

Induction of urokinase-type plasminogen activator by interleukin- 1β and tumor necrosis factor- α in human lung microvascular endothelial cells

Katsuyuki KOBAYASHI, Kimiko TAKAHASHI, Hisanaga YAGYU, Koji KISHI,
Hiroyuki NAKAMURA, Katsuhiko NARUSHIMA, Takeshi MATSUOKA

Fifth Department of Internal Medicine, Tokyo Medical University, Ibaraki, Japan

Abstract

In order to clarify the role of human lung microvascular endothelial cells (HLMECs) at inflammatory sites such as those of acute lung injury, the effects of interleukin (IL)- 1β or tumor necrosis factor (TNF)- α on urokinase-type plasminogen activator (uPA) secretion and uPA mRNA expression in HLMECs were examined by means of ELISA and competitive RT-PCR. The amount of uPA secreted by HLMECs was significantly increased by treatment with both IL- 1β and TNF- α . The amount of uPA mRNA expression in HLMECs was significantly and dose-dependently (0.1, 1, 10 ng/ml) increased by treatment with IL- 1β or TNF- α , and both IL- 1β and TNF- α increased uPA mRNA expression at 4, 8, and 12 hrs after the cytokines addition. These results suggest that the increase of the uPA secretion by the cytokines stimulation is caused by the protein synthesis in HLMECs, and that HLMECs may produce uPA mRNA in the early stage of inflammatory diseases such as acute lung injury to secrete uPA for tissue repair and remodeling.

Introduction

Vascular endothelial cells play four major roles: 1) control of the transport of the nutritional materials of blood¹⁾; 2) modulation of vascular tone via production of endothelin, nitric oxide, or prostacyclin²⁾³⁾⁴⁾; 3) leukocyte transmigration⁵⁾; and 4) maintenance of blood fluidity via production of plasminogen activators and other fibrinolytic enzymes⁶⁾. Takahashi et al. reported that human lung microvascular endothelial cells (HLMECs) produced and secreted more urokinase-type plasminogen activator (uPA) than human hepatic endothelial cells, human umbilical endothelial cells, or angioma endothelial cells⁷⁾. These findings indicate that there is a greater requirement for uPA in the lungs than in other organs, and that uPA is therefore likely to play a unique role in the lung.

uPA is secreted as a single chain proenzyme of 55 kDa molecular weight that consists of 411 amino acids and still retains a low level of enzymatic activity⁸⁾⁹⁾¹⁰⁾. A cleavage at the peptide bond Lys 158-Ile 159 of pro-urokinase by plasmin or kallikrein leads to double-chain uPA as a high-molecular-weight urokinase consisting of an N-terminal part of 22 kDa molecular weight and a C-terminal part of 33 kDa molecular weight. An additional cleavage at the peptide bond Lys 135-Lys 136 and Arg 156-Phe 157 of uPA by plasmin leads to low-molecular-weight urokinase of 31.5 kDa. Activated uPA can bind to the uPA receptor (uPAR) found in the cell membrane of some types of cells to restrict uPA activity to the cell surface, and convert the zymogen plasminogen to plasmin, which degrades extracellular matrix components, including fibronectin and collagenase, allowing cell proliferation

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Correspondence and reprint requests to: Katsuyuki Kobayashi, MD, Fifth Department of Internal Medicine Kasumigaura Hospital, Tokyo Medical University 3-20-1, Chuou, Ami, Inashiki, Ibaraki 300-0395 Japan

Phone: +81-298-87-1161 FAX: +81-298-88-3463

E-mail: katsuk@tokyo-med.ac.jp

and migration¹¹⁾¹²⁾¹³⁾.

Though endothelial cells secrete urokinase-type plasminogen activator (uPA) and tissue-type plasminogen activator (tPA) in inflammation, uPA expression is markedly increased while tPA expression is decreased in comparison to the levels in normal endothelial cells¹⁴⁾. It has also been reported that lavage fluid from the normal lung contains a large amount of uPA antigen, but this enzyme activity was not detected in the bronchoalveolar lavage fluid of patients with acute respiratory distress syndrome¹⁵⁾. These observations suggest that uPA plays an important role in extravascular processes such as tissue remodeling and cell migration¹⁶⁾, and may also serve a protective role in surfactant function¹⁵⁾.

