Increased secretion of urokinase-type plasminogen activator by human lung microvascular endothelial cells

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HISANAGA YAGYU,[1] AND TAKESHI MATSUOKA[1]
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Takahashi, Kimiko, Yasuhide Uwabe, Yoshio Sawasaki, Toshio Kiguchi, Hiroyuki Nakamura, Kosuke Kashiwabara, Hisanaga Yagyu, and Takeshi Matsuoka. Increased secretion of urokinase-type plasminogen activator by human lung microvascular endothelial cells. *Am. J. Physiol.* 275 (*Lung Cell. Mol. Physiol.* 19): L47–L54, 1998.—Human lung microvascular endothelial cells (HLMECs) secreted 1.5–15 times more urokinase-type plasminogen activator (uPA) antigen than human hepatic microvascular endothelial cells, human umbilical vein endothelial cells (HUVECs), angioma endothelial cells, and lung fibroblasts. All of these cells also secreted a 100-fold greater amount of plasminogen activator inhibitor-1 than of uPA antigen, and uPA activities were not detected in the culture medium. The expression of uPA mRNA in HLMECs was higher (100-fold) compared with HUVECs, angioma endothelial cells, and lung fibroblasts. HLMECs secreted uPA antigen on both the luminal and basal sides of the cells. On the other hand, HLMECs secreted a 10- to 15-fold lower amount of tissue-type plasminogen activator than HUVECs, mostly on the luminal side. After stimulation with interleukin (IL)-1β, HLMECs secreted a six- to ninefold amount of uPA antigen. In contrast, no stimulatory effect was observed in HUVECs even under high IL-1β concentrations. The secretion of uPA and plasminogen activator inhibitor-1 from HLMECs was also enhanced by tumor necrosis factor-α and IL-2. These results suggest that HLMECs may contribute not only to the patency of lung vessels but also to the maintenance of alveolar functions through the production and secretion of uPA, especially in the presence of inflammatory cytokines.

tissue-type plasminogen activator; plasminogen activator inhibitor-1; human umbilical vein endothelial cells

THE INNER SURFACE OF BLOOD VESSELS is lined with vascular endothelial cells that differ in morphologies and properties according to the tissues served by the vessels (21). An examination of such tissue specificity may facilitate our understanding of endothelial functions in different organs. Four major functions of vascular endothelial cells are known: *1*) control and transport of nutritional materials from blood (31); *2*) modulation of vascular tone by producing endothelin, nitric oxide, or prostacyclin (15, 27, 32); *3*) leukocyte transmigration (8); and *4*) maintenance of blood fluidity by producing fibrinolytic enzymes such as plasminogen activators (PAs) (16).

PAs are serine proteases and have been classified into two groups, urokinase type (uPA) and tissue type (tPA). tPA is strongly connected with fibrinolysis, and its activity is increased by the coexistence of a fibrin fragment (25), whereas uPA is related to tissue remodeling (1). Increased amounts of uPA facilitate uPA turnover, leading to remodeling of injured tissues including alveoli. Most secreted uPA binds to the uPA receptor (uPAR) found in the cell membrane of some types of cells and restricts uPA activity to the cell surface (20). The bound uPA is believed to activate plasmin, which degrades matrix components surrounding cells, thereby allowing cell migration. When uPAR-uPA is inactivated by the binding of PA inhibitor-1 (PAI-1), a uPAR-uPA-PAI-1 complex is internalized, and uPA-PAI-1 is degraded (17). The uPAR recycles and becomes available for binding to uPA again (9). It can be speculated that a large amount of uPA production implies a fast uPA turnover cycle, particularly at sites of tissue injury undergoing repair.

Lavage fluid from normal lung contains a lot of uPA antigen, but the enzyme activity has not been detected in lavage fluid from patients with adult respiratory distress syndrome (ARDS), suggesting that uPA may also serve a protective role in surfactant function (3). Sources of uPA in the lung have been reported to be macrophages (6) and epithelial cells (18). Previously, Takahashi et al. (28) reported that bovine lung microvascular endothelial cells produced and secreted >10 times as much uPA as bovine aortic, hepatic, and adrenal microvascular endothelial cells and lung fibroblasts, and uPA antigen and activity were detected only in those lung cells. Bovine lung microvascular endothelial cells secreted uPA on both sides of the cell layer, i.e., on the luminal surface and on the basal surface attached to basement membrane.

Of course, when the role of human lung diseases is considered, human lung microvascular endothelial cells (HLMECs) are most appropriate for study. In a report (7), uPA production has been described as one of the properties of microvascular endothelial cells derived from human lung. However, the isolation and culture conditions of human microvascular endothelial cells are much more complicated than those of the cells from other species (2, 22). Isolation of human microvascular endothelial cells has been limited so far. For these reasons, neither the species nor the tissue specificity of human microvascular endothelial cells has been fully investigated yet. In this report, we examined the uPA production of HLMECs and compared the amount with those of endothelial cells obtained from the other human tissues. The effect of inflammatory cytokines on the secretion of uPA and PAI-1 by these cells was also examined.

