FIG. 6. GATA-3 expression is induced by PPARγ ligand, rosiglitazone. Rosiglitazone (30 mg/kg) or control vehicle in triplicate was administered by daily gavage 1 day before administration of 2.5% DSS. Western blot analysis was performed using whole colon tissue obtained from day 7 of DSS treatment. Equal amounts of protein were loaded per lane.

and inducing Th2-associated cytokines and transcription factors in a model of colitis associated with disruption of the intestinal epithelial cell barrier. Previous experiments in the DSS model of colitis have revealed different inflammatory responses when administered chronically (29,40,41), or acutely, as performed here. Whereas the chronic form of DSS colitis has been demonstrated to represent a Th1- and Th2-associated inflammation (41), the acute form of DSS administration has been shown to represent a predominantly Th1 model of inflammation (42). Thus in the latter case, the TZDs appear to shift the cytokine balance to a noninflammatory Th2 response. Therefore, based on our findings in the DSS model of acute colitis, and previously described studies of colitis associated with the TNBS model (38), PPARγ likely plays an important role in down-regulating Th1 intestinal inflammation.

The increase in susceptibility to colitis in the PPARγ heterozygote, as also observed here, also suggests that PPARγ normally provides protection against intestinal inflammation and injury. Another potential possibility is that a lack of PPARγ results in constitutively increased production of innate inflammatory mediators leading to increased inflammation. However, against this latter hypothesis is the fact that no increase in endogenous inflammation was observed in the heterozygotic mice.

During the initiation phase of DSS colitis, intestinal macrophages/dendritic cells appear to play a particularly critical role by driving inflammation through the production of mediators associated with innate immunity (43), such as iNOS (44). DSS-stimulated macrophages also can abnormally activate $CD4^+$ T cells (45,46) promoting the development of a Th1 response. It is very likely that IL-12 plays a crucial role in this outcome. In this regard, PPARγ has been shown to inhibit IL-12 expression by macrophage/dendritic cells (34,47), and DSS colitis can be exacerbated through the presence of excess IL-12 by increasing IFNγ levels (48). Moreover, our observation that there was no effect by PPARγ agonist ligands on acute DSS colitis when administered the day after colitis onset is consistent with the concept that PPARγ activation primarily impacts the initiation phase of the inflammation. In support of this finding, studies in the predominant Th1-driven TNBS model of colitis also suggested that PPARγ activation resulted in greater inhibitory effects when initiated the day before, rather than the day of, TNBS administration (38).

Based on these findings, it can be suggested that activation of macrophage/dendritic cells is the major factor in influencing the development of the acute inflammatory colitis associated with DSS administration. This hypothesis is supported by the observation that DSS colitis can occur in severe combined immunodeficiency mice (49) and recombinase activase gene-deficient mice (39), which lack both T and B cells. T cells may, however, potentially play a role in augmenting or reducing the continued inflammation initiated by activation of the antigen-presenting cells (50). Support of this modulatory function of T cells in the DSS colitis model is noted by inhibitory effects of TNFα blockade, albeit macrophages may be a source of this cytokine as well (51,52). Since PPARγ activation can directly reduce the levels of TNFα by inhibition of (NFκB) complex activity (26,30), the observed effects of PPARγ ligation in reducing DSS-induced inflammation as shown here may be through this mechanism as well as direct inhibition of macrophage/dendritic cell function.

Another potential mechanism for the reduction in inflammation and cytokine shift away from a Th1 inflammatory state towards a Th2 environment may be through alterations in T-cell differentiation. As suggested, the development of a Th1 cell response is most likely curtailed by the effects of PPARγ on the macrophage/dendritic cell's ability to produce IL-12. Since sustained TNFα levels can induce IL-12 production, PPARγ-associated reduction of TNFα levels may also indirectly reduce IL-12 and contribute to the development of a Th1 response (53). Also, IL-12 maintains activated Th1 cells, and a reduction of IL-12 could lead to an increase in Th1 apoptosis and affect the Th1/Th2 cell populations (54,55). Alternatively, PPARγ activation could directly result in driving naïve cells to develop into Th2 cells. Very little is known about the effects of PPARγ activation on Th2 cytokine production or T-cell differentiation. In one study, the PPARγ activator prostanoid, 15-δPGJ_2 (47), has been shown to enhance IL-10 inhibition of macrophages. In addition, IL-4 has been repeatedly demonstrated to induce PPARγ expression (56–60). Our findings would then suggest that Th2 cytokines may be regulated by, and also potentially regulate, PPARγ. Consistent with this shift from Th1 to Th2 cytokine production in the presence of PPARγ-activation is our detection of increased levels of the transcription factor GATA-3 in the PPARγ-treated mice. Along with

c-Maf, GATA-3 can influence the differentiation of naïve T cells into Th2 cells (33). Along with increasing Th2 development, PPARγ activation may concomitantly inhibit Th1 development, as PPARγ can directly inhibit NFAT activity, which is also involved in Th1 cell differentiation (36).

In the current study, we also observed differences in the clinical efficacy of the TZD class of molecules in the acute DSS-induced colitis model. The different dose responses observed with the PPARγ ligands as shown here may be due to either their affinity levels for PPARγ or with differences in the conformations induced by the different TZD ligands on the PPARγ receptor. For example, in vitro studies have indicated that pioglitazone has a stronger affinity to PPARγ then does rosiglitazone (61). We noted, however, the opposite effects on colitis in our studies suggesting the latter mechanism may be dominant. Similarly, even though relatively equivalent doses of troglitazone were used, this TZD clearly had significantly less of a clinical effect. This difference in efficacy between troglitazone and rosiglitazone has been observed in other studies and is confirmed in the present report (29,38). Troglitazone has affinities for both PPARγ and PPARα (62), which may contribute to its hepatotoxicity in clinical use. Thus, it is becoming increasingly evident that different PPARγ agonist ligands can have different clinical effects despite binding to a common receptor (63). The different effects require interpretation in terms of potential future therapy of these agonists in IBD.

CONCLUSION

In conclusion, PPARγ activation represents a novel mechanism for reducing the development of a Th1 inflammatory response and shifting it towards a Th2 response. Based on the data presented here and previous studies by others, it can be suggested that PPARγ activation may affect a number of pathways involved with down-regulating intestinal tissue inflammation associated with excessive Th1 cytokine production. These probably include inhibition of NFκB activation, down-regulation of Th1 cytokines, up-regulation of Th2 cytokines, and regulation of Th1 and Th2 cell differentiation. Furthermore, these effects vary with respect to the specific TZD analog studied, suggesting significant differences may exist between these agents and their potential use in human inflammatory conditions such as IBD.

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Targets of Transcriptional Regulation by Two Distinct Type I Receptors for Transforming Growth Factor-β in Human Umbilical Vein Endothelial Cells

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Transforming growth factor-β (TGF-β) plays a crucial role in vascular development and homeostasis by regulating many transcriptional targets. Activin receptor-like kinase 5 (ALK-5) is a TGF-β type I receptor expressed in various TGF-β-responsive cells. In contrast, ALK-1 functions as a TGF-β type I receptor in endothelial cells, and is responsible for human hereditary hemorrhagic telangiectasia (HHT) type II. ALK-5 and ALK-1 mediate TGF-β signals through distinct Smad proteins, i.e., Smad2/Smad3 and Smad1/Smad5, respectively. To identify target genes of ALK-1 and ALK-5 in endothelial cells, we conducted oligonucleotide microarray analysis. Human umbilical vein endothelial cells (HUVEC) were infected with recombinant adenoviruses carrying a constitutively active form of ALK-1 or ALK-5. ALK-5 inhibited the proliferation, network formation, and tube formation of HUVEC and induced their apoptosis, whereas ALK-1 did not exhibit significant effects on HUVEC in vitro. mRNAs were extracted from HUVEC and used for hybridization of oligonucleotide arrays representing approximately 7,000 human genes. Northern blot and quantitative real-time polymerase chain reaction (PCR) analyses were also performed for some of these genes, confirming the validity of this microarray analysis. We found that ALK-1 specifically upregulated Smad6, Smad7, Id1, Id2, endoglin, STAT1, and interleukin 1 receptor-like 1. ALK-5, in contrast, upregulated PlGF, SM22α, connexin 37, βIG-H3, and LTBP1. ALK-1 downregulated Smad1, CXCR4, Ephrin-A1, and plakoglobin, whereas ALK-5 downregulated claudin 5 and integrin β_5. These results revealed some new targets of TGF-β in endothelial cells, and differences in transcriptional regulation patterns between ALK-1 and ALK-5.
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Transforming growth factor-β (TGF-β) belongs to a large superfamily of related polypeptides, including activins, bone morphogenetic proteins (BMPs), growth/differentiation factors, and anti-Müllerian hormone (Heldin et al., 1997). TGF-β is involved in diverse biological processes, such as cell proliferation, migration, differentiation, survival, and cell–cell and cell–matrix interaction. TGF-β plays a very important role in the development of the vascular system, affecting functions of both endothelial cells and periendothelial cells (Carmeliet, 2000). The cellular responses mediated by TGF-β are complex and can be stimulatory or inhibitory, depending on cell type and conditions. TGF-β generally inhibits proliferation and migration of endothelial cells, but potently stimulates extracellular matrix (ECM) production. Importantly, TGF-β is essential for the recruitment and regulation of pericytes and smooth muscle cells, which lead to vascular maturation and stabilization (Pepper, 1997).

TGF-β superfamily members bind to heteromeric complexes of type I and II transmembrane serine/threonine kinase receptors (Heldin et al., 1997). Upon ligand binding, type II receptors recruit and transphosphorylate type I receptors, which subsequently activate downstream signal mediators, Smads. Only one TGF-β

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type II receptor (TβR-II) is present in mammals, whereas two type I receptors appear to serve as TGF-β type I receptors. Activin receptor-like kinase 5 (ALK-5) is a TGF-β type I receptor ubiquitously expressed in TGF-β-responsive cells. ALK-5 mediates TGF-β signaling through phosphorylating Smad2/Smad3. In addition, another type I receptor, ALK-1, is expressed in endothelial cells and binds to TGF-β and possibly other ligands (Lux et al., 1999; Oh et al., 2000). ALK-1 signals are transduced through Smad1/Smad5, which are major downstream mediators of BMPs. Thus, in endothelial cells, ALK-5 and ALK-1 mediate TGF-β signaling through distinct transcriptional regulation of target genes.

Previous investigations have shown that mutations of the ALK-1 gene are linked to human hereditary hemorrhagic telangiectasia (HHT) type II, also known as Osler–Rendu–Weber syndrome (Johnson et al., 1996). In addition, endoglin, an accessory receptor modulating TGF-β signals, has been reported to be responsible for HHT type I (McAllister et al., 1994). HHTs are autosomal dominant disorders, characterized by recurrent bleeding in mucosal telangiectasias, and arteriovenous malformations in lung, brain, and liver (Jacobson, 2000). HHT patients have dilated blood vessels with thin walls, and exhibit abnormal arteriovenous fusion and shunting.