Interleukin (IL)-1 β and tumor necrosis factor (TNF)- α are known to appear during earliest-phase inflammatory lesion development¹⁷⁾, and can alter PA expression in endothelial cells to favor uPA production and extracellular activities¹⁸⁾¹⁹⁾. In this study, the effects of IL-1 β or TNF- α on uPA secretion and uPA mRNA expression in HLMECs were examined by means of ELISA and competitive RT-PCR in order to clarify the role of HLMECs at inflammatory sites such as those of acute lung injury.

Methods

Materials

Collagenase, EDTA, sulfuric acid, chloroform, isopropanol, ethanol, Tris, acetic acid, agarose and ethidium bromide were from Wako Pure Chemicals (Tokyo, Japan); newborn calf serum was from Mitsubishi Kasei (Tokyo, Japan); medium 199, endothelial-SFM, trypsin, TRIzol, 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); the competitive DNA construction kit, competitive RNA transcription kit and one step RNA PCR kit were from TaKaRa Biochemical (Tokyo, Japan); 24-well plates, 60-mm-diameter plastic culture dishes and 96-well microtest tissue culture plates were from Falcon (Lincoln Park, NJ); 6-well plates were from Costar (Cambridge, MA); the uPA ELISA kit was from Monozyme (Hoerholm, Denmark); and recombinant human IL-1 β and TNF- α were from Genzyme (Cambridge, MA).

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 under the permission of the ethics committee in Tokyo Medical University. The surgery was performed by the

Surgical Service of Kasumigaura Hospital, Tokyo Medical University (Ibaraki, Japan) and Mito Saiseikai General Hospital (Ibaraki, Japan). HLMECs were isolated according to the previously described method⁷⁾. In brief, the sections were digested with 0.1% collagenase, and obtained cells 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 μ g/ml of streptomycin) and incubated at 37°C to separate the endothelial cell-rich suspension from contaminating macrophages. After 1 hr, the cells floating in the dish were collected and seeded onto the fibronectin-coated dish (50 ng/cm²). 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.

Measurement of uPA antigens

The uPA antigens were determined by conventional ELISA methods according to the manuals of the kit. In brief, monoclonal mouse anti-human uPA antibody was coated on 96-well microtiter plates at 4°C overnight, and then samples were added to the plates to allow binding with the immobilized antibody at 4°C overnight again. Biotinylated monoclonal mouse anti-human uPA antibody was added to each well to react with the bound uPA antigen for 1 hr at room temperature, and peroxidase-conjugated streptavidin was again added to the wells at room temperature.

After 1 hr 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 were expressed as the means \pm SD. Mean values per 104 cells were determined in triplicate wells for each experiment.

Competitive RT-PCR

To obtain total RNA, cells cultivated in 6-well plates were lysed by the addition of TRIzol. The RNA was extracted with chloroform, isopropanol and ethanol by centrifugation after each addition according to the manufacturer's instructions. The extracted RNA was dissolved in distilled water, and the absorbance was

measured with a spectrophotometer (UV-1600; Shimadzu, Kyoto, Japan) at 260 nm to estimate the amount of total RNA.

The set of primers specific for human pro-uPA from a transformed human endothelial cell line, from which an amplified fragment of 459 bp was obtained, consisted of a forward primer beginning at 769 bp with a nucleotide sequence of 5'-GCCTTGCTGAAGATCCGTTCCAAGGAGGGC-3' and a reverse primer beginning at 1198 bp with a nucleotide sequence of 5'-CAGGCCATTCTCTTCCTTGGTGTGACTGCG-3'.

The 383 bp competitor DNA for uPA that has the sense and antisense uPA primer sequences at both its ends was made with a competitive DNA construction kit using λ DNA 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.

