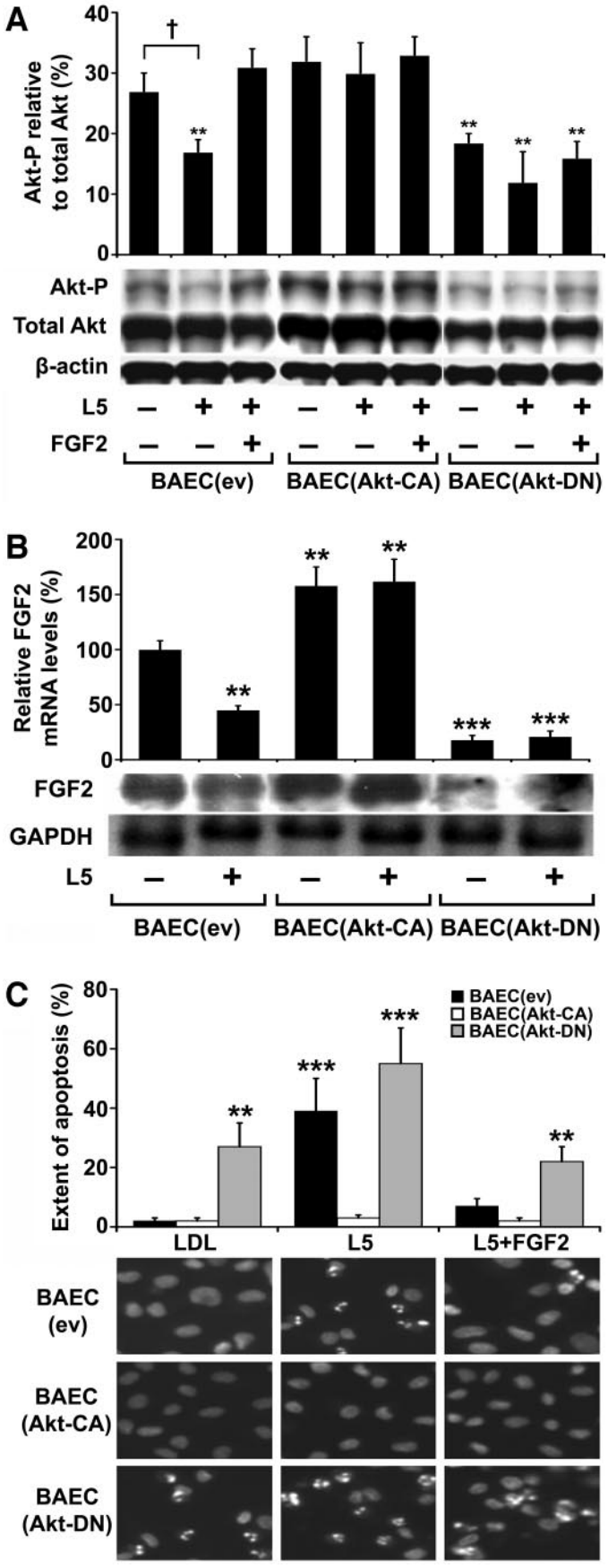

FIG. 4. Effects of Akt-CA and Akt-DN on Akt phosphorylation, FGF2 expression, and apoptosis in BAECs exposed to L5. *A*: β-Actin and phosphorylated Akt levels in the cell extracts were determined by Western blots in transfected BAECs at 24 h after treatment with PBS, L5, or a combination of L5 and FGF2. †$P < 0.05$ between each other ($n = 3$). *B*: FGF2 mRNA levels were examined by Northern blot analysis in transfected BAECs 24 h after PBS or L5 treatment. *C*: Extent of apoptosis in transfected BAECs treated with L5 or L5 in combination with FGF2 for 24 h. **$P < 0.01$, ***$P < 0.001$ vs. BAECs treated with normal LDL ($n = 3$) in all panels.

essential to both FGF2 transcription and Akt phosphorylation. The significance of this positive feedback mechanism was further assessed in BAEC(Akt-DN) and BAEC(Akt-CA) exposed to 50 μg/ml L5. In BAEC(Akt-DN), spontaneous apoptosis was intensified and could not be rescued by FGF2 (Fig. 4*C*). In contrast, L5 was unable to induce apoptosis in BAEC(Akt-CA), which is strengthened by its artificially reinforced Akt-FGF2 survival loop.

