Protease-activated receptor (PAR)-1 and PAR-2 participate in the cell growth of alveolar capillary endothelium in primary lung adenocarcinomas

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Protease-Activated Receptor (PAR)-1 and PAR-2 Participate in the Cell Growth of Alveolar Capillary Endothelium in Primary Lung Adenocarcinomas

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BACKGROUND. Cell growth can be induced via elicitation of protease-activated receptors (PAR) with serine proteases such as thrombin and trypsin.

METHODS. To understand whether PAR are involved in tumor vessel formation in the neoplastic cell-bearing alveolar walls, immunohistochemical and reverse transcriptase-polymerase chain reaction analyses were performed using the lung tissues from 16 patients with primary lung adenocarinomas.

RESULTS. In microdissected tumor alveolar walls, the expressions of PAR-1 and PAR-2 mRNA were increased by 10-fold ($P < 0.05$) and 16-fold ($P < 0.01$), respectively, as compared with normal alveolar walls. Confocal microscopy revealed that tumor capillary endothelial cells in alveolar walls lost thrombomodulin expression. Instead, the expression of PAR-2 often became obvious at the normal border. Both PAR-1 and PAR-2 were expressed in the microvessel endothelial cells in tumors. Trypsin mRNA was expressed in 7 of the 16 cancer cell-bearing tissue specimens in contrast to 1 of the 14 normal alveolar walls. Immunohistochemically, trypsin was positive in the neoplastic cells from 10 patients and in lung adenocarcinoma cell lines (A549, HLC-1, LC-2, and PC-14). An in vitro assay showed a significant increase in idoxuridine (IdU) or bromodeoxyuridine uptake in human pulmonary artery endothelial cells and human umbilical cord vein endothelial cells after treatments with α-thrombin or activating peptides; SFLLRN for PAR-1 and SLIGKV for PAR-2, respectively.

CONCLUSIONS. Thus, proliferation of alveolar capillary endothelial cells is initialized in part by PAR activation with serum thrombin and neoplastic cell-released trypsin. These results suggest a synergistic effect of PAR with vascular endothelial growth factor in alveolar angiogenesis. ***Cancer*** **2003;97:703–13.**

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KEYWORDS: protease-activated receptor, serine protease, angiogenesis, vascular endothelial cell, lung adenocarcinoma.

Angiogenesis is induced by a variety of proliferating stimuli to vascular endothelial cells, including basic fibroblast growth factor, vascular endothelial growth factor (VEGF), hepatocyte growth factor, and platelet-derived growth factor. These factors leading to tumor vessel formation[1] are frequently released from neoplastic cells or their surrounding mesenchymal cells. The normal alveolar capillary endothelium is morphologically characterized by a very thin and nonfenestrated cytoplasm. The cytoplasm of these endothelial cells, as demonstrated immunohistochemically, lacks the expression of von Willebrand factor and is covered fully by thrombomodulin, which exerts anticoagulant function as a thrombin receptor.[2] These capillary endothelial cells are quiescent in a normal environment. In primary

lung adenocarcinomas, angiogenesis in the alveolar walls is associated with increased expression of $VEGF_{165}$ mRNA and its protein is derived from the neoplastic cells.[3] In addition, KDR (the VEGF receptor) mRNA was simultaneously up-regulated in the capillary endothelial cells, whereas their phenotype, as demonstrated by immunohistochemistry and ultrastructure, clearly changed to the type of bronchial capillaries.[4] However, VEGF is not the sole determinant of capillary endothelial cell growth.

Protease-activated receptors (PAR-1 to PAR-4), G-protein–coupled receptors, were found in a variety of cells in mammalian tissues.[5,6] They are not stimulated by the conventional ligand-receptor interaction, but they require partial proteolytic cleavage of the NH_2 terminus by serine proteases such as thrombin, trypsin, and mast cell tryptase. The consequence of PAR-1 activation by thrombin[7] will lead to proliferation of mesenchymal cells such as endothelial cells,[8,9] procollagen synthesis in smooth muscle cells,[10] and up-regulation of interleukin-8 release from fibroblasts.[11] The PAR-2 receptor is distributed systemically in the human body.[12] Airway contraction/relaxation is modulated by PAR-2 with elicitation by trypsin in bronchial epithelial cells.[13] There is increasing evidence that PAR family members play pivotal roles in cell physiology and in wound healing.[5,6] However, it is not known whether the PAR family is expressed in any cells of the alveolar walls, such as alveolar epithelial cells, septal fibroblasts, capillary endothelial cells, and pericytes. To understand the role of PAR in alveolar wall angiogenesis, we analyzed the mRNA expression and immunohistochemical localization of PAR family members in alveolar wall tissues covered with neoplastic cells of primary lung adenocarcinomas. This is the first study to indicate that serine proteases are actively involved in the angiogenesis of alveolar walls. We suggest that there may be synergistic effect with VEGF[14] in primary lung adenocarcinomas.

MATERIALS AND METHODS

Patients and Tissues

Fresh frozen tissues were obtained from 16 patients who underwent a lobectomy for a solitary primary adenocarcinoma. All tumors originated in the lung periphery.[2–4] The normal lung tissue samples were taken from distant areas from isolated tumor nodules and 14 samples were histologically normal. The current study focused mainly on the alveolar walls with or without surface spreading of neoplastic cells. Using the World Health Organization guidelines,[15] the primary lung adenocarcinomas in the current study comprised two histologic patterns, i.e., bronchoalveolar carcinoma and papillary adenocarcinoma. Tumor tissue specimens were cut into small pieces for different preparation. For example, frozen sections without fixation were used for mRNA analysis, frozen sections with brief fixation in 4% buffered paraformaldehyde were used for immunofluorescence staining, and fresh tissues with fixation in 10% buffered formalin were used for paraffin embedding. For immunohistochemical studies, the frozen or paraffin sections were reacted with primary antibodies as described below. For mRNA analysis, normal and neoplastic cell-spreading alveolar walls were microdissected using the laser capture microdissection method (LCM 100 Image Archiving Workstation, Arcturus Engineering, Mountain View, CA).