Competitor RNA (1×10^5 to 1×10^7 copies) was added to each total RNA (100 ng) sample from the cultured endothelial cells, and RT-PCR was carried out using one step RT-PCR kit according to the manufacturer's manual under the following conditions: 1 cycle at 50°C for 30 min; 1 cycle at 94°C for 2 min; and 25 cycles at 94°C for 30 sec, 65°C for 30 sec, and 72°C for 1 min (TaKaRa Thermal Cycler MP). The PCR reaction products were separated by electrophoresis on a 2.0% agarose-Tris-acetate-EDTA gel. The gel was stained with ethidium bromide and analyzed using a FluorImager SI (Amersham Pharmacia Biotech, Buckinghamshire, England).

Cytokine treatment

The monolayers of HLMECs were treated with endothelial-SFM for 4 hrs in order to subtract the effect of the serum. After the treatment, 1 ng/ml of IL-1 β or TNF- α was added to HLMECs. Before the cytokines addition or after an additional 12 and 24 hrs of incubation, the culture medium was collected, and cell numbers were counted. The amount of uPA antigen in the conditioned medium was measured by the ELISA method. In separate examinations, 0.1, 1, or 10 ng/ml of IL-1 β or TNF- α were added to the cultures of HLMECs. After 12 hrs of incubation, total RNA was extracted from HLMECs, and uPA mRNA was measured by the method described in competitive RT-PCR. Similarly, 1 ng/ml of IL-1 β or TNF- α was added to the cultures of HLMECs. After either 4, 8, or 12 hrs of incubation, total RNA was extracted from HLMECs, and competitive RT-PCR was carried out.

Results

The confluent HLMECs expressed the typical endothelial cell structure consisting of cobblestone

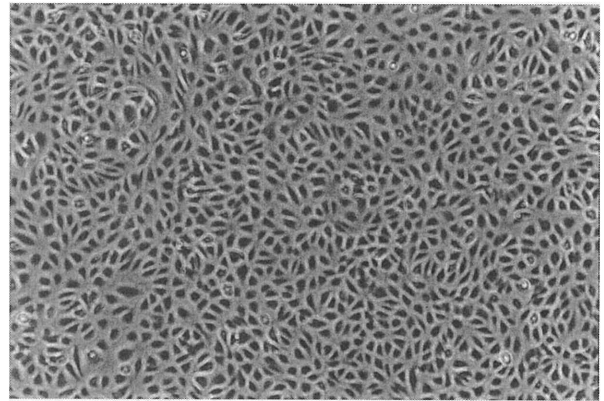


Fig. 1 Phase-contrast microphotograph of a confluent monolayer of HLMECs. Magnification, $\times 260$. These cells expressed typical endothelial cell structures with cobblestone monolayers.

monolayers (Fig. 1). These cells were also positive for factor VIII antigen, which is known to be the most remarkable marker of endothelial cells (data not shown).

Effect of IL-1 β or TNF- α on uPA secretion

The confluent monolayers of HLMECs were treated with 1 ng/ml of IL-1 β or TNF- α for 12 or 24 hrs. IL-1 β stimulation resulted in significant (13- to 20-fold) increases in the secretion of uPA antigen by HLMECs (Fig. 2A). The increase of uPA antigen by IL-1 β reached a peak after 12 hrs, and tended to decrease after 24 hrs. TNF- α also stimulated a significant (3.5- to 4-fold) increase in secretion of uPA antigen by HLMECs (Fig. 2B). The amount of uPA antigen stimulated with TNF- α increased continuously to 24 hrs.

Induction of uPA mRNA by IL-1 β and TNF- α

To further define the regulation of uPA by IL-1 β or TNF- α , total RNA fractions were obtained from HLMECs and analyzed by the competitive RT-PCR method as described in the Methods section. The amount of uPA mRNA in HLMECs induced by all concentrations (0.1, 1, 10 ng/ml) of IL-1 β was significantly (1.3- to 1.6-fold) increased in a dose-dependent manner (Fig. 3). On the other hand, there was no significant increase in the amount of uPA mRNA induced by 0.1 ng/ml TNF- α , while there was a significant increase (1.3- to 1.4-fold) in the amount of uPA mRNA induced by 1 and 10 ng/ml of TNF- α (Fig. 4). The time-course experiments demonstrated a marked upregulation of uPA mRNA by 1 ng/ml of IL-1 β or TNF- α which was evident at 4 hrs and peaked at 8 hrs (Figs. 5 and 6).

