Akt, a serine-threonine kinase, regulates multiple cellular processes in vascular cells. We have previously documented that Akt activates integrins and Akt1 deficiency results in matrix abnormalities in skin and blood vessels in vivo. Based on these observations, we hypothesized that Akt1 is necessary for integrin activation and matrix assembly by fibroblasts. In this study, using various cell systems, we show that Akt1 is essential for the inside-out activation of integrins in endothelial cells and fibroblasts, which in turn, mediates matrix assembly. Fibronectin is a major extracellular matrix component of the skin and the vascular basement membrane, which possesses binding sites for many integrins and extracellular matrix proteins. Akt1−/− fibroblasts and NIH fibroblasts expressing dominant negative Akt1 (K179M-Akt1) showed impaired fibronectin assembly compared with control fibroblasts. In contrast, expression of constitutively active Akt1 (myrAkt1) resulted in enhanced fibronectin assembly. Although increased fibronectin assembly by myrAkt1-expressing human foreskin fibroblasts was abolished by treatment with anti-integrin β1 blocking antibodies, treatment with β1-stimulating antibodies rescued the impaired fibronectin assembly that was due to lack of Akt activity. Finally, expression of myrAkt1 corrected the phenotype of Akt1−/− fibroblasts thus showing that Akt1 regulates fibronectin assembly through activation of integrin αβ1.

Protein kinase B (Akt) is a multitask kinase, which regulates various cellular processes in vascular cells such as cell survival, cell cycle, metabolism, protein synthesis, and transcriptional regulation (1–3). It has been previously shown that Akt is involved in the activation of integrins (4), and integrin activation is a key step necessary for the adhesion and migration of endothelial cells and fibroblasts, which regulates assembly of extracellular matrix (ECM) (5). Growth factor activation of endothelial cells also depends on its ability to attach to the ECM proteins (6) and in the absence of ECM engagement, endothelial cells undergo apoptosis by a process termed “anoikis” (7). Expression of constitutively active Akt blocks anoikis in the absence of cell attachment (8). Attachment of vascular cells to matrix proteins is dependent on integrin activation and clustering, which leads to assembly of actin filaments and focal adhesion formation (9).

Integrins comprise a large family of heterodimeric ECM receptors made of an α and a β subunit that integrate the ECM to the intracellular cytoskeleton to mediate cellular adhesion and migration (10, 11). Most of the matrix proteins such as fibronectin and collagen bind to several integrins (10). Conversely, individual integrin dimers recognize several ECM proteins (12). The stimuli received along with growth factor-mediated activation of signaling pathways regulate various cellular processes such as adhesion and migration of cells via activation of integrins. This unique ability of the integrins to regulate the attachment of cells to ECM proteins is termed “inside-out signaling” (13). In addition, ligand binding is transduced from the extracellular domain to the cytosol by “outside-in signaling” (14). Thus, integrins can transduce signals through the cell membrane in either direction.

A number of signaling pathways synergistically regulate the highly complex process of integrin activation (15). Cell adhesion, migration, survival, and proliferation all depend on integrin signaling through FAK, Src, and PI3K pathways activated downstream of growth factor stimulation and ECM attachment (16). SYF cells (lacking Src family kinases Src, Yes, and Fyn) treated with PI3K inhibitors decrease adhesion and fibronectin matrix assembly (17). Akt, a major downstream target of PI3K signaling, regulates recycling of integrins αβ1 and αβ3 from the lagging edge to the lamellipodia in fibroblasts (18), leading to enhanced migration. Translocation of integrin αβ1β3 from focal contacts to and along the ECM contacts, is also necessary for early fibronectin fibrillogenesis (19). It has been shown that mutation of a specific amino acid in integrin β1 (W775A) selectively inhibits activation of Akt in fibroblasts in response to bFGF, with no detectable inhibition of activities of other
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kinases (20), suggesting that Akt controls fibronectin-mediated signals. Although Akt can directly regulate integrin activation via phosphorylation of threonine residue 753 of integrin the cytoplasmic tail of β1 (21, 22), a recent study suggests that Akt phosphorylates girdin, a cytoskeletal protein, and thus regulates cytoskeletal re-arrangement and cell migration in COS7 cells (23). Overall, PI3K–Akt signaling-mediated integrin activation is necessary for the adhesion and migration of vascular cells on various matrix proteins, which in turn, regulates angiogenesis.

Akt signaling is complicated due to the presence of three different Akt isoforms with >85% homology in the structure and substrate specificity (3), but which differ in their functions in vivo as evidenced by the studies using animal models (1). Information about individual roles of each isoform at the cellular levels in vascular cells is only beginning to emerge. Our study, using Akt1 knock-out mice, showed that Akt1 is the major Akt isoform in endothelial cells and accounts for nearly 70% of the total Akt activity (2). Absence of Akt1 results in a series of ECM defects in vivo, including reduced levels of collagen in skin and impaired laminin content within the vascular basement membrane (2). Because secretion and assembly of matrix proteins is mainly dependent on integrin activation, our results indicate that Akt1 signaling might be important for integrin activation in endothelial cells and fibroblasts. In this study we sought to investigate the roles of Akt1, the predominant isoform of Akt in vascular cells, in the regulation of integrin activation, ECM recognition, migration, and fibronectin assembly by endothelial cells and fibroblasts. To do this, we used wild-type (WT) and Akt1−/− endothelial cells and fibroblasts and assessed their ability to adhere and migrate on different matrix proteins. Wild-type and Akt1−/− fibroblasts, along with NIH 3T3 fibroblasts expressing various forms of Akt1 such as WT-Akt1, constitutively active (myrAkt1) and dominant negative (DN-Akt1) were used to delineate the importance of Akt1 in controlling the matrix recognition by the cells and the assembly of fibronectin matrix. Additionally, integrin β1-stimulating antibodies, blocking antibodies, and antibodies that detect only the active form of integrin β1 were used to demonstrate that Akt1-mediated effects were via the ability of Akt1 to activate integrin β1. Altogether, our data demonstrate that Akt1 is an important mediator of integrin activation in endothelial cells and fibroblasts and regulates matrix recognition, migration, and organization of fibronectin.

