C5b-9-induced Endothelial Cell Proliferation and Migration Are Dependent on Akt Inactivation of Forkhead Transcription Factor FOXO1*

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Migration and proliferation of aortic endothelial cells (AEC) are critical processes involved in angiogenesis, atherosclerosis, and postangioplasty restenosis. Activation of complement and assembly of the C5b-9 complement complex have been implicated in the pre-lesional stage of atherosclerosis and progression of the atherosclerotic lesion. We have shown that C5b-9 induces proliferation and activates phosphatidylinositol 3-kinase (PI3K), but it is unknown whether this can lead to activation of Akt in AEC, a major downstream target of PI3K, or if C5b-9 can induce the migration of AEC, a critical step in angiogenesis. In this study, we show that C5b-9 induces AEC proliferation and migration and also activates the PI3K/Akt pathway. C5b-9 activates Akt as shown by in vitro kinase assay and phosphorylation of Ser-473. C5b-9-induced cell cycle activation was inhibited by pretreatment with LY294002 (PI3K inhibitor), SH-5 (Akt inhibitor), or transfection with Akt siRNA. These data suggest that the PI3K/Akt pathway is required for C5b-9-induced cell cycle activation. FOXO1, a member of forkhead transcription factor family, was phosphorylated at Ser-256 and inactivated after C5b-9 stimulation as shown by a decrease in DNA binding and cytoplasmic relocalization. Cytoplasmic relocalization was significantly reduced after pretreatment with LY294002, SH-5, or transfection with Akt siRNA. Silencing FOXO1 expression using siRNA stimulated AEC proliferation and regulated angiogenic factor release. Our data indicate that C5b-9 regulation of the cell cycle activation in AEC through Akt pathway is dependent on inactivation of FOXO1.

Endothelial cell activation and proliferation play a prominent role in angiogenesis, atherosclerosis, postangioplasty restenosis, and other inflammatory vascular diseases (1–3). In atherosclerosis, activation of endothelial cells triggers platelet aggregation, leukocyte adhesion and transmigration, and proliferation of vascular smooth muscle cells (1). Inflammatory processes activated by humoral and cellular immune effectors are thought to be involved in the pathogenesis of atherosclerosis (4). Activation of complement and generation of C5b-9 have been implicated in the prelesional stage of atherosclerosis and the progression of atherosclerotic lesions (5, 6). Complement is activated by cholesterol-containing lipids derived from atherosclerotic lesions and by enzymatically modified low density lipoproteins (7, 8). In addition, C5b-9 deposition has been found in human atherosclerotic lesions (9, 10).

Activation of the complement cascade leads to formation and deposition of the terminal complement complexes C5b-7, C5b-8, and C5b-9 on cellular membranes (11). The membrane attack complex, C5b-9, can cause cell death by lysis. However, when the number of C5b-9 molecules is limited on host cell membranes, it can activate signaling pathways leading to cell cycle activation and cell survival (12). The PI3K pathway was shown to be involved in C5b-9-induced cell survival and cell cycle activation (13–15).

One major downstream target of PI3K is the serine/threonine kinase Akt (16, 17). Akt itself phosphorylates a multitude of substrates involved in cell cycle activation and cell survival functions (18–21). One such target is the FOXO transcription factor family. Forkhead box (FOX) superfamily of transcription factors are classified by their conserved DNA binding region characterized by three α-helices, a wing-like structure, and a C-terminal basic region (22). Forkhead box subclass O (FOXO) factors are subfamily members of FOX and consist of the following: FOXO1 (FKHR), FOXO3a (FKHRL-1), and FOXO4 (AFX) (23–26). FOXO members bind to the consensus DNA sequence of TTGTTTAC (27). Through their function as transcription factors, FOXO proteins regulate the expression of cell cycle regulators such as the cyclin-dependent kinase inhibitor, p27 (28–30), and pro-apoptotic genes, such as Fas and Bim (28, 31). Akt inactivates FOXO proteins through phosphorylation at three distinctive sites, leading to their unbinding from DNA, nuclear exclusion (24), and subsequent degradation (32–34). In part, this leads to decreased

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3 The abbreviations used are: PI3K, phosphatidylinositol 3-kinase; FOXO, forkhead box subclass O; PIGF, placenta growth factor; AEC, aortic endothelial cell; TCC, terminal complement complex; BrdUrd, bromodeoxyuridine; HRP, horseradish peroxidase; VEGF, vascular endothelial growth factor; GRO, growth regulated oncogene; IL-6, interleukin-6; IL-8, interleukin-8; RANTES, regulated on activation normal T-cells expressed and secreted; siRNA, short interfering RNA; FACS, fluorescence-activated cell sorter; PBS, phosphate-buffered saline; FBS, fetal bovine serum; NHS, normal human sera; siCTR, control siRNA; EGM, endothelial cell growth media; EMSA, electrophoretic mobility shift assay; EC, endothelial cells.
C5b-9-induced Endothelial Cell Proliferation

expression of p27 (29, 35), allowing cyclin-dependent kinases to become activated and for cell cycle progression to occur.

