Induction of α2-antiplasmin inhibits E-cadherin processing mediated by the plasminogen activator/plasmin system, leading to suppression of progression of oral squamous cell carcinoma via upregulation of cell-cell adhesion

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Abstract. The plasminogen activator/plasmin system is one of the main protease systems involved in tumor cell invasion and metastasis. Our previous study has shown that plasmin degrades E-cadherin and promotes cell dissemination by downregulation of E-cadherin-mediated cell-cell adhesion in oral squamous cell carcinoma (SCC) cells. To examine the effect of downregulation of the plasminogen activator/plasmin system by α2-antiplasmin (α2-AP) on cell-cell adhesion mediated by E-cadherin in oral SCC cells, the oral SCC cell line SCCKN was stably transfected with α2-AP cDNA. Induction of α2-AP expression led to the inhibition of the proteolysis of E-cadherin by plasminogen activator/