Immunohistochemical Stainings

Immunoperoxidase staining

Deparaffinized tissue sections were stained by an avidin-biotin complex immunoperoxidase method.[2–4] The sections were treated with 0.3% hydrogen peroxide in methanol for 15 minutes at 20°C to suppress endogenous peroxidase activity and incubated for 10 minutes in 10% normal rabbit serum to prevent nonspecific binding of the primary antibody. Incubation with the primary antibody for PAR-1 (mouse monoclonal [mm] IgG, WEDE15, ImmunoTech, Marseille, France, 1:100) was performed overnight at 4 °C. The sections were reacted with 3,3′-diaminobenzidine and counterstained with hematoxylin.

Immunofluorescence staining

Fresh tissues were embedded in OCT compound, snap frozen in acetone dry ice, and stored at −80 °C until use. An indirect single or double immunofluorescence method was applied on 4-μm–thick sections as described previously.[2–4] Briefly, the sections, as well as the cells ($1–5 \times 10^5$ in eight-well chamber slides fixed with acetone) of cancer and endothelial cell lines described below, were incubated overnight at 4 °C with a combination of mm IgG antibody or rabbit polyclonal (rp) IgG antibody against different antigens. The primary antibodies comprised thrombomodulin (mm IgG, TM1009, Dako, Carpinteria, CA, 1:100), trypsin (mm IgG, Chemicon, Temecula, CA, 1:150), thrombin (mm IgG, T4, Biogenesis, Poole, England, 1:100), and PAR-2 (rp IgG, Teijin, Tokyo, Japan, 1:150). After washing, the sections were incubated with fluorescein isothiocyanate (FITC)-labeled goat antirabbit IgG (Vector Laboratories, Burlingame, CA) for a single stain. For double staining, FITC-labeled goat antirabbit and Texas red-labeled horse antimouse IgG were incubated as secondary antibodies for 60 minutes at room temperature in the dark. Nuclear counterstaining was done at room temperature with 0.01% TOTO-3 iodide

(Molecular Probes, Eugene, OR). The sections were evaluated by a confocal laser scanning microscope (model TC-SP, Leica, Heidelberg, Germany) equipped with argon and argon-krypton laser sources. In the resulting preparations, red fluorescence represented thrombomodulin, thrombin, or trypsin and green fluorescence depicted PAR-2. The nuclei appeared as blue.

Immunohistochemical Control Procedures

In the two negative control procedures, 1) the primary antibody was omitted from the staining procedure and 2) the primary antibody was replaced by corresponding amounts of normal immunoglobulin from the same animal species. Both control procedures consistently gave negative results.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Analyses

The detailed procedure for RT-PCR was described in previous papers.[3,4] Briefly, total RNA was extracted using an RNeasy mini kit (Qiagen, Hilden, Germany) from microdissected normal and neoplastic cell-spreading alveolar walls and from adenocarcinoma and endothelial cell lines. Complementary DNA was synthesized from total RNA primed with oligo (dT) using superscript II reverse transcriptase (Gibco BRL Rockville, MD) at 42 °C for 1 hour. The RT-PCR analysis was carried out using 1 μL of the cDNA products. The primer sequences for thrombin were forward, 5′-TGGGTACTGCGACCTCAACTAT-3′; reverse, 5′-CAGACACACAGGGTGAATGTAGTC-3′; the product size, 601 bp, and for trypsin: forward, 5′-CTCCTGATCCTTACCTTTGTGG-3′; reverse, 5′-AGGGTAGGAGGCTTCACACTTAG-3′; the product size, 519 bp. The primer sequences for PAR-1 were forward, 5′-CAGTTTGGGTCTGAATTGTGTCG-3′; reverse, 5′-TGCACGAGCTTATGCTGCTGAC-3′; the product size, 592 bp, and for PAR-2; forward, 5′-TGGATGAGTTTTCTGCATCTGTCC-3′; reverse, 5′-CGTGATGTTCAGGGCAGGAATG-3′; the product size, 491 bp.

Following one cycle of denaturation at 94 °C for 10 minutes, PCR was performed at 94 °C for 45 seconds, 60 °C for 45 seconds for thrombin and trypsin (for PAR-1 and PAR-2, annealing was done at 58 °C for 45 seconds), and extension at 72 °C for 2 minutes for all primers. Following 18–25 cycles of PCR for endothelial cells or 40 cycles for the others, the reactions were stopped by chilling to 4 °C. The PCR products were electrophoresed on 2% agarose gels and visualized by ethidium bromide staining. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control. Semiquantitative measurements of each mRNA were done based on the standard curves constructed for each PCR product and GAPDH. The analyses were done using Molecular Imager FX and Quantity One software package (Bio-Rad, Hercules, CA).

Human Lung Adenocarcinoma Cell Lines

Four human lung carcinoma cell lines were purchased from the Riken Cell Bank (Tokyo, Japan) and used with the following culture media: A549 (Dulbecco's modified Eagle medium + 10% fetal bovine serum [FBS]), HLC-1 (Ham F12 and 10% FBS), LC-2/ad (Ham F12, RPMI 1640, 15% FBS, and 25 mM HEPES), and PC-14 (RPMI 1640 and 10% FBS). They were derived from human lung adenocarcinomas—A549 was derived from alveolar cancer cells and the others were derived from bronchogenic carcinomas with high (HLC-1), moderate (LC-2/ad), and poor (PC-14) differentiation.