In this study, we investigated the mechanisms of C5b-9-induced AEC proliferation. In addition, we show for the first time that C5b-9 induces cell migration through the release of soluble factors. For C5b-9-induced endothelial cell proliferation, we studied the requirement of the PI3K/Akt pathway and the role of FOXO1. We show that PI3K/Akt is required for endothelial cell cycle activation by C5b-9, and this leads to the inactivation of FOXO1. C5b-9 induces phosphorylation of FOXO1, unbinding from DNA, and cytoplasmic localization of FOXO1. All of these effects require Akt as shown by using SH-5, a pharmacological inhibitor of Akt, and Akt siRNA. In conclusion, we show that C5b-9 induces AEC proliferation and migration through PI3K/Akt-dependent phosphorylation and inactivation of FOXO transcription factors.

MATERIALS AND METHODS

Chemicals and Reagents—The following kinase inhibitors were used: LY294002 and wortmannin (Biomol, Plymouth Meeting, PA) and SH-5 (Axxora LLC, San Diego, CA). BCA were used: LY294002 and wortmannin (Biomol, Plymouth Chemicals Co, Tokyo, Japan) prevents C5b-9 assembly in serum by binding to C5 (36, 37). Alternatively, complement-deficient serum (1/10) and replacement of the missing component (10 μg/ml) were added after antibody sensitization. The sublytic serum complement was titrated previously by using limited doses of antibody and excess NHS and then measuring lactate dehydrogenase release as an indication of cell death (38). We also assembled C5b-9 by treating cells sequentially with C5b6, C7, and C8/C9, as described previously (36).

Cell Proliferation Assay—BrdUrd incorporation was performed using the BrdUrd flow kit (Pharmingen) as described previously (39). In brief, AEC were stimulated with C5b6, C5b-7, C5b-8, and C5b-9 assembled from purified components for 24 h and then labeled with 10 μM BrdUrd for 1 h. AEC were harvested by trypsinization and then permeabilized with Cyto-fix/Cytoperm buffer provided by the manufacturer. BrdUrd and DNA were stained with fluorescein isothiocyanate-conjugated anti-BrdUrd and 7-amino-actinomycin D, respectively, according to the manufacturer’s instructions. Two-color flow cytometric analysis enumerates and characterizes cells that are actively synthesizing DNA in S-phase. Flow cytometric analysis was performed using a BD Biosciences flow cytometer and Consort-40 software. Cell number was determined using a Cell Titer 96 nonradioactive cell proliferation assay kit (Promega, Madison, WI) as described previously (13).

Assay for DNA Synthesis—DNA synthesis was tested as described previously (13). Briefly, AEC treated with antibody and deficient serum were incubated with 1 μCi of [3H]thymidine in endothelial cell basal media without serum and growth supplements for 18 h at 37 °C. After washing in ice-cold PBS, cells were lysed in 0.3 M NaOH. Lysates were precipitated with 20% trichloroacetic acid and then filtered through a Whatman GF/A glass fiber filter (Whatman, Maidstone, UK). The radioactivity on the dried filter was counted by liquid scintillation. In some experiments AEC were pretreated 30 min with wortmannin (100 nM) or LY294002 (25–50 μM) to inhibit PI3K and SH-5 (10 μM) to inhibit Akt. The dose of each inhibitor was predetermined, and used in all experiments.

Cell Migration Assay—It has been shown previously that C5b-9 assembly induces the release of growth factors and cytokines (40). To determine whether these released growth factors or cytokines may induce AEC migration, conditioned media from AEC after C5b-9 assembly were used as a chemoattractant. Serum- and growth factor-starved AEC were stimulated with C5b-9 generated from purified components for 6 h. The conditioned media were then used as a chemoattractant. To measure cell migration, Innocyte 96-well cell migration assay (EMD Biosciences Inc., San Diego, CA) was used following the manufacturer’s instructions. Briefly, the conditioned media were added to the lower chamber, whereas trypsinized AEC were added to the upper chamber of the cell migration chamber. The cells were allowed to migrate through an 8-μm pore size membrane for 18 h. The cells were detached from the membrane and quantitated by labeling with calcein-AM.

