Overexpression of Urokinase-type Plasminogen Activator in Human Gastric Cancer Cell Line (AGS) Induces Tumorigenicity in Severe Combined Immunodeficient Mice

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The significance of urokinase-type plasminogen activator (uPA) expression in gastric cancer development was tested by using a human uPA cDNA transfection approach and an *in vivo* severe combined immunodeficient (SCID) mouse model. The AGS gastric cancer cell line, which has urokinase-type plasminogen-activator receptor (uPAR) but lacks uPA, was transfected with a plasmid containing human uPA cDNA and injected into the backs of SCID mice. Compared with the parent AGS cells, uPA protein secretion in AGS-2-, AGS-4-, and AGS-8-transfected cells increased by 26.1-, 34.6-, and 4.8-fold, respectively ($P<0.05$). mRNA expression levels of uPA in the AGS-4 clone were much stronger than those in AGS-2 and AGS-8 clones. After the cancer cells (2×10^6) were injected s.c. into the SCID mice, a palpable mass was observed at the injection site at around 140 days post-injection, followed by accelerated growth of the xenograft up to 180 days post-injection only in the high uPA-producing clone (AGS-4). These results suggest that continuous and high production of uPA by tumor cells is one of the important factors reflecting the malignancy of gastric cancer cells.

Key words: uPA — Transfection — SCID — AGS — Gastric cancer

Tumor progression involves modulation of the tumor cell adhesion during migration and metastasis, as well as degradation of the extracellular matrix (ECM) during invasion. In particular, the urokinase-type plasminogen activator (uPA) and its specific cell surface receptor, uPAR play important roles in tumor invasion and metastasis. uPA is expressed and secreted as an inactive single-chain proenzyme (pro-uPA), which is converted by limited proteolysis into the enzymatically active two-chain uPA.[1, 2] This, in turn, cleaves plasminogen, generating the broad-spectrum proteolytic enzyme plasmin.[3] Plasmin degrades ECM and activates a group of metalloproteinases, contributing to the focal proteolytic events that enable invasion and metastasis. Functional uPA activity depends not only on the concentration of uPA, but also on uPAR density and the concentration of uPA inhibitors, types 1 and 2 (PAI-1 and PAI-2).[4]

Tissue levels of uPA, uPAR, and PAI-1 are higher in several malignant tumors than those of the corresponding adjacent normal tissues.[5, 6] Expression levels of uPA or uPAR are associated with invasiveness and poor prognosis in breast and stomach cancers.[7, 8] Many studies have shown that inhibition of uPA activity or uPA binding to uPAR results in the inhibition of tumor growth and reduced or abolished formation of metastasis.[9, 10] In addition, uPA has been directly associated with cell proliferation, migration, and chemotaxis,[11–13] all events that could have significant impacts on the tumor cell dissemination. Most likely, uPA facilitates cancer invasion by stimulating ECM degradation.

Gastric cancer still remains one of the most common causes of cancer-related deaths in the world. In Korea, gastric cancer is the most frequently seen malignancy and the most important cause of cancer-related deaths. Increased levels of uPA, uPAR, and PAI-1 have been observed in gastric cancer tissues by immunohistochemistry; these increases have been considered to have a prognostic value.[14, 15]

In our study, the *in vivo* severe combined immunodeficient (SCID) mice inoculation method was used to investigate the effect of uPA cDNA transfection on the tumorigenicity of the AGS cell line, which originally lacks uPA while processing uPAR. Our results demonstrate that increased tumorigenicity and shortened survival of the SCID mice, probably due to pulmonary metastasis, are associated with uPA transfection.

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MATERIALS AND METHODS

uPA plasmid and cell line Expression vector pRc/CMV (Invitrogen, Carlsbad, CA) containing the entire coding region of 1.309 kb human uPA cDNA was a gift from Dr. John Sipley. A human gastric cancer cell line AGS[16] was maintained in RPMI 1640 supplemented with heat-inactivated 10% fetal bovine serum (FBS), 100 U/ml of penicillin, and 100 μM/ml of streptomycin.

Transfection and selection AGS cells (2.5×10^6) were transfected with 10 μg of plasmid DNA by electroporation in 0.4-cm Bio Rad cuvettes at 250 V, 960 μF, using a Bio Rad Gene Pulser with a capacitance extender, as previously described.[17] Transfected clones were selected by culturing the cells in RPMI 1640 medium containing 200 μg/ml of Neomycin analog G418 (Boehringer Mannheim, Indianapolis, IN). After 7 days, discrete neomycin-resistant colonies were cloned and allowed to grow as monoclonal-cell populations.