Discussion

uPA production and activity in endothelial cells are regulated by cytokines^{20,21}, growth factors²⁰, and hormones^{21,22}. IL-1 β and TNF- α are known as important inflammatory cytokines and coexist in the early

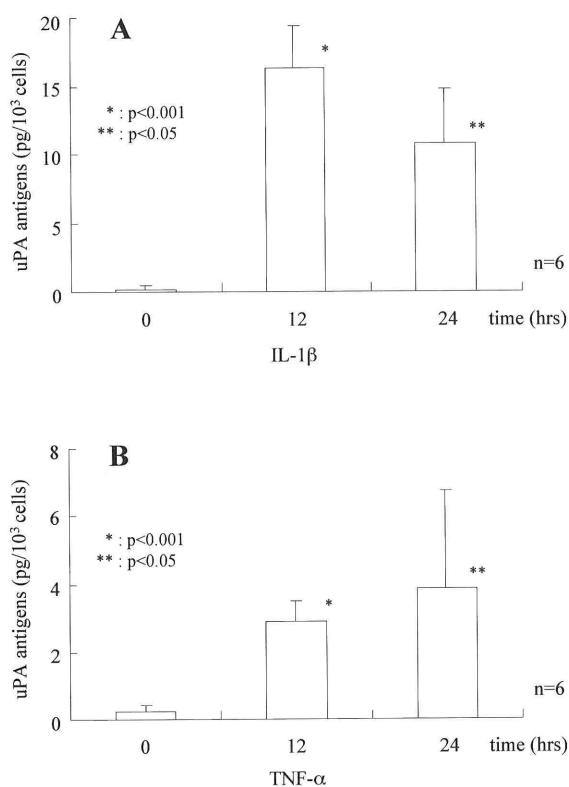


Fig. 2 Effect of IL-1 β (A) or TNF- α (B) on secretion of uPA by HLMECs. Cells were treated with endothelial-SFM. Four hours after the treatment, 1 ng/ml of IL-1 β or TNF- α was added to the cells. The medium was collected before and 12 and 24 hrs after the addition of cytokines, and cell numbers were counted. The amount of uPA antigen in the medium was measured by the ELISA method. Values are the means \pm SD. P values were compared to the amount of antigens secreted by cytokine-untreated HLMECs.

stage of inflammation¹⁷). Accordingly, we here examined the effects of IL-1 β or TNF- α on uPA secretion and uPA mRNA expression in HLMECs. HLMECs were obtained from human lung samples within 1–2 mm of the periphery.

Stimulation with either IL-1 β or TNF- α increased the amount of uPA antigen and uPA mRNA from HLMECs (Fig. 2, 3 and 4), and time-course experiments demonstrated a marked upregulation of uPA mRNA by these cytokines that was evident at 4 hrs (Figs. 5 and 6). These results show that the increase in uPA secretion by the cytokines stimulation is occurred via protein synthesis in HLMECs. The induction of uPA by low concentrations of cytokines (0.1, 1, or 10 ng/ml of IL-1 β or TNF- α) indicates that HLMECs secrete uPA even in surrounding tissue of inflammatory sites and in the early stage of inflammatory lung diseases such as acute lung injury. In this study, the exposure to cytokines was brief, and the concentration of cytokines used was much lower than that known to induce morphological change or detachment of the cells, because low-concentration