Human Endothelial Cells

Normal human pulmonary artery endothelial cells (HPAEC), human umbilical cord vein endothelial cells (HUVEC), and human aortic endothelial cells (HAEC) were obtained from Clonetics (Walkersville, MD). They were cultured on gelatin-coated dishes in an endothelial cell basal medium (EBM) (Clonetics) supplemented with EGM SingleQuots (BioWhittaker, Walkersville, MD). Cells were maintained in a tissue culture incubator (5% CO_2-95% air, 95% humidity) at 37 °C temperature. Only four to eight passages of the cell lines were used for the experiments.[16]

In Vitro Endothelial Cell Proliferation Assay with α-Thrombin and Activating Peptides for PAR-1 (SFLLRN) and PAR-2 (SLIGKV)

Recombinant human tumor necrosis factor-alpha (TNF-α) was obtained from R&D Systems (Minneapolis, MN) and human plasma thrombin from Sigma Bachem (St. Louis, MO). The synthetic activating peptides, SFLLRN and SLIGKV, were purchased from Bachem (Bubendorf, Switzerland). DNA synthesis in endothelial cells was analyzed after the treatment of α-thrombin or SLIGKV with the DNA-IdU labeling and detection kit (Takara Shuzou, Shiga, Japan). The HPAEC and HUVEC were seeded into 48-well dishes at 5×10^3 cells per well for 2 days and cultured in serum-reduced medium (one-forth of the usual medium) for 18 hours. Alpha-thrombin was applied to the cells in serum-free EBM (0.25% bovine serum albumin) for 12 hours. Cells were labeled with 10 μmol/L IdU for 12 hours.

After fixation, anti-IdU and horseradish peroxidase-conjugated antimouse IgG antibodies were used for detecting incorporated IdU. All processes were performed according to the manufacturer's manual (Takara, Chiba, Japan). The IdU incorporation rate (percent control) gave the normalized data at each

time point against the untreated control, which was set to 100%. The proliferation rates of SFLLRN-treated cells were determined by enzyme-linked immunosorbent assay (ELISA) using the bromodeoxyuridine (BrdU) labeling and detection kit III (Boehringer Mannheim, Mannheim, Germany). Cells were placed as described above and cultured in serum-reduced medium for 18 hours. The endothelial cells were treated for 12 hours at the indicated concentration of SFLLRN and further incubated for 12 hours with the presence of BrdU (10 μmol/L). Then, cells were fixed in 70% ethanol (0.5 M HCl) for 30 minutes at -20 °C and processed for ELISA using a peroxidase (POD)-conjugated antibody against BrdU according the manufacturer's instructions. To compare the results with those following up-regulation of PAR-2 mRNA expression, a 10-ng/mL TNF-α pretreatment[16] was done for 24 hours and SLIGKV was added to HPAEC and HUVEC. DNA uptake was measured in the same way as in α-thrombin.

Statistics

The experiments were repeated three times. Data are presented as means $\pm$ SD. Analysis was performed using the Student's t test for unpaired observations (two-tailed), and significance was considered at P value less than 0.05.

RESULTS

Immunohistochemical and Confocal Microscopic Observations

An immunohistochemical reaction for thrombomodulin was consistently found along cell membranes in the normal alveolar capillary endothelial cells. However, expression of thrombomodulin was negative in most tumor cell-bearing capillary endothelial cells. Some neoplastic cells were faintly positive for the antigen in one tumor tissue specimen (data not shown). The reactions for PAR-1 and PAR-2 were not usually found in any cells located in the normal alveolar walls. However, the relatively hypertrophic cytoplasm of Type II alveolar epithelial cells was occasionally positive for these receptor antigens. The cytoplasm of neoplastic cells overlying the alveolar walls was often reactive for PAR-1 (Fig. 1A) and PAR-2 (Fig. 1B,C) in all patients examined. Groups of neoplastic cells in the alveolar walls were unreactive for each antigen. Subjacent to neoplastic cells in the alveolar walls, the plasma membrane of capillary endothelial cells was occasionally reactive for PAR-1. The PAR-1–reactive endothelial cells occurred densely in dilated microvessels in fibrotic alveolar walls.

At the border areas between normal and tumor-spreading alveolar walls, thrombomodulin was demonstrated along the capillary endothelial surface linings in the alveolar walls with no tumor cell invasion. However, the expression of thrombomodulin was often lost along the cytoplasmic segments of the endothelial cells located beneath the neoplastic cells. Confocal dual immunofluorescent study demonstrated that a positive reaction occurred segments of the capillary loops that were unreactive for thrombomodulin (Fig. 1B). Alveolar capillary endothelial cells were also reactive for PAR-2 beneath the continuous arrangement of neoplastic cells that were frequently reactive for the antigen as well (Fig. 1C). Microvessels deep in the scar tissue of tumors were uniformly reactive for PAR-1 and PAR-2, whereas they totally lacked the reaction for thrombomodulin.

Trypsin was immunohistochemically demonstrated in the normal bronchial basal cells, whereas normal alveolar wall cells were not. The cytoplasm of neoplastic cells was often positive for trypsin in 10 of 16 patients (Fig. 1D). The neoplastic cells that were fully reactive for trypsin often showed less reactivity for PAR-2 (Fig. 1D). Thrombin was never retrieved in any neoplastic cells or cells in the alveolar walls in paraffin or frozen sections. In all cell lines of primary lung adenocarcinomas (A549, HLC-1, LC-2ad, and PC-14), the immune reactions for PAR-1, PAR-2 (Fig. 2), and trypsin (Fig. 3) were positive. However, the fluorescence intensity of PAR-2 varied among the cell lines and the reaction in HLC-1 was quite faint compared with the others. Thrombin was not reactive in any of the cell lines.