Angiogenic Factor Protein Array—To identify which growth factors are released after C5b-9 stimulation, RayBio® human angiogenesis antibody array 1.1 (RayBiotech Inc. Norcross, GA) was used to detect the expression of the most important angiogenic factors (angiogenin, epidermal growth factor, ENA-78,
basic fibroblast growth factor, GRO, interferon-γ, insulin-like growth factor-1, IL-6, IL-8, leptin, MCP-1, platelet-derived growth factor-BB, PIGF, RANTES, transforming growth factor-β, TIMP-1, thrombopoietin, VEGF, and VEGF-D) following the manufacturer’s instructions. Briefly, AEC were cultured in serum- and growth factor-free media for 18 h. They were treated with C5b-9 or C5b6 assembled from purified components for 1 h. The conditioned media were incubated with the array overnight at 4 °C. The array was washed with Wash Buffer I, three times at room temperature, and then Wash Buffer II, two times at room temperature. Biotin-conjugated anti-angiogenic factors primary antibodies were added at room temperature for 1 h. The array was washed, and HRP-conjugated streptavidin was added and incubated at room temperature for 2 h. After washing, the array was developed using ECL, and the proteins were quantitated by densitometry scanning and expressed as the C5b-9/C5b6 ratio.

In Vitro Kinase Assays—The activities of PI3K and Akt were determined by in vitro kinase assays as described previously (15). For Akt kinase, the cell lysates (100 μg of protein) were immunoprecipitated with anti-Akt IgG (4 μg) and protein A/G-agarose (10 μl). Agarose beads were then incubated in a reaction buffer with 1 μCi of [γ-32P]ATP, Akt-specific peptide (RPRAATF) (10 μM) (Upstate Biotechnology, Inc.), and 40 μM of cAMP-dependent protein kinase inhibitor peptide (Upstate Biotechnology, Inc.) for 10 min at 30 °C. Supernatants (5 μl) were spotted on P81 phosphocellulose paper (Whatman, Maidstone, UK). After washing three times in 0.75% phosphoric acid and in acetone, the radioactivity of each P81 paper square was counted by liquid scintillation counting.

Western Blotting—For experiments using either SH-5 or Akt siRNA, cells were kept in basal media with 0.5% FBS for 18 h. Treated cells were washed twice with ice-cold PBS and then lysed in lysis buffer (Cell Signaling Technology Inc.) supplemented with 1 mm phenylmethylsulfonyl fluoride and 25 nM calyculin A. 20–30 μg of protein were loaded, electrophoresed using SDS-PAGE, and transferred to nitrocellulose membrane. The membrane was blocked in Tris-buffered saline containing 0.5% Tween 20 and 1% bovine serum albumin. The membrane was incubated with the primary antibody, followed by incubation with 1:10,000 dilution of HRP-conjugated secondary antibody (Santa Cruz Biotechnology). Bands were visualized by ECL and quantitated by densitometric scanning.

siRNA Transfection—AEC were transfected with a final concentration of 25 nM siRNA using TransIT siQuest reagent (Mirus, Madison, WI) for 6 h and then transfected again for another 24 h. Cells were then cultured in basal media supplemented with FBS and growth factors for 18 h.

siRNA Target Sequences—siRNA were synthesized by Qiagen (for Akt and CTR siRNA) and by Biopolymer/Genomics core facility at University of Maryland, Baltimore (FOXO1 siRNA). The validated target sequences were as follows: for Akt siRNA (siAkt), 5′-CCTGCCCTTCTACAACCAGGA-3′ (41); for FOXO1 siRNA (siFOXO), 5′-GAGGGTGGCCCTACTTCAAG-3′ (29); and for control siRNA (siCTR), 5′-AATTCTCCGAACGTGT-CACGT-3′.

C5b-9-induced Endothelial Cell Proliferation

Nuclear/Cytoplasmic Extraction Preparation and DNA Binding Assay—To prepare nuclear and cytoplasmic extracts, NE-PER kit was used (Pierce). Briefly, cells were washed with PBS, trypsinized, and resuspended in cytoplasmic extraction reagent I (CERI) and incubated on ice. Cytoplasmic extraction reagent II (CERII) was then added, and the extracts were further incubated on ice. The extracts were then centrifuged for 15 min, and the cytoplasmic fraction was transferred to a new tube. Nuclear extraction reagent was added to the pellet, and the nuclear pellet was incubated on ice for 40 min while vortexing every 10 min. The extract was centrifuged for 15 min, and the nuclear fraction was transferred to a new tube. CER I and nuclear extraction reagents were supplemented with 1 mm phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 mM NaVO₄, 1 mM NaF, and 25 mM calyculin A. DNA binding assay was performed using EMSA “Gel-Shift” kit (Panomics Inc., Redwood City, CA) following the manufacturer’s protocol. Briefly, 2.5 μg of nuclear extract was mixed with biotin-labeled double-stranded oligonucleotides containing the FKHR (FOXO1) consensus binding site 5′-CAAAAACAA-3′. The reaction was incubated at 20 °C for 30 min, and DNA-protein complexes were resolved on 6% TBE-polyacrylamide gel. The DNA-protein complexes were transferred to nitrocellulose membrane and immobilized by UV irradiation. The membrane was then incubated with streptavidin-HRP, and DNA-protein complexes were detected by ECL.