METHODS

Materials. Collagenase, EDTA, sulfuric acid, chloroform, isopropanol, ethanol, Tris, acetic acid, agarose, and ethidium bromide were from Wako Pure Chemicals (Tokyo, Japan); dispase was from Godo Shusei (Tokyo, Japan); newborn calf serum was from Mitsubishi Kasei (Tokyo, Japan); medium 199, keratinocyte-SFM medium, trypsin, TRIzol, *Taq* polymerase, 2′-deoxynucleoside 5′-triphosphate mixture, DNA ladder, penicillin-streptomycin mixture, and Fungizone were from GIBCO BRL (Grand Island, NY); human recombinant basic fibroblast growth factor was from Intergen (Purchase, NY); human cellular fibronectin was from Fibrogenex (Chicago, IL); competitive DNA construction kit and competitive RNA transcription kit were from Takara Biomedicals (Tokyo, Japan); 24-well plates and cell culture inserts (pore size 0.45 μm) were from Falcon (Lincoln Park, NJ); 60-mm-diameter plastic culture dishes were from Nunc (Naperville, IL); 6-well plates were from Costar (Cambridge, MA); 5(6)-carboxyfluorescein-*N*-hydroxysuccinimide ester and 96-well microtiter plates were from Dai-Nippon Pharmaceutical (Osaka, Japan); anti-human factor VIII complex was from Immunotech (Marseille, France); avidin-biotin complex staining kit was from Vector Laboratories (Burlingame, CA); Spectrozyme UK was from American Diagnostica (Greenwich, CT); uPA ELISA kit was from Monozyme (Hoersholm, Denmark); PAI-1 and tPA ELISA kits were from Biopool (Uema, Sweden); recombinant human interleukin (IL)-1β and tumor necrosis factor (TNF)-α were from Genzyme (Cambridge, MA); and IL-2 was from Takeda Pharmaceutical (Osaka, Japan). T-primed first-strand kit (Ready-To-Go) was purchased from Pharmacia (Uppsala, Sweden). Human pro-uPA was obtained from Abbott Laboratories (Abbott Park, IL) courtesy of Dr. Jack Henkin.

Cell culture. Small sections of human lung within 1–2 mm of the periphery were obtained from normal regions of lungs of patients undergoing resection for solitary lung tumors. The surgery was performed by the Surgical Service of Kasumigaura Hospital, Tokyo Medical College (Ibaraki, Japan). HLMECs were isolated according to the modified method previously described (28). In brief, the sections were digested with 0.1% collagenase, and cells obtained were seeded onto plastic dishes in growth medium (medium 199 supplemented with 20% newborn calf serum, 10 ng/ml of basic fibroblast growth factor, 100 U/ml of penicillin, and 100 μg/ml of streptomycin) and incubated at 37°C to separate the endothelial cell-rich suspension from contaminating macrophages. After 1 h, the cells floating in the dish were collected and seeded onto the fibronectin-coated dish (50 ng/cm^2). The lung tissue debris of the collagenase digestion was further digested with 0.05% trypsin-0.02% EDTA, and the cells obtained from this digestion were seeded directly onto a new fibronectin-coated dish. After cell attachment, the culture was rinsed with PBS, 0.02% EDTA was added to release nonendothelial cells from the surface of the plates, and, finally, those nonendothelial cells that were still on the plate were detached from the surface with a small piece of silicon rubber connected to a syringe. After the endothelial cell colonies grew large enough, they were subcultured with a rubber policeman and seeded onto new fibronectin-coated dishes.

Hepatic tissues were obtained from livers of patients undergoing resection for solitary liver tumors, and microvascular endothelial cells were cultivated with the same method as the lung microvascular endothelial cells. The angioma endothelial cells were isolated from venous forearm angioma tissue by treatment with 0.1% collagenase. Human umbilical vein endothelial cells (HUVECs) were cultivated with 500 IU/ml of dispase or 0.1% collagenase with the same method as in a previous report (29). Lung fibroblasts, which were nonendothelial and long-shaped cells, were obtained during the preparation of HLMECs. The culture medium was changed twice a week. Cells in younger generations (*passages 2–7*) were used for each examination.

The confluent cell monolayers on the fibronectin-coated 24-well plates were washed twice with serum-free medium, after which keratinocyte-SFM containing bovine pituitary extract and epidermal growth factor was added according to the manufacturer's directions. After 12 h of incubation at 37°C, the conditioned medium was collected for determination of uPA and PAI-1. The remaining cells were detached with 0.05% trypsin-0.02% EDTA and counted.

To examine the secretion amount of uPA and PAI-1 from freshly isolated human lung cells, cells cultivated for 1 wk after the separation were seeded onto fibronectin-coated 24 well-plates. The 12-h conditioned medium was collected and uPA and PAI-1 antigens were determined by the same method described in *Measurement of uPA, tPA, and PAI-1 antigens.*

Measurement of uPA, tPA, and PAI-1 antigens. The uPA, tPA, and PAI-1 antigens were determined by conventional ELISA methods according to the manuals of the kits. In brief, monoclonal mouse anti-human uPA, tPA, or PAI-1 antibody was coated on 96-well microtiter plates at 4°C overnight, and samples were added to the plates to allow binding with the immobilized antibody at 4°C overnight again. Biotinylated monoclonal mouse anti-human uPA, tPA, or PAI-1 antibody was added to each well to react with the bound uPA, tPA, or PAI-1 antigen for 1 h at room temperature, and peroxidase-conjugated streptavidin was again added to the wells at room temperature. After 1 h of incubation, the contents of the plate were allowed to react for exactly 30 min with *o*-phenylenediamine, the substrate for peroxidase. The plate was measured at 490 nm by a microplate reader (model 3550-UV, Bio-Rad, Tokyo, Japan) after the reaction was terminated by the addition of 1 N sulfuric acid. Data are expressed as means ± SD. Mean values per 10^4 cells were determined in triplicate wells for each experiment, and every experiment was repeated at least three times.