RT-PCR in the Microdissected Tissues of Alveolar Walls and Cancer Cell and Endothelial Cell Lines

In the microdissected normal alveolar wall tissues from the 14 patients, PAR-1, PAR-2, and trypsin mRNA were detectable in 4 (28%; mean/GAPDH $\pm$ SE: 0.011 $\pm$ 0.006), 5 (36%; 0.063 $\pm$ 0.023), and 1 (7%; 0.099) patient, respectively. In contrast, these mRNA levels were demonstrated in 14 (87%; 0.111 $\pm$ 0.034), 15 (88%; 0.983 $\pm$ 0.167), and 7 (43%; 0.468 $\pm$ 0.164) of the 16 adenocarcinoma patients, respectively. Compared with normal alveolar walls, the mean expression of the above mRNA levels indicate a 9.6 ($p < 0.05$), 15.6 ($p < 0.01$), and 4.7-fold increase in the neoplastic cell-bearing alveolar walls, respectively (Fig. 4A,B). The concordance rates between immunohistochemical and RT-PCR results for PAR-1, PAR-2, and trypsin were 70%, 87%, and 50%, respectively.

The RT-PCR analysis demonstrated that PAR-1 and PAR-2 mRNA were detected in each of the adenocarcinoma cell lines. Trypsin mRNA was expressed in LC-2 and PC-14, but was not detectable in the A549 and HLC-1 cell lines (Fig. 4C). Thrombin mRNA was not detected in any of the cell lines. Compared with

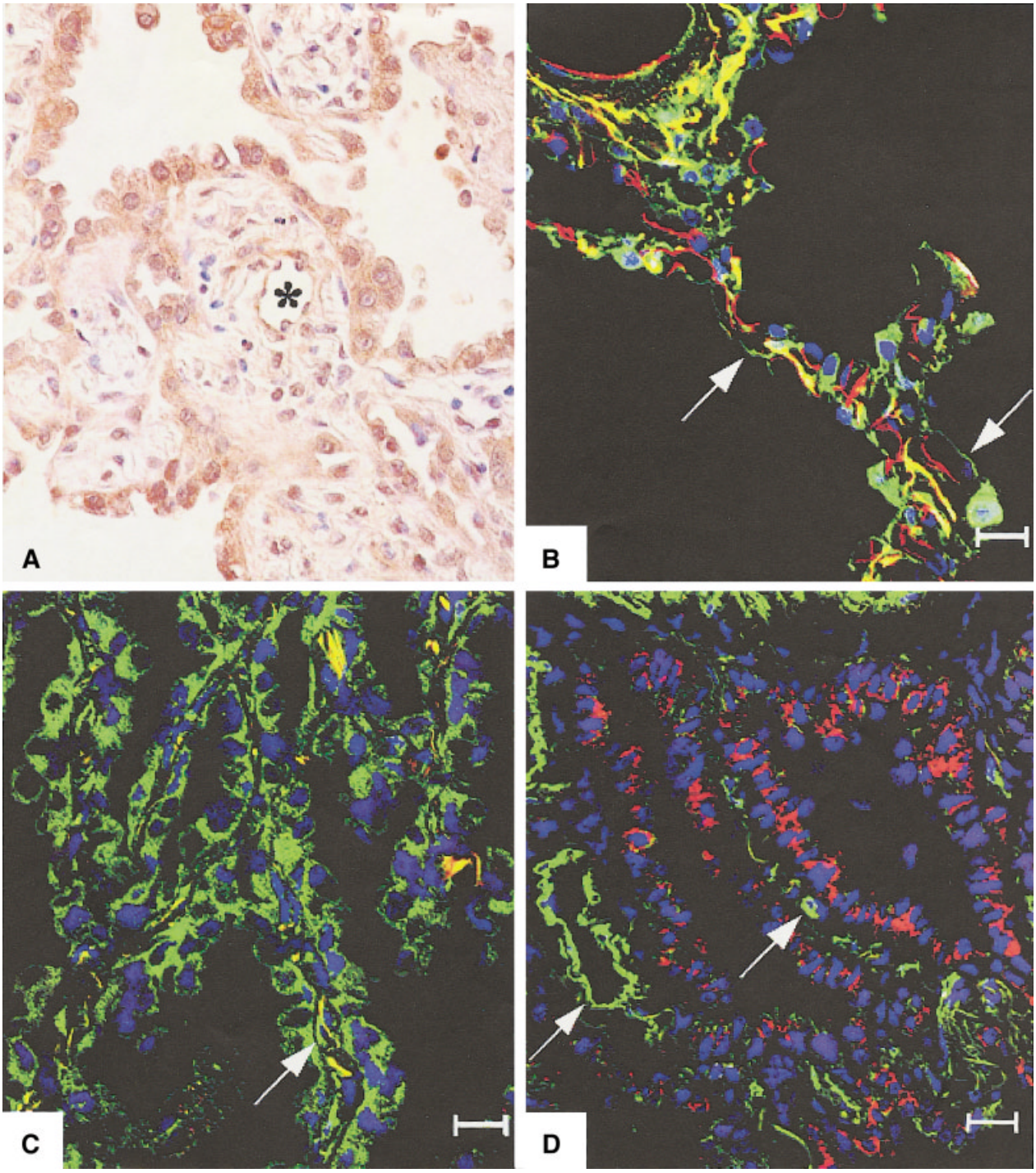


FIGURE 1. Immunohistochemical single staining for protease-activated receptor (PAR)-1 and dual stainings for PAR-2 (green) and thrombomodulin (TM) (red); (B and C), and for PAR-2 (green) and trypsin (red) in (D) in primary lung adenocarcinomas. (A) The PAR-1 reaction is shown in the neoplastic cells spreading on alveolar walls and in subjacent alveolar capillary endothelial cells (*). (B) Isolated neoplastic cells located along the alveolar walls are reactive for PAR-2. Note that the positive reaction for TM is retained along some portions of the cell membrane in alveolar capillary endothelia, but that the remaining capillary loops change their immunohistochemical property to PAR-2 (arrows). (C) Most tumor cells lining the alveolar walls stain positively for PAR-2. In addition, the capillaries (arrow) situated within the alveolar walls are reactive for the antigen, but not for TM. (D) Neoplastic cells of adenocarcinoma are strongly reactive for trypsin, but not for PAR-2. The PAR-2 positive reaction is evident in the endothelial cells of microvessels (left arrow) as well as of alveolar capillaries (right arrow). Scale bar = 20 μm (B–D); magnification $\times$ 200 (A).