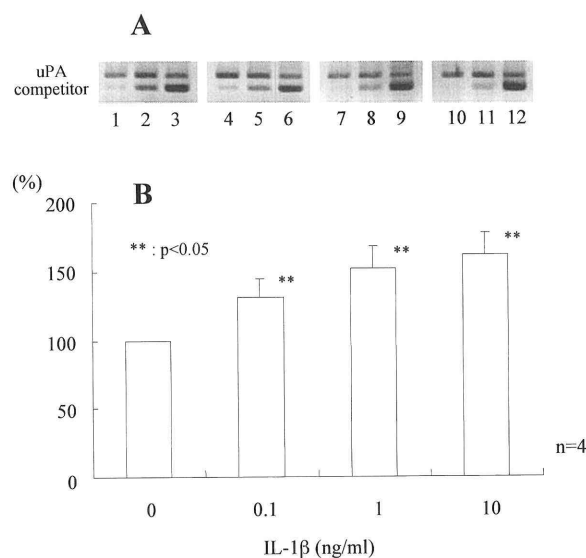


Fig. 3 Effect of IL-1 β on induction of uPA mRNA in HLMECs. Cells were treated with endothelial-SFM with or without each concentration (0.1, 1, 10 ng/ml) of IL-1 β for 12 hrs. After the stimulation, the amount of uPA mRNA was examined by competitive RT-PCR. A: Competitive RT-PCR was performed on coexistence of competitor RNA for uPA (383 bp) with 1 × 10⁵ (lanes 1, 4, 7, 10), 1 × 10⁶ (lanes 2, 5, 8, 11), and 1 × 10⁷ (lanes 3, 6, 9, 12) copies of added competitor. Lanes 1–3: without IL-1 β ; Lanes 4–6: 0.1 ng/ml IL-1 β ; Lanes 7–9: 1 ng/ml IL-1 β ; Lanes 10–12: 10 ng/ml IL-1 β . B: The amount of uPA mRNA was calculated with a FluorImager SI, and compared to the uPA mRNA value without the cytokine. Values are the means \pm SD. P values were compared to the amount of uPA mRNA expressed by cytokine-untreated HLMECs.

cytokine stimulation reflects the early inflammation stage and the circumference of the inflammation itself.

Although there have been many investigations on uPA expression using various vascular endothelial cells, the results have differed according to the type of vascular endothelial cells^{7,23}). In a study by Takahashi et al., HLMECs produced and secreted a much larger amount of uPA than liver vascular endothelial cells or human umbilical vein vascular endothelial cells⁷). These findings indicate that there is tissue specificity in the roles of each type of vascular endothelial cell. They also show that vascular endothelial cells of the lung should be used for examinations of lung disease. Furthermore, endothelial cells from the peripheral lung should be used when studying the physiological response between vascular endothelial cells and alveoli pulmonis or interstitial tissue.

The vascular endothelial cell regulates fibrinolytic activity not only of the intravascular spaces by secreting uPA, but also on the cell surface by expression of uPAR which exists on endothelial cells themselves²⁴) and macrophages²⁵). The combination of uPA and uPAR