Studies by gene targeting have revealed that disruption of components of the TGF-β signaling pathway, including TGF-β1 (Dickson et al., 1995), TβR-II (Oshima et al., 1996), and ALK-5 (Larsson et al., 2001), resulted in severe vascular abnormalities in mice. In addition, mice lacking endoglin (Li et al., 1999), ALK-1 (Oh et al., 2000; Urness et al., 2000), or Smad5 (Chang et al., 1999; Yang et al., 1999) exhibit similar phenotypes in vascular tissues, although they are not identical to those of TGF-β1-, TβR-II-, and ALK-5-null mice. These mice have overlapping vascular phenotypes characterized by distended vessels and defects of smooth muscle cell differentiation. It has been reported that the formation of primary capillary plexuses is relatively normal, whereas sprouting and branching of vessels are impaired in these mice (Larsson et al., 2001).

TGF-β signaling is thus essential for vascular development and maturation, but the mechanisms of transcriptional regulation of this signaling have not been clearly defined. To determine targets of TGF-β mediated by the two type I receptors, ALK-1 and ALK-5, we performed oligonucleotide microarray analysis. Human umbilical vein endothelial cells (HUVEC) infected with a constitutively active form of ALK-1 or ALK-5 were used in this study. This approach allowed us to identify novel target genes of TGF-β, and provided insights into the regulation of vascular development by ALK-1 and ALK-5.

MATERIALS AND METHODS

Cell culture and adenovirus infection

HUVEC were obtained from Sanko Junyaku (Tokyo, Japan), and cultured on type I collagen-coated dishes at 37°C in a 5% CO_2 atmosphere in endothelial basal medium (EBM) containing 2% fetal bovine serum (FBS) and endothelial cell growth supplements (Clonetics Corp., East Rutherford, NJ). HUVEC were used between passages 2 and 5. Recombinant adenoviruses with β-galactosidase as a control, or with a constitutively active form of ALK-1 or ALK-5, were infected into HUVEC at a multiplicity of infection (m.o.i.) of 100 pfu/cell except for growth and tube formation assays (see below). Appropriate m.o.i. had been previously determined by β-galactosidase assay.

Growth assay

Cells were seeded at a density of 1×10^5 cells per well in 6-well plates and cultured as described above. The next day, cells were infected with adenoviruses at a m.o.i. of 50 and 100 pfu/cell. After 48 h, cells were trypsinized and the numbers of cells were counted by a Coulter counter. The experiments were performed in duplicate.

DNA fragmentation assay

HUVEC infected with adenoviruses containing β-galactosidase or constitutively active ALK-1 or ALK-5 at a m.o.i. of 100 pfu/cell were cultured for 48 h as described above. Cells were lysed with 20 mM Tris-HCl, pH 7.5, 10 mM EDTA, and 0.5% Triton X-100, and the lysates were treated with 0.2 mg/ml proteinase K and 0.1 mg/ml RNase for 1 h at 42°C to remove protein and RNA. DNA was extracted from the lysates and subjected to electrophoresis on a 2% agarose gel.

Network formation assay

Cells were cultured and infected with adenoviruses at a m.o.i. of 100 pfu/cell as described above. Forty-eight hours after infection, cells were trypsinized and resuspended in Dulbecco's modified Eagle's medium (DMEM) with 5% FBS. Cell suspension was seeded at a density of 3×10^5 cells per dish on a 35-mm Matrigel (Becton Dickinson Labware, Bedford, MA). Cells on Matrigel dishes were incubated for 12 h and examined by phase-contrast microscopy.

Tube formation assay

Cells were trypsinized and suspended at a final concentration of 1×10^6 cells/ml in a 7:2:1 mixture of type I collagen solution (Nitta Gelatin, Osaka), five times DMEM and 0.05 N NaOH solution containing 2.2% $NaHCO_3$ and 200 mM HEPES. Five hundred microliters of this preparation was placed in 24-well plates and incubated for 30 min at 37°C in a humidified incubator to allow polymerization. After gel formation, EBM containing 2% FBS, endothelial cell growth supplements, and 20 ng/ml of vascular endothelial growth factor (VEGF; R&D Systems, Minneapolis, MN) were added and adenoviruses were infected at a m.o.i of 200 pfu/cell. Appropriate m.o.i. in collagen gels had been previously determined by β-galactosidase assay. Cells immersed in collagen gels were cultured for 7 days and examined by phase-contrast microscopy.

Immunoblotting analysis

Cells were infected with adenoviruses as described above. The cells were solubilized in a buffer containing 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 1% aprotinin, and 1 mM phenylmethyl sulfonyl fluoride, and the cell lysates were subjected to SDS–PAGE. Proteins were electrotransferred to FluoroTrans

W membranes (Pall), immunoblotted with anti-HA 3F10 (Roche Diagnostics-Gelman, Ann Arbor, MI), anti-phospho-Smad1/5/8, or anti-phospho-Smad3 antibodies (Sugizaki, T., unpublished data), and detected using an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech, Piscataway, NJ).

RNA extraction

Forty-eight hours after infection with recombinant adenoviruses, total RNAs were extracted from HUVEC using ISOGEN (Nippon Gene, Toyama). mRNAs were isolated from total RNAs with oligotex dT-30 Super latex beads (TaKaRa Biochemicals, Tokyo), following the manufacturer's protocol.

Oligonucleotide microarray analysis

We conducted oligonucleotide microarray analysis using GeneChip (Affymetrix, Santa Clara, CA) as described previously (Akiyoshi et al., 2001). Two micrograms of mRNAs were used for the preparation for probes hybridized to a GeneChip array containing approximately 7,000 human genes. From measured hybridization intensities, average difference and absolute call were calculated by GeneChip software of Affymetrix. Average difference directly reflects gene expression level, and absolute call determines whether gene expression is present or absent compared with mismatch probe intensity as a control for cross-hybridization. Fold change was also calculated by taking the difference between control and sample average difference intensities. However, it should be noted that fold change does not necessarily match the ratio of average differences, since the mathematical equations used to determine it take other factors into consideration. A negative fold change value indicates that gene expression has decreased to below the control level. In this analysis, we considered twofold change as the threshold for significant change in accordance with the manufacturer's recommendations. To more accurately detect genes with significant change in expression, we excluded genes whose average differences were less than 100 and absolute calls were absent after increase or before decrease. However, we extracted important genes involved in vascular function whose intensities were less than 100 or absolute calls were absent. Detailed information is available at the following URL: (http://www2.genome.rcast.u-tokyo.ac.jp/database/db.asp).

Northern blotting

Two micrograms of mRNAs extracted from HUVEC were electrophoresed on a 1% formaldehyde-agarose gel. The RNAs were transferred to a Hybond-N membrane (Amersham Pharmacia Biotech) and cross-linked by UV irradiation. α^{32}P-dCTP-labeled probes were synthesized with a Ready-To-Go Kit (Amersham Pharmacia Biotech) and hybridized to the membrane at 65°C overnight in a solution containing five times SSC, five times Denhardt's solution, 0.5% SDS, and 100 μg/ml salmon sperm DNA. The membrane was washed for 20 min at 65°C in two times SSC with 0.1% SDS, then in one times SSC with 0.1% SDS, and in 0.1 times SSC with 0.1% SDS. The blots were exposed to imaging films and analyzed with a FUJI BAS 2500. The membrane was stripped by washing in 50% formamide and one times SSC at 65°C for at least 3 h, and then used for successive hybridization as described above. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probe was used as an internal control for RNA loading.

Quantitative real-time reverse transcriptase-polymerase chain reaction (RT-PCR) analysis

HUVEC were infected with recombinant adenoviruses carrying LacZ, ALK-1 or ALK-5, or treated with 5 ng/ml of TGF-β3 (R&D Systems). Total RNA was isolated, and first-strand cDNA was synthesized using the Superscript First-Strand Synthesis System (Invitrogen, Carlsbad, CA) with random hexamer primers. Quantitative real-time RT-PCR analysis was performed using GeneAmp 5700 Sequence Detection System (Applied Biosystems, Foster City, CA). One microliter of the diluted first strand cDNA product was amplified in triplicate in a 25-μl reaction containing 12.5 μl of SYBR Green PCR Master Mix (Applied Biosystems) and 10 pmol of each primer. The primer sequences are available upon request. The PCR program consisted of the following two steps. The first step was an initial denaturation where the reaction was incubated for 10 min at 95°C. In the second step, DNA was amplified for 35 cycles of 15 sec at 95°C, and 1 min at 60°C. The data generated from each PCR reaction were analyzed using GeneAmp 5700 SDS software (Applied Biosystems).

RESULTS AND DISCUSSION

Effects of ALK-1 and ALK-5 on HUVEC in vitro

Since TGF-β is a potent inhibitor of cell growth and an inducer of apoptosis, we first examined the effects of ALK-1 and ALK-5 on the growth and apoptosis of HUVEC in vitro. Forty-eight hours after infection of ALK-5-adenovirus, growth of HUVEC was significantly inhibited compared to the control cells infected with the adenovirus carrying β-galactosidase (Fig. 1A). On the other hand, ALK-1 inhibited the growth of HUVEC less potently than ALK-5. DNA ladder formation was detected only in the ALK-5-infected HUVEC, indicating that ALK-5 but not ALK-1, induced apoptosis of HUVEC (Fig. 1B).

We next examined the effects of ALK-1 and ALK-5 on differentiation of HUVEC. TGF-β inhibited the formation of tube-like structure of HUVEC in type I collagen gels (data not shown). TGF-β also inhibited the network formation of bovine aortic endothelial cells in Matrigel, although this was not remarkable in HUVEC (data not shown). We determined the network formation of HUVEC in Matrigel and the formation of tube-like structure in type I collagen gels using adenoviruses carrying constitutively active ALK-1 or ALK-5. As shown in Figure 1C,D, ALK-5 inhibited both the network and tube formation of HUVEC in vitro. In contrast, ALK-1 did not significantly affect them of these cells.