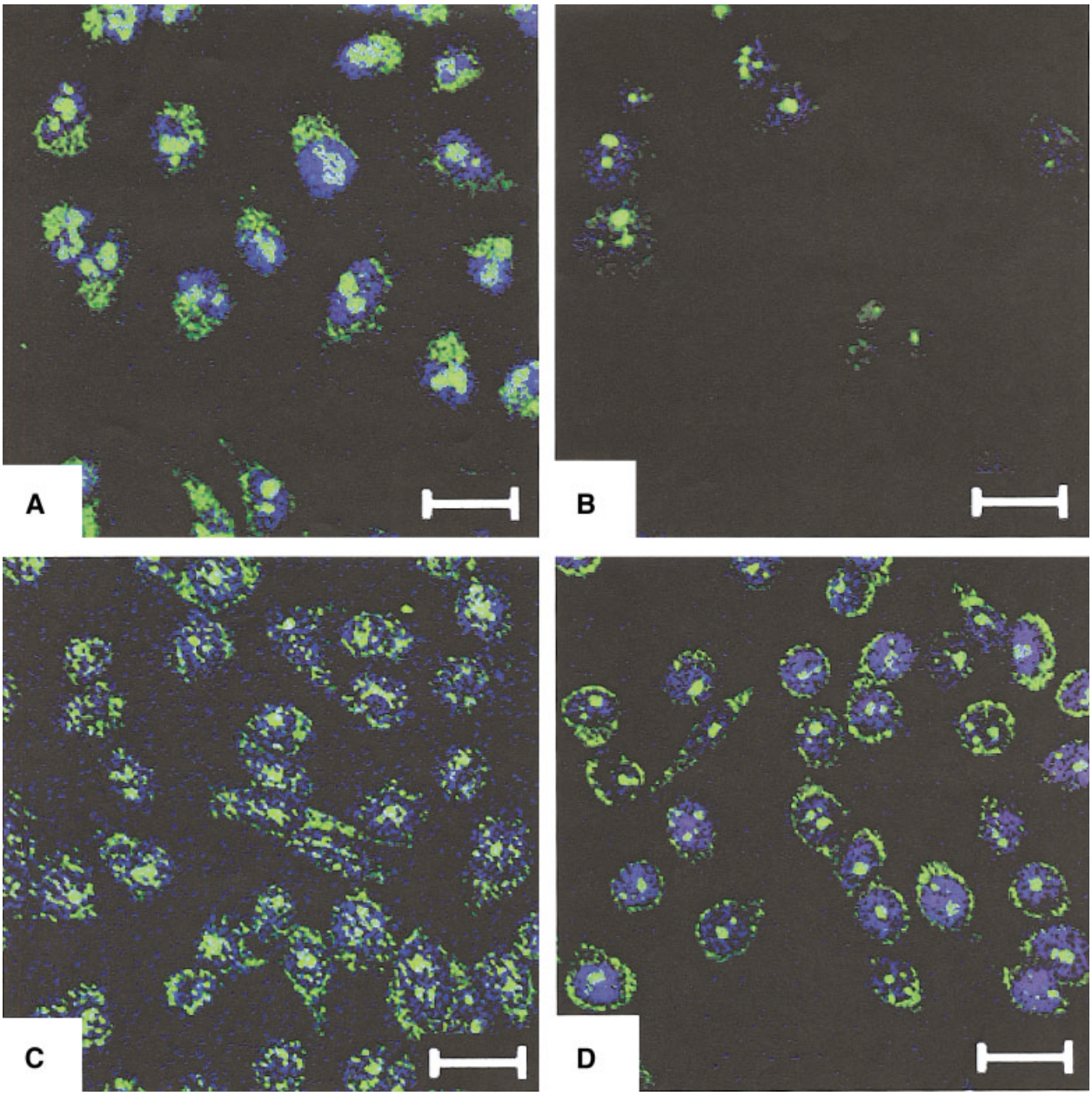


FIGURE 2. Immunofluorescence staining of protease-activated receptor (PAR)-2 in the primary lung adenocarcinoma cell lines. A-549 (A), HLC-1 (B), LC-2 (C), and PC-14 (D) cells are reactive for PAR-2, although the intensities are variable. Scale bars = 20 μm.

the mRNA levels in HPAEC, the expression ratios of mean PAR-1 mRNA in HUVEC and HAEC were 108% and 122%, respectively, whereas mean PAR-2 mRNA ratios were 105% and 53%, respectively (Fig. 4D).

In Vitro Assays of Growth Activity of Endothelial Cells in Response to α-Thrombin and the Activating Peptides, SFLLRN and SLIGKV

Thrombin induced significant growth activity in HPAEC and HUVEC in a dose-dependent manner (up to 230% in HPAEC compared with nonstimulated cells; Fig. 5A). The activating peptide, for PAR-1, SFLLRN, induced a 1.7-fold growth activity in HPAEC, but did not exert significant DNA uptake in HUVEC (Fig. 5B). To up-regulate PAR-2 mRNA in endothelial cells, pretreatment with TNF-α resulted in a significant DNA uptake in HPAEC (Fig. 6A) and HUVEC (Fig. 6B) of about 143% and 124%, respectively. However, DNA uptake was not significant if there was no pretreatment with TNF-α.