Measurement of uPA activity. uPA activity in the serum-free conditioned medium was measured with the chromogenic substrate for uPA, Spectrozyme UK, after activation of pro-uPA by plasmin treatment as described in a previous report (28).

Determination of the directionality of uPA and tPA secretion by HLMECs and HUVECs. HLMECs or HUVECs were seeded onto the fibronectin-coated membrane (pore size 0.45 μm) of the cell culture insert for 24-well plates. After the cells reached confluency, 12-h serum-free conditioned medium using keratinocyte-SFM was collected from both the upper and lower chambers. The cells were detached with 0.05% trypsin-0.02% EDTA from each cell culture insert membrane and counted. The amount of uPA and tPA antigens in the conditioned medium was measured by the method described in *Measurement of uPA, tPA, and PAI-1 antigens.* To confirm that the cell layer was at confluency, pro-uPA was labeled with fluorescence and was added to the upper chamber (1 ng/well). After 12 h of incubation, the fluorescence in the lower chamber was measured with Fluoroskan II (Dai-Nippon Pharmaceutical) with an excitation wavelength of 485 nm and an emission wavelength of 538 nm. The confluency of the cell layer was also confirmed by scanning electron microscopy (30).

RT-PCR. To obtain total RNA, cells cultivated in 60-mm-diameter dishes were lysed by the addition of TRIzol. The RNA was extracted with chloroform, isopropanol, and ethanol by centrifugation after each addition according to the manu-

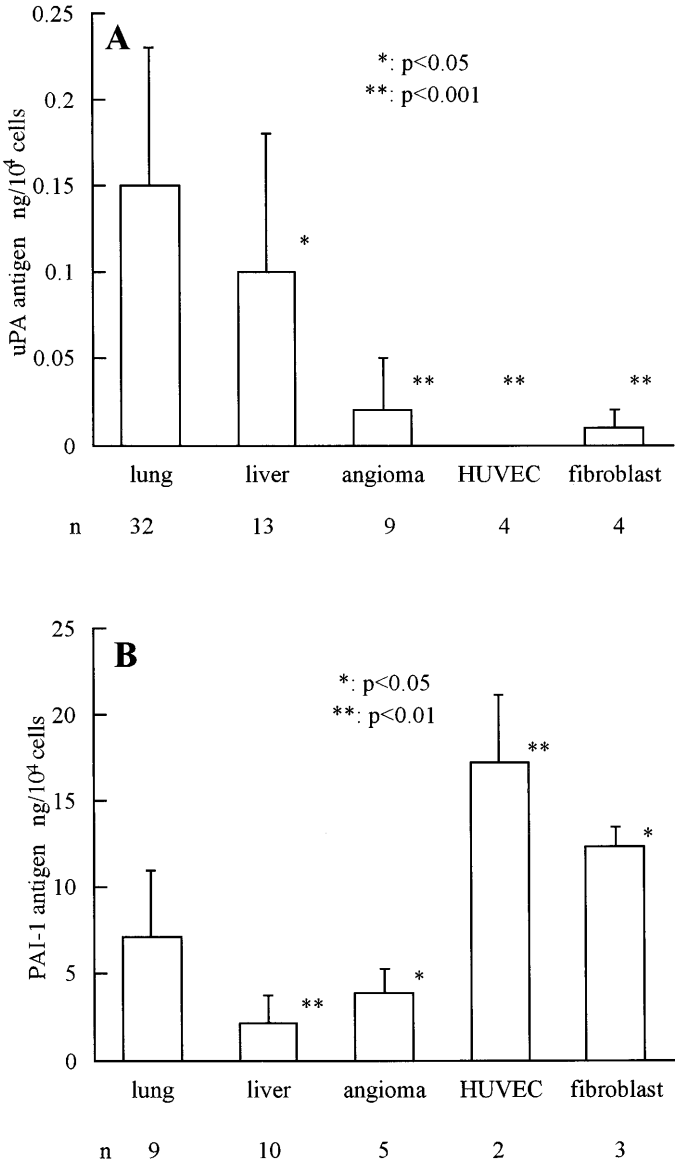

Fig. 1. Amount of urokinase-type plasmogen activator (uPA; *A*) and plasminogen activator inhibitor-1 (PAI-1; *B*) antigens secreted from human lung microvascular endothelial cells (HLMECs), liver microvascular endothelial cells, angioma endothelial cells, human umbilical vein endothelial cells (HUVECs) and fibroblasts. Confluent cultures of each cell type were washed and added to serum-free medium. After 12 h of incubation, conditioned medium was collected, and uPA and PAI-1 antigens were measured by ELISA method. Values are means ± SD; *n*, no. of samples. *P* values compared amount of antigens secreted by HLMECs.