OxLDL mimics the inhibitory effects of L5 on EC survival. Because oxLDL also induces EC apoptosis, we examined whether oxLDL mimics L5 in inhibiting Akt phosphorylation. At 24 h, oxLDL markedly decreased Akt phosphorylation. In contrast, FGF2 increased the phosphorylated Akt level by almost twofold and completely prevented oxLDL-mediated reduction in phosphorylated Akt levels (Fig. 5*A*). Compared with PBS and LDL, oxLDL markedly reduced FGF2 mRNA levels, but the inhibition was prevented by 10 ng/ml FGF2 (Fig. 5*C*). OxLDL also induced apoptosis in ~50% of treated BAECs (Fig. 5*D*). Simultaneous addition of FGF2 attenuated oxLDL-induced apoptosis in a concentration-dependent manner. At 10 ng/ml FGF2, very few oxLDL-treated cells were apoptotic. In contrast, 50 ng/ml VEGF failed to attenuate oxLDL-induced apoptosis. These results illustrate that oxLDL, though known to be different from L5, possesses similar cytotoxic properties by disturbing the Akt-FGF2 pathway.

Importance of endogenous FGF2 on transcription feedback. Because other growth factors can also activate PI3K, we examined whether endogenous FGF2 by itself is adequate to regulate its own expression. Stably transfected BAEC lines that over- or underexpress FGF2 were established and designated as BAEC[FGF2(+)] and BAEC[FGF2(−)], respectively. As expected, immunocytochemical staining of BAEC[FGF2(+)] revealed significantly increased levels of intracellular FGF2 compared with parental BAECs (Fig. 6*A*). In contrast, almost no FGF2 staining was detected in BAEC[FGF2(−)], demonstrating the effectiveness of the antisense FGF2 RNA in inhibiting FGF2 protein expression. Western blot analyses also showed that intracellular FGF2 protein was greatly increased in BAEC[FGF2(+)] and markedly reduced in BAEC[FGF2(−)] compared with BAECs or BAECs transfected with empty vector (Fig. 6*B*). Nuclear run-on assays confirmed that rates of FGF2 gene transcription were greatly increased in BAEC[FGF2(+)] compared with BAECs and BAECs transfected with the empty vector. The antisense FGF2 RNA in BAEC[FGF2(−)] was expected to reduce FGF2 protein levels only by inhibiting FGF2 translation; therefore, it is important to note that FGF2 transcription was also severely impaired in these cells. The results indicate that FGF2 transcription is dependent on sufficient supply of endogenously produced FGF2.

FGF2 modulation affects Akt phosphorylation and sensitivity to apoptosis. We also examined whether reducing endogenous FGF2 alone can impair Akt phosphorylation. At 24 h, unfractionated, normolipidemic LDL (50 mg/ml) slightly elevated the phosphorylated Akt level compared with PBS treatment (Fig. 7*A*). In contrast, L5 caused a significant suppression in Akt phosphorylation.