Oligonucleotide microarray analyses of genes regulated by ALK-1 and ALK-5

We next performed oligonucleotide microarray analyses of genes in HUVEC regulated by ALK-1 and ALK-5. When ALK-1-adenovirus was infected into HUVEC and the cell lysates were analyzed by immunoblotting, we found that ALK-1 protein was expressed from 8

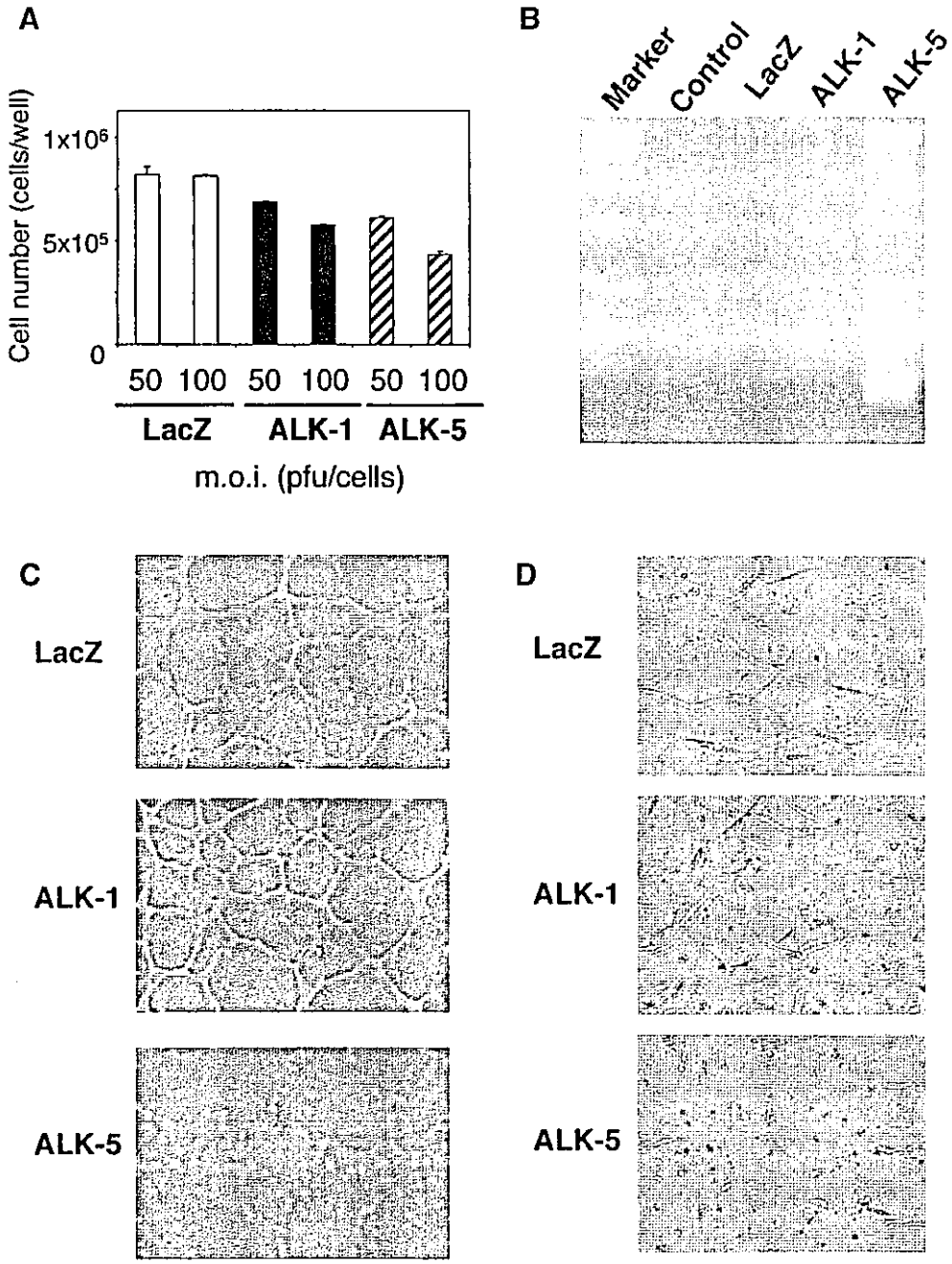


Fig. 1. Biological effects of activin receptor-like kinase 1 (ALK-1) and activin receptor-like kinase 5 (ALK-5) on human umbilical vein endothelial cells (HUVEC) in vitro. **A**: Growth inhibitory effects of ALK-1 and ALK-5 on HUVEC. Cells were infected with recombinant adenoviruses carrying LacZ as a control, or a constitutively active form of ALK-1 or ALK-5 at a m.o.i. of 50 and 100 pfu/cell. After 48 h, cell number was determined by a Coulter counter. **B**: Induction of apoptosis by ALK-5 in HUVEC. DNA from HUVEC was analyzed by agarose gel electrophoresis and visualized by ethidium bromide staining. Migration of DNA molecular weight standards is shown in the left lane. HUVEC without adenovirus infection was used as control. **C**: Network formation of HUVEC. Cells were infected with adenoviruses at a m.o.i. of 100 pfu/cell. Forty-eight hours after infection, cells were seeded on a 35 mm-Matrigel and examined by phase-contrast microscopy after 12 h. **D**: Tube-like structure formation of HUVEC. Cells were suspended in type I collagen solution, placed in 24-well plates, and incubated for 30 min at 37°C to allow polymerization. After gel formation, adenoviruses were infected at a m.o.i of 200 pfu/cell. Cells immersed in collagen gels were cultured for 7 days and examined by phase-contrast microscopy.

to 48 h after infection (Fig. 2A). Phosphorylation of Smad1/5/8, substrates of ALK-1, was detected at relatively later periods and detected most prominently at 48 h (Fig. 2A). Expression of ALK-5 and phosphorylation of its substrate Smad3 were detected from 8 to 48 h (Fig. 2B). Because of the phosphorylation profile of Smad1/5/8, we analyzed RNAs from HUVEC 48 h after infection of adenoviruses.

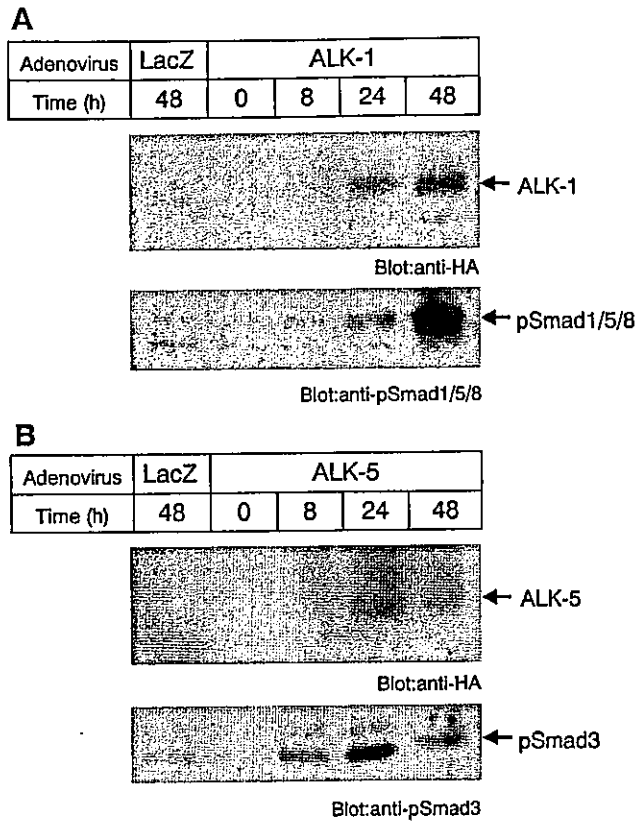


Fig. 2. Expression of activin receptor-like kinase 1 (ALK-1) and activin receptor-like kinase 5 (ALK-5), and phosphorylation of Smads in human umbilical vein endothelial cells (HUVEC) infected with the ALK-1- or ALK-5-adenoviruses. HUVEC infected with recombinant adenoviruses carrying a constitutively active form of ALK-1 (A) or ALK-5 (B) at a m.o.i. of 100 pfu/cell were harvested at different time periods, and analyzed by immunoblotting using anti-HA antibody to detect ALK-1 (A) or ALK-5 (B), or anti-phospho-Smad1/5/8 (A) or anti-phospho-Smad3 (B) antibodies.

Gene expression profiles regulated by ALK-1 and ALK-5 are listed in Tables 1–5. Several genes showed two results in GeneChip analysis, indicating that they were examined by two probe sequences covering different regions of the genes. The discrepancy between the two results may have depended on the intensities of mismatch probes used as a control for cross-hybridization, since the GeneChip intensities were calculated by taking the difference between perfect match and mismatch probes.

We identified 46 genes upregulated by ALK-1 (Table 1) and 52 genes upregulated by ALK-5 (Table 2) from among approximately 7,000 genes. On the other hand, 54 genes were downregulated by ALK-1 (Table 3) and 49 genes by ALK-5 (Table 4). Among identified genes, eight genes were induced by both ALK-1 and ALK-5 (Tables 1 and 2), whereas 14 genes were repressed by both (Tables 3 and 4). We also extracted other genes possibly involved in vascular function (Table 5). Detailed inspection of the total data suggested that ALK-1 or ALK-5 regulated most genes in an almost independent manner, and that the regulated genes were involved in various cellular functions. This may be one possible explanation for the lack of redundancy between ALK-1- and ALK-5-deficient mice, although these two types of knockout mice exhibited vascular defects with certain similarities.

Using the criteria used for this analysis, we could not detect genes exhibiting reciprocal change in regulation of expression by ALK-1 and ALK-5, suggesting that ALK-1 and ALK-5 do not control most genes in a competitive fashion. However, by lowering the threshold of significant expression level or fold change, we identified the KIAA0018 gene, whose intensity was increased by ALK-1 and decreased by ALK-5 (Table 1). On the other hand, five genes, including placental growth factor (PlGF), microfibril-associated protein 2, KIAA0123, chloride channel kidney B, and Ephrin-A1, were upregulated by ALK-5 but downregulated by ALK-1 (Tables 2 and 3).

Microarray analysis of the adenovirus-infected HUVEC was performed one time in the present study. In order to confirm the validity of the microarray analysis, we also performed Northern blot analysis (Fig. 3A–C) and quantitative real-time RT-PCR of some of the genes regulated by ALK-1 or ALK-5 (see below, Fig. 4). The results obtained from Northern blots and real-time RT-PCR correlated well with those from GeneChip intensities, which supported the validity of our criteria for screening genes in this microarray.

Genes upregulated by ALK-1

High expression of ALK-1 gene was detected in HUVEC without stimulation (Table 1). Moreover, expression of ALK-1 was strongly enhanced by infection with ALK-1-adenovirus, confirming the validity of this analysis.

ALK-1 induced inhibitory Smads, i.e., Smad6 and Smad7 (Table 1). These I-Smads can thus act as negative feedback regulators of TGF-β signaling (Nakao et al., 1997; Ishida et al., 2000). In contrast to previous reports (Afrakhte et al., 1998), neither Smad7 nor Smad6 was significantly induced by ALK-5. They may be transiently upregulated early and disappear under the conditions used in the present study (measurement 48 h after adenovirus infection). Northern blot analysis confirmed the upregulation of Smad6 and Smad7 by ALK-1 (Fig. 3A).