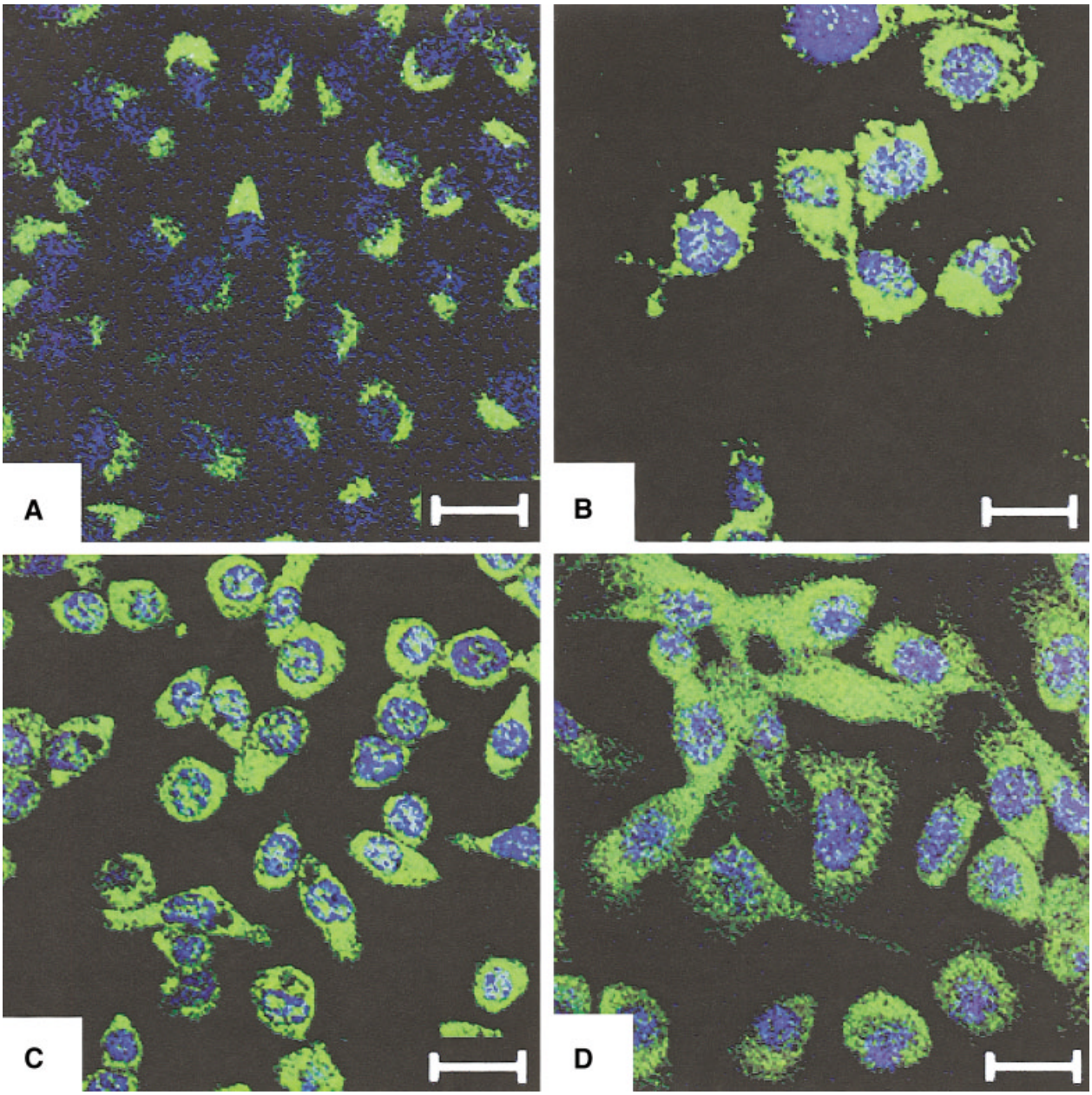
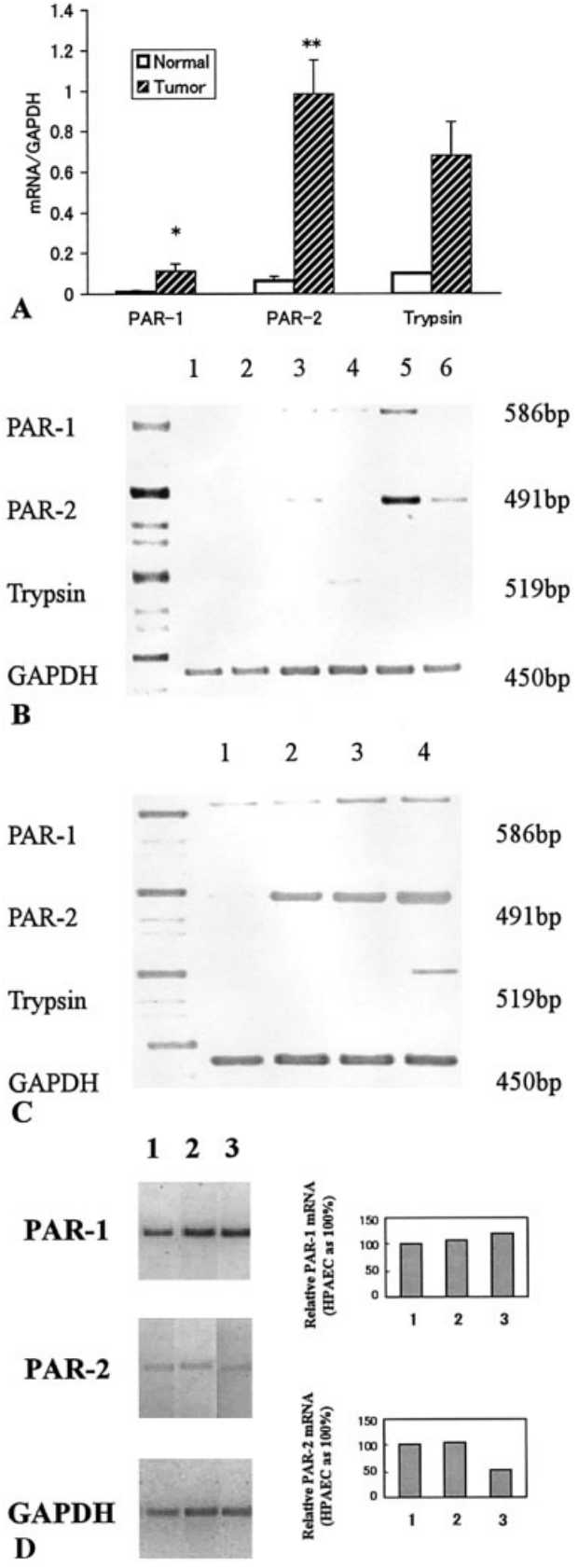


FIGURE 3. Immunofluorescence staining for trypsin in the cancer cell lines. The cytoplasm of the cells in the four different cell lines is fully reactive for trypsin. Scale bars = 20 μm.