EXPERIMENTAL PROCEDURES

Reagents and Antibodies—All organic chemicals of analytical grade were obtained from Sigma or Fisher unless otherwise specified. Sterile media and PBS for cell culture were obtained from the media core services at the Lerner Research Institute. Rabbit polyclonal anti-Akt, -Akt1, -pT308-Akt, and -pS473-Akt were purchased from Cell Signaling (Danvers, MA). Anti-fibronectin and -β-actin antibodies and purified laminin were purchased from Sigma. Integrin β1-blocking antibody (MAB2253Z) and HUTS-4 antibody were purchased from Chemicon (Temecula, CA). Anti-β1 antibody TS2/16 was obtained from Pierce. WOW-1 Fab was a kind gift from Dr. S. J. Shattil, Scripps Research Institute, La Jolla, CA. Purified fibronectin was purchased from BD Biosciences (San Jose, CA). Vitronectin and fibrinogen was purchased from Calbiochem. Anti-rabbit and anti-mouse Alexa Fluor antibodies were purchased from Invitrogen.

Western Blot Analysis—Cell lysates were prepared using lysis buffer (20 mM Tris-HCl, pH 7.4, 1% Triton X-100, 3 mM EGTA, 5 mM EDTA, phosphatase inhibitors (10 mM sodium pyrophosphate, 5 mM sodium orthovanadate, 5 mM sodium fluoride, and 10 μM okadaic acid), protease inhibitor mixture (Roche Diagnostics, Basel, Switzerland) and 1 mM phenylmethylsulfonyl fluoride. SDS-PAGE and Western blotting were performed as described previously (24).

Animals—Akt1−/− mice were generated as previously described (25) and were maintained in the 129 R1/C57BL/6 background. Sex- and age-matched 8- to 12-week-old WT and Akt1−/− mice were used in the study. All protocols were performed under institutionally approved procedures.

Isolation of Primary ECs and Mouse Embryonic Fibroblasts—The microvascular ECs were isolated from lungs using a previously described protocol (2). Briefly, lungs were minced and digested using a 3 mg/ml collagenase-dispase mixture (Roche Diagnostics) for 4 h at room temperature. The rest of the procedures were performed as described previously (2). Mouse embryonic fibroblasts were collected from 11- to 14-day-old embryos. Fetuses were removed from amniotic sacs and cleared of the heads, viscera, and limbs. Embryos were transferred to a sterile container containing DMEM pre-warmed to 37 °C. Embryos were then washed and chopped to 3-mm3 cubes until the washes were clear of blood cells. Cubes were digested using a 3 mg/ml collagenase-dispase mixture for 4 h at room temperature. After enzymatic digestion, the supernatant was collected through a tissue strainer (Fisher Scientific). The filtrate was centrifuged, and the pellet was washed in PBS. The cells were then resuspended in DMEM culture medium with 10% fetal bovine serum and plated on plastic.

Cell Lines and Cell Culture—NIH 3T3 fibroblasts were obtained from ATCC (Manassas, VA) and were transfected with WT-Akt1, myrAkt1, and DN-Akt1 (Akt1 K179M) using FuGENE 6 transfection reagent as per the manufacturer’s protocol. Expression of exogenous Akt1 is performed at near to physiological levels to prevent any non-physiological effects of Akt1 due to overexpression. Approximately 60–70% transfection efficiency was obtained in NIH 3T3 cells as observed by GFP staining. Primary human foreskin fibroblasts (HFFs), passages 11–16, were obtained from MetroHealth Hospital, Cleveland, OH. HFFs for blocking antibody experiments were transfected by retroviral infection and subjected for puromycin selection. HUVECs and HFFs for WOW-1 and HUTS-4 experiments, respectively, were infected with adenoviruses expressing GFP (control) and myrAkt1. For reconstitution of Akt1 in Akt1−/− endothelial cells and MEF, cells were infected with adenovirus encoding GFP or myrAkt1. Fibroblasts were cultured in DMEM with 10% fetal bovine serum, whereas endothelial cells were cultured in DMEM/F-12, supplemented with l-glutamine, 90 μg/ml heparin, 50 μg/ml endothelial cell growth supplement (BD Biosciences), and 10% fetal bovine serum.
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WOW-1 and HUTS-4 Binding Assay—Activation-dependent ligand WOW-1 Fab (with His tag) was used to determine the activation status of integrins. To analyze WOW-1 binding, serum-starved ECs were incubated with 1 μg/ml MnCl₂, followed by addition of WOW-1 Fab in adherent conditions for 30 min. Cells were washed in PBS and further incubated with anti-His Alexa 555 antibody for another 30 min. After washing, cells were isolated using cell scrapers, vortexed and washed two more times in PBS. Cells were then fixed in 2% paraformaldehyde. Essentially the same protocol was used for HUTS-4 binding assays except that HFFs were used for the analysis and that anti-mouse Alexa 647 antibody was used for probing. FACS was performed using FACSCalibur (BD Biosciences), and the data were analyzed using CellQuest or FlowJo software. Only intact cells were analyzed, excluding the cell debris.

Fibronogen Binding Assay—The analyses of fibrogen binding and integrin expression were performed as previously described (26). To analyze fibrogen binding, endothelial cells were stimulated with Mn²⁺. Alexa 633-labeled fibrogen was added at a final concentration of 200 nM for 45 min. Cells were fixed in 1% Paraformaldehyde followed by FACS analysis using FACSCalibur (BD Biosciences), and the data were analyzed using CellQuest software.

Cell Adhesion Assay—Cell adhesion assays were performed as previously described (4). Mouse lung ECs and mouse embryonic fibroblasts, within 1–2 passages, were suspended in serum-free medium (DMEM/F-12) and then stimulated with 20 ng/ml VEGF and FGF, respectively. The cells in suspension were immediately transferred to ligand-coated 12-well plates at 1 × 10⁵ cells/well. After incubation at 37 °C for 42 min, the wells were washed three times. Cells were then fixed in 70% methanol and stained with 1% toluidine blue in 1% borax, and adherent cells were examined microscopically and quantified.