ELISA for uPA antigen levels uPA level in serum-free, RPMI 1640 medium was measured using a commercially available ELISA kit (product No. 894, American Diagnostica, Greenwich, CT) as previously described.[18] The kit is based on a double-antibody sandwich technique, in which two different monoclonal antibodies are used for the capture and detection of the antigen. Cells (1×10^5) in 1.0 ml of complete culture medium were inoculated into each well of a 6-well plate and further cultured for 24 h in a s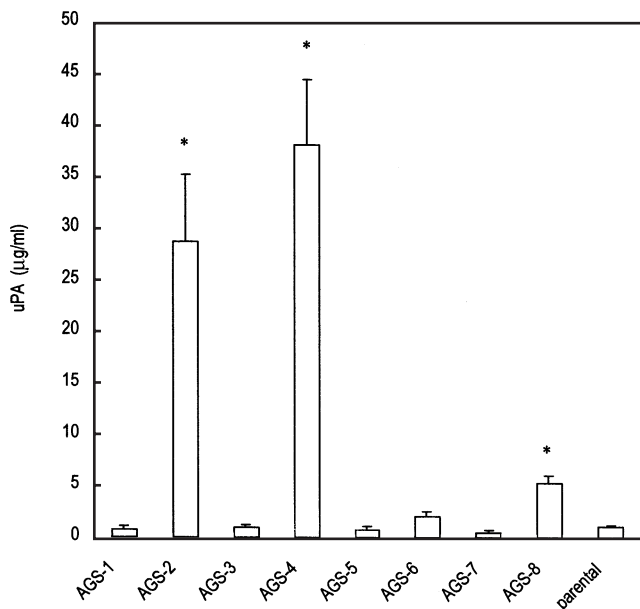erum-free medium. The serum-free medium samples were prepared by removing the supernatants from the wells, filtering them through a 0.2-μm filter, and storing them at $-80°C$ until analysis. Absorbances were calibrated using purified human uPA, and all ELISA results were expressed as micrograms of protein per milliliter. Means±standard deviations for four separate trials are shown in Fig. 1.

Northern blot analysis Total RNA was extracted from confluent cultures by the standard guanidinium thiocyanate-phenol-chloroform technique.[19] Twenty micrograms of total RNA was electrophoresed on 1% denaturing agarose gel containing 7.5% formaldehyde and transferred onto a nylon membrane (Amersham, Arlington Heights, IL) through capillary transfer with 20× SSC solution. Baked nylon membrane was hybridized with ^{32}P-labeled probes[20] corresponding to uPA using the random primer method. After washing, the membrane was dried and autoradiographed. The membrane was then striped and rehybridized with a ^{32}P-labeled glyceraldehyde-3′-phosphate dehydrogenase (GAPDH) probe to check the amount of transferred RNA in each lane.

Tumor growth in SCID mice Six-week-old C.B. 17 *scid/scid* (SCID) mice were housed and maintained in the animal facility of the Korea Research Institute of Bioscience and Biotechnology (Taejon, Korea) under specific pathogen-free conditions with continuous microbiological monitoring. Sterilized commercial diet (PMI, Brentwood, MO) and water were given *ad libitum*. To investigate the effect of uPA transfection on the tumorigenicity, invasion, and lung metastasis of AGS cells, transfected uPA-expressing and non-transfected control AGS cells (2×10^6 cells in 100 μl of PBS) were inoculated s.c. into the backs of SCID mice. The mice (8 in each group) were inspected daily, and the tumor size was measured three times weekly in two dimensions with calipers. The tumor volumes were calculated based on the formula: length $\times$ (width)^2/2.

All mice were sacrificed either when they fell into coma due to tumor or 6 months post-inoculation. Subcutaneous tumor masses, lungs, livers, spleens, and kidneys were fixed in 10% neutral-buffered formalin, embedded in paraffin, and stained with hematoxylin and eosin for histopathological examination.