Immunohistochemistry—AEC were cultured on plastic slides and stimulated with C5b-9 for 4 h. The slides were washed in PBS, and the cells were fixed for 20 min in acetone with 3% hydrogen peroxide to inhibit endogenous peroxidase. Slides were washed in PBS and incubated overnight at 4 °C with anti-FOXO1 antibody diluted 1:50 in PBS and then with goat antirabbit HRP-conjugated IgG (Jackson ImmunoResearch) for 1 h at room temperature. The reaction was developed using Nova RED (Vector Laboratories) as a substrate, and the slides were then washed in distilled water and counterstained with Harris’s hematoxylin (Sigma) and mounted with permanent mount.

RESULTS

C5b-9 Induces AEC Proliferation—To show that C5b-9 was responsible for cell cycle activation (Fig. 1), AEC were exposed to anti-HLA I antibody + C7-deficient serum (C5b6), C8-deficient serum (C5b-7), C9-deficient serum (C5b-8), or basal media (unstimulated). To assemble C5b-9, C7-deficient serum reconstituted with purified C7 was used. C5b-9 caused a 3.6-fold increase in [3H]thymidine incorporation compared with C5b6 (Fig. 1A), whereas C5b-7 and C5b-8 induced a smaller increase. To show that C5b-9 induces not only S-phase induction but also G₂/M-phase entry, AEC were exposed to C5b-9 assembled from purified components and then pulsed with BrdUrd for 1 h, and cell cycle induction was assayed by FACs analysis (Fig. 1B). About 99% of unstimulated cells were arrested in the G₀/G₁-phases of the cell cycle after 24 h of starvation (Fig. 1B). Treatment of cells with C5b6 failed to induce S-phase entry, whereas C5b-7 and C5b-8 caused 8–9% of cells to enter S-phase with no G₂/M-phase entry. When
cells were treated with C5b-9, a significant number of cells progressed from G0/G1-phase to S-phase (22%) and G2/M-phase (30%) (Fig. 1B). C5b-9 also induced a 2.2-fold increase in cell number over the C5b6 level after 48 h of stimulation, as determined by MTS cell proliferation assay (Promega, Madison, WI) (data not shown).

**C5b-9 Induces AEC Migration and Growth Factor Release**

One consequence of AEC activation and proliferation is the process of angiogenesis. In angiogenesis, AEC migrate before new blood vessel formation. Because C5b-9 can induce AEC to release pro-angiogenic factors fibroblast growth factor, platelet-derived growth factor (40), and IL-8 (42), we used C5b-9-stimulated AEC conditioned medium in a chemotactic chamber to test cell migration. AEC were stimulated with C5b-9 assembled from purified components, and the conditioned media were used as a chemoattractant in a cell migration assay. Media from C5b-9-treated cells induced a 2-fold increase in cell migration compared with media from unstimulated or C5b6-treated cells (Fig. 2) suggesting that pro-angiogenic factors released may be implicated in C5b-9-induced AEC migration. EGM, which contains FBS and growth factors, induced the largest increase in AEC migration (Fig. 2).

To determine what soluble factors are released after C5b-9 stimulation of AEC, an antibody array detecting factors involved in angiogenesis was used. The conditioned media from AEC stimulated with C5b-9 assembled from purified components in serum- and growth factor-free media were used to probe the antibody array. Table 1 lists the factors that were detected and showed at least a 1.5-fold increase in C5b-9-treated EC compared with C5b6-treated EC. Besides the known angiogenic factors induced by C5b-9, we identified a new factor that was induced, placenta growth factor (PlGF), which is a member of VEGF family involved in AEC proliferation and migration (43). Using a PlGF enzyme-linked immunosorbent assay (R & D Systems, Minneapolis, MN), we verified the results obtained in the protein array. Our results show that C5b-9 induced a 5.4-fold increase over C5b6 levels (67.1 pg/ml in C5b-9-treated cells versus 12.6 pg/ml in C5b6-treated cells). These data suggest that multiple angiogenic factors are induced by C5b-9 and may be involved in C5b-9-induced AEC prolifera-
tion and migration. To test the involvement of the VEGF receptor 2 in C5b-9-induced signaling, we used an antibody to phosphorylated VEGF receptor 2 at Tyr-1175. VEGF receptor 2 was not phosphorylated after stimulation with C5b-9 (data not shown), suggesting that the receptor is not involved in C5b-9 mediated effects on AEC.