In Vitro “Wound Healing” Assay—Wild-type and Akt1 knock-out EC or fibroblasts were plated at about 90% confluence in 6-well culture plates. After 12 h, the endothelial monolayers were wounded by two perpendicular strokes across the diameter of the well with a 2.5-mm-wide cell scraper. The media containing the dislodged cells was then aspirated, and the plates were rinsed with PBS. Fresh 2% serum-containing medium was added to the plates along with VEGF for endothelial cells, while serum-free medium with FGF was added to the plates for fibroblasts. The cells were incubated at 37 °C. 12 h after wounding, plates were fixed with 70% methanol and stained with toluidine blue. The width of the wound was visualized with an inverted microscope at 10× magnification and was quantified using Image Pro-Plus software. A total of 20 random measurements were analyzed for each of four wounds at each time point. Distance between groups of cells is measured. Percentage of recovery was calculated using the equation, % recovery = [1 – (T₁ – T₉)] × 100, where T₀ is the wounded area at time 0 and T₁ is the wounded area after 12 h.

Boydren Chamber Migration Assay—We performed cell migration assays using Transwell plates in which plate membranes were pre-coated with appropriate matrix proteins. Endothelial cells from wild-type or Akt1−/− mice were incubated with or without VEGF for 4 h at 37 °C. The cells were fixed with 1% paraformaldehyde and stained with toluidine blue. We microscopically quantified migration (magnification, 200×).

Analysis of Fibronectin Assembly—A previously described protocol was used for the analysis of fibronectin assembly (27). Fibroblasts were grown on glass coverslips with or without matrix coating and then overnight incubation in serum-free medium, the conditioned media was collected and the confluent monolayer was fixed in 1% paraformaldehyde then probed with anti-fibronectin antibody overnight at 4 °C. The cells were probed with labeled secondary antibody (Alexa Flour 486, Invitrogen) and mounted using 4',6-Diamidino-2-phenylindole-containing Vectashield (Vector Laboratories, Burlingame, CA). The slides were photographed using a fluorescence microscope.

To assay assembly of exogenous fibronectin in the presence and absence of inhibitors of PI3K (10 μM LY294002) and Akt (1 μM SH-5), we standardized the optimum concentration of fibronectin required for our study and compared it with the control (no exogenous fibronectin) in a short-term assay. NIH 3T3 cells (1 × 10⁶/well in a 12-well plate) were added to glass coverslips and were incubated to reach a monolayer. After 1 h, each well (except control) was supplemented with 10 μg of fibronectin in the presence and absence of PI3K and Akt inhibitors and further incubated for 4 h. The coverslips were then stained in 2% paraformaldehyde and stained with anti-fibronectin antibodies and processed as above.

Treatment with Integrin β₁-stimulating and -blocking Antibodies—Integrin β₁-stimulating antibody (TS2/16) binds to the extracellular domain of integrin β₁ on the fibroblasts, thus inducing conformation changes that activate integrin β₁. In contrast, integrin β₁-blocking antibodies bind to the extracellular domain of integrin β₁ on the membrane surface of fibroblasts preventing attachment to their respective ligands. We incubated HFFs with TS2/16 in the presence and absence of SH5 for 1 h with 10 μg/ml fibronectin added to each well then further incubated them for 4 h. Integrin β₁ blocking antibody was used at a concentration of 1 μg/ml and incubated for 3 h at 37 °C in a CO₂ incubator. Treatments were repeated every 3 h to a total of four treatments in 12 h. The coverslips were fixed in 1% paraformaldehyde and subjected to immunostaining with anti-fibronectin antibody.

Statistical Analysis—All the data are presented as means ± S.D. We performed all the analyses using two-sample t tests with a two-tailed distribution, and the significance was set at 0.05 levels (marked with an asterisk wherever data is statistically significant).

RESULTS

Akt1 Mediates Extracellular Matrix Recognition and Migration of ECs—Several studies indicate that Akt is involved in the regulation of integrin signaling. It was originally believed that Akt primarily mediates cellular responses induced by integrin ligation upon attachment to extracellular matrix, known as outside-in signaling (3). However, some of the most recent reports emphasize the possible role of Akt in modulation of integrin functional activity or inside-out integrin signaling (1, 2, 28). To substantiate the role of Akt in integrin-mediated cellular responses we used WT and Akt1−/− mouse lung endothelial
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Migration of endothelial cells is necessary for tube formation and neo-vascularization in vivo. Knowing that Akt1 signaling directly affects integrin activation, we next determined the role of Akt1 in endothelial migration in a two-dimensional migration assay, namely the endothelial monolayer wound healing assay. This method is designed to assess directed migration of WT and Akt1−/− endothelial cells and to heal the scratch wound created in the monolayer. Our data show that deficiency of Akt1 substantially impaired the migration of endothelial cells on matrix proteins such as fibronectin, vitronectin, and fibrinogen, but not on laminin, suggesting that Akt1 activity is necessary for the optimal directed migration of endothelial cells (Fig. 1B and supplemental Fig. S1B). Quantification of the data showed that the percentage of recovery attained by Akt1−/− endothelial cells plated on fibronectin, vitronectin, and fibrinogen was at least 2-fold lower compared with the WT (Fig. 1B). Because we did not observe a difference on laminin, we set up...
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A. Confluent monolayers of HUVECs were serum-starved overnight and treated with 50 ng/ml insulin-like growth factor-I or 20 ng/ml bFGF. Lysates prepared at different time points (0, 5, and 15 min) after treatment with insulin-like growth factor-I and bFGF were subjected to Western analysis for pS473 Akt and total Akt. C. HUVECs were either kept in suspension or plated on fibronectin or fibrinogen and were treated with either control (0.01% BSA in PBS) or 20 ng/ml VEGF for 15 min. Lysates were prepared and subjected for Western analysis for p473/Akt/P308 Akt and total Akt. Akt1 mediates activation of integrins.

B. Confluent monolayers of HUVECs were either kept in suspension or plated on fibronectin or fibrinogen and were treated with either control (0.01% BSA in PBS) or 20 ng/ml VEGF for 15 min. Lysates were prepared and subjected for Western analysis for pS473 Akt and total Akt.