Inhibitor of DNA binding 1 (Id1) and Id2 were specifically induced by ALK-1 (Table 1). Id3 was highly expressed in HUVEC, although neither ALK-1 nor ALK-5 significantly affected its expression (Table 5). The expression of Id4 was very low in the presence and absence of ALK-1 or ALK-5 (Table 5). Northern blot analysis confirmed the levels of expression of Id genes (Fig. 3B). Id proteins are helix-loop-helix proteins, which dominant-negatively antagonize other basic helix-loop-helix (bHLH) transcription factors including MyoD, and regulate growth and differentiation of diverse types of cells (Norton et al., 1998). Disruption of both Id1 and Id3 genes results in vascular abnormalities with dilated vessels without sprouting or branching of capillaries (Lyden et al., 1999). These vessel deformities appear similar to those in ALK-1- or ALK-5-deficient mice. Recently, Id genes have been shown to be direct targets of BMP signaling essentially mediated by Smad1/Smad5 (Hollnagel et al., 1999) and to play important roles in

TABLE 1. Genes upregulated by ALK-1

Accession number	Description	Control	ALK-1		ALK-5	
		Intensity	Intensity	Fold change	Intensity	Fold change
Receptors and related genes						
D12763	Interleukin 1 receptor-like 1 (IL1RL1)	−61	755	>36.3	16	>4.3
L17075	ALK-1	235	2,020	8.8	181	−1.3
X72012	Endoglin	917	2,376	2.7	1,155	1.2
Growth factors and related genes						
X17094	Furin/PACE	20	160	>7.1	95	>4.3
Cell–cell and cell–matrix interaction genes						
L27624	Tissue factor pathway inhibitor 2	31	100	3.2	289	9.4
D29992	Tissue factor pathway inhibitor 2	81	174	2.1	590	6.3
L25851	Integrin αE	37	123	2.3	85	2.4
Intracellular regulators						
Cytoskeleton						
M98343	EMS1	30	144	4.8	67	2.3
M61764	Tubulin gamma 1	114	309	2.7	188	1.6
Cell cycle related genes						
U21090	DNA polymerase delta-2 regulatory subunit (POLD2)	16	100	>4.7	47	>2.3
U96915	Sin3-associated polypeptide 18-kD (SAP18)	50	147	3.0	167	3.4
X13293	MYBL2	65	180	2.8	105	1.6
J04611	Lupus autoantigen p70	131	348	2.7	301	2.3
X02596	Breakpoint cluster region (BCR)	75	198	2.6	148	2.0
X59798	Cyclin D1	163	206	2.0	175	1.7
X14850	H2AX histone	188	384	2.0	261	1.4
Signal transduction and related genes						
M97936	STAT1	−47	348	>18.2	−47	>1.0
M97935	STAT1	231	514	2.2	306	1.1
U59914	Smad6	22	272	>11.9	38	>1.7
AF010193	Smad7	39	203	6.5	62	1.6
M97796	Id2	55	311	5.7	48	−1.2
S78825	Id1	311	1,339	4.0	386	1.1
U59877	RAB31	−40	137	5.5	151	6.1
Differentiation and development related genes						
D78611	Mesoderm specific transcript (MEST)	128	547	3.6	200	1.2
Others						
X71874	Proteasome subunit MECL1	29	119	4.1	23	−1.3
D17793	Aldo-keto reductase B	56	259	3.9	90	1.6
S79639	Exostoses 1 (EXT1)	43	153	3.6	167	3.9
U32907	37 kDa leucine-rich repeat protein	63	244	3.4	247	3.5
M65292	H factor-like 1	255	755	3.0	304	1.2
U40572	Syntrophin beta 2	68	200	2.9	106	1.6
M83186	COX7A1	70	101	2.7	108	1.0
M55621	N-acetylglucosaminyltransferase 1	48	124	2.6	72	1.5
U45285	T cell immune regulator 1	63	281	2.6	195	1.6
D28473	Isoleucyl-tRNA synthetase	52	136	2.6	98	2.8
M62762	ATPase H+ transporting lysosomal	257	791	2.4	509	1.4
U28811	Golgi apparatus protein 1	167	397	2.4	214	1.3
U40369	Spermidine/spermine N1-acetyltransferase	110	305	2.3	64	−1.3
D13643	KIAA0018/Seladin-1	85	138	2.2	−32	<−4.4
X03100	Human mRNA for SB class II histocompatibility antigen alpha-chain	48	107	2.2	94	1.9
D11094	Proteasome 26S subunit ATPase 2	140	297	2.1	168	1.2
M31166	Pentraxin 3	113	235	2.1	62	−1.8
U19796	Melanoma-associated antigen recognized by T lymphocytes	82	171	2.1	95	1.2
X92814	Rat HREV107-like protein	78	163	2.1	46	−1.7
X04654	Small nuclear ribonucleoprotein 70 kD	101	214	2.1	222	2.2
L38503	Glutathione S-transferase theta 2 (GSTT2)	114	217	2.0	231	2.2
M32886	Sorcin	60	119	2.0	111	1.9
U61263	Human acetolactate synthase homolog	113	107	2.0	94	−1.1
U81802	Phosphatidylinositol 4-kinase catalytic beta	100	135	2.0	82	1.2

Low or negative values of the intensity indicate that the gene expression levels were low or absent. Negative values of the fold change indicate that the expression of the gene decreased below the control levels. A greater than sign (>) indicates that the fold change likely represents an overestimation, since the intensity of the gene was below a certain threshold in the control sample. Consequently, the fold change was increased to an arbitrary, low value by the GeneChip software. A less than sign (<) indicates that the fold change likely represents an underestimation, since the intensity of the gene was below a certain threshold in the control sample. The fold change was decreased to an arbitrary, high value by the GeneChip software.

TABLE 2. Genes upregulated by ALK-5

Accession number	Description	Control	ALK-1		ALK-5	
		Intensity	Intensity	Fold change	Intensity	Fold change
Receptors and related genes						
X66945	Fibroblast growth factor receptor 1 (FGFR1)	71	69	−1.0	170	2.4
Y09392	Tumor necrosis factor receptor superfamily member 12	84	79	−1.1	114	2.4
Growth factors and related genes						
J03242	Insulin-like growth factor 2 (IGF2)	185	94	−1.6	796	3.8
X54936	Placental growth factor (PlGF)	90	−8	<−5.3	214	2.4
M34057	Latent transforming growth factor beta binding protein 1 (LTBP1)	126	247	1.6	338	2.1
Cell–cell and cell–matrix interaction genes						
L27624	Tissue factor pathway inhibitor 2	31	100	3.2	289	9.4
D29992	Tissue factor pathway inhibitor 2	81	174	2.1	590	6.3
M77349	βIG-H3	117	139	1.0	591	7.1
M96789	Connexin 37	230	347	1.5	505	2.7
U19718	Microfibril-associated protein 2	94	−112	<−4.5	123	2.1
Intracellular regulators						
Cytoskeleton						
M95787	SM22α	−32	−107	>1.7	114	>11.4
J04029	Keratin 10	28	86	3.0	139	3.7
M83216	Caldesmon 1	72	94	−1.2	219	2.3
J02854	Myosin regulatory light chain 2 smooth muscle isoform	51	76	1.5	114	2.2
M19267	Tropomyosin 1	455	497	1.1	973	2.1
Z24727	Tropomyosin 1	638	609	−1.0	1,280	2.0
Cell cycle related genes						
U96915	Sin3-associated polypeptide 18-kD (SAP18)	50	147	3.0	167	3.4
X05855	H3 histone family 3A	67	122	1.8	165	2.5
U11791	Cyclin H	36	98	2.7	105	2.4
U31814	Histone deacetylase 2 (HDAC2)	78	103	1.3	176	2.3
X94232	APC-binding protein RP1/EB2	100	151	1.5	231	2.3
J04611	Lupus autoantigen p70	131	348	2.7	301	2.3
X63692	DNA methyltransferase 1	51	69	2.0	103	2.1
X02596	Breakpoint cluster region (BCR)	75	198	2.6	148	2.0
Signal transduction and related genes						
X54637	Tyrosine kinase 2	27	35	1.3	104	3.9
X12953	RAB2	63	111	1.8	315	3.4
X70683	SOX4	104	135	−1.0	248	2.4
U37146	Nucleor receptor corepressor 2/SMART	245	329	1.0	500	2.0
X07767	Protein kinase cAMP-dependent catalytic alpha	71	102	1.4	139	2.0
U78313	MyoD family inhibitor	50	54	1.1	135	2.0
Others						
D50913	KIAA0123	−8	−232	<−10.8	202	>10.1
S79639	Exostoses 1 (EXT1)	43	153	3.6	167	3.9
U32907	37 kDa leucine-rich repeat protein	63	244	3.4	247	3.5
U06155	Human chromosome 1q subtelomeric sequence D1S553	43	60	1.4	146	3.4
Z30644	Chloride channel kidney B	78	11	<−3.9	266	3.4
X87212	Cathepsin C	187	254	1.4	464	2.8
U16954	ALL1-fused gene from chromosome 1q	176	319	1.8	474	2.7
U55206	Gamma-glutamyl hydrolase	43	69	3.0	110	2.6
U86602	Nucleolar protein p40	40	97	2.4	103	2.6
M19961	COX5B	232	317	1.4	283	2.4
Y50939	Amyloid beta precursor protein-binding protein 1	48	85	1.8	117	2.4
U45328	Ubiquitin-conjugating enzyme E21 (UBE21)	146	211	1.4	328	2.3
U27460	UDP-glucose pyrophosphorylase 2	67	95	1.4	183	2.3
L38503	Gluathione S-transferase theta 2 (GSTT2)	114	217	2.0	231	2.2
X01630	Argininosuccinate synthetase	50	96	−1.9	109	2.2
X79865	Mitochondrial ribosomal protein L12	60	84	2.9	135	2.2
X04654	Small nuclear ribonucleoprotein 70 kD	101	214	2.1	222	2.2
X91911	Glioma pathogenesis-related protein	104	171	1.2	266	2.1
U26312	Chromobox homolog/HP1-gamma	53	93	1.8	108	2.1
U20499	Sulfotransferase family 1A phenol-preferring member 3	60	138	2.3	128	2.1
L12723	Heat-shock 70-kD protein 4	54	99	1.3	146	2.0
M23114	ATP2A2	77	134	1.7	155	2.0
X81003	Protein phosphatase 1, regulatory (inhibitor) subunit 11	46	107	1.8	101	2.0
J02621	High mobility group protein 14 (HMG 14)	261	459	1.3	648	2.0

Values are shown as in Table 1.