Immunohistochemistry Replicate paraffin sections of the tumor masses were mounted on Probe-On slides (Fisher Scientific, Pittsburgh, PA), deparaffinized, and rehydrated. Unlabeled mouse monoclonal antibody directed against uPA (product No. 3689, American Diagnostica) was used as the primary antibody. The standard avidin-biotin-peroxidase complex (ABC) method was used according to the manufacturer's protocol (Zymad, San Francisco, CA) to detect the antigen using 3,3-diaminobenzidine as the chromogen. The control procedure included omission of the primary antibody and substitution of an isotype-matched

Fig. 1. The quantitation of uPA protein levels by ELISA in transfected clones and parental AGS cells. * $P<0.05$ relative to the parental group.

irrelevant antibody. Mammary gland tumor tissue was used as a positive control.

Statistical analysis Data were analyzed for statistical significance by using Student's *t* test.

RESULTS

Expression of uPA by plasmid transfection To determine whether uPA-transfected clones can produce uPA in AGS cells, a construct containing the *Xba*I/*Hin*dIII fragment of sense uPA cDNA (1309 bp) was transfected into AGS cells. Approximately 300 neomycin-resistant clones were screened, among which 8 clones (AGS-1, -2, -3, -4, -5, -6, -7, and -8) were selected.

Levels of uPA protein production of the eight selected clones and the parent clone were analyzed by an ELISA method. Compared to the parent clone, the amounts of uPA protein produced in AGS-2, -4, and -8 were increased significantly ($P<0.05$) by 26.1–(28.7 ± 6.4 μg/1×10^5 cells), 34.6–(38.1 ± 6.3 μg/1×10^5 cells), and 4.8-fold (5.3 ± 0.8 μg/1×10^5 cells), respectively. Over-production of uPA was not noted in AGS-1, -3, -5, -6, -7, or -9 (Fig. 1).

To confirm uPA mRNA expression in AGS-2, -4, and -8 and parent AGS cells, northern blot analysis using a ^{32}P-labeled uPA probe was performed. uPA mRNA transcript migrating between 18S and 28S rRNA was expressed only in AGS-2, -4, and -8, but not in the parent clone (Fig. 2). The mRNA expression level of uPA in AGS-4 cells was higher than those of AGS-2 and -8, an indication of a correlation between the degree of mRNA and protein expression.

Tumor growth in SCID mice To investigate and compare the *in vivo* tumorigenicity and metastatic potential of the high uPA-producing clone (AGS-4), low uPA-producing clone (AGS-8), and non-transfected AGS cells, each clone (2×10^6) was injected s.c. into SCID mice. A palpable mass was first noted at the injection site at around 140 days post-injection and continued to grow up to 180 days in the mice treated with the AGS-4 clone (Figs. 3 and 4). The estimated 50% survival time of SCID mice bearing AGS-4 tumors was 172.5 days. However, mice treated

with the AGS-8 clone or parent AGS did not develop any palpable tumor mass and lived until the end of the experimental period (Fig. 5).

Histopathology and immunohistochemistry Histologically, s.c. nodules consisted of compact sheets of pleomor-
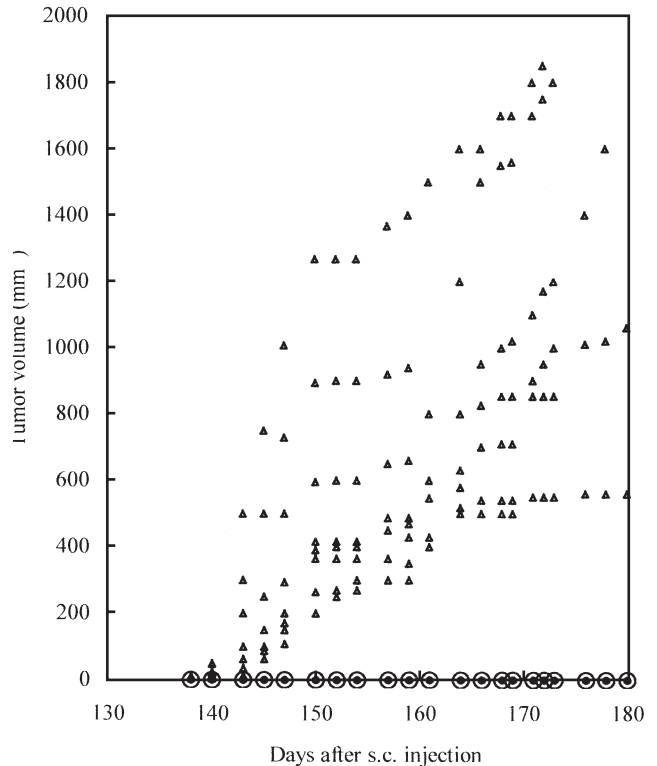