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B. Confluent monolayers of HUVECs were either kept in suspension or plated on fibronectin or fibrinogen and were treated with either control (0.01% BSA in PBS) or 20 ng/ml VEGF for 15 min. Lysates were prepared and subjected for Western analysis for pS473 Akt and total Akt.

Akt1 Mediates Activation of Integrins in ECs and Fibroblasts—In ECs, Akt activation occurred upon treatment with various growth factors, including insulin-like growth factor-I, bFGF, and VEGF (Fig. 2, A and B). A time-dependent increase in phosphorylation of Akt at Ser-473 and Thr-308 was observed after treatment with VEGF, and the presence of extracellular matrix was not required (Fig. 2B). Direct comparison of Akt phosphorylation in WT and Akt1/−/− ECs revealed that VEGF-induced phosphorylation of total Akt was substantially reduced in the absence of Akt1 (2). Interactions with matrix proteins such as fibronectin and fibrinogen resulted in the activation of Akt even in the absence of VEGF (Fig. 2C). This shows that both growth factors and integrin signaling lead to Akt activation. As anticipated, phosphorylation of Akt was completely abolished by the treatment with PI3K inhibitors LY294002 and wortmannin, showing that Akt was activated downstream of PI3K (Fig. 2C).

Knowing that integrin-mediated cell adhesion is impaired in Akt1/−/− ECs, we sought to determine whether endothelial Akt is involved in integrin inside-out signaling (integrin activation). Accordingly, we used WOW-1 Fab, known to specifically detect the active conformers of integrin α5β1 and αvβ3, as a reporter of integrin activation in these studies (4). Transfection of HUVECs with constitutively active Akt1 (myrAkt1) resulted in at least a 2-fold increase in WOW-1 Fab binding to ECs in suspension (p < 0.001) (Fig. 2D and supplemental Fig. S4A). This indicates that Akt1 activity is sufficient for induction of integrin activation on ECs. Likewise, analysis of soluble ligand binding to fibroblasts expressing myrAkt1 and DN-Akt1 as well as WT and Akt1/−/− MEF by a fibrinogen binding assay in the presence of Mn2+ revealed that integrin activity appears to be impaired upon DN-Akt1 expression (p < 0.0004) and enhanced upon myrAkt1 expression (~2 fold; p < 0.002) (Fig. 2E). The lack of Akt1 in fibroblasts from Akt1/−/− mice also resulted in impaired fibrinogen binding (~50% inhibition) in the presence and absence of bFGF (p < 0.006 and p < 0.001, respectively) (Fig. 2F). Taken together, these data...
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FIGURE 3. Akt1−/− fibroblasts exhibit impaired adhesion and migration to fibronectin. A, wild-type and Akt1−/− MEFs (10^5 cells/well) were introduced into 12-well plates coated with BSA, fibronectin, or vitronectin (1 µg/ml) and were allowed to attach by incubating for 45 min at 37 °C in the presence of 20 ng/ml bFGF. Adhered cells were fixed in 70% methanol and stained using toluidine blue and visualized in a phase-contrast microscope. B, wild-type and Akt1−/− MEFs were plated at ~90% confluency in 6-well culture plates coated with BSA, fibronectin, or vitronectin and allowed to form a monolayer. Monolayers were wounded across the diameter of the well with a 2.5-mm-wide cell scraper. The media and dislodged cells were aspirated, and the monolayers were analyzed microscopically at 12 h (5× magnification). C, a time course effect of scratch wound assay on fibronectin (1 µg/ml) at 3, 6, 9, and 12 h. D, monolayers of WT MEFs were serum-starved overnight and were then treated with VEGF (20 ng/ml), bFGF (20 ng/ml), 1 µM VEGFR2 inhibitor (SU1498), and/or 10 µM LY294002, and the lysates were subjected to Western analysis. E, Western blots of lysates from the wild-type and Akt1−/− MEFs, probed with antibodies to Akt1, Akt2, Akt3, panAkt, and β-actin. F, Western analysis of lysates from the wild-type and Akt1−/− MEFs after treatment with bFGF (20 ng/ml) at time intervals of 0, 5, 10, 15, 30, and 60 min probed for pS473 Akt and panAkt. G, histogram showing band densitometry analysis of the above data analyzed using Kodak molecular imaging software (version 4.0).

Adhesion to fibronectin both in the absence and the presence of stimulation with the major growth factor bFGF (supplemental Fig. S2A and Fig. 3A) (p values were <0.00002 and <0.002, respectively). Because no differences in integrin expression were found between WT and Akt1−/− fibroblasts (supplemental Fig. S3A and Fig. 3, B and C), these data indicate that integrin activity might be impaired by the lack of Akt1 signaling. At the same time, no differences in fibroblast attachment to plastic (data not shown) and BSA (control substrate) were found between WT and Akt1−/− cells (Fig. 3A and supplemental Fig. S2A). Although integrin αvβ3 is expressed at a low level on fibroblasts as evidenced by Western blot (data not shown), Akt1−/− fibroblasts adhered to vitronectin to the same extent as WT cells (Fig. 3A and supplemental Fig. S2A).

Fibroblasts are important for repair of wounds in vivo, because migration of fibroblasts to the wound area is a necessary step for effective wound healing in skin. We performed in vitro wound healing assays for fibroblasts plated on BSA, fibronectin, and vitronectin. Akt1−/− fibroblasts did not show significant impairment in migration on BSA (non-ligand control) and vitronectin compared with WT (Fig. 3B and supplemental Fig. S2B). At the same time, quantification analysis showed that the percentage of wound recovery on fibronectin was lower compared with WT (~25% slower; p < 0.002) (Fig. 3B). In another experiment, we determined a time course of wound healing by Akt1−/− fibroblasts on fibronectin. A significant impairment in wound healing by Akt1−/− fibroblasts (~2-fold decrease) was noted at all time points, compared with WT (Fig. 3C and supplemental Fig. S2C). Collectively, our data show that Akt1 is necessary for the recognition of extracellular matrix by fibroblasts.