TABLE 3. Genes downregulated by ALK-1

Accession number	Description	Control	ALK-1		ALK-5	
		Intensity	Intensity	Fold change	Intensity	Fold change
Receptors and related genes						
L06797	CXCR4/fusin/neuropeptide Y receptor Y3	121	17	<−5.6	9.3	−1.1
M60459	Erythropoietin receptor	148	56	−2.6	58	−2.6
X52425	Interleukin 4 receptor (IL4R)	146	139	−2.0	290	1.6
Growth factors and related genes						
Z49269	Small inducible cytokine subfamily A member 14 (SCYA14)	552	288	−2.4	179	−3.1
M26683	MCP1/SCYA2	468	202	−2.3	421	−1.1
Cell–cell and cell–matrix interaction genes						
M30269	Nidogen/enactin	253	50	−3.9	123	−2.1
M57730	Ephrin-A1	208	55	−3.8	432	1.9
Z68228	Plakoglobin/catenin gamma	744	224	−3.3	659	−1.1
U41767	A disintegrin and metalloprotease15 (ADAM15)	924	271	−2.4	376	−2.5
M32334	ICAM2	2,300	1,044	−2.2	1,429	−1.6
Z48481	MMP14/MT1-MMP	323	182	−2.1	150	−2.0
Intracellular regulators						
Cytoskeleton						
Y00503	Keratin 19	123	18	<−3.1	4	<−3.7
Cell cycle related genes						
M60974	GADD45A	113	43	−2.6	70	−1.6
U50079	Histone deacetylase 1 (HDAC1)	265	216	−2.3	221	−1.2
D50405	Histone deacetylase 1 (HDAC1)	231	79	−2.1	117	−1.4
X98260	M-phase phosphoprotein 11/ZRF1	213	108	−2.0	97	−1.8
Signal transduction and related genes						
M35416	Ras-like protein B (RALB)	129	17	<−4.5	22	<−4.3
U59423	Smad1	131	44	−3.4	129	−1.0
X69699	Paired box gene 8 (PAX8)	267	123	−2.3	213	−1.5
J04152	Tumor-associated calcium signal transducer 2	190	82	−2.3	132	−1.4
Z31560	SOX2	115	55	−2.1	53	−2.1
Others						
J02874	Fatty acid binding protein 4 (FABP4)	166	40	−6.6	258	1.2
U83115	Absent in melanoma 1 (AIM1)	221	35	−5.4	148	−1.5
L42373	Protein phosphatase 2 regulatory subunit B (B56) alpha	190	0	<−4.1	52	−1.4
S53911	CD34	662	160	−4.1	257	−2.6
U41518	Aquaporin 1	395	134	−3.8	144	−2.7
U72649	B-cell translocation gene 2	296	121	−3.4	485	1.1
L42176	Four and a half LIM domains 2 (FHL2)	288	85	−3.3	315	1.3
M34516	Human omega light chain protein 14.1	118	36	−3.3	94	−1.3
S82597	GalNAc transferase 1	324	77	−3.1	181	−1.3
U10991	G2 protein	194	65	−3.0	115	−1.3
U44755	Small nuclear RNA activating protein complex polypeptide 2	164	57	−2.9	83	−2.0
U88629	ELL-related RNA polymerase II (ELL2)	100	34	−2.9	71	−1.4
L43579	Soares fetal liver spleen 1 NFLS	112	40	−2.8	20	<−5.0
D28416	Esterase D	481	143	−2.5	312	−1.1
D83777	KIAA0193	220	89	−2.5	203	−1.1
X55740	Nucleotidase 5-prime	121	48	−2.5	68	−1.8
D87460	Paralemmin	225	94	−2.4	178	−1.3
M12886	T-cell antigen receptor beta subunit	300	126	−2.4	295	−1.0
M94856	Fatty acid binding protein 5 (FABP5)	576	239	−2.4	780	1.4
D49824	Major histocompatibility complex class I B/HLA-B	360	156	−2.3	117	−3.1
M23254	Calpain 2	913	328	−2.3	835	−1.1
Z18951	Caveolin 1	1,985	738	−2.3	1,099	−1.5
U09820	ATRX	141	51	−2.3	80	−1.4
D61391	Phosphoribosyl pyrophosphate synthetase protein 1	150	68	−2.2	132	−1.1
U77604	Glutathione S-transferase microsomal 2 (MGST2)	406	187	−2.2	262	−1.5
J03069	MYCL2	658	317	−2.1	284	−1.2
M16405	Cholinergic receptor muscarinic 4	152	117	−2.1	95	−1.6
U21931	Human fructose-1, 6-bisphosphatase 1 (FBP1)	103	48	−2.1	−18	−1.5
AB006782	Galectin 9	566	270	−2.1	554	−1.4
D85527	LIM domain kinase 2	144	71	−2.0	146	−1.5
M59807	Natural killer cell transcript 4	1,262	621	−2.0	1,024	−1.2
X52008	Glycine receptor alpha-2 subunit	500	144	−2.0	185	−1.3
Y07867	Pirin	113	57	−2.0	105	−1.1
J02960	Beta-2-adrenergic receptor	260	131	−2.0	133	−2.0

Values are shown as in Table 1.

TABLE 4. Genes downregulated by ALK-5

Accession number	Description	Control	ALK-1		ALK-5	
		Intensity	Intensity	Fold change	Intensity	Fold change
Receptors and related genes						
D49410	Interleukin 3 receptor alpha (IL3RA)	197	148	−1.3	50	−2.9
M60459	Erythropoietin receptor	148	56	−2.6	58	−2.6
Growth factors and related genes						
Z49269	Small inducible cytokine subfamily A member 14 (SCYA14)	552	288	−2.4	179	−3.1
Cell–cell and cell–matrix interaction genes						
AF000959	Claudin 5	1,108	891	−1.2	350	−3.2
U41767	A disintegrin and metalloprotease 15 (ADAM15)	924	271	−2.4	376	−2.5
Z54367	Plectin 1	325	241	−1.4	192	−2.3
M30269	Nidogen/enactin	253	50	−3.9	123	−2.1
X53002	Integrin β5	364	256	−1.4	169	−2.1
Z48481	MMP14/MT1-MMP	323	182	−2.1	150	−2.0
Intracellular regulators						
Cytoskeleton						
Y00503	Keratin 19	123	18	<−3.1	4	<−3.7
X82207	Actin-related protein 1B	375	115	−1.9	103	−2.1
Signal transduction related genes						
M35416	Ras-like protein B (RALB)	129	17	<−4.5	22	<−4.3
M12174	Ras homolog gene family member B/RHOB	272	384	1.4	165	−2.5
M31158	Protein kinase cAMP-dependent regulatory type II beta	102	127	−1.3	45	−2.3
D13988	GDP dissociation inhibitor 2	426	223	−1.9	158	−2.2
Z31560	SOX2	115	55	−2.1	53	−2.1
M38591	S100 calcium-binding protein A10	1,611	914	−1.8	806	−2.0
M34667	Phospholipase C gamma 1	130	90	−1.4	66	−2.0
U44755	Small nuclear RNA activating protein complex polypeptide 2	164	57	−2.9	83	−2.0
Others						
U80184	Flightless I homolog	110	58	−1.9	−4	<−5.9
L43579	Soares fetal liver spleen 1NFLS	112	40	−2.8	20	<−5.0
L02648	Transcobalamin II	278	157	−1.8	61	−4.5
AB001325	Aquaporin 3	259	229	−1.1	81	−3.2
D49824	Major histocompatibility complex class I B/HLA-B	360	156	−2.3	117	−3.1
L16895	Lysyl oxidase	105	97	−1.1	39	−2.7
U41518	Aquaporin 1	395	134	−3.8	144	−2.7
Z84497	Bromodomain-containing 2	432	223	−1.5	126	−2.7
S53911	CD34	662	160	−4.1	257	−2.6
X90872	Gp25L2	307	267	−1.2	82	−2.6
X98248	Sortilin	187	130	−1.4	71	−2.6
J03263	Lysosome-associated membrane protein 1 (LAMP1)	368	239	−1.5	147	−2.5
S72024	Eukaryotic translation initiation factor 5A (EIF5A)	285	218	−1.0	83	−2.5
D83018	NEL-like 2 (NELL2)	125	83	−1.5	51	−2.4
M55153	Transglutaminase 2	397	291	−1.5	166	−2.4
U92015	Human clone 1437890 defective mariner transposon Hsmar2	104	86	−1.2	34	−2.4
U31929	Nuclear receptor subfamily 0 group B member 1/DAX1	219	195	−1.1	36	−2.4
D49387	Leukotriene B4 12-hydroxydehydrogenase	301	226	−1.5	146	−2.3
U90552	Butyrophilin subfamily 3 member A1	239	91	−1.7	104	−2.3
AC000064	Human BAC clone RG083M05 from 7q21-7q22	249	188	−1.3	90	−2.2
M63379	Clusterin	3,664	2,197	−1.7	1,631	−2.2
U54644	Tubby homolog	385	273	−1.4	178	−2.2
AF008937	Syntaxin 16	113	84	−1.1	44	−2.1
D28137	Bone marrow stromal cell antigen 2	288	173	−1.1	136	−2.1
S72487	ESTs moderately similar to S72487	144	83	−1.7	90	−2.1
U17077	BENE protein	207	268	1.3	100	−2.1
U31342	Nucleobindin 1	218	164	−1.3	103	−2.1
M37457	ATP1A3	298	204	−1.5	142	−2.1
Z35093	Surfeit 1 (SURF1)	230	143	−1.3	116	−2.0
J02960	Beta-2-adrenergic receptor	260	131	−2.0	133	−2.0

Values are shown as in Table 1.

TABLE 5. Genes involved in vascular function

Accession number	Description	Control	ALK-1		ALK-5	
		Intensity	Intensity	Fold change	Intensity	Fold change
Cell cycle regulators						
U66838	Cyclin A1	11	32	>1.9	4	<−1.3
X51688	Cyclin A2	46	73	1.0	20	−1.1
M25753	Cyclin B1	85	83	−1.0	66	−1.3
M74091	Cyclin C	17	6	<−1.2	24	>1.5
X59798	Cyclin D1	163	206	2.0	175	1.7
D13639	Cyclin D2	5	14	<−1.5	4	<−1.1
M92287	Cyclin D3	145	166	1.1	168	1.2
M74093	Cyclin E1	−52	−67	<−1.7	−33	>1.8
Z36714	Cyclin F	39	−82	<−2.3	−65	<−2.5
X77794	Cyclin G1	495	411	−1.2	423	−1.2
U47414	Cyclin G2	17	22	>1.2	9	<−1.3
U11791	Cyclin H	36	98	2.7	105	2.4
D50310	Cyclin I	576	363	−1.6	512	−1.1
X05360	CDC2/CDK1	13	36	>2.0	14	>1.4
M81933	CDC25A	41	58	1.4	12	−1.2
S78187	CDC25B	200	241	1.2	137	−1.5
L26584	CDC25C	29	1	<−2.2	74	2.6
M68520	CDK2	7	33	>2.1	27	>1.9
U37022	CDK4	161	206	1.3	139	1.0
X66365	CDK6	−71	−58	>1.6	−32	>2.7
L20320	CDK7	58	57	−1.0	88	1.5
U09579	p21	273	150	−1.8	280	1.0
U10906	p27	19	−9	>1.3	89	1.1
U22398	p57	14	8	<−1.3	5	<−1.4
U26727	p16	−30	−22	>1.3	−8	>1.9
L36844	p15	70	60	−1.2	35	−2.0
U40343	p19	135	118	−1.1	73	−1.3
M22898	p53	119	61	−1.4	106	−1.1
U47677	E2F1	54	47	−1.1	27	−2.0
L22846	E2F2	24	41	1.7	28	1.1
D38550	E2F3	104	98	−1.1	99	−1.0
U15641	E2F4	−26	−33	<−1.3	17	>2.8
U31556	E2F5	61	−2	−1.7	30	−2.0
L41870	RB1	14	13	>1.5	18	>1.5
L41870	RB1	20	−1	<−1.9	17	<−2.0
L14812	p107	52	46	−1.1	51	−1.0
X76061	p130	−8	14	>1.8	11	>1.8
L23959	Dp-1	−155	−122	>2.4	−106	>3.1
L40386	Dp-2	79	78	−1.0	31	1.1
L00058	c-Myc	83	84	1.1	114	1.4
Growth factors and related genes						
M27281	VEGF	27	2	<−1.3	13	<−1.6
U43142	VEGF-C	−50	−11	>5.1	−44	>2.5
X54936	PlGF	90	−8	<−5.3	214	2.4
D13628	Angiopoietin 1	17	23	<−1.1	6	<−1.7
M19989	PDGF-A	5	−3	<−1.3	61	−1.1
M12783	PDGF-B	11	−18	<−2.3	13	>1.1
M27968	Basic FGF	−5	−19	<−1.6	2	>1.3
Receptors and related genes						
S77812	VEGFR1/Flt-1	110	80	−1.4	50	−1.3
L04947	VEGFR2/Flk-1/KDR	33	−31	<−3.8	−14	<−3.1
X69878	VEGFR3/Flt-4	249	129	−1.9	273	1.1
L06139	Tie2/TEK	57	136	1.6	129	1.8
X60957	Tie1	887	523	−1.7	665	−1.3
L06797	CXCR4	121	17	<−5.6	93	−1.1
D50683	TβR-II	863	487	−1.8	642	−1.3
X72012	Endoglin	917	2,376	2.7	1,155	1.2
L07594	Betaglycan	21	31	>1.7	19	>1.2
Z37976	LTBP2	467	297	−1.6	696	1.5
M21574	PDGFRA	18	53	1.2	25	>1.3
J03278	PDGFRB	33	45	1.4	45	1.4
X66945	FGFR1	71	69	−1.0	170	2.4
J02958	HGF receptor	18	47	>2.3	35	>1.8
Cell–matrix interaction genes						
Z74616	Collagen type I alpha 2	42	24	−1.4	84	3.0
M26576	Collagen type IV alpha 1	1,529	928	−1.6	2,889	1.9
X05610	Collagen type IV alpha 2	1,506	1,210	−1.2	2,656	1.8
M92642	Collagen type XVI alpha 1	−41	−24	>1.7	97	>7.0
L22548	Collagen type XVIII alpha 1	495	392	−1.8	508	1.0
X02761	Fibronectin 1	4,090	3,513	−1.2	2,862	−1.4
X02761	Fibronectin 1	2,420	3,631	1.4	3,869	1.5