Fig. 3. Rates of tumor development in SCID mice inoculated with 2×10^6 cells of AGS-4 (△), AGS-8 (●), and parent AGS (○) cells. Each group consisted of 8 mice.
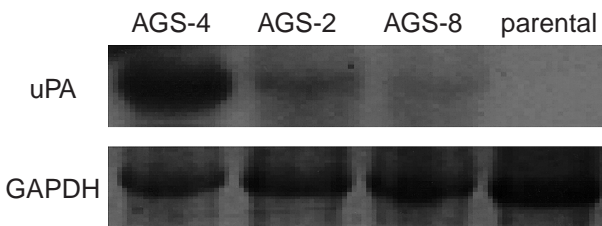

Fig. 4. SCID mouse injected with high-uPA-producing clone. Note well-demarcated s.c. neoplastic mass formation at the right chest region.

Fig. 2. Northern blot analysis of uPA mRNA expression in human uPA-transfected AGS cells.

phic neoplastic cells (Fig. 6). Among the eight mice inoculated, pulmonary metastasis was observed in seven (Fig. 7), though no evidence of metastasis was noted in the liver, spleen, and kidney. On immunohistochemistry, most tumor cells were strongly positive for uPA (Fig. 8).

DISCUSSION

Expression patterns of uPA and uPAR differ depending on the type of tumor involved.[21, 22] In our previous study,

differential uPA and uPAR expressions at both mRNA and protein levels were observed in four human gastric-cancer cell lines (AGS, Hs746T, SNU-1, and SNU-5), among which AGS and SNU-5 produce functionally active uPAR, but lack uPA secretion, therefore can not form tumors on the modified chorioallantoic membranes (CAM) of chick embryos. Hs746T, which expressed both uPA and uPAR, showed tumorigenicity and high invasiveness on CAM of chick embryos.[23] Local invasion of tumor cells and angio-
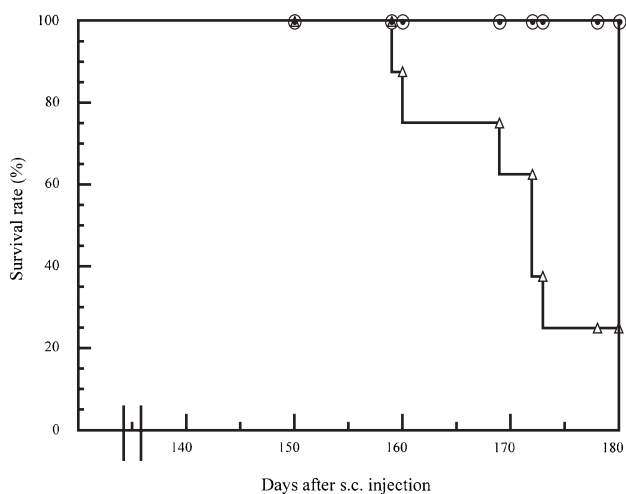

Fig. 5. Survival rate in SCID mice inoculated with 2×10^6 cells of AGS-4 (△), AGS-8 (●), and parent AGS cells (○). Each group consisted of 8 mice.

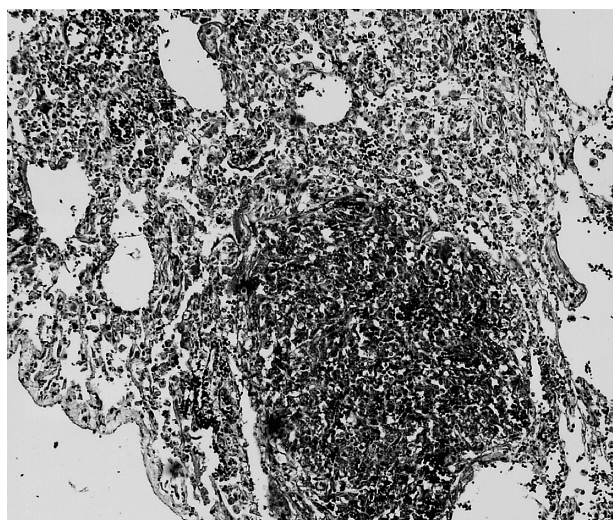
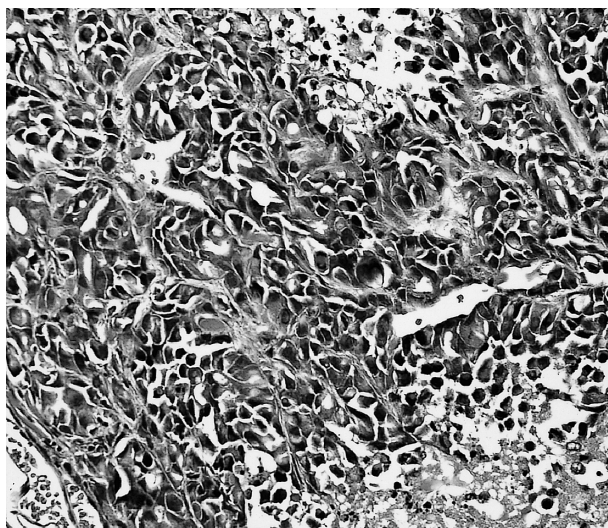Fig. 7. SCID mouse injected with high-uPA-producing clone. Note well-demarcated focus of pulmonary metastasis. H&E. ×100.