As a next step, we sought to determine what growth factors are able to activate Akt signaling in fibroblasts. Treatment with bFGF but not with VEGF stimulated phosphorylation and activation of Akt in mouse embryonic fibroblasts (MEFs) as detected by Western blotting using pS473 Akt antibodies (Fig. 3D). This activation was PI3K-dependent, because it was completely abolished by treatment with inhibitor LY294002 (Fig. 3E).
blasts showed decreased levels of total Akt phosphorylation compared with WT (Fig. 3G).

Deficiency of Akt1 Leads to Impaired Fibronectin Matrix Assembly by Fibroblasts—As discussed above, integrin activation and several integrin-dependent functions, including cell adhesion and migration, appeared to be impaired in Akt1−/− cells. It is known that, besides matrix recognition, integrin receptors actively participate in matrix assembly and remodeling (29, 30). In fact, changes in extracellular matrix assembly are anticipated as a direct consequence of altered integrin activation (29, 30). We have recently shown that Akt1-deficient mice are characterized by a series of deficiencies in extracellular matrix, which lead to impaired skin development and compromised maturation of blood vessels (1, 2). Knowing that fibroblasts are the major cell type contributing to secretion, assembly, and remodeling of extracellular matrix, we sought to determine the role of Akt1 in fibronectin matrix assembly by fibroblasts.

To this end, we isolated primary fibroblasts from WT and Akt1−/− embryos, and assessed the assembly of fibronectin matrix. The staining for fibronectin revealed that WT fibroblasts were able to lay down a substantial amount of well organized fibronectin fibrils (Fig. 4A). At the same time, fibronectin assembly by Akt1−/− fibroblasts was significantly impaired (Fig. 4A). Image analysis revealed that WT fibroblasts deposited 2-fold more fibronectin than Akt1−/− fibroblasts (p < 0.0001) (Fig. 4B). Thus, it appears that absence of Akt1 results in impaired assembly of fibronectin matrix.

To further assess the role of Akt1 in the process of secretion and assembly of fibronectin, we transfected NIH 3T3 fibroblasts (with 60–70% transfection efficiency as evidenced by the number of GFP-positive cells/field) with vector control (pBabe-puromycin) and Akt1 variants such as WT-Akt1, myrAkt1, and DN-Akt1 and analyzed the role of Akt1 in assembly of fibronectin. Monolayers were fixed in 1% paraformaldehyde and were stained with anti-fibronectin antibody to study matrix assembly. Bar = 20 μm. E, histogram showing quantification of the above data as analyzed by Image ProPlus.

FIGURE 4. Deficiency of Akt1 results in impaired fibronectin assembly by fibroblasts. A, equal numbers of WT and Akt1−/− MEFs (1 × 10^5/well) were plated in 12-well plates onto glass coverslips and allowed to form a monolayer. The monolayer was fixed using 1% paraformaldehyde and stained using anti-fibronectin antibodies. Fluorescent images of fibronectin meshwork created by WT and Akt1−/− MEFs were visualized using a fluorescence microscope. Bar = 20 μm. B, these results were quantified using Image ProPlus software for the percentage of the total area positive for fibronectin and were normalized to the number of nuclei counted per field (three different fields taken from four sets of samples). C, NIH 3T3 cells were transfected with control vector (pBabe-Puromycin), WT-Akt1, myrAkt1, and DN-Akt1. 36 h after transfection, lysates were prepared and subjected to Western analysis for pS473Akt and Akt1. D, NIH 3T3 cells were transfected with control vector, WT-Akt1, myrAkt1, and DN-Akt1 and were used to study the role of Akt1 in assembly of fibronectin. Monolayers were fixed in 1% paraformaldehyde and were stained with anti-fibronectin antibody to study matrix assembly. Bar = 20 μm. E, histogram showing quantification of the above data as analyzed by Image ProPlus.

3D). At the same time, VEGFR2 inhibitor SU1498 did not have any effect on Akt phosphorylation in fibroblasts. Akt1−/− fibroblasts exhibited modest increases in the levels of both Akt2 and Akt3 as compared with the WT fibroblasts (Fig. 3E). The total levels of phosphorylated Akt in Akt1−/− fibroblasts were significantly lower compared with that of WT (Fig. 3, F and G). A minimal level of active Akt was present in WT fibroblasts in untreated condition (Fig. 3, F and G), which was even lower in Akt1−/− fibroblasts. Treatment with bFGF resulted in increased Akt phosphorylation right from the 5-min time point, reaching a maximum in 15 min. At all time points observed, Akt1−/− fibroblasts showed decreased levels of total Akt phosphorylation compared with WT (Fig. 3G).
fected with Akt1 constructs, without dramatic changes in the levels of total Akt1 (Fig. 4C). Even though DN-Akt1 can be phosphorylated at Ser-473 (Fig. 4C), the kinase is still inactive due to a mutation in the kinase domain (K179M). Results show that introduction of either WT-Akt1 or myrAkt1 into NIH 3T3 fibroblasts substantially increased assembly of fibronectin (Fig. 4D). Although both WT-Akt1 and myrAkt1 stimulated assembly and deposition of fibronectin fibrils, DN-Akt1 significantly inhibited this process (Fig. 4D). The difference in fibronectin expression between myrAkt1- and WT-Akt1-transfected cells was not dramatic, indicating that even a modest increase in Akt1 activation over the baseline levels is sufficient for integrin activation and up-regulation of fibronectin assembly. In contrast, transfection with DN-Akt1 inhibited the ability of NIH 3T3 cells to mediate fibronectin assembly (Fig. 4D). Additionally, DN-Akt1-expressing cells showed impaired attachment to fibronectin fibrils leading to clumping of partially assembled fibronectin fibrils, indicating that activation of integrin is impaired by reduced Akt1 activity (Fig. 4, C and D). Quantification results revealed that the amount of assembled fibronectin per cell doubled upon transfection with Akt constructs (WT-Akt1 and myrAkt1) ($p < 0.003$ and $p < 0.008$, respectively), whereas DN-Akt1 caused significant reduction in fibronectin assembly ($p < 0.007$) (Fig. 4E). Taken together, these results demonstrate that Akt1, an important downstream target of growth factor signaling, regulates fibronectin assembly in fibroblasts.