PI3K Is Induced by C5b-9 and Is Required for Cell Cycle Activation—We showed previously that C5b-9 induces PI3K activation in smooth muscle cells (13), and now we wanted to confirm its induction by C5b-9 in AEC. As shown in Fig. 3A, C5b-9 induced PI3K activity with a maximum of 3.8-fold at 20 min over C5b6 levels. The C5b-9 induced activation was inhibited by wortmannin, a specific PI3K inhibitor (Fig. 3B). To determine cell cycle activation, $[^{3}H]$thymidine incorporation was measured. Pre-treatment with wortmannin abolished C5b-9 $[^{3}H]$thymidine incorporation indicating PI3K was required for cell cycle activation (Fig. 3C).

Activation of Akt Is Required for C5b-9 Cell Cycle Activation—We then assessed Akt activation, a major downstream target for PI3K. C5b-9 increased Akt activity 3-fold over the C5b6 level at 10 min (Fig. 4A), and this was also inhibited by wortmannin (Fig. 4B) but not by rapamycin (data not shown). Phosphorylation of Ser-473 is indicative and required for Akt activation (44, 45). Although there was no basal level of phosphorylation, C5b-9 induced phosphorylation at Ser-473 as assessed by Western blotting using an antibody that specifically recognizes phosphorylation of Ser-473 on Akt (Fig. 4C). SH-5 is an inhibitor of Akt activity and was able to significantly reduce this phosphorylation (Fig. 4C). To test if Akt is required for cell cycle activation by C5b-9, AEC were pretreated with SH-5 and then stimulated with C5b-9, and $[^{3}H]$thymidine incorporation was measured (Fig. 4D). SH-5 abolished the increase in $[^{3}H]$thymidine incorporation, providing evidence for the requirement of Akt. To confirm that endogenous Akt is required for cell cycle activation, siRNA targeting Akt was used to transfect AEC (Fig. 4E). By using a validated Akt siRNA target sequence (41), we were able to knock down Akt expression in AEC by 80%. Nonsilencing siCTR had no effect on Akt expression (Fig. 4F, left panel). Akt knockdown using siRNA also significantly decreased $[^{3}H]$thymidine incorporation induced by C5b-9 (Fig. 4F, right panel). These data clearly show that Akt activity is required for C5b-9-induced cell cycle activation. The effect of Akt on PI GF release was determined by enzyme-linked immunosorbent

### TABLE 1

| Growth factor | C5b-9/C5b6 ratio | Function |
|--------------|------------------|----------|
| Epidermal growth factor | 2.6 | Growth factor |
| ENA-78 | 3 | Chemokine |
| GRO | 1.6 | Chemokine |
| IL-6 | 2 | Cytokine |
| IL-8 | 2.8 | Cytokine |
| MCP-1 | 4 | Chemokine |
| PlGF | 2.9 | Growth factor |
| RANTES | 1.6 | Chemokine |
| Angiogenin | 1.5 | Growth factor |

#### FIGURE 3. C5b-9 induction of PI3K is required for cell cycle activation. A, activation of PI3K by C5b-9. AEC exposed to C5b-9 (solid circle) or C5b6 (open circle) were examined for PI3K activity at the indicated times. C5b-9 increased PI3K activity 3.8-fold over the C5b6 level at 20 min ($p < 0.001$). Results are shown as mean ± S.E. from two experiments performed in duplicate. B, effect of wortmannin on PI3K activation. AEC were pretreated with wortmannin (100 nM) for 45 min and then exposed to C5b-9 for 15 min. Unstimulated cells (unstim.) and cells treated with C5b-9 or C5b6 were also included in the experiment. The autoradiogram is one of three experiments with similar results showing phosphorylated phosphatidylinositol (PI'P3) separated on TLC. C, requirement of PI3K for cell cycle activation. AEC pretreated with 100 nM wortmannin (wort.) for 45 min were exposed to serum C5b-9 for 18 h in the presence of 1 μg/ml $[^{3}H]$thymidine. Thymidine incorporation induced by C5b-9 was effectively abolished by wortmannin.
assay using cells transfected with Akt siRNA. Knockdown of Akt in AEC dramatically reduced levels of released PlGF in response to C5b-9 (0.14 and 4.4 pg/ml in C5b-9- and C5b-9-treated cells, respectively, compared with 17.1 pg/ml PlGF in control siRNA-transfected cells).