facturer's instructions. The extracted RNA was dissolved in distilled water, and the absorbance was measured with a spectrophotometer (UV-1600, Shimazu, Kyoto, Japan) at 260 nm to estimate the amount of total RNA. To prepare template cDNA, equal amounts of total RNA were added to tubes of a T-primed first-strand kit including *Not* I-$(dT)_{18}$ primer, 2′-deoxynucleoside 5′-triphosphates, and RT, and the tubes were shaken at 37°C. After 60 min of incubation, uPA sequences were subsequently amplified by PCR with 1 μl of cDNA template, 0.5 U/ml of *Taq* polymerase, and 1 μM sense and antisense oligomer primers in a total volume of 10 μl. The set of primers specific for human pro-uPA from a transformed human endothelial cell line (29, 33), from which an amplified fragment of 459 bp was obtained, consisted of a forward primer beginning at bp 769 with a nucleotide sequence of 5′-GCCTTGCTGAAGATCCGTTCCAAGGAGGGC-3′ and a reverse primer beginning at bp 1198 with a nucleotide sequence of 5′-CAGGCCATTCTCTTCCTTGGTGTGACTGCG-3′. RT-PCR was carried out under the following conditions: 1 cycle at 95.0°C for 3 min; 30 cycles at 95.0°C for 45 s, 65.0°C for 45 s, and 72.0°C for 3 min; and 1 cycle at 72.0°C for 5 min (Program Temp Control System PC-800, ASTEC, Fukuoka, Japan). The PCR reaction products (3–7 μl) were separated by electrophoresis on a 2.0% agarose-Tris-acetate-EDTA gel and stained with ethidium bromide.

For quantitative analysis of uPA mRNA in human endothelial cells, a competitive RT-PCR method was used (13). The 383-bp competitor DNA for uPA that has the sense and antisense uPA primer sequences at its both ends was made

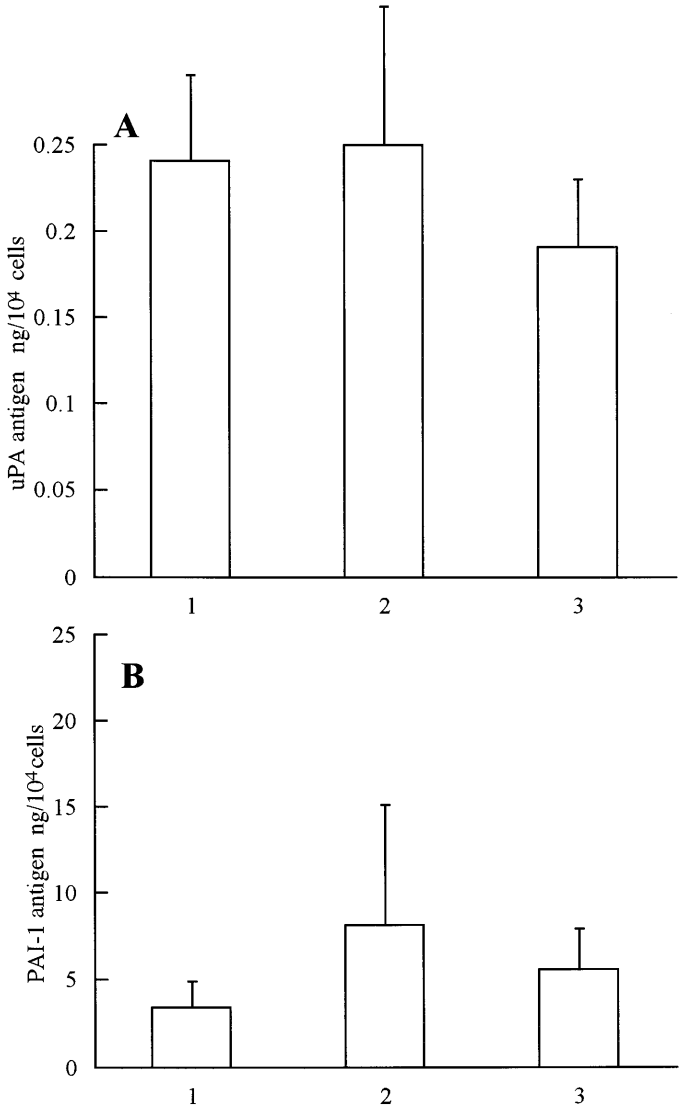

Fig. 2. Amount of uPA (*A*) and PAI-1 (*B*) antigens secreted from freshly isolated human lung cells. One-week-old cultured human lung cells were inoculated onto fibronectin-coated 24-well plates and added to serum-free medium. After 12 h of incubation, conditioned medium was collected, and uPA and PAI-1 antigens were measured by ELISA method. Freshly isolated human lung cells were not pure microvascular endothelial cells. Because they also included alveolar epithelial cells and fibroblasts, experiments were carried out 3 times (nos. on *x*-axis). Values are means ± SD.