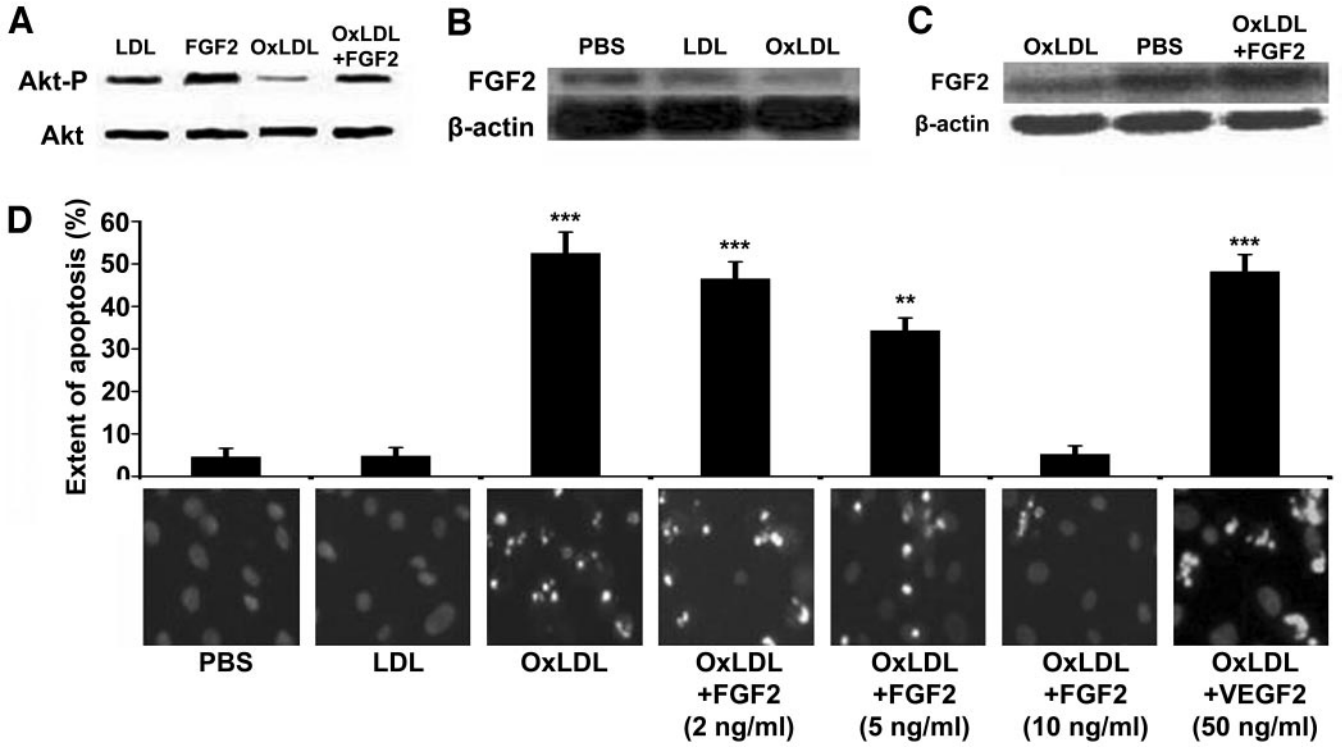

FIG. 5. Differential effects of FGF2 and oxLDL on Akt phosphorylation, FGF2 expression, and apoptosis in BAECs. *A*: Western blot of phosphorylated Akt levels in BAECs treated with normolipidemic LDL, FGF2, oxLDL, or a combination of oxLDL and FGF2. *B*: Northern blot of FGF2 mRNA levels in BAECs treated for 24 h with PBS, LDL, or oxLDL. *C*: Northern blot of FGF2 mRNA levels in BAECs treated with oxLDL, PBS, or a combination of oxLDL and FGF2. *D*: Apoptosis visualized by epifluorescence microscopy was evaluated at 24 h in BAECs treated with PBS, LDL, oxLDL, oxLDL and FGF2, or oxLDL and VEGF. *$P < 0.05$, ***$P < 0.001$ vs. PBS ($n = 3$).

Of importance, increased FGF2 transcription in BAEC[FGF2(+)] was accompanied by a twofold increase in phosphorylated Akt level. In sharp contrast, Akt phosphorylation was severely suppressed in BAEC[FGF2(−)], a change similar to that seen in L5-treated BAECs. Most importantly, FGF2 augmentation in BAEC[FGF2(+)] protected Akt phosphorylation in the presence of L5. Knowing that exogenous FGF2 preserves BAEC survival, we characterized how the endogenous FGF2 transfectants performed in our apoptosis assay, especially when challenged with L5. BAECs transfected with empty vector, [FGF2(−)], or [FGF2(+)] were exposed to 50 μg/ml LDL or L5 for 24 h. As expected, BAEC(ev) control subjects were healthy after LDL administration but highly apoptotic in the L5 group. BAEC[FGF2(−)] transfectants struggled to survive whether they exposed to LDL or L5, while BAEC[FGF2(+)] transfectants were resistant to L5-induced apoptosis (Fig. 7*B*).

DISCUSSION

Type 2 diabetes is equivalent to cardiovascular disease for increasing cardiovascular risk even in the absence of elevated LDL cholesterol (2). However, LDL cholesterol remains the primary target for cardiovascular risk reduction in all patients (1). This implies that diabetic LDL, irrespective of plasma LDL cholesterol concentrations, may exhibit certain properties similar to those of nondiabetic, hypercholesterolemic LDL. Here, we demonstrate that the L5 circulating in diabetic patients without elevated LDL cholesterol is similar to hypercholesterolemic L5, as previously described by us (3). In essence, diabetic L5 induces EC apoptosis by disrupting a novel FGF2 autoregulatory mechanism.