C5b-9 Induces Phosphorylation of FOXO1 Protein—FOXO1 is a member of the FOXO family of forkhead transcription factors that regulate the expression of genes involved in cell cycle activation and survival. It is regulated by phosphorylation at Thr-24, Ser-256, and Ser-319 by Akt (24, 46). AEC were stimulated with serum C5b-9 for 10 min and then lysed and immunoblotted with anti-phospho-FOXO1 (Ser-256) (Fig. 5A). Pretreatment with SH-5 abolished phosphorylation of FOXO1 induced by C5b-9. C5D did not induce phosphorylation of FOXO1. These data suggest that phosphorylation of FOXO1 is because of C5b-9 assembly. To confirm that phosphorylation of FOXO1 requires Akt activity, AEC were transfected with a nonsilencing siCTR or Akt siRNA (siAkt) and then stimulated with serum C5b-9 for 10 min (Fig. 5B). In control siRNA-transfected cells, there was a 4.3-fold increase in FOXO1 phosphorylation over unstimulated levels and a 2.4-fold increase over C5D-treated cells. However, in Akt siRNA-transfected cells, there was no increase in phosphorylated FOXO1 compared with unstimulated cells and C5D-treated cells. Unstimulated and C5D-treated cells for both control siRNA and Akt siRNA showed low levels of FOXO1 phosphorylation because of the presence of 2% FBS that was required in order for Akt-depleted cells to survive. This phosphorylation could be because FOXO1 is either very sensitive to the remaining Akt activity after knockdown in expression or there is an unidentified kinase that phosphorylates this site in AEC.

C5b-9 Induces FOXO1 Inactivation by DNA Unbinding and Cytoplasmic Relocalization—Phosphorylation of FOXO1 at Ser-256 by Akt leads to FOXO1 unbinding from DNA (47). To determine whether C5b-9 induces FOXO1 to unbind from DNA, EMSA was performed using biotinylated double-stranded DNA probe

FIGURE 4. C5b-9 induction of Akt is required for cell cycle. A, activation of Akt by C5b-9. AEC exposed to C5b-9 (●) or C5b6 (□) using purified components were examined for Akt activity at the indicated times. C5b-9 increased Akt activity 3.4-fold over the C5b6 level at 10 min (p < 0.001). Results are shown as mean ± S.E. from two experiments, performed in duplicate. B, effects of wortmannin on Akt activation. AEC pretreated with wortmannin (100 nM) were exposed to C5b-9 using purified components. Akt activity was then measured. Wortmannin (wort.) abolished C5b-9 induced Akt activation. C, inhibition of Akt activation by SH-5. AEC were pretreated with SH-5 (10 μM) for 3 h and then exposed to serum C5b-9. The cells were then lysed, and 100 μg of total protein were immunoprecipitated with anti-Akt IgG. Western blotting was performed using an IgG anti-Akt phosphorylated at Ser-473. SH-5 was able to significantly decrease Akt phosphorylation. Phosphorylated Akt with respect to total Akt was quantified by densitometric scanning (lower panel). D, effect of SH-5 on thymidine incorporation. AEC were pretreated with 10 μM SH-5 for 3 h and then exposed to serum C5b-9 and 1 μCi/ml of [3H]thymidine for 18 h. SH-5 decreased thymidine incorporation induced by C5b-9. E, Akt knockdown using siRNA. AEC were transfected with 25 nm siRNA targeting Akt (siAkt) or a nonsilencing siCTR for 48 h. Western blotting was performed to show the knockdown (80%) in expression by siAkt. F, Akt siRNA inhibits thymidine incorporation. AEC transfected with control siRNA or Akt siRNA for 24 h were starved in serum- and growth factor-free basal media for 18 h. The cells were exposed to serum C5b-9 and 1 μCi/ml of [3H]thymidine for 18 h. Transfection with Akt siRNA abolished the increase in thymidine incorporation by C5b-9.
C5b-9-induced Endothelial Cell Proliferation

A

B

FIGURE 5. C5b-9 induces phosphorylation of FOXO1. A, effect of C5b-9 on FOXO1 phosphorylation. AEC were starved 18 h in basal media supplemented with 0.5% FBS and then pretreated with 10 μM SH-5 for 3 h. These cells were then exposed to serum C5b-9 or C5D for 10 min and lysed. Cell lysates were examined by immunoblotting using an IgG anti-FOXO1 phosphorylated at Ser-256. Akt expression was knocked down by siRNA. Akt siRNA abolished the increase in cytoplasmic FOXO1 compared with unstimulated cells. However, Akt siRNA was able to effectively block relocalization of FOXO1 to the cytoplasm in response to serum C5b-9.