Akt1 Regulates Assembly of Exogenous Fibronectin Matrix—A number of factors might contribute to matrix assembly by fibroblasts, including an ability of the cells to respond to stimuli and secrete ECM proteins (5). To demonstrate that Akt mediates the final step of fibronectin matrix assembly, which is dependent on the recognition of fibronectin but independent from fibronectin synthesis, we assessed the ability of cells to assemble exogenous fibronectin into the fibrillar structures. Fibroblasts were allowed to adhere on glass coverslips for 1 h in the presence and absence of control vehicle (0.05% Me$_2$SO) or inhibitors of PI3K (LY294002) and Akt (SH5), followed by the addition of exogenous fibronectin. In the absence of exogenous fibronectin only negligible amount of fibronectin matrix assembly was observed, whereas cells supplemented with exogenous fibronectin showed $\sim$35% of the total area positive for fibronectin fibrils (Fig. 5, A and B). Pre-treatment with PI3K (LY294002) and Akt (SH5) inhibitors showed significant inhibition of fibronectin matrix assembly ($p < 0.004$ and $p < 0.0006$, respectively) (Fig. 5, A and B). Like-
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wise, Akt1$^{+/−}$ fibroblasts exhibited impaired assembly of exogenous fibronectin matrix compared with WT ($p < 0.003$) (Fig. 5, C and D), thus showing that Akt1 is the major candidate downstream of PI3K involved in the regulation of matrix assembly. We further confirmed these results using NIH 3T3 fibroblasts expressing myrAkt1 and DN-Akt1, which showed enhanced ($\sim 30\%$ increase, $p < 0.0003$) and impaired ($\sim 25\%$ decrease, $p < 0.002$) fibronectin matrix assembly compared with control, respectively (Fig. 5, E and F). These data demonstrate that Akt controls the final step of matrix assembly, which, in turn, is known to be dependent on the recognition of fibronectin by activated integrins.

Akt1-dependent Fibronectin Matrix Assembly Is Mediated by Activation of Integrin $\beta_1$.—Previous reports show that integrin $\alpha_\text{v}\beta_1$ is the major receptor for fibronectin in fibroblasts and ECs and is responsible for fibronectin matrix assembly (29). To demonstrate the role of integrin $\alpha_\text{v}\beta_1$ in Akt1-dependent fibronectin recognition and assembly, HFFs were transfected with control GFP and myrAkt1 via adenovirus infection, then integrin activation was measured by means of HUTS-4 binding (31). The data show that expression of active Akt1 in HFFs enhanced its ability to bind to HUTS-4 ($p < 0.005$) (Fig. 6A and supplemental Fig. S4), indicating that Akt1 regulates activation of integrin $\beta_1$. Additionally, pre-treatment of HFFs with TS2/16, a $\beta_1$-integrin-stimulating antibody, corrected the impaired adhesion of SH5-treated HFFs to fibronectin ($p < 0.005$) (Fig. 6, B and C).

Next, we assessed whether there is a direct link between Akt1-dependent integrin activation and fibronectin assembly. To this end, we transfected HFFs with myrAkt1 and DN-Akt1 using retroviral infection (Fig. 7, A and B). Fibronectin assembly was then assessed in the transfected cells in the presence or absence of $\beta_1$-blocking antibodies. Representative images of fibronectin assembly and quantitative results are shown in Fig. 7, C and D, respectively. Untransfected cells were able to support fibronectin assembly into matrix at a relatively modest level ($\sim 14\%$ of total area was fibronectin-positive). Treatment with $\beta_1$ integrin blocking antibody decreased the fibronectin-positive area to 7.5% ($p < 0.001$). Transfection with myrAkt1 resulted in a 2-fold increase in the fibronectin-positive area, and this increase was completely inhibited by treatment with integrin $\beta_1$-blocking antibody ($p < 0.009$). In addition, the area of fibronectin assembly by fibroblasts expressing constitutively active Akt1 was brought back to the basal level observed in the control ($p < 0.004$) (Fig. 7D). This indicates that myrAkt1-stimulated matrix assembly is mediated by activation of $\beta_1$ integrin.

In contrast, inhibition of Akt signaling by the expression of an inactive form of Akt1 resulted in down-regulation of fibronectin assembly, compared with control ($p < 0.001$) (Fig. 7D). The residual level of matrix assembly ($\sim 10\%$ of the total area) by DN-Akt1-expressing fibroblasts was further inhibited by integrin $\beta_1$-blocking antibody ($\sim 6\%$ of the total area; $p < 0.001$) (Fig. 7D). Together, our data demonstrate that Akt1 regulates fibronectin matrix assembly via activation of integrin $\beta_1$. To the other end, we utilized $\beta_1$ integrin-specific antibody TS2/16, which is known to activate this integrin (32). Activation of $\beta_1$ integrins by exogenously added TS2/16 was able to correct defective cell adhesion resulting from treatment with SH5 ($p < 0.0005$) (Fig. 7, C and D). Importantly, integrin activation was able to override the inhibition of fibronectin assembly caused by SH5 treatment. Akt inhibitor SH5 impaired fibronectin assembly by fibroblasts, and this response was completely

![FIGURE 6. Akt1 is essential for the activation of $\beta_1$ integrins in fibroblasts. A, HFFs were infected with adenovirus encoding control GFP or myrAkt1 and were then subjected to FACS analysis for HUTS-4 antibody binding, which specifically binds to the active form of integrin $\beta_1$. Bar = 50 $\mu$m. B, HFFs were treated with SH-5 (1 $\mu$g/ml) and/or integrin $\beta_1$-stimulating antibodies (TS2/16, 1 $\mu$g/ml) for 30 min in suspension and were subjected to adhesion assay on fibronectin coated wells. Bar = 50 $\mu$m. C, quantification of HFFs adhered to fibronectin-coated wells after treatment in suspension with SH-5 (1 $\mu$g/ml) and/or integrin $\beta_1$-stimulating antibodies (TS2/16, 1 $\mu$g/ml) for 30 min.