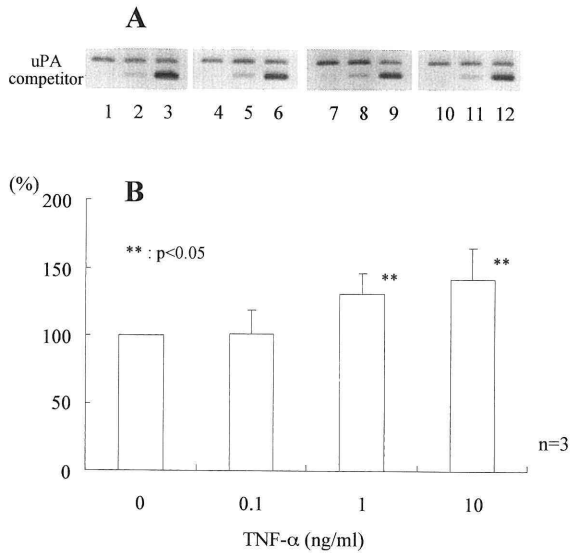


Fig. 4 Effect of TNF- α on induction of uPA mRNA in HLMECs. Cells were treated with endothelial-SFM with or without each concentration (0.1, 1, 10 ng/ml) of TNF- α . The amount of uPA mRNA was examined by the same method described in the legend of Fig. 3. Competitive RT-PCR was performed on coexistence of competitor RNA for uPA (383 bp) with 1×10^5 (lanes 1, 4, 7, 10), 1×10^6 (lanes 2, 5, 8, 11), and 1×10^7 (lanes 3, 6, 9, 12) copies of added competitor. Lanes 1-3: without TNF- α ; Lanes 4-6: 0.1 ng/ml TNF- α ; Lanes 7-9: 1 ng/ml TNF- α ; Lanes 10-12: 10 ng/ml TNF- α . B: The amount of uPA mRNA was calculated with a FluorImager SI, and compared to the uPA mRNA value without the cytokine. Values are the means \pm SD. P values were compared to the amount of uPA mRNA expressed by cytokine-untreated HLMECs.

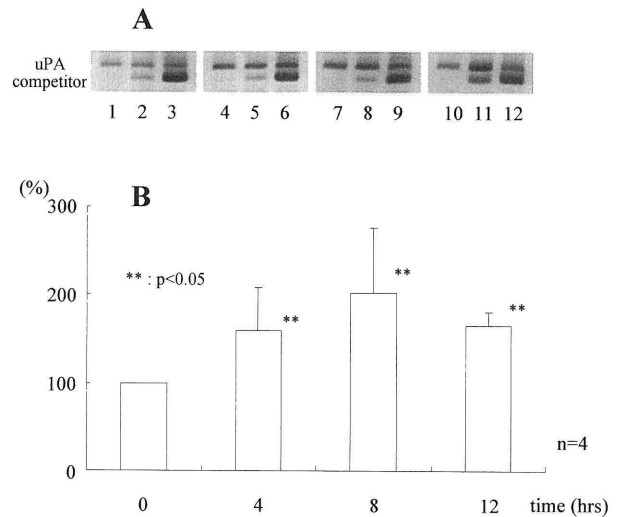


Fig. 5 Time-course of uPA mRNA induction by IL-1 β in HLMECs. Cells were treated with endothelial-SFM medium with 1 ng/ml of IL-1 β . The amount of uPA mRNA was examined by competitive RT-PCR at 4, 8, and 12 hrs after the cytokine addition. A: Competitive RT-PCR was performed on coexistence of competitor RNA for uPA (383 bp) with 1×10^5 (lanes 1, 4, 7, 10), 1×10^6 (lanes 2, 5, 8, 11), and 1×10^7 (lanes 3, 6, 9, 12) copies of added competitor. Lanes 1-3: before IL-1 β addition; Lanes 4-6: at 4 hrs after IL-1 β addition; Lanes 7-9: at 8 hrs after IL-1 β addition; Lanes 10-12: at 12 hrs after IL-1 β addition. B: The amount of uPA mRNA was calculated with a FluorImager SI, and compared to the uPA mRNA value without the cytokine. Values are the means \pm SD. P values were compared to the amount of uPA mRNA expressed by cytokine-untreated HLMECs.

activates various enzymes or growth factors, and regulates the proteolytic digestion of the cell circumference for angiogenesis, cell proliferation and migration through the intracellular signal transduction systems²⁶⁾²⁷⁾³⁸⁾ in order to assist tissue repair at the inflamed sites.

Hepatocyte growth factor (HGF), also known as scatter factor, is a plasminogen-like protein with mitogenic and chemotactic effects on a wide variety of cells²⁹⁾. HGF is secreted as a single chain precursor devoid of biological activity (pro-HGF) by mesenchymal cells, and the pro-HGF is activated by uPA³⁰⁾. This activation would contribute to the repair of wounded cells and tissues at inflammatory sites. It has also been reported that uPA activates pro-HGF³¹⁾, and

that HGF from fibroblasts promotes DNA synthesis and proliferation of rat alveolar type II cells³²⁾. We reported that HLMECs secreted uPA not only to the vascular side but also to the basement membrane⁷⁾. Together, these reports indicate that uPA from HLMECs binds to uPAR except on the cell surface of HLMECs or intravascular macrophages, and that such bound uPA activates pro-HGF from not only HLMECs themselves but also epithelial cells and fibroblasts to assist tissue repair at the interstitium and alveolar spaces in lung injury. However, further detailed examinations are needed before we can discuss a possible relationship between the present results and the pathogenesis of lung inflammation.