Cell Cycle Activation Is Mediated through FOXO1 Inactivation—To test if FOXO1 inactivation is required for cell cycle activation, FOXO1 was inactivated by silencing expression using a validated siRNA (29). Akt siRNA was able to knock down expression by 97% in AEC (Fig. 7A). Knockdown of FOXO1 expression in serum- and growth factor-deprived AEC induced a significant increase in S- and G2/M-phase cells (Fig. 7B) indicating that inactivation of FOXO1 alone is sufficient to drive the cell cycle. The FOXO siRNA-induced AEC proliferation was such that we were unable to detect an additive effect of C5b-9 (Fig. 7B). These data suggest that inactivation of FOXO1 is the major pathway for C5b-9-induced cell cycle activation.

Effect of FOXO Knockdown on C5b-9-induced Angiogenic Factor Release—To identify FOXO-regulated angiogenic factors that might mediate the effect of C5b-9 on AEC, we examined the angiogenic factor release profile by knocking down FOXO expression using siRNA. The conditioned media from transfected cells stimulated with C5b-9 assembled from purified components were used to probe angiogenic factor array. The array was quantitated by densitometric scanning, and C5b-9-induced release was calculated by determining the C5b-9/C5b6 ratio for each factor. C5b-9-induced release was used to calculate the ratio of FOXO siRNA (siFOXO) to siCTR-transfected cells (Table 2). Knockdown of FOXO expression resulted in changes of C5b-9 release in angiogenic factors. Silencing FOXO inhibits C5b-9 release of IL-6, PlGF, and RANTES, suggesting an involvement of FOXO in this process. On the other hand, the release of epidermal growth factor, ENA-78, GRO, and angiogenin was increased after silencing FOXO. In addition, the release of IL-8 and MCP-1 did not exhibit a dramatic change indicating that the release of these factors is FOXO-independent (Table 2). Taken together these data show that FOXO transcription factors regulate angiogenesis-related factors and support its important role in C5b-9-induced AEC proliferation and migration.

DISCUSSION

Here we report for the first time that C5b-9-induced cell cycle activation in AEC is mediated through Akt-induced inactivation of FOXO1. Although C5b-9 is known to activate...
the PI3K/Akt signaling pathway (14, 15), its downstream targets required for cell cycle activation are not yet known. C5b-9 induces expression of proteins that regulate the cell cycle (39, 48) and down-regulates the expression of p27, a potent cell cycle inhibitor, in AEC (data not shown) and other cells (11, 49). In addition, the expression of p27 is down-regulated by FOXO1 upon phosphorylation by Akt (25, 35). Therefore, we chose to assess the effects of C5b-9 on Akt kinase and FOXO1 in this study. Our data clearly indicate that activation of PI3K and Akt is required for the C5b-9-induced cell cycle activation and progression, as shown by the use of inhibitors and of Akt siRNA. In addition, the role of Akt in regulating the FOXO1 transcription factor was investigated. Akt is known to phosphorylate the following three consensus sites of FOXO1: Thr-24, Ser-256, and Ser-319 (47). FOXO1, when phosphorylated at these sites, dissociates from the DNA and is exported into the cytoplasm, thus losing its transcriptional activity of cell cycle inhibitory genes (47). The Ser-256 site is critical to inactivate FOXO1 and is required for effective phosphorylation at Thr-24 and Ser-319 (47, 50, 51). Phosphorylated FOXO1 is released from DNA, and this is followed by cytoplasmic relocalization, binding to 14-3-3 protein, and degradation (50, 52, 53). We found that Ser-256 phosphorylation occurred as early as 10 min after exposure to C5b-9. In addition, FOXO1 was present as the FOXO1-DNA complex in growth factor-deprived AEC, and this association was significantly reduced in response to C5b-9. In addition, FOXO1 was present as the FOXO1-DNA complex in growth factor-deprived AEC, and this association was significantly reduced in response to C5b-9. In addition, FOXO1 was present as the FOXO1-DNA complex in growth factor-deprived AEC, and this association was significantly reduced in response to C5b-9. In addition, FOXO1 was present as the FOXO1-DNA complex in growth factor-deprived AEC, and this association was significantly reduced in response to C5b-9.