TABLE 5. (*Continued*)

Accession number	Description	Control	ALK-1		ALK-5	
		Intensity	Intensity	Fold change	Intensity	Fold change
X03168	Vitronectin	−23	−67	<−2.9	−2	>1.9
U77846	Elastin	10	72	>1.5	88	>3.1
M10321	von Willebrand factor	3,661	3,761	1.0	3,140	−1.2
X14787	Thrombospondin 1	113	107	−1.1	147	1.3
L12350	Thrombospondin 2	38	20	<−1.8	18	<−1.2
L38969	Thrombospondin 3	−15	−31	<−1.7	−13	>1.1
Z19585	Thrombospondin 4	2	−43	<−3.0	−10	<−1.5
M14219	Decorin	30	43	1.4	51	1.7
M34276	Plasminogen	76	55	−1.4	52	−1.4
K03021	t-PA	−56	−23	>2.4	60	>6.0
X02419	u-PA	30	33	−1.3	27	−1.1
U09937	u-PAR	193	50	−1.2	132	1.0
J03764	PAI-1	2,477	2,228	−1.1	3,053	1.2
X54925	MMP1	−20	−8	>1.3	−3	>1.5
M55593	MMP2	2,428	2,429	−1.2	2,867	1.1
X05232	MMP3	−17	−24	<−1.3	−7	>1.4
L22524	MMP7	11	3	<−1.4	13	>1.1
J05556	MMP8	23	39	1.3	35	<−1.1
J05070	MMP9	22	3	<−18	28	>1.2
X07820	MMP10	19	40	>1.9	57	>2.7
X57766	MMP11	280	228	−1.5	209	−1.3
L23808	MMP12	−39	−15	>2.0	−13	>2.1
X75308	MMP13	42	11	<−1.8	6	<−2.0
Z48481	MMP14/MT1-MMP	323	182	−2.1	150	−2.0
Z48482	MMP15/MT2-MMP	−179	−235	<−3.5	−68	>5.9
D83646	MMP16/MT3-MMP	101	50	−2.1	57	−1.6
X89576	MMP17	−11	−19	<−1.3	−34	>1.5
X92521	MMP19	55	31	−1.8	29	−1.9
D11139	TIMP1	6	17	>1.4	6	>1.5
M32304	TIMP2	264	290	1.1	152	−1.7
D45917	TIMP3	11	22	>1.3	15	>1.4
U76456	TIMP4	−6	−22	<−1.7	9	<−1.8
X68742	Integrin α1	14	5	<−1.7	12	<−1.4
J02963	Integrin αIIb	22	14	<−1.3	13	<−1.4
M59911	Integrin α3	68	65	−1.7	79	−1.4
X16983	Integrin α4	11	9	>1.1	−8	<−1.3
X06256	Integrin α5	916	1,228	1.1	1,162	1.3
X53586	Integrin α6	85	41	−2.1	64	−1.3
X74295	Integrin α7	146	99	−1.5	82	−1.2
L36531	Integrin α8	84	69	−1.1	70	−1.1
D25303	Integrin α9	−0	89	>2.9	−46	<−1.5
U40279	Integrin αD	130	129	−1.0	113	−1.1
L25851	Integrin αE	37	123	2.3	85	2.4
Y00796	Integrin αL	14	45	>2.3	20	>1.2
J03925	Integrin αM	−4	−10	<−1.2	7	>1.1
M14648	Integrin αV	10	3	<−1.6	13	>1.1
M34189	Integrin β1	8	22	>1.6	14	<−1.1
M15395	Integrin β2	9	6	<−1.1	19	>1.1
M35999	Integrin β3	−3	32	>2.5	−2	>1.0
S70348	Integrin β3	26	14	<−1.5	15	>1.1
X53587	Integrin β4	−79	−29	>3.2	−55	>2.4
X53002	Integrin β5	364	256	−1.4	169	−2.1
M35198	Integrin β6	2	−4	<−1.3	7	>1.2
S80335	Integrin β7	15	41	>2.2	17	>1.1
M73780	Integrin β8	13	65	>2.5	19	>1.3
Cell–cell interaction genes						
L34657	PECAM1	1,531	924	−1.7	1,063	−1.4
M30257	VCAM1	0	26	>1.9	0	<−1.1
M24283	ICAM1	−14	−3	>1.5	11	>2.1
X52947	Connexin 43	163	132	1.1	301	1.8
X79981	VE-cadherin	1,737	1,744	1.0	2,310	1.2
D21255	OB-cadherin	38	62	2.3	23	−1.4
U03100	Catenin alpha 1	240	248	1.0	291	1.2
M94151	Catenin alpha 2	−10	−5	>1.1	2	>1.5
X87838	Catenin beta 1	118	84	−1.4	91	−1.1
Z68226	Plakoglobin/catenin gamma	744	224	−3.3	659	−1.1
M57730	Ephrin-A1	208	55	−3.8	432	1.9
U14187	Ephrin-A3	204	94	−1.4	205	1.0
U26403	Ephrin-A5	12	34	>1.2	2	<−1.4
U09303	Ephrin-B1	86	77	−1.1	67	−1.3
U81262	Ephrin-B2	41	52	1.6	52	1.2
U66406	Ephrin-B3	−5	47	1.6	99	3.3

(*Continued*)

TABLE 5. (*Continued*)

Accession number	Description	Control	ALK-1		ALK-5	
		Intensity	Intensity	Fold change	Intensity	Fold change
M18391	Eph-A1	51	−17	<−1.9	35	1.1
M59371	Eph-A2	−5	26	<−2.1	21	>2.2
M83941	Eph-A3	10	3	<−1.5	8	<−1.1
L36645	Eph-A4	138	49	−2.8	82	−1.7
L36644	Eph-A5	54	2	<−3.3	2	<−2.0
L36642	Eph-A7	−18	−3	>1.7	1	>1.9
L40636	Eph-B1	21	2	>1.0	37	−1.2
L41939	Eph-B2	149	74	−1.2	94	−1.1
X75208	Eph-B3	68	38	−1.8	50	−1.4
U07695	Eph-B4	486	907	1.4	575	−1.1
Cytoskeleton						
X13839	Actin alpha 2	98	48	−1.9	89	1.1
X00351	Actin beta	4,774	4,685	−1.0	4,222	−1.1
M63391	Desmin	−124	−41	>2.2	−63	>3.7
Z19554	Vimentin	6,622	5,281	−1.3	4,511	−1.5
Transcription factors						
U59423	Smad1	131	44	−3.4	129	−1.0
U68018	Smad2	45	29	−1.5	22	−1.2
U68019	Smad3	23	23	−1.0	17	<−1.3
U44378	Smad4	18	19	>1.1	9	<−1.4
U59913	Smad5	14	−3	<−1.4	5	<−1.1
U59914	Smad6	22	272	>11.9	38	>1.7
AF010193	Smad7	39	203	6.5	62	1.6
M97936	STAT1	−47	348	>18.2	−47	>1.0
M97935	STAT1	231	514	2.2	306	1.1
U18671	STAT2	66	21	>1.1	25	>1.3
L29277	STAT3	256	209	−1.2	207	−1.2
L78440	STAT4	110	144	1.7	78	−1.2
U43185	STAT5A	−27	40	>2.2	2	>2.2
U47686	STAT5B	62	58	−1.4	47	−1.7
X56677	MyoD	51	71	−1.4	2	<−3.1
S78825	Id1	−11	69	>5.1	−28	>1.0
S78825	Id1	311	1,339	4.0	386	1.1
M97796	Id2	55	311	5.7	48	−1.2
X69111	Id3	966	1,559	1.6	1,174	1.2
U28368	Id4	−2	14	>1.7	8	>1.5
J04101	Ets-1	−167	−213	<−3.0	−174	<−1.3
U22431	HIF-1α	78	67	−1.2	151	1.2
M69238	HIF-1β/ARNT	−23	53	<−1.5	−2	>1.9
U81984	HIF-2α/EPAS1	212	160	−1.3	115	−1.8
X67235	HEX	100	107	1.1	152	1.5
U11732	TEL	54	33	−1.6	27	−2.1
X61755	HOX3D	123	−12	<−5.7	95	−1.5
S57212	MEF2C	61	131	−1.1	102	1.0
M64497	COUP-TFII	218	326	1.5	278	1.3
M98833	Fli-1	98	78	−1.2	67	−1.5
M68891	GATA2	−11	62	>4.2	58	−2.7
M77810	GATA2	17	36	>1.8	−8	<−2.1
L34357	GATA4	−13	−11	>1.1	−21	<−1.3
U66075	GATA6	−14	11	>2.5	−2	>1.5
D43968	RUNX1/PEBP2αB/AML1	30	−15	<−2.9	2	<−2.2
Others						
M93718	Nitric oxide synthase 3	779	800	1.4	573	−1.5
X01677	Glyceraldehyde-3-phosphate dehydrogenase	4,621	3,610	−1.3	3,001	−1.5

Values are shown as in Table 1.

the differentiation of neuronal cells (Nakashima et al., 2001). The present findings suggest that Id1 through 3 may also be critical regulators of endothelial cells, and possibly control vascular functions as downstream effectors of ALK-1. In agreement with the present finding, Goumans et al. (2002) recently reported that Id1 is induced by ALK-1 in endothelial cells.