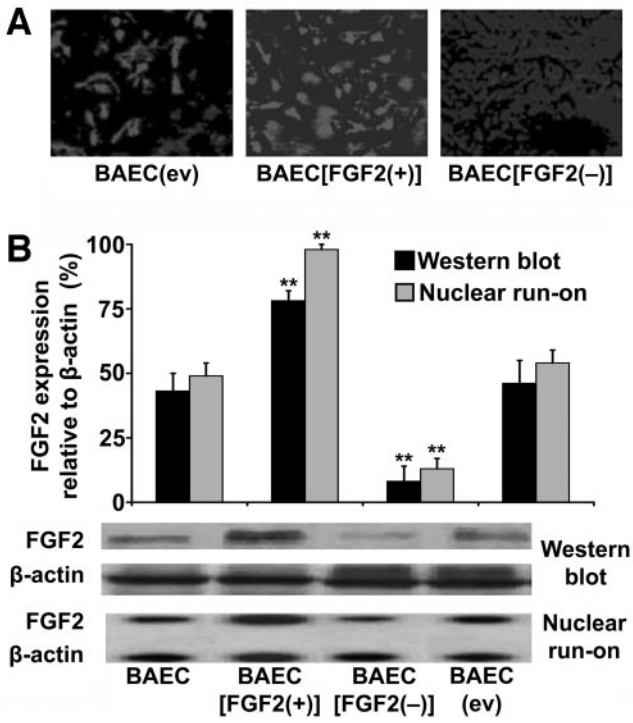

FIG. 6. Effects of sense and antisense treatments on FGF2 transcription and translation and Akt phosphorylation in BAECs. *A*: Intracellular FGF2 protein was detected by immunostaining in FGF2-transfected BAECs. *B*: FGF2 protein levels were assayed by Western blot analysis, and FGF2 transcription was evaluated by nuclear run-on analysis in transfected BAECs. **$P < 0.01$ vs. BAEC Western blot or nuclear run-on ($n = 3$).

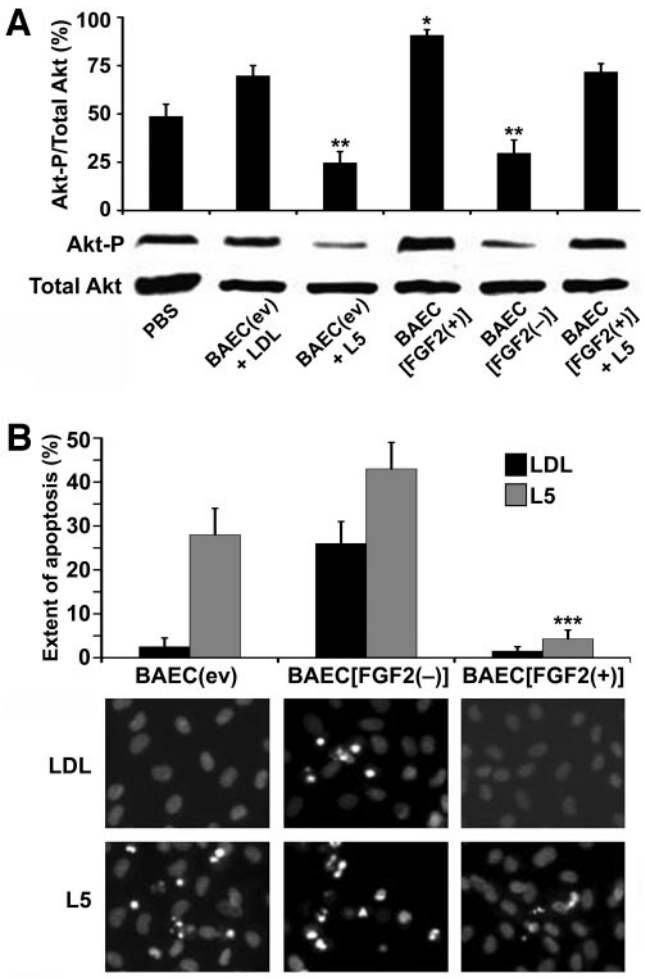

FIG. 7. FGF2 transcriptional modulation of Akt phosphorylation and sensitivity to apoptosis. *A*: Phosphorylated Akt levels were assessed by Western blot analysis in BAECs treated with PBS, normolipidemic LDL, or L5 and in transfected BAECs treated with L5. *$P < 0.05$, **$P < 0.01$ vs. BAEC plus PBS ($n = 3$). *B*: Extent of apoptosis in transfected BAECs treated with LDL or L5 for 24 h. ***$P < 0.001$ vs. vector control BAECs treated with L5 ($n = 3$).