**FIGURE 6. Inactivation of FOXO1 by C5b-9.** A, C5b-9 induces dissociation of FOXO1 from the DNA-FOXO1 complex. AEC were cultured in serum and growth factor medium for 18 h and then stimulated with C5b-9 for the indicated periods of times. Nuclear extracts from these cells were analyzed by EMSA using biotinylated double-stranded DNA probes for FOXO1. In unstimulated (unstim.) cells (0 h), FOXO1 showed the FOXO1-DNA complex, although addition of excess cold competitor (cold comp.) displaced the labeled complex. Treatment with C5b-9 induced a significant decrease in FOXO1 binding at 1 and 2 h after stimulation. B, localization of FOXO1 by immunohistochemistry. AEC plated onto plastic chamber slides were grown in basal media free of serum and growth factors for 18 h and then stimulated with serum C5b-9 or C5D for 4 h. Nuclear and cytoplasmic fractions were extracted using NE-PER kit from Pierce. Immunoblotting was carried out using anti-FOXO1 IgG and anti-β-tubulin as a loading control. Serum C5b-9 was able to induce a significant increase in cytoplasmic FOXO1 levels with a corresponding decrease in nuclear levels. Pretreatment with LY294002 decreased this effect indicating the requirement of PI3K. Densitometric quantitation of FOXO1 expression was performed using Ne-PER from Pierce. Immunoblotting was carried out using anti-FOXO1 IgG and anti-β-tubulin as a loading control. Serum C5b-9 was able to induce a significant increase in cytoplasmic FOXO1 levels with a corresponding decrease in nuclear levels. Pretreatment with LY294002 decreased this effect indicating the requirement of PI3K. D, effect of Akt siRNA on FOXO1 localization in the cytoplasm. AEC were transfected with Akt siRNA (siAkt) or siCTR and then cultured in basal media containing 2% FBS for 18 h. Cytoplasmic fractions were examined for FOXO1 expression. Cells stimulated with C5b-9 showed an increase at 4 h in cytoplasmic FOXO1 levels, and this was abolished by siAkt, which effectively knocked down Akt protein levels as shown using anti-Akt antibody. Cytoplasmic levels of FOXO1 were expressed as ratio to tubulin (lower panel).
cytoplasmic relocalization after 30 min of exposure (54). The kinetics suggest that in addition to phosphorylation induced by Akt pathway, the C5b-9 cytoplasmic relocalization of FOXO1 might require additional stimuli. Accelerated cytoplasmic transfer of FOXO1 requires phosphorylation at Ser-319, in addition to Ser-24 and Thr-256 (55). Also, other kinases, such as serum- and glucocorticoid-inducible kinase and DYRK1a (24), phosphorylate Ser-319, Ser-322, Ser-325, and Ser-329 to promote nuclear exclusion (53). Therefore, inactivation of FOXO1 may require sustained activation of multiple signaling pathways. We can speculate that a multistep regulation is involved in C5b-9- induced inactivation of FOXO1. The release of growth factors by C5b-9 may provide the sustained signal.

We wanted to determine what other steps in angiogenesis, beside AEC proliferation, may be effected by C5b-9. Because C5b-9 is present in human atherosclerotic lesions (10) and induces AEC proliferation, we postulated that C5b-9 may act as a pro-angiogenic factor by inducing not only AEC proliferation but also migration. Our exploration to assess the migration of AEC using purified components to assemble C5b-9 revealed interesting and encouraging results. We show for the first time that C5b-9 was able to induce AEC migration. This process may involve regulation of FOXO1 as overexpression of constitutively active FOXO1 mutant in human umbilical vascular EC inhibited migration, although silencing of FOXO1 increased migration (56). Also, FOXO1-deficient mice were reported to exhibit abnormal angiogenesis (57), although endothelial cell migration and tube formation were inhibited in mice with constitutively active FOXO1 and FOXO3a (56). Therefore, C5b-9-mediated inactivation of FOXO1 may regulate AEC migration and proliferation.

Multiple soluble angiogenic factors are released by C5b-9 from AEC. Some of the factors identified by us using protein array have angiogenic activity and are known to be released by cells when exposed to sublytic C5b-9 (42, 58–60). We also identified a new angiogenic factor released by C5b-9, PlGF, a member of the vascular endothelial growth factor family. C5b-9-induced PlGF release was inhibited after transfection with Akt and FOXO1 siRNA indicating that Akt and FOXO1 are involved in growth factor release in addition to cell cycle activation. This factor has been shown to induce neovascularization in vitro and induce endothelial cell migration and proliferation (43). Local adenoviral delivery of PlGF promotes atherogenic neointima formation in hypercholesterolemic rabbits, and PlGF is required for macrophage accumulation in early atherosclerotic lesions in ApoE−/− mice (61). Isolated fragments of atherosclerotic plaque possess angiogenic activity (62), and it was shown that neovascularization or angiogenesis promotes atherosclerosis (63). Moreover, C5b-9 was shown to play an essential role in the development of laser-induced choroidal angiogenesis in mice possibly through the up-regulation of angiogenic factors (64). Taking these facts into account, it is reasonable to presume that C5b-9 may have pro-angiogenic potential through the release of angiogenic soluble factors.

In conclusion, we have shown that C5b-9 signaling is linked to FOXO transcription factors and angiogenic factors release in AEC. Our data suggest FOXO1 plays a major role in regulation of C5b-9-induced AEC proliferation and might represent a therapeutic target for regulating atherosclerotic related angiogenic events.

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C5b-9-induced Endothelial Cell Proliferation