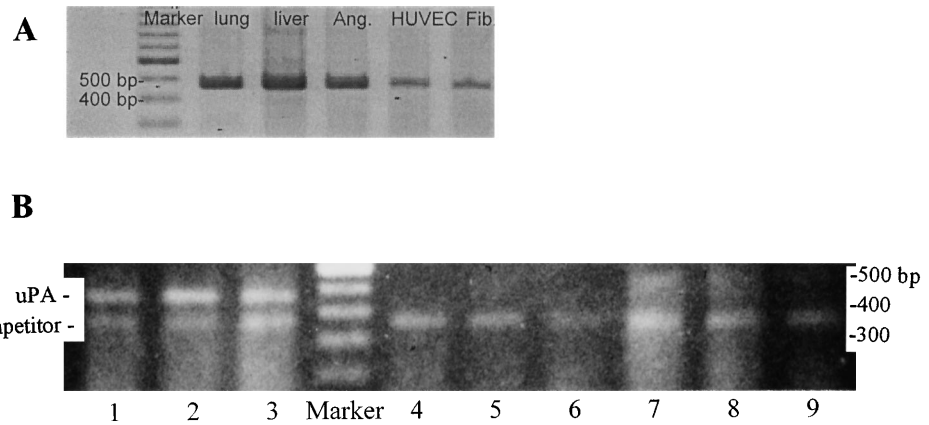

Fig. 3. Detection of uPA mRNA in HLMECs, liver microvascular endothelial cells, angioma endothelial cells (Ang.), HUVECs, and lung fibroblasts (Fib.). RT-PCR was performed on total RNA as described in METHODS. *A:* a single band is seen in each lane, which has an identical no. of base pairs (459 bp) as the predicted size of fragment given by primers specific to uPA mRNA. HUVECs express about one-half the amount of amplified uPA mRNA as HLMECs as determined by image analyzer. *B:* competitive RT-PCR was also performed on coexistence of competitor RNA for uPA (383 bp) with 9.6×10^5 (*lanes 1, 4,* and *7*), 9.6×10^6 (*lanes 2, 5,* and *8*), and 9.6×10^7 (*lanes 3, 6,* and *9*) copies of added competitor. *Lanes 1–3:* HLMECs; *lanes 4–6:* human liver endothelial cells; *lanes 7–9:* HUVECs.

with a competitive DNA construction kit using λDNA as a template. The competitor RNA was also made with a competitive RNA transcription kit using the competitor DNA according to the manufacturer's manual. The competitor RNA (9.6×10^5 to 9.6×10^7 copies) was added to each total RNA (1.56 ng) sample from the cultured endothelial cells, and RT-PCR was carried out with the same procedures as described above.

Cytokine treatment. After the monolayers of human lung endothelial cells were treated with one of the inflammatory cytokines (IL-1β, 1 ng/ml; IL-2, 0.1 ng/ml; or TNF-α, 1 ng/ml) for 10 h, they were maintained in serum-free medium (without the cytokines) for 12 more h and collected, and the cell number in each well was counted. The amount of uPA and PAI-1 antigens in the conditioned medium was measured as described in *Measurement of uPA, tPA, and PAI-1 antigens.* In separate examinations, 0.1, 1.0, or 10.0 ng/ml of IL-1β were added to the cultures of HLMECs and HUVECs. After 12 h of incubation, the conditioned medium was collected, and the amount of uPA antigen was measured. Data are expressed as mean ± SD. The mean values per 10^4 cells were determined in triplicate or duplicate for each experiment. For statistical analysis, Student's *t*-test was used.

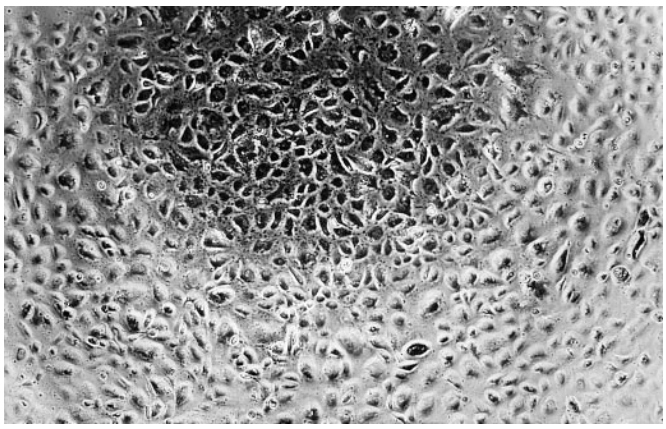

Fig. 4. Phase-contrast microphotograph of confluent monolayer of HLMECs on membrane of cell culture insert. Magnification, ×500.

RESULTS

The confluent monolayer of isolated pure cultures of HLMECs showed a typical cobblestone monolayer, and the representative endothelial marker, von Willebrand factor, was detected as in other endothelial cells but not in fibroblasts (data not shown). With the use of these cells, the amount of uPA and PAI-1 antigens secreted into the culture medium was determined. The amount of uPA antigen detected in HLMEC medium was significantly higher (>1.2–1.9 times) than in the medium of hepatic microvascular endothelial cells ($P < 0.05$), angioma endothelial cells ($P<0.001$), HUVECs ($P<0.001$), and lung fibroblasts ($P<0.001$; Fig. 1*A*). The amount of PAI-1 antigen secreted from the HLMECs was significantly higher than that secreted by the hepatic microvascular ($P<0.01$) and angioma endothelial cells ($P<0.05$) but lower than that secreted by HUVECs ($P<0.01$) and lung fibroblasts ($P<0.05$; Fig. 1*B*). In all cases, the amount of PAI-1 antigens secreted exceeded the amount of uPA secreted by ~100-fold. In fact, uPA activity was not detected at all in the conditioned medium when measurements were made with chromogenic substrate (data not shown). The freshly isolated human lung endothelial cells (the purity of endothelial cells was >90%) secreted comparable amounts of uPA and PAI-1 as the purified and serially passaged HLMECs (Fig. 2).

To examine uPA mRNA levels, total RNA fractions were obtained from various endothelial cells and lung fibroblasts and analyzed by the RT-PCR method described in METHODS. A difference in the expression of uPA mRNA was observed among the cells. The same result was observed in all five experiments. uPA mRNA was clearly detected in unstimulated HLMECs and hepatic microvascular endothelial cells, but the expression level was much lower in HUVECs and lung fibroblasts (Fig. 3*A*). The estimated amount of uPA mRNA in the RNA preparation from HLMECs (1.56 ng