Exogenous FGF2 stimulates proliferation and prevents apoptosis induced by various factors in ECs and many cell types (17,29). Because some other growth factors exhibit similar effects (30), it was not appreciated how critical endogenous FGF2 is in securing EC survival. Our findings indicate that L5 inhibits FGF2 transcription with resultant caspase-3 activation and EC apoptosis, which can be prevented by restoring the FGF2-Akt pathway.

Exogenous FGF2 is known to induce expression of many genes (17), but how FGF2 expression is regulated has not been fully described. FGF2 exerts most of its effects by activating the PI3K-Akt pathway (31). Our findings indicate that FGF2 is autoregulated in an autocrine manner through a positive feedback mechanism that involves Akt activation. L5 disrupts this FGF2 autoregulatory mechanism, resulting in EC apoptosis. In early atherosclerosis, minimal apoptosis of vascular EC occurs at sites with low shear stress (32,33). In advanced atherosclerosis, apoptotic ECs contribute to thrombosis by activating tissue factor (34). Thus, protection of this FGF2-PI3K-Akt autoregulatory loop is important for the prevention or deceleration of atherothrombosis.

Electronegative LDL, including L5 and LDL(−), is mildly oxidized in vivo. Their presence strongly supports the role of LDL modification in atherosclerosis (35). Recently, electronegative LDL has also been located in atherosclerotic lesions (36). Under in vitro settings, minimally modified LDL may closely resemble L5 (37). The effects of L5 could be reproduced by oxLDL. Thus, it is not the degree of oxidation but the altered properties of these lipoproteins that are responsible for the adverse effects of modified LDL.

It should be noted that other growth factors, including VEGF, platelet-derived growth factor, epidermal growth factor, and nerve growth factor, can also activate PI3K (25,29,38). Thus, L5 may also act by downregulating these other growth factors. However, our data show that correcting the deficit in FGF2 alone provided complete protection against L5. Failure of VEGF to prevent L5-induced apoptosis suggests that VEGF is unable to substitute for FGF2 in regulating all FGF2-dependent effects. In contrast, FGF2 enhancement may indirectly activate VEGF-regulated bioactivities by stimulating VEGF expression (39).

Treatment of BAECs with wortmannin decreased FGF2 expression, leading to spontaneous apoptosis in the absence of L5. When the effect of wortmannin is overcome by FGF2, FGF2 expression is restored, strongly suggesting that FGF2 expression is an Akt-dependent process. Phosphorylated Akt levels were markedly increased in BAEC(Akt-CA) and severely decreased in BAEC(Akt-DN). The changes in phosphorylated Akt level led to changes in FGF2 expression in the same direction. Thus, although wortmannin may also inhibit other kinases (40), the role of Akt activation in FGF2 expression is confirmed. In BAEC(Akt-DN), apoptosis was not prevented by FGF2 because there was almost no Akt available to convey the FGF2 signaling. In contrast, the Akt-FGF2 signaling was so strong in BAEC(Akt-CA) that L5 was unable to induce apoptosis.

While Akt can also be activated by other growth factors, our findings in the BAEC[FGF2(+)] and BAEC[FGF2(−)] settings proved that endogenous FGF2 plays an essential role in this process. Baseline expression of FGF2 was high in BAEC[FGF2(+)] and almost nonexistent in BAEC[FGF2(−)]. Antisense RNA should inhibit translation but not transcription of the targeted gene. It is therefore important to emphasize that FGF2 transcription was indeed markedly suppressed in BAEC[FGF2(−)]. A major

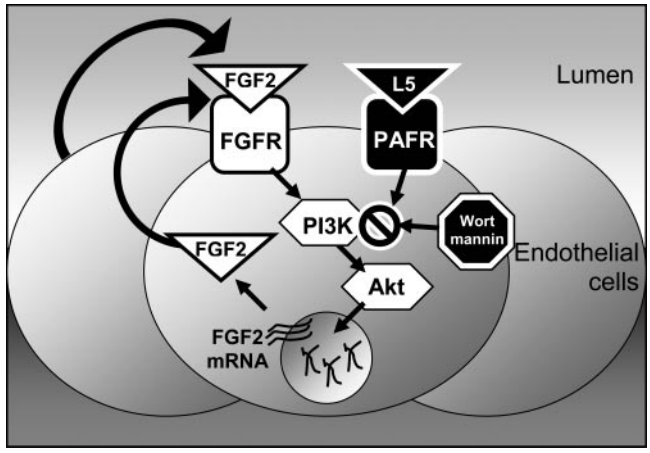

FIG. 8. A schematic diagram of the FGF2-PI3K-Akt autoregulatory loop in vascular ECs. Arrows indicate stimulation or direction of signaling pathway or changes. FGFR, FGF receptor; PAFR, PAF receptor.