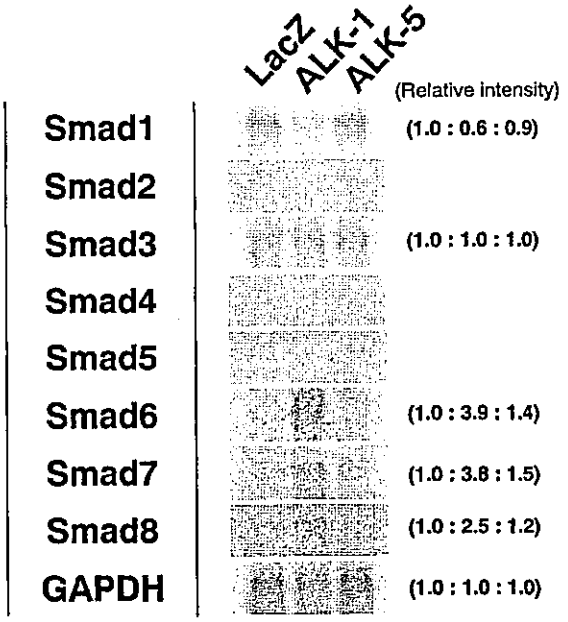
Interestingly, ALK-1 specifically induced endoglin, and expression of endoglin was very high in HUVEC (Table 1). Northern blot analysis yielded a similar result (Fig. 3C). Endoglin is a transmembrane glycoprotein structurally similar to TGF-β type III receptor (also termed betaglycan) and modulates TGF-β signaling by binding to TGF-β in association with TβR-II (Guerrero-Esteo et al., 2002). Mutations of the human endoglin gene are responsible for HHT type I (McAllister et al., 1994). Endoglin-deficient mice exhibit vascular anomalies characterized by enlarged and immature vessels similar to those in ALK-1-null mice (Li et al., 1999; Urness et al., 2000; Oh et al., 2000). Since abnormalities in the ALK-1 and endoglin genes are linked to HHTs (Johnson et al., 1996; McAllister et al., 1994), they may cooperatively regulate the binding of TGF-β superfamily ligands.

A

GeneChip intensity

LacZ	ALK-1	ALK-5
131	44	129
45	29	22
23	23	17
18	19	9
14	-3	5
22	272	38
39	203	62

Northern blotting



B

GeneChip intensity

LacZ	ALK-1	ALK-5
311	1339	386
55	311	48
966	1559	1174
-2	14	8

Northern blotting

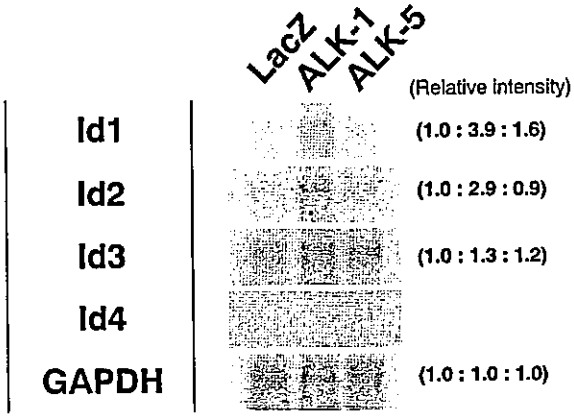


Fig. 3. GeneChip intensity and Northern blotting in HUVEC. A: The expression of Smad genes. B: The expression of Id genes. C: The expression of genes involved vascular function. Human umbilical vein endothelial cells (HUVEC) were infected with recombinant adenoviruses carrying LacZ as a control, or a constitutively active form of activin receptor-like kinase 1 (ALK-1) or activin receptor-like kinase 5 (ALK-5). Total RNAs were extracted from HUVEC 48 h after adenoviruses infection, and subjected to Northern blotting. GAPDH cDNA probe was used as an internal control for RNA loading. GeneChip intensity directly reflects gene expression level. A negative value indicates that the intensity of perfect match probe was below that of mismatch probe as a cross-hybridization control, suggesting that the gene expression was low or absent. Relative intensities of the bands in Northern blot analyses were calculated using LacZ-infected cells as controls. (Continues.)

Signal transducer and activator of transcription 1 (STAT1) was upregulated by ALK-1 but not by ALK-5 (Table 1). This result was confirmed by Northern blot analysis (Fig. 3C). We also found that other STAT proteins, including STAT2 through STAT5, were not affected by ALK-1 or ALK-5 (Table 5). Probes for STAT6 were not present in this array. STAT proteins are activated by tyrosine phosphorylation and modulate diverse cellular functions as transcriptional regulators (Darnell et al., 1994). Certain growth factors or cytokines, including interferon-α, interferon-γ, epidermal growth factor, platelet-derived growth factor (PDGF), and interleukin 6 (IL-6), have been reported to activate STAT1. A previous study also demonstrated that STAT1 is phosphorylated by a mutant form of angiopoietin receptor, Tie2, resulting in venous malformations due to abnormally increased tyrosine kinase activity (Korpelainen et al., 1999). Recently, VEGF

C

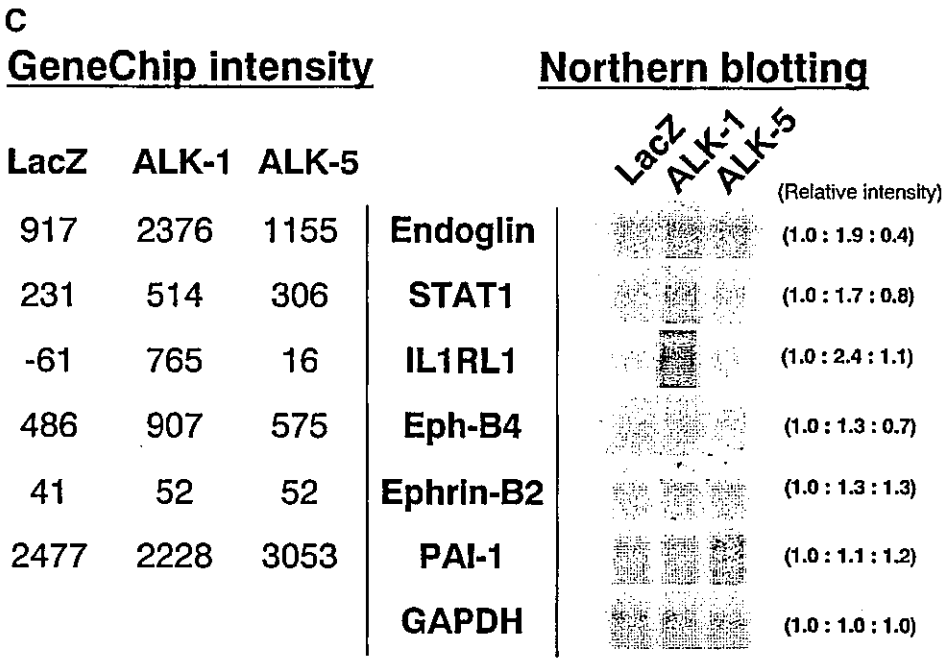


Fig. 3. (*Continued*)

was shown to activate STAT1 in bovine endothelial cells through VEGF receptor 2 (VEGFR2/Flk-1/KDR) (Bartoli et al., 2000). These results suggest that signaling cross talk may exist between TGF-β and other important endothelial ligands including angiopoietins and VEGF through STAT1.

Interleukin 1 receptor-like 1 (IL1RL1) was strongly induced by ALK-1, in both GeneChip (Table 1) and Northern blot analysis (Fig. 3C). IL1RL1 is structurally very similar to IL-1 receptor (Tominaga, 1989) and belongs to a cluster of genes, including IL1R1, IL1R2, IL1RL2, and IL18R1 (Dale and Nicklin, 1999). The physiological ligands for IL1RL1 and its signal transduction pathway have not been determined. IL1RL1 has been suggested to be involved in the growth of cells, but its role in regulation of endothelial cells remains to be elucidated.

Genes upregulated by ALK-5

Enhancement of ALK-5 gene expression was not detected in HUVEC infected with the ALK-5 adenoviruses in this array (data not shown), since the probe for ALK-5 in this microarray was designed to cover only the untranslated region of ALK-5, which is missing in the recombinant adenovirus cDNA. However, expression profiles of many genes were changed by ALK-5 in comparison with those of control or ALK-1, and some of the genes exhibited profiles similar to those stimulated by TGF-β in HaCaT keratinocytes (Akiyoshi et al., 2001), confirming the validity of analysis using the ALK-5 adenovirus (see below).

PlGF, which belongs to the VEGF family (Neufeld et al., 1999), was upregulated by ALK-5 and possibly downregulated by ALK-1, although its intensity before decrease was less than 100 (Table 2). PlGF stimulates vascular development by amplifying VEGF responses through VEGFR1/Flt-1. Loss of PlGF in mice impaired growth and migration of endothelial cells, and inhibited recruitment of smooth muscle cells and monocytes/macropharges under pathological conditions (Carmeliet et al., 2001). The intensity of VEGFR1/Flt-1 was decreased from 110 to 50 by ALK-5 and slightly decreased to 80 by ALK-1, but fold changes were −1.4 and −1.3, indicating no significant changes (Table 5). These results suggest the possibility that TGF-β can affect endothelial cells by regulating the expression of PlGF, but it depends largely on whether ALK-5 or ALK-1 dominantly mediates TGF-β signals and on the phases of vasculogenesis and angiogenesis.

ALK-5 specifically induced SM22α, a highly expressed gene in smooth muscle cells (Solway et al., 1995), and its differentiation marker during development (Table 2). In addition, caldesmon 1 and myosin regulatory light chain 2, which are expressed in smooth muscle cells, were upregulated by ALK-5 (Table 2). ALK-5 also increased the expression of tropomyosin 1 (Table 2). Induction of these genes was not observed in ALK-1-infected HUVEC. A recent study has shown that VEGFR2/Flk-1-positive endothelial precursor cells among embryonic stem cells can differentiate into both endothelial cells and smooth muscle cells (Yamashita et al., 2000). The present findings suggest possible effects of TGF-β on differentiation and transformation of mature endothelial cells by ALK-5, which lead to vascular stabilization.