DISCUSSION

In the normal pulmonary circulation, coagulant activity of thrombin is inhibited by a complex formation of thrombin with thrombomodulin that is constitutively expressed on the surface of alveolar capillary endothelial cells.[2] Blood coagulation is accelerated in the lung tissues with primary lung adenocarcinoma. This is in accordance with previous results that showed that thrombomodulin expression was lost in alveolar capillary endothelial cells, but that von Willebrand factor was expressed in the cytoplasm.[3] Induction of von Willebrand factor can be promoted by the activation of endothelial PAR-2.[17] This reaction is in good accord with the new synthesis of endothelial PAR-2 in the alveolar capillaries demonstrated in the current study. Expression of PAR-2 could be generated by inflammatory mediators[18] such as TNF-α. The release of TNF-α from neoplastic cells suppressed transcription of the thrombomodulin gene[19] and its expression[20] in vascular endothelial cells in vitro. Without thrombo-



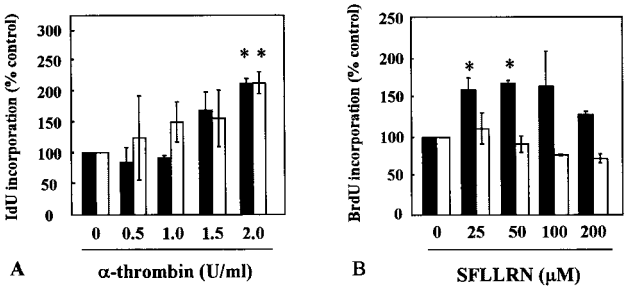


FIGURE 5. (A) IdU incorporation induced by α-thrombin in human pulmonary artery endothelial cells (HPAEC; black bars) and human umbilical cord vein endothelial cells (HUVEC; open bars). The HPAEC and HUVEC were made quiescent by incubation in serum-reduced medium for 18 hours. Quiescent endothelial cells were stimulated with 0.5, 1.0, 1.5, and 2.0 U/mL α-thrombin for 12 hours. To measure DNA synthesis, cells were further incubated in the presence of IdU for 12 hours. The percentage of IdU incorporation is expressed as mean $\pm$ SD ($P < 0.05$). (B) Incorporation of BrdU in HPAEC (black bars) and HUVEC (open bars) stimulated by the SFLLRN peptide. Quiescent HPAEC and HUVEC were treated with 25, 50, 100, and 200 μM SFLLRN in endothelial basal medium (EBM; 0.25% bovine serum albumin [BSA]) for 12 hours. The EBM (0.25% BSA) were preincubated with 5 μM amastatin for 30 minutes before the addition of SFLLRN. To measure DNA synthesis, cells were incubated with BrdU for 12 hours. The percentage of BrdU incorporation is expressed as mean $\pm$ SD ($P < 0.05$).

modulin expression, endothelial cells showed an efficient mitogenic response to thrombin.[21]

However, it is unknown whether PAR family members and serine proteases are expressed in the normal alveolar wall cells. We found higher expressions of PAR-1 and PAR-2 mRNA in the microdissected tissues of alveolar walls with neoplastic cells than without neoplastic cells. The expression in the normal walls depended on the presence of hypertrophic Type

FIGURE 4. Expression of protease-activated receptor (PAR)-1, PAR-2, and trypsin mRNA in microdissected alveolar walls and in primary lung adenocarcinoma cell lines. (A) Average expression of PAR-1, PAR-2, and trypsin mRNA/glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in the normal and tumor cell-spreading alveolar walls (*$P < 0.05$, **$P < 0.01$). (B) Gel electrophoresis of PAR-1, PAR-2, and trypsin mRNAs obtained from the normal control walls (lanes 1 and 2) and the tumor cell-bearing walls (lanes 3–6). (C) Gel electrophoresis of PAR-1, PAR-2, and trypsin mRNA in primary lung adenocarcinoma cell lines, including A549 (lane 1), HLC-1 (lane 2), LC-2 (lane 3), and PC-14 (lane 4). (D) Gel electrophoresis of PAR-1, PAR-2, and GAPDH reverse transcription-polymerase chain reaction products in cultured human pulmonary artery endothelial cells (lane 1), human umbilical cord vein endothelial cells (lane 2), and human aortic endothelial cells (lane 3). The relative expression of PAR-1 and PAR-2 mRNA normalized by GAPDH mRNA is also shown on the right.

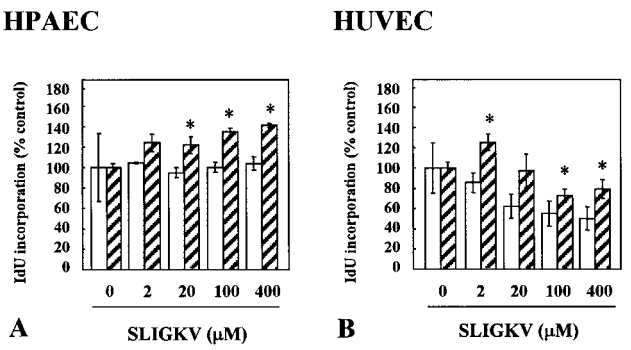


FIGURE 6. IdU incorporation induced by SLIGKV in tumor necrosis factor (TNF)-α–treated human pulmonary artery endothelial cells (A) and human umbilical cord vein endothelial cells (B) Cells preincubated with (stippled bars) or without (open bars) TNF-α pretreatment for 24 hours before the addition of SLIGKV. Cells were stimulated with 2, 20, 100, and 400 μM of SLIGKV. IdU was added for 12 hours and quantified by enzyme-linked immunosorbent assay. Data are expressed as mean $\pm$ SD ($P < 0.05$).