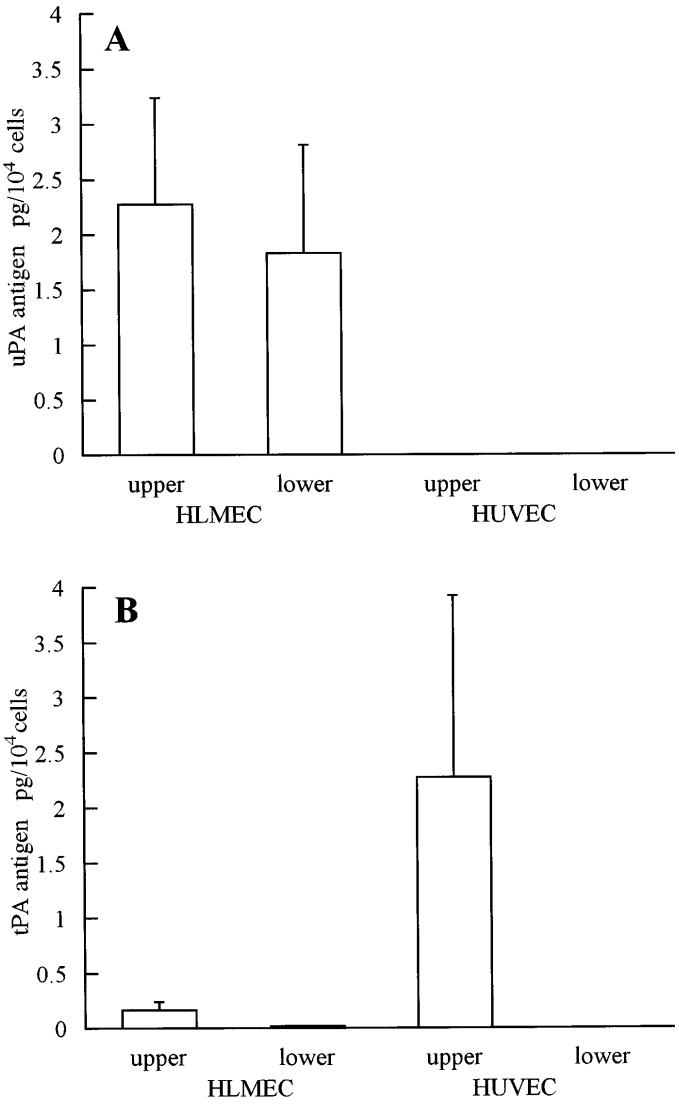

Fig. 5. Directionality of uPA (*A*) and tissue-type plasminogen activator (tPA; *B*) secretion by HLMECs and HUVECs. Confluent monolayers of HLMECs or HUVECs on membranes of cell culture inserts were fed with serum-free medium. After 12 h, amount of uPA and tPA antigens in upper and lower chambers was measured. Values are means ± SD; $n = 10$ samples for uPA and 3 samples for tPA.

as total RNA) was 9.6×10^7 copies but was 9.6×10^5 copies in that from HUVECs by competitive RT-PCR (Fig. 3*B*). The uPA mRNA in liver microvascular endothelial cells was $< 9.6 \times 10^5$ copies and in angioma cells and fibroblasts was not detected in these experiments.

For determination of possible direction specificity in uPA secretion, HLMECs were cultured on the fibronectin-coated polystyrene membrane of cell culture inserts. The cells expressed as a confluent monolayer on the membrane (Fig. 4). After 12 h of incubation in serum-free conditioned medium, the conditioned medium was collected from both the upper and lower chambers. uPA antigens were found in both chambers (Fig. 5*A*). To examine whether the secreted uPA to the upper chamber passed through the endothelial cell layer or whether the cells secreted uPA in both directions after the HLMECs reached confluency on the membrane of the cell culture insert, an excess amount of fluorescence-labeled single-chain uPA was added to the upper chamber. Neither the fluorescence nor the increase in uPA antigen in the lower chamber was detected after 12 h of incubation (data not shown). On the other hand, tPA antigen was found mainly in the upper chamber (100% HUVECs; 85% HLMECs; Fig. 5*B*).

Alteration in the uPA amount secreted from both HLMECs and HUVECs was measured after treatment with different concentrations of IL-1β. After 12 h of stimulation with IL-1β, the amount of uPA antigen secreted by HLMECs increased in a dose-dependent manner, and the increase reached almost sixfold with the concentration as low as 0.1 ng/ml (Fig. 6*A*). In contrast, the amount of uPA antigen in HUVEC-conditioned medium was unaffected by IL-1β treatment. On the other hand, a 10- to 15-fold amount of tPA