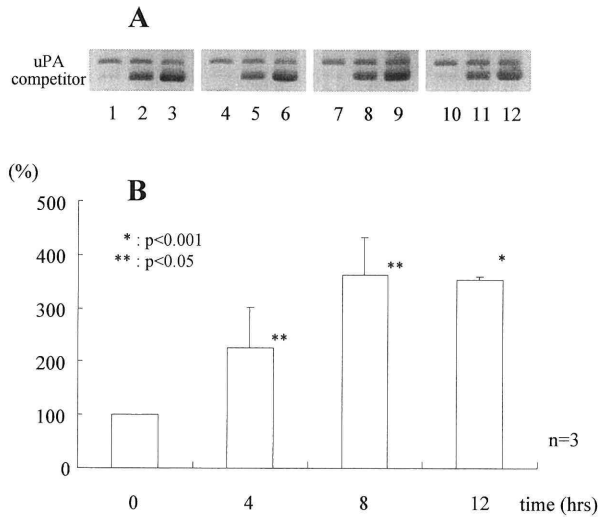


Fig. 6 Time-course of uPA mRNA induction by $\text{TNF-}\alpha$ in HLMECs. Cells were treated with endothelial-SFM medium with 1 ng/ml of $\text{TNF-}\alpha$. The amount of uPA mRNA was examined by the same method as described in the legend of Fig. 5. A: Competitive RT-PCR was performed on coexistence of competitor RNA for uPA (383 bp) with 1×10^5 (lanes 1, 4, 7, 10), 1×10^6 (lanes 2, 5, 8, 11), and 1×10^7 (lanes 3, 6, 9, 12) copies of added competitor. Lanes 1-3: before $\text{TNF-}\alpha$ addition; Lanes 4-6: at 4 hrs after $\text{TNF-}\alpha$ addition; Lanes 7-9: at 8 hrs after $\text{TNF-}\alpha$ addition; Lanes 10-12: at 12 hrs after $\text{TNF-}\alpha$ addition. B: The amount of uPA mRNA was calculated with a FlourImager SI, and compared to the uPA mRNA value without the cytokine. Values are the means \pm SD. P values were compared to the amount of uPA mRNA expressed by cytokine-untreated HLMECs.

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ヒト肺微小血管内皮細胞における IL-1 β および TNF- α 刺激後の uPA 誘発の検討

小林 克行 高橋 君子 柳 生 久 永
岸 厚 次 中 村 博 幸 成 島 勝 彦
松 岡 健

東京医科大学内科学第5講座

【要旨】 急性肺傷害などの炎症の場におけるヒト肺微小血管内皮細胞 (HLMECs) の役割を明らかにするため、HLMECs からの uPA 分泌と uPA mRNA の発現に対する IL-1 β および TNF- α 刺激の影響を ELISA 法と competitive RT-PCR 法を用いて検討した。HLMECs から分泌される uPA は IL-1 β または TNF- α 刺激によって明らかに増加した。HLMECs での uPA mRNA 発現は IL-1 β または TNF- α 刺激に対し明らかに増加し、それらの刺激量(0.1, 1, 10 ng/ml)に依存した mRNA 発現の増加が認められた。また、それぞれのサイトカイン刺激の 4, 8, 12 時間後で uPA mRNA 発現の増加が認められた。これらの結果は、IL-1 β および TNF- α 刺激によって HLMECs より分泌される uPA は HLMECs 自体によってタンパク質合成されたものであり、HLMECs は組織修復やリモデリングに必要な uPA を分泌するために、急性肺傷害などの炎症性疾患の早期に uPA mRNA を産生していることを示唆するものである。

〈Key words〉 ウロキナーゼ型プラスミノゲンアクティベーター, 急性肺傷害, ヒト肺微小血管内皮細胞