II alveolar epithelial cells, although the etiology of this reactive hypertrophy was not clear. Nevertheless, quiescent Type II alveolar epithelial cells might express a minimal amount of mRNA for these receptors. According to Cocks et al.,[13] PAR-2 expression is localized in the normal human bronchial epithelial and peribronchial smooth muscle cells. Bronchial epithelial PAR-2 can exert a protective effect against bronchoconstriction in humans and animals, which was demonstrated by the in vitro activation of PAR-1, PAR-2, and PAR-4.[22] In addition, growth activity was shown in lung fibroblasts via stimulation of PAR-2 by mast cell tryptase.[23] With regard to vascular cells, PAR-1 activation of endothelial cells by thrombin led to relaxation in pulmonary arteries.[24] The PAR-2 activation in human vascular endothelial cells mediates their mitogenic response in vitro.[25] Both PAR-1 and PAR-2 are closely related to cell growth of endothelial cells and exert other physiologic functions of epithelial and mesenchymal cells.

The expressions of PAR-1 and PAR-2 on the alveolar capillary endothelial cells shown in the current study suggest a functional transition of the capillaries from a quiescent to an alert state. Endothelial PAR-1 expression was dense in the microvessels in fibrotic alveolar walls. In contrast, faint expression of PAR-2 occurred along the cell membranes of alveolar capillaries in and around tumor cell-bearing alveolar walls. The difference in the localization of the two receptors might depend on tissue preparation. For example, the reaction for PAR-1 was performed on formalin-fixed paraffin sections, whereas the reaction for PAR-2 was performed on fresh frozen sections. It is noteworthy that most microvessels in cancer scars were exclusively reactive for both antigens, indicating that these receptors might activate mesenchymal cells to promote collagen deposit[26] in areas of neoplastic cell proliferation. Heterogenous reactivities in microvascular endothelial cells would be in good accord with the results demonstrated for permeability function[27] and granulocyte-macrophage–colony-stimulating factor expression[28] in the lung capillaries.

A major contribution to the higher expression of PAR-1 and PAR-2 mRNA may be the number of neoplastic cells spreading on alveolar walls. Alveolar capillary endothelial cells should confer some contribution to the levels of PAR-1 and PAR-2 as the receptor proteins were newly expressed in the tissues. In the current study, thrombin was never retrieved from any of the cell types found in the alveolar walls. The RT-PCR analysis failed to detect thrombin mRNA. This contradicts a previous study[6] that reported that thrombin is synthesized in tumor cells. However, thrombin is well generated in primary adenocarinomas under hypercoagulability conditions. Thus, PAR-1 expression in alveolar capillary endothelial cells might be elicited by thrombin for their own cell growth. This assumption is proved by the current experiments involving HPAEC and HUVEC, which expressed PAR-1 and PAR-2 mRNA at similar levels in vitro. Following α-thrombin treatment of HPAEC and HUVEC, IdU uptake reached 220% above the control level in a dose-dependent manner. In addition, the activating peptide (SFLLRN) for PAR-1 induced increased cell growth in HPAEC. The HPAEC and HUVEC also showed an increase in BrdU uptake when stimulated with SLIGKV after pretreatment with TNF-α to upregulate PAR-2 mRNA. These results confirmed the potential growth activity of the endothelial cells via elicitation of G protein-coupled receptors in the human alveolar capillaries that were terminal branches of the pulmonary arteries.

Trypsin (ogen) is a major protease activator of PAR-2 expression. Trypsin was originally found in pancreatic acinar cells. However, the distribution of trypsin occurs throughout the body, including the skin, esophagus, small intestine, lung, kidney, liver, extrahepatic bile duct, and neuronal cells.[29] The distribution pattern of trypsin might suggest that its physiologic function is related to PAR-2 expression. The current study demonstrated that trypsin was not expressed by any of the cell types in the normal alveolar walls. Neoplastic cells in primary lung adenocarcinomas were reactive for trypsin in 44% of the patients in the current study. Kawano et al.[30] showed that trypsin was expressed in 56% of lung adenocarcinomas and far less frequently (0–12%) in other types of lung tumors. The diversity of trypsin expression in

tumor cells is consistent with variation in the expression levels of trypsin mRNA in the cell lines. Cells derived from less differentiated adenocarcinomas (LC-2 and PC-14) expressed trypsin mRNA, but neither A549 nor HLC-1 cells expressed it. The current study also showed marked variability in the immunohistochemical expression of trypsin in neoplastic cells in the same patient. Selected tumor cells exerted proteolytic activity for the activation of PAR-2, which led to cell growth in adjacent capillary endothelial cells.

Previous studies[3,4] demonstrated a significant up-regulation of KDR and $VEGF_{165}$ mRNA in neoplastic cell-spreading alveolar walls. Our study confirmed that capillary endothelial cells were often reactive for PAR-1, as was KDR in the previous study.[4] Tsopanoglou and Maragoudakis[14] showed that exposure of HUVEC to thrombin induced up-regulation of KDR mRNA and sensitized the cells for mitogenic activity lead by VEGF in a dose-dependent manner. These results suggest that thrombin plays a critical role as an active accelerator of VEGF function for the tumor vessel formation in primary lung adenocarcinomas.

In conclusion, alveolar capillary endothelial cells were activated for angiogenesis in a unit compartment of the alveolar wall. Proliferation of alveolar capillary endothelial cells was initialized in part by PAR activation with serum thrombin and neoplastic cell-released trypsin. Alveolar angiogenesis might be synergistically promoted with tumor-derived VEGF.

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