Fig. 6. SCID mouse injected with high-uPA-producing clone. Note sheets of pleomorphic neoplastic cells in the s.c. area. H&E. ×400.
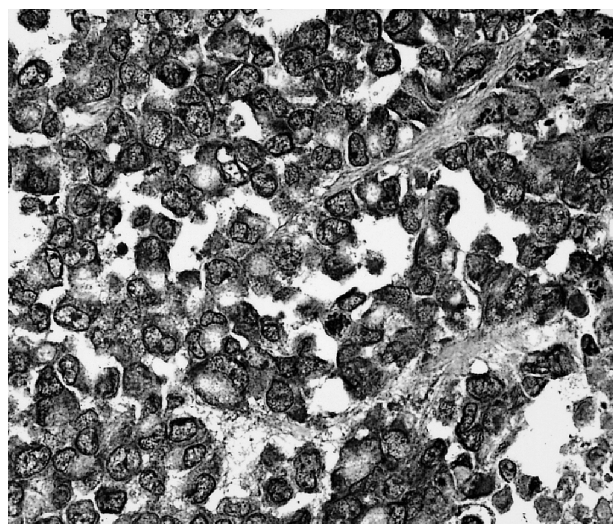

Fig. 8. The tumor cells in the s.c. area are positive for uPA. ABC. ×400.

genesis are prerequisite for tumor growth *in vivo*. They can be accomplished by the degradation of ECM, which also assists tumor mass expansion and endothelial cell migration. Many studies have shown the involvement of uPA in these processes. uPA, especially cell surface receptor-bound uPA, plays a major role and, therefore, indirectly can contribute to tumor growth. Because concurrent expression of uPA and uPAR is required for efficient invasion and angiogenesis, the AGS cells that could not produce functionally active uPA, but only have uPAR, were not tumorigenic or invasive in SCID mice.

It has been reported that HT29 human colon carcinoma cell line producing uPAR but not uPA, when transfected with uPA cDNA, developed the capability to invade artificial basement membrane.[24] It has also been reported that T24 human bladder carcinoma cell line producing only a minimal amount of uPA was noninvasive in Matrigel invasion assays; however pretreatment of the cells with high-molecular-weight uPA markedly increased the invasiveness.[25] However, as both previous studies had been performed *in vitro*, we compared the tumorigenicities of high uPA-producing (AGS-4) clone, low uPA-producing (AGS-8) clone, and parent AGS cells in our *in vivo* SCID mice model.

We observed tumor formation only in SCID mice injected with the high uPA-producing clone at about 140 days after injection, after which the growth rate of the tumor accelerated. One of the important steps for tumor progression and invasion is the destruction of ECM that separates the epithelial and stromal compartments by serine proteinases such as uPA. Though it took a long period for tumors to appear in the mice, the results of this study suggest that the tumor formation in SCID mice was due to continuous high-level secretion of uPA by AGS cells, leading to destruction of the ECM and eventually to local invasion and pulmonary metastasis. However, there is a possibility of clonal selection during the tumor formation. Diverse tumor progression, as shown in Fig. 3, may suggest this.

A recent study has demonstrated that 25 patients with high uPA activities had lower survival rates than 46 patients with low uPA activities.[26] We also observed that 75% of SCID mice injected with a high uPA-producing clone died, whereas all SCID mice injected with either a low uPA-producing clone or parent AGS cells lived until the end of the experimental period.

In conclusion, this study demonstrates that continuous high-level production of uPA by tumor cells is an important factor determining the malignancy of cancer cells.

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