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restored by TS2/16 treatment (Fig. 7, C and D). Thus, we established a causative link between Akt-dependent activation of integrin β₁ and fibronectin assembly into matrix by fibroblasts. Reconstitution of Akt1 in Akt1−/− ECs and Fibroblasts Corrected the Impaired Migration and Fibronectin Matrix Assembly—Because we observed defective integrin activation and fibronectin...
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A

B

C

FIGURE 8. Reconstitution of Akt1 in Akt1−/− ECs and fibroblasts rescues the impaired migration on fibronectin and impaired fibronectin assembly, respectively. A, WT and Akt1−/− ECs were infected with adenovirus expressing GFP or myrAkt1, respectively, and were subjected to 12 h scratch wound assay on fibronectin. B and C, WT and Akt1−/− MEFs were infected with adenovirus expressing GFP or myrAkt1 and subjected to assembly of exogenously supplied fibronectin. Cells were fixed, stained for fibronectin, and analyzed using microscopy and Image ProPlus analysis.

In parallel, expression of myrAkt1 in ECs and fibroblasts can save the phenotype. To do this, we expressed myrAkt1 in Akt1−/− ECs and fibroblasts and determined whether restored Akt1 activity is able to rescue the impaired ability of Akt1−/− cells to migrate on fibronectin and mediate fibronectin assembly. Our data show that reconstitution of Akt1 in Akt1−/− ECs resulted in enhanced migration on fibronectin (p < 0.006) while expression of control GFP in WT and Akt1−/− cells had no effect (Fig. 8A and supplemental Fig. S5). In parallel, expression of myrAkt1 in Akt1−/− fibroblasts corrected their impaired ability to perform assembly of exogenous fibronectin into functional matrix (p < 0.0003) (Fig. 8, B and C).

DISCUSSION

In this study, we tested the hypothesis that Akt1, a major downstream target of PI3K activity, mediates extracellular matrix recognition as well as its assembly. We report that deficiency of Akt1 results in impaired adhesion and migration of ECs to various matrix proteins, including fibronectin, vitronectin, and fibrinogen. Soluble ligand binding studies revealed that Akt controls integrin activation. Likewise, an absence of Akt1 in fibroblasts resulted in impaired adhesion and migration on fibronectin, a major ligand for α5β1 integrin. Importantly, not only recognition of but assembly of fibronectin was also impaired by Akt1 null fibroblasts, cells expressing a dominant negative Akt or upon treatment with Akt inhibitor. In contrast, expression of constitutively active Akt1 in fibroblasts enhanced fibronectin assembly, which was inhibited by anti-integrin antibodies. Using antibodies that specifically detect the active form of integrin β1 in fibroblasts, we were able to show that activation of integrin β1 is inhibited in the absence of Akt1. Moreover, the impaired cell migration and fibronectin assembly exhibited by the Akt1−/− cells was corrected by pre-treatment of these cells with TS2/16, antibodies that specifically activate β1 integrins upon binding. Thus, the defective fibronectin assembly in the absence of Akt1 was due to the impaired ability of the fibroblast’s integrins to recognize this matrix protein. Altogether, our results show that Akt1 is necessary for integrin activation on ECs and fibroblasts, a process facilitating extracellular matrix assembly, as well as migration and adhesion to it. These findings provide a molecular mechanism explaining a series of extracellular matrix abnormalities observed in Akt-deficient mice (1, 2) as well as in animals overexpressing myrAkt for a prolonged time (33).

Phosphorylation and activation of Akt in ECs occurs downstream of PI3K following treatment with growth factors (including bFGF, insulin-like growth factor-I, or VEGF) or by plating the cells on extracellular matrix proteins such as fibronectin or fibrinogen. Akt signaling is complicated due to the presence of three different isoforms with similar substrate specificities (34), but which differ in their function due to differential localization and levels of expression (1). Most of the studies are centered on the functional importance of overall Akt activity in various cell types without sufficient consideration of the roles of individual Akt isoforms, despite the obvious significance for specific therapeutic targeting. Our recent study showed that absence of Akt1 in ECs resulted in a nearly 70% reduction in basal Akt activity (2). Being the predominant isoform, Akt1 might regulate multiple functions in ECs as well as in fibroblasts. In this report we demonstrate that the absence of Akt1 activity affects the ability of ECs to recognize ECM proteins via integrins. Our results show that Akt1−/− ECs adhere to fibronectin, vitronectin, and fibrinogen with lower affinity compared with WT. Impaired adhesion of Akt1−/− ECs on extracellular matrix indicates defective inside-out activation of α5β1 and αvβ3 integrins on ECs. Experiments with soluble ligand or the genetically engineered probe (WOW-1) for the active conformers of αvβ3 and α5β1 on ECs and HUTS-4 for
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α5β1 in fibroblasts showed that Akt1, indeed, regulates integrin activation or inside-out signaling in ECs. Using a series of Akt constructs, we have demonstrated that Akt activation is directly linked to the functional activity of several integrins and, as a consequence, to the ability of cells to interact with extracellular matrix components, such as fibrinogen, fibronectin, and vitronectin, but not laminin. Interestingly, VEGF stimulation had no effect on cellular interactions with laminin, indicating that laminin receptors might exist in an active state and do not require stimulation with growth factors for their activation. Consequently, the lack of Akt signaling did not affect cellular interactions with laminin. Overall, these results clearly show that Akt1 is an essential intermediate signaling molecule involved in the regulation of inside-out activation of several integrins on ECs and fibroblasts.

A number of studies have shown that inhibition of PI3K in ECs results in cell detachment from matrix and anoikis (1, 3). Because anoikis is known to be integrin-dependent, it appears that the PI3K pathway might control integrin function and, as a consequence, the susceptibility of cells to apoptosis. This study documents that inhibition of Akt activity in ECs results in decreased affinity of integrins to ECM proteins, which eventually might make cells vulnerable to apoptosis. Our findings predict the possibility of increased apoptotic events in endothelium in the absence of Akt. Thus, the Akt pathway plays an anoikis/apoptosis protective role via regulation of integrin activity.