Connexin 37, a gap junction protein, was induced by ALK-5 (Table 2). Connexin 37 is expressed in blood vessels and vascular endothelial cells, and functions as a cell–cell channel and adhesion molecule (Reed et al., 1993). The induction of connexin 37 by ALK-5 may affect intercellular communication and adhesion of endothelial cells, possibly leading to both activation

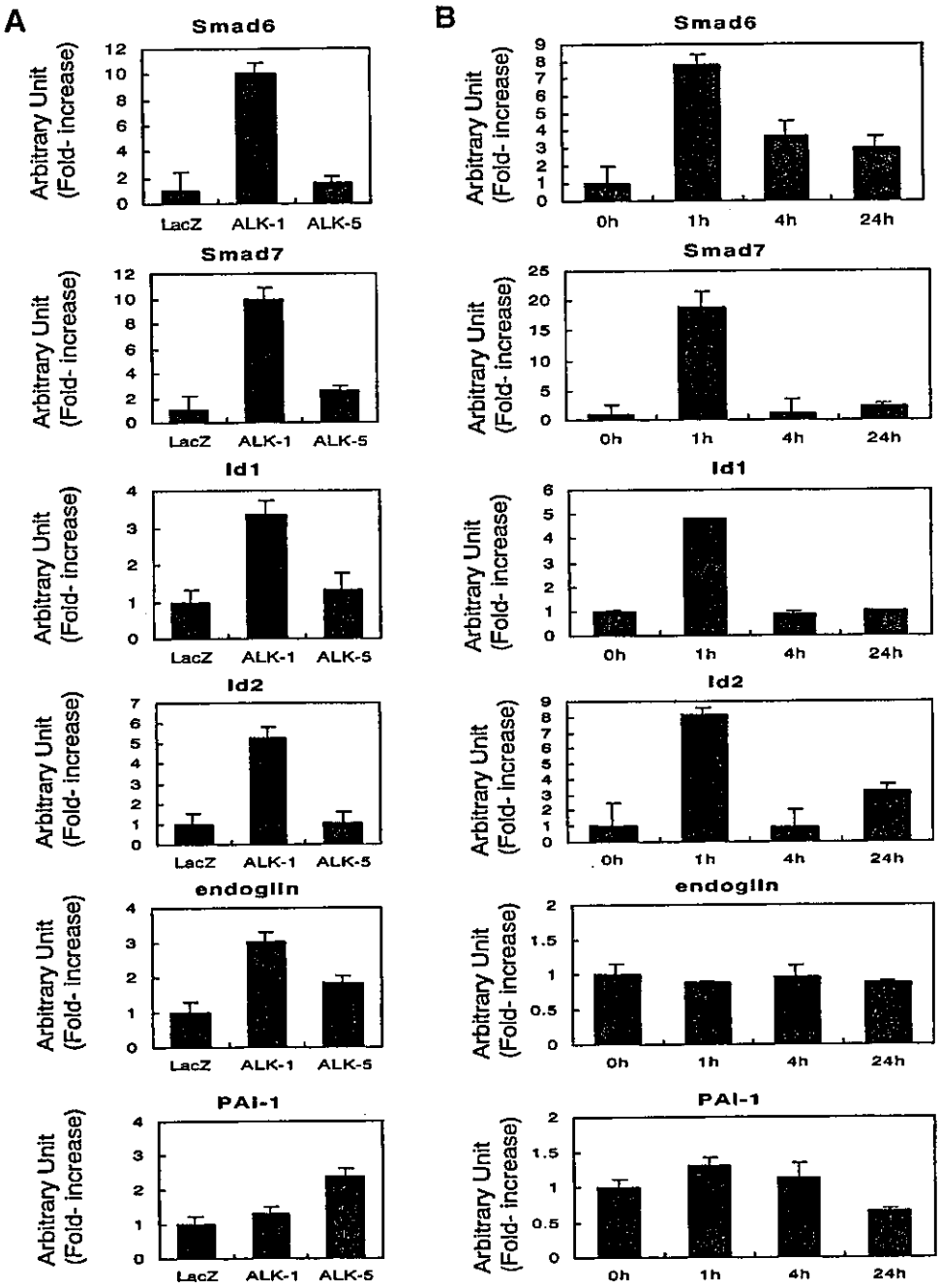


Fig. 4. Expression levels of TGF-β-target genes analyzed by quantitative real-time RT-PCR. A: Expression levels of some of the activin receptor-like kinase 5 (ALK-5) and activin receptor-like kinase 1 (ALK-1) target genes identified by the oligonucleotide microarray were analyzed by quantitative real-time RT-PCR. B: Human umbilical vein endothelial cells (HUVEC) were treated with 5 ng/ml TGF-β3 for the indicated times, and expression levels of the ALK-5 and ALK-1 target genes examined in (A) were analyzed by quantitative real-time RT-PCR.

and stabilization of vessels depending on the phase of vascular development. In the early phase, cell–cell communication and adhesion are required for cell fusion, network formation, and sprouting and branching of vessels. On the other hand, during the late phase of vascular formation, enhancement of cell contacts may inhibit endothelial cell proliferation and migration, and plasma extravasation, leading to vascular maturation.

βIG-H3, also termed TGF-β-induced 68-kD (Skonier et al., 1992), was upregulated by ALK-5 (Table 2). Induction of βIG-H3 was also observed in HaCaT keratinocyte cells treated with TGF-β (Akiyoshi et al., 2001). βIG-H3 is a secreted protein with the Arg-Gly-Asp (RGD) motif found in many ECM proteins, and modulates cell adhesion and growth (Skonier et al., 1994; Billings et al., 2002). Cell–matrix interaction is required for the

regulation of cell growth, survival, migration, and tube morphogenesis; and ECM accumulation leads to vascular maturation. The induction of βIG-H3 by ALK-5 may contribute to these functions, which would be consistent with TGF-β-mediated vascular responses.

ALK-5 induced latent TGF-β binding protein 1 (LTBP1) (Table 2), which is a component of the high-molecular-weight complex of latent TGF-β. LTBP1 regulates secretion and matrix-interaction of the latent TGF-β complex (Saharinen et al., 1999). We also found that furin was upregulated by ALK-5 and ALK-1, although the GeneChip intensity of furin with ALK-5 was slightly less than 100 (Table 1). Furin has been reported to be induced by TGF-β and to proteolytically process the pro-form of TGF-β (Blanchette et al., 1997). The induction of LTBP1 by ALK-5, together with the induction of furin, may positively regulate TGF-β signaling. In this study, neither ALK-5 nor ALK-1 affected levels of expression of TGF-β1 through β3 (data not shown). TβR-II was highly expressed in HUVEC but did not exhibit significant change over twofold with either ALK-1 or ALK-5 (Table 5).

Genes downregulated by ALK-1

ALK-1 repressed Smad1, a downstream signal mediator of ALK-1 (Table 3). Northern blot analysis confirmed this result, although the reduction of Smad1 gene expression was less remarkable in the Northern blotting analysis (Fig. 3A). This is consistent with negative feedback regulation of ALK-1 signaling. Other R-Smads, including Smad5, were not affected by ALK-1 (Table 5) and not detected by Northern blot analysis under the conditions tested (Fig. 3).

Chemokine (C-X-C) receptor 4 (CXCR4), also called fusin or neuropeptide Y receptor Y3, was downregulated by ALK-1 (Table 3). Other neuropeptide Y receptors were not affected by ALK-1 in this array (data not shown). CXCR4 is a G protein-coupled receptor containing 7-transmembrane regions, implicated in immune function, organ development, and cancer metastasis in response to its ligand, SDF-1 (Bleul et al., 1996). CXCR4 is expressed in developing vascular endothelial cells. Mice lacking CXCR4 have exhibited defective formation of the large vessels in the gastrointestinal tract, although vasculogenesis and angiogenesis were normal in other vascular tissues (Tachibana et al., 1998). SDF-1 and CXCR4 are positive regulators of vascularization, but their effects on endothelial cell functions remain to be determined. Further investigations will be needed to determine whether the downregulation of CXCR4 by ALK-1 is essential for vascular development or maturation.

The expression of Ephrin-A1 was decreased by ALK-1 and probably was increased by ALK-5, although the fold change induced by ALK-5 was 1.9 (Table 3). Eph proteins, which belong to the receptor tyrosine kinase family and their ligands, Ephrins, play critical roles in neural and vascular development through bi-directional intercellular signaling and cell–cell adhesion (Frisen et al., 1999). In endothelial cells, tumor necrosis factor-α has been shown to induce Ephrin-A1 (Holzman et al., 1990). Ephrin-A1 binds Eph-A receptors (Eph-A1 through 7), most of which were not expressed at significant levels in HUVEC (Table 5). Probes for Eph-A6 were not present in this array. A previous study reported that endothelial cells expressing Eph-B4 differentiate into veins, whereas those expressing Ephrin-B2 differentiate into arteries during development (Wang et al., 1998). Ephrin-B2 is also expressed in arterial smooth muscle cells and possibly affects communication between periendothelial cells and endothelial cells (Shin et al., 2001). Inspection of expression patterns of Ephrins and Eph proteins revealed high expression of Eph-B4 and low expression of Ephrin-B2 in HUVEC (Table 5), consistent with the evidence that Eph-B4 is a marker gene for venous endothelial cells. The intensity of Eph-B4 was increased from 486 to 907 by ALK-1. Northern blot analysis of Eph-B4 and Ephrin-B2 revealed results similar to those obtained with the GeneChip (Fig. 3C). We also found that Ephrin-B3 was increased 3.3-fold by ALK-5, although the intensity induced by ALK-5 was 99 (Table 5). Ephrin-B3 is highly expressed in forebrain and is considered important for brain development (Tang et al., 1997). The roles of Ephrin-A1 and Ephrin-B3 in HUVEC have not been determined, but our findings suggest that ALK-1 and ALK-5 may control the regulation of Ephrins and Eph proteins and induce vascular development and stabilization.

ALK-1 repressed expression of plakoglobin, also termed desmoplakin 3 or γ-catenin (Table 3). Plakoglobin is a cytoplasmic component of desmosomes and adhering junctions (Mathur et al., 1994) and mediates intracellular signals through the connection of cadherin to actin filaments (Knudsen and Wheelock, 1992). Plakoglobin-deficient mice have been reported to exhibit impaired adhesion of myocardial cells and ventricular rupture as a result of defects in desmosomes (Ruiz et al., 1996). The repression of expression of plakoglobin by ALK-1 may result in detachment of endothelial cells. During the initial phase of angiogenesis, activation and detachment of endothelial cells are required for invasion, migration, and proliferation. It is thus possible that ALK-1 may positively regulate these processes, although many other adhesive proteins are also involved in cell-cell interaction.

Genes downregulated by ALK-5

In contrast to the repression of Smad1 by ALK-1, Smad3, which acts downstream of ALK-5 signaling, was not affected by ALK-5 in GeneChip (Table 5) and Northern blot analyses (Fig. 3A). Another R-Smad mediating ALK-5 signals, Smad2, could not be detected by Northern blot analysis under the conditions tested (Fig. 3A).

ALK-5 specifically repressed claudin 5 (Table 4), a component of tight junction proteins expressed in endothelial cells (Morita et al., 1999). Only claudin 10 was also detected in this array, but its level of expression was very low (data not shown). Tight junctions constitute seals around cells and function as a physical barrier between the intravascular lumen and extracellular space. Claudin 5 thus plays an important role in endothelial cell adhesion and vascular permeability. Its repression by ALK-5 may permit plasma extravasation through tight junctions, affecting migration of endothelial cells and monocytes/macrophages into the extravascular space. Reduced cell adhesion may lead to