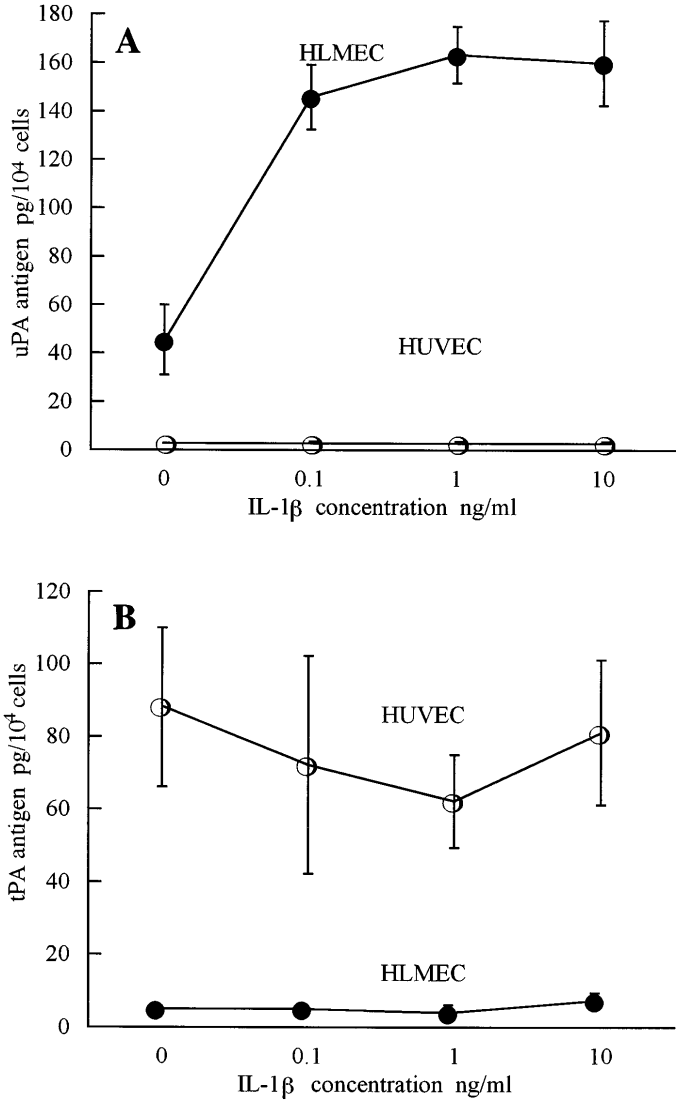

Fig. 6. Dose response of uPA (*A*) and tPA (*B*) secretion by HLMECs (•) and HUVECs (○) treated with interleukin (IL)-1β in serum-free keratinocyte-SFM. After 12 h of incubation, culture medium was collected, and amount of uPA and tPA antigens was measured. Values are means ± SD; $n = 6$ samples.

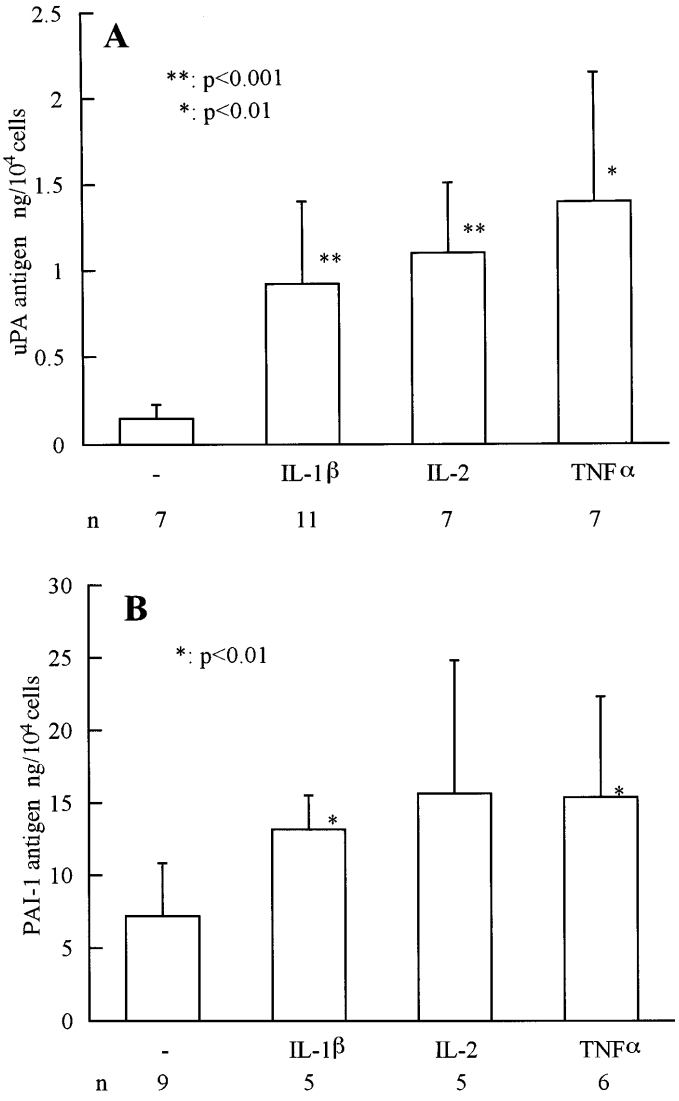

Fig. 7. Effect of cytokines on secretion of uPA (*A*) and PAI-1 (*B*) by HLMECs. Cells were treated with 1 ng/ml of IL-1β, 0.1 ng/ml of IL-2, or 1 ng/ml of tumor necrosis factor (TNF)-α for 10 h, and medium was changed to serum-free keratinocyte-SFM. After an additional 12 h of incubation, medium was collected, and cells were counted. Amount of uPA and PAI-1 antigens in conditioned medium was measured by ELISA method. Values are means ± SD; *n*, no. of samples. *P* values compared amount of antigens secreted by cytokine-untreated HLMECs.

was detected in HUVEC-conditioned medium compared with that in HLMECs (Fig. 6*B*). But the amount of tPA secretion did not respond to the addition of IL-1β. The confluent monolayers of HLMECs were treated with low levels of IL-1β, TNF-α, or IL-2 for 10 h. The three cytokines stimulated significant (6- to 9-fold) increases in the secretion of uPA antigens by HLMECs (IL-1β and IL-2, $P < 0.001$; TNF-α, $P < 0.01$; Fig. 7*A*). The amount of PAI-1 secreted by HLMECs was also significantly increased by treatment with IL-1β and TNF-α ($P < 0.01$; Fig. 7*B*). No significant changes in cell number or morphology were observed after the treatments.