An important feature of ECs is their ability to form capillary tube-like structures both in vitro and in vivo (35). Formation of capillary tubes is dependent on cellular ability to migrate on various matrix proteins (36). The process of endothelial migration, in turn, is triggered by growth factor stimulation and mediated by activated integrins (4). In endothelial monolayer wound healing assays Akt1−/−ECs were unable to directionally migrate and heal the wounds on fibronectin, vitronectin, and fibrinogen. Thus, Akt1 signaling is necessary to regulate directional migration of ECs on fibronectin, vitronectin, and fibrinogen. As expected from results of our adhesion experiments, no difference between migration of WT and Akt1−/−ECs was observed when gelatin and laminin were used as a substrate, even at high concentrations, implying that laminin receptors might be active independently of the Akt pathway. Altogether, our results show that Akt1 activity controls inside-out activation of integrins and subsequent adhesion and migration of ECs on ECM proteins.

It is important to emphasize that integrins are not only able to recognize extracellular matrix but also contribute to its assembly (5). Our recent studies showed that secretion and organization of collagen and expression of laminin are impaired in Akt1 deficient mice (1, 2). These defects lead to the formation of leaky blood vessels with basement membrane abnormalities and collagen-deficient skin (2). Interestingly, skin defects are even more severe in double Akt1/Akt2 knock-out mice resulting in translucent skin (37). Because fibroblasts are known to secrete and organize extracellular matrix and the latter response is known to be integrin-dependent (5), we hypothesized that abnormalities observed in Akt1 knock-out mice could be due to impaired ability of the Akt1−/−fibroblasts to bind to and organize matrix proteins as a result of impaired integrin activation. Hence, we focused on the role of Akt1 in the activation of integrins in fibroblasts. In mouse embryonic fibroblasts, bFGF but not VEGF stimulated activation of Akt. Despite the modest increase in the levels of Akt2 and Akt3 in Akt1−/−fibroblasts, an overall level of total Akt phosphorylation and expression was decreased indicating the lack of compensation by other Akt isoforms. As was observed in ECs, a reduction in Akt activity was expected to cause impaired matrix recognition by the fibroblasts. Indeed, Akt1−/−fibroblasts exhibited impaired adhesion on fibronectin, a specific ligand for α5β1, a major integrin receptor expressed on fibroblasts, when compared with WT cells. Moreover, Akt1−/−fibroblasts exhibited impaired migration on this substrate. This indicates that abnormal adhesion and migration of Akt1−/−fibroblasts are due to impaired activation of integrin α5β1.

Extracellular matrix acts as “informational glue,” and integrins sense the changes in matrix and convey bi-directional signals, which, in turn, regulate cell behavior. ECM proteins such as fibronectin or collagen, present in skin and the basement membrane of the blood vessels, form distinct protein networks that show tissue-specific alterations in composition and architecture (38). Fibronectin is a high molecular weight multidomain glycoprotein present in plasma and intercellular spaces and has multiple functions as ligand for various integrins, including α5β1 and α6β1 (39). Fibroblasts mediate organization of soluble fibronectin into a highly structured, disulfide cross-linked pericellular matrix containing other matrix components (29). Fibronectin binds to various β1 integrins such as α5β1, α5β1, α5β1, α5β1, α5β1, and α5β1 (10). Among these integrin α5β1 has been shown to be the most important for the assembly of fibronectin matrix (29). Integrin α5β1 binds newly secreted soluble fibronectin and links it to the actin cytoskeleton, which is an important step in fibronectin fibrillogenesis (40, 41). Because integrin-dependent functions such as adhesion and migration were impaired by the lack of Akt1 in fibroblasts, one might predict that the ability of these cells to assemble fibronectin fibrils would be impaired as well. Using multiple approaches we have documented that, compared with WT, fibroblast assembly by Akt1−/−fibroblasts is impaired. Similarly, we also observed impaired fibronectin assembly by NIH 3T3 fibroblasts expressing DN-Akt1 (kinase dead-Akt1). In sum, our results show that Akt1 mediates fibronectin matrix assembly by the fibroblasts through regulation of fibronectin recognition and migration.

Another important factor that determines effective matrix assembly is the amount of matrix secreted by the vascular cells (42). Even though ECs and smooth muscle cells are able to secrete low levels of matrix proteins (43), fibroblasts are the major source of extracellular matrix in skin and blood vessels (5, 29). A short-term assay using exogenously provided fibronectin allowed us to differentiate between the effects of Akt1 on integrin activity from the direct regulation of fibronectin secretion. This analysis documented that the Akt pathway regulates fibronectin assembly. This shows that Akt1-regulated fibronectin recognition and migration can determine the course of fibronectin assembly by the fibroblasts. The latter process is
essential for wound healing and numerous pathological conditions dependent on tissue remodeling and repair.

The modular structure of fibronectin consists of 12 type-I modules, two type-II modules, and 15–17 type-III modules (29). It has been shown previously that the 70-kDa N terminus (with 5 type-I repeats), which binds to collagen and fibrinogen/fibrin (44, 45), and the integrin αβ1 binding sequence Arg-Gly-Asp (RGD) in the repeat-III10 domain of fibronectin (44, 45) are essential for its proper assembly. Importantly, we were able to show that impaired fibronectin assembly by SH5 treatment in HFFs could be corrected by pre-treatment with anti-β1 integrin-stimulating antibodies and that enhanced fibronectin assembly caused by expression of myrAkt1 was inhabitable by anti-β1 integrin-blocking antibodies. Thus, Akt1 regulates fibronectin assembly into matrix by modulation of functional activity of β1 integrin on fibroblasts. Finally, we show that reconstitution of Akt1−/− ECs and fibroblasts with active Akt1 corrected the abnormalities in migration and fibronectin assembly, respectively, thus demonstrating that Akt1 is necessary for activation of integrins, cell migration, and matrix assembly. Our finding that the Akt pathway regulates recognition as well as organization of extracellular matrix has numerous implications in physiology and pathophysiology and opens new avenues for the development of therapeutic strategies focused on tissue repair.

Acknowledgments—We thank L. Mavrakis and A. Money for supplying human foreskin fibroblasts and HUVECs, harvested through the Birthright Services Dept. at the Cleveland Clinic Foundation and Perinatal Clinical Research Center at MetroHealth Hospital. We also acknowledge the technical assistance by Joseph Mudd, summer student, in the retroviral infection of human foreskin fibroblasts.

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