function of FGF2 as an autocrine is to induce its own expression (17). Under our mitogen-free culture conditions, FGF2 appears to be the primary stimulator for its own transcription. In BAEC[FGF2(−)], reduction in intracellular FGF2 protein leads to eventual reduction in FGF2 transcription. Downregulation of FGF2 was always accompanied by a reduction in Akt phosphorylation. Likewise, augmented FGF2 expression was always accompanied by enhanced Akt phosphorylation. Thus, by manipulating Akt and FGF2 expression, we have proven that Akt phosphorylation depends on a continuous supply of endogenous FGF2 and that FGF2 transcription is dependent on Akt phosphorylation.

We previously reported that the apoptotic signals of L5 are transduced by the PAF receptor and that hydrolysis of PAF-like lipids contained in L5 abolishes the apoptotic activity (3). Combining our previous and current findings, we postulate that FGF2 transcription is regulated by a novel FGF2-PI3K-Akt autoregulatory loop and that L5 impairs EC survival by disrupting this signaling loop (Fig. 8). Our findings suggest that L5 may play a role in diabetes-associated atherosclerosis. By disrupting the FGF2-PI3K-Akt loop, L5 can potentially impair the FGF2-dependent reendothelialization and collateral formation essential for tissue perfusion (19,20). A prospective study is warranted to determine the clinical significance of L5 or electronegative LDL in patients with diabetes.