DISCUSSION

The results of the present study clearly show that uPA is actively produced and secreted by microvascular endothelial cells from human lung and liver, but endothelial cells from large vessels produced less uPA (Figs. 1*A* and 3). Although the freshly isolated human lung endothelial cells were not pure endothelial cells (purity ~90%), they secreted comparable amounts of uPA and PAI-1 as the purified and serially passaged HLMECs (Fig. 2). We also found that there was a distinct difference between HLMECs and HUVECs on the reactivity of uPA and tPA production when stimulated by IL-1β (Fig. 6). It has been reported that HUVECs in primary culture produce almost exclusively tPA but progressively produce uPA after multiple passages (5). In the present experiments, endothelial cells (including HUVECs) were in younger generations (*passages 2–7*). Therefore, we expected to find little or no uPA activity in the HUVEC-conditioned medium. HLMECs secreted lower amounts of tPA than HUVECs. It suggested that uPA may work in a different way from tPA in vivo.

Many reports have demonstrated the heterogeneity of endothelial cells (21), especially with regard to angiogenesis (34), vulnerability to pathological conditions (26), and the distribution of membrane proteins (10). Interestingly, although a clear difference was found in the amount of uPA antigen, such a tissue specificity was not so obvious from the results of RT-PCR experiments, and slight but not negligible bands of uPA mRNA were detected in HUVECs (Fig. 3). In competitive RT-PCR, a semiquantitative RT-PCR method, the uPA mRNA expression in HLMECs was 100-fold higher than that in HUVECs. This result was the reflection of the ELISA data (Fig. 1). But there was less expression of uPA mRNA than the level assumed from the ELISA value in human liver microvascular endothelial cells. These discrepancies might reflect a difference in the turnover of mRNA, the posttranscriptional modulation such as processing or translation rate, the size of membrane and intracellular uPA pools, or the secretion rate of uPA.

All cells examined here also secreted about a 100-fold higher amount of PAI-1 than of uPA (Fig. 1*B*); the amount of uPA activated may be regulated by inhibitors to ensure that the quantity of tissues or proteins degraded is not excessive (4). Almost all uPA activity on the cell surface and in the culture medium might bind PAI-1 and be inactivated. Takahashi et al. (28) reported that bovine lung endothelial cells secreted almost negligible antigens of PA inhibitors. The differences could be construed as evidence for species specificity (11), which emphasizes the importance of the study using human cells for the interpretation of clinical significance, indicating that the use of animal cells may be misleading.

As in bovine cells (28), human lung endothelial cells secreted uPA antigen not only from the luminal surface but also from the surface attached to the basement membrane (Fig. 5*A*). Spontaneous leakage of uPA, which was secreted into the upper chamber, was not

present when the fluorescence-labeled uPA was added to the upper chamber (data not shown). The basement membrane of lung microvascular endothelial cells is in immediate contact with alveolar epithelial cells. uPA antigen was detected on the normal alveolar surface (3), and macrophages (6) or epithelial cells (18) have been suggested as a possible source of uPA. From the present findings, it seems likely that HLMECs may also supply uPA to alveolar surfaces through basement membrane-directed secretion as do bovine lung microvascular endothelial cells (28). It has been postulated that fibrinogen or other plasma proteins that leak into the alveolar spaces and interfere with the function of surfactant may normally be removed by uPA. In contrast, tPA from both HLMECs and HUVECs was secreted into the upper chamber (Fig. 5*B*). It suggests that the physical role of uPA may be different from that of tPA.

Secretion of uPA from HLMECs was potentiated with IL-1β, whereas no stimulation was found in HUVECs even under high IL-1β concentrations (Fig. 6*A*). In contrast, the secreted amount of tPA from HLMECs and HUVECs was not changed by the IL-1β concentration (Fig. 6*B*). The different response to this cytokine may also represent the tissue specificity of endothelial cells. Other cytokines, TNF-α and IL-2, also increased the secretion of uPA and PAI-1 from HLMECs (Fig. 7). In these experiments, the exposure to cytokines was brief, and the concentration of cytokines used in these experiments was much lower than that inducing morphological change or detachment of the cells (19, 23). Kiguchi et al. (14) reported that such minimal exposure to cytokines induced intercellular adhesion molecule-1 expression in bovine lung microvascular endothelial cells, which was potentiated by subthreshold hypoxia. IL-1β and TNF-α are known to be important inflammatory cytokines and coexist at sites of inflammation (23). Tissue-constructing cells at inflammatory sites are often damaged and need repair (19, 23). The actions of these cytokines on endothelial cells that promote leukocyte adhesion and activation are likely to be important in the development of acute inflammatory responses (14, 23). After the adhesion of leukocytes to endothelial cells, the leukocytes migrate into the surrounding tissues to support tissue repair (23). For this migration and the subsequent angiogenesis as a tissue repair mechanism, uPA is a very important protease involved in the degradation of connective tissues (12). The increased secretion of uPA and PAI-1 by inflammatory cytokines might play a significant role in acute and chronic lung inflammatory diseases. For example, uPA secreted from HLMECs might be directed to the cells that express uPAR, including endothelial cells themselves and macrophages. The uPA-uPAR binding would induce the migration of these cells and might help tissue repair at the inflamed sites. This postulate is based on the findings that ARDS is characterized by morphological and biological damage to lung microvascular endothelial cells. These cells provide protection against the development of acute lung injury and also participate in the repair of alveolar tissue after injury (3, 12, 24). However, further detailed examinations are needed before we can discuss a possible relationship between the present results and the pathogenesis of lung inflammation.

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