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REFERENCES

1. Grundy SM, Cleeman JI, Merz CN, Brewer HB Jr, Clark LT, Hunninghake DB, Pasternak RC, Smith SC Jr, Stone NJ: Implications of recent clinical trials for the National Cholesterol Education Program Adult Treatment Panel III guidelines. *Arterioscler Thromb Vasc Biol* 24:e149–e161, 2004
2. Nesto RW: Beyond low-density lipoprotein: addressing the atherogenic lipid triad in type 2 diabetes mellitus and the metabolic syndrome. *Am J Cardiovasc Drugs* 5:379–387, 2005
3. Chen CH, Jiang T, Yang JH, Jiang W, Lu J, Marathe GK, Pownall HJ, Ballantyne CM, McIntyre TM, Henry PD, Yang CY: Low-density lipoprotein in HC human plasma induces vascular endothelial cell apoptosis by inhibiting fibroblast growth factor 2 transcription. *Circulation* 107:2102–2108, 2003
4. Yang CY, Raya JL, Chen HH, Chen CH, Abe Y, Pownall HJ, Taylor AA, Smith CV: Isolation, characterization, and functional assessment of oxidatively modified subfractions of circulating low-density lipoproteins. *Arterioscler Thromb Vasc Biol* 23:1083–1090, 2003
5. Tai MH, Kuo SM, Liang HT, Chiou KR, Lam HC, Hsu CM, Pownall HJ, Chen HH, Huang MT, Yang CY: Modulation of angiogenic processes in cultured endothelial cells by low density lipoproteins subfractions from patients with familial hypercholesterolemia. *Atherosclerosis* 186:448–457, 2006
6. Avogaro P, Bon GB, Cazzolato G: Presence of a modified low density lipoprotein in humans. *Arteriosclerosis* 8:79–87, 1988
7. Demuth K, Myara I, Chappey B, Vedie B, Pech-Amsellem MA, Haberland ME, Moatti N: A cytotoxic electronegative LDL subfraction is present in human plasma. *Arterioscler Thromb Vasc Biol* 16:773–783, 1996
8. Ziouzenkova O, Asatryan L, Sahady D, Orasanu G, Perrey S, Cutak B, Hassell T, Akiyama TE, Berger JP, Sevanian A, Plutzky J: Dual roles for lipolysis and oxidation in peroxisome proliferation-activator receptor responses to electronegative low density lipoprotein. *J Biol Chem* 278:39874–39881, 2003
9. Benitez S, Sanchez-Quesada JL, Ribas V, Jorba O, Blanco-Vaca F, Gonzalez-Sastre F, Ordonez-Llanos J: Platelet-activating factor acetylhydrolase is mainly associated with electronegative low-density lipoprotein subfraction. *Circulation* 108:92–96, 2003
10. Sanchez-Quesada JL, Benitez S, Ordonez-Llanos J: Electronegative low-density lipoprotein. *Curr Opin Lipidol* 15:329–335, 2004
11. Asatryan L, Hamilton RT, Isas JM, Hwang J, Kayed R, Sevanian A: LDL phospholipid hydrolysis produces modified electronegative particles with an unfolded apoB-100 protein. *J Lipid Res* 46:115–122, 2005
12. Guevara NV, Chen KH, Chan L: Apoptosis in atherosclerosis: pathological and pharmacological implications. *Pharmacol Res* 44:59–71, 2001
13. Choy JC, Granville DJ, Hunt DW, McManus BM: Endothelial cell apoptosis: biochemical characteristics and potential implications for atherosclerosis. *J Mol Cell Cardiol* 33:1673–1690, 2001
14. Rigla M, Sanchez-Quesada JL, Ordonez-Llanos J, Prat T, Caixas A, Jorba O, Serra JR, de Leiva A, Perez A: Effect of physical exercise on lipoprotein(a) and low-density lipoprotein modifications in type 1 and type 2 diabetic patients. *Metabolism* 49:640–647, 2000
15. Yang CH, Chen HH, Huang MT, Raya JL, Yang JH, Chen CH, Gaubatz JW, Pownall HJ, Taylor AA, Ballantyne CM, Jenniskens FA, Smith CV: Pro-apoptotic low-density lipoprotein subfractions in type II diabetes atherosclerosis. *Atherosclerosis* 193:283–291, 2007
16. Klagsbrun M, Edelman ER: Biological and biochemical properties of fibroblast growth factors: implications for the pathogenesis of atherosclerosis. *Arteriosclerosis* 9:269–278, 1989
17. Chen CH, Poucher SM, Lu J, Henry PD: Fibroblast growth factor 2: from laboratory evidence to clinical application. *Curr Vasc Pharmacol* 2:33–43, 2004
18. Folkman J, Klagsbrun M, Sasse J, Wadzinski M, Ingber D, Vlodavsky I: A heparin-binding angiogenic protein–basic fibroblast growth factor–is stored within basement membrane. *Am J Pathol* 130:393–400, 1988
19. Heil M, Schaper W: Influence of mechanical, cellular, and molecular factors on collateral artery growth (arteriogenesis). *Circ Res* 95:449–458, 2004
20. Chen CH, Walterscheid JP: Plaque angiogenesis versus compensatory arteriogenesis in atherosclerosis. *Circ Res* 99:787–789, 2006
21. Bochkov VN, Philippova M, Oskolkova O, Kadl A, Furnkranz A, Karabeg A, Afonyushkin T, Gruber F, Breuss J, Minchenko A, Mechtcheriakova D, Hohensinner P, Rychli K, Wojta J, Resink T, Erne P, Binder BR, Leitinger N: Oxidized phospholipids stimulate angiogenesis via autocrine mechanisms implicating a novel role for lipid oxidation in the evolution of atherosclerotic lesions. *Circ Res* 99:900–908, 2006
22. Kanda S, Hodgkin MN, Woodfield RJ, Wakelam MJ, Thomas G, Claesson-Welsh L: Phosphatidylinositol 3′-kinase-independent p70 S6 kinase activation by fibroblast growth factor receptor-1 is important for proliferation but not differentiation of endothelial cells. *J Biol Chem* 272:23347–23353, 1997
23. Gratton JP, Morales-Ruiz M, Kureishi Y, Fulton D, Walsh K, Sessa WC: Akt down-regulation of p38 signaling provides a novel mechanism of vascular endothelial growth factor-mediated cytoprotection in endothelial cells. *J Biol Chem* 27:30359–30365, 2001
24. Rommel C, Clarke BA, Zimmermann S, Nunez L, Rossman R, Reid K, Moelling K, Yancopoulos GD, Glass DJ: Differentiation stage-specific inhibition of the Raf-MEK-ERK pathway by Akt. *Science* 286:1738–1741, 1999
25. Chavakis E, Dernbach E, Hermann C, Mondorf UF, Zeiher AM, Dimmeler S: Oxidized LDL inhibits vascular endothelial growth factor-induced endothelial cell migration by an inhibitory effect on the Akt/endothelial nitric oxide synthase pathway. *Circulation* 103:2102–2107, 2001
26. Abid MR, Guo S, Minami T, Spokes KC, Ueki K, Skurk C, Walsh K, Aird WC: Vascular endothelial growth factor activates PI3K/Akt/forkhead signaling in endothelial cells. *Arterioscler Thromb Vasc Biol* 24:294–300, 2004
27. Trivier E, Kurz DJ, Hong Y, Huang HL, Erusalimsky JD: Differential regulation of telomerase in endothelial cells by fibroblast growth factor-2 and vascular endothelial growth factor-a: association with replicative life span. *Ann N Y Acad Sci* 1019:111–115, 2004
28. Chang PY, Luo S, Jiang T, Lee YT, Lu SC, Henry PD, Chen CH: Oxidized low-density lipoprotein downregulates endothelial basic fibroblast growth factor through a pertussis toxin-sensitive G-protein pathway: mediator role of platelet-activating factor-like phospholipids. *Circulation* 104:588–593, 2001
29. Karsan A, Yee E, Poirier GG, Zhou P, Craig R, Harlan JM: Fibroblast growth

factor-2 inhibits endothelial cell apoptosis by Bcl-2-dependent and independent mechanisms. *Am J Pathol* 151:1775–1784, 1997
30. Kumar P, Miller AI, Polverini PJ: p38 MAPK mediates gamma-irradiation-induced endothelial cell apoptosis, and vascular endothelial growth factor protects endothelial cells through the phosphoinositide 3-kinase-Akt-Bcl-2 pathway. *J Biol Chem* 279:43352–43360, 2004
31. Raffioni S, Bradshaw RA: Activation of phosphatidylinositol 3-kinase by epidermal growth factor, basic fibroblast growth factor, and nerve growth factor in PC12 pheochromocytoma cells. *Proc Natl Acad Sci U S A* 89:9121–9125, 1992
32. Dimmeler S, Haendeler J, Galle J, Zeiher AM: Oxidized low-density lipoprotein induces apoptosis of human endothelial cells by activation of CPP32-like proteases: a mechanistic clue to the 'response to injury' hypothesis. *Circulation* 95:1760–1763, 1997
33. Tricot O, Mallat Z, Heymes C, Belmin J, Leseche G, Tedgui A: Relation between endothelial cell apoptosis and blood flow direction in human atherosclerotic plaques. *Circulation* 101:2450–2453, 2000
34. Mallat Z, Benamer H, Hugel B, Benessiano J, Steg PG, Freyssinet JM, Tedgui A: Elevated levels of shed membrane microparticles with procoagulant potential in the peripheral circulating blood of patients with acute coronary syndromes. *Circulation* 101:841–843, 2000
35. Steinberg D, Witztum JL: Is the oxidative modification hypothesis relevant to human atherosclerosis? Do the antioxidant trials conducted to date refute the hypothesis? *Circulation* 105:2107–2111, 2002
36. Damasceno NR, Sevanian A, Apolinario E, Oliveira JM, Fernandes I, Abdalla DS: Detection of electronegative low density lipoprotein (LDL(−)) in plasma and atherosclerotic lesions by monoclonal antibody-based immunoassays. *Clin Biochem* 39:28–38, 2006
37. Subbanagounder G, Leitinger N, Schwenke DC, Wong JW, Lee H, Rizza C, Watson AD, Faull KF, Fogelman AM, Berliner JA: Determinants of bioactivity of oxidized phospholipids: specific oxidized fatty acyl groups at the sn-2 position. *Arterioscler Thromb Vasc Biol* 20:2248–2254, 2000
38. Chaudhary LR, Hruska KA: The cell survival signal Akt is differentially activated by PDGF-BB, EGF, and FGF-2 in osteoblastic cells. *J Cell Biochem* 81:304–311, 2001
39. Seghezzi G, Patel S, Ren CJ, Gualandris A, Pintucci G, Robbins ES, Shapiro RL, Galloway AC, Rifkin DB, Mignatti P: Fibroblast growth factor-2 (FGF-2) induces vascular endothelial growth factor (VEGF) expression in the endothelial cells of forming capillaries: an autocrine mechanism contributing to angiogenesis. *J Cell Biol* 141:1659–1673, 1998
40. Ferby IM, Waga I, Hoshino M, Kume K, Shimizu T: Wortmannin inhibits mitogen-activated protein kinase activation by platelet-activating factor through a mechanism independent of p85/p110-type phosphatidylinositol 3-kinase. *J Biol Chem* 271:11684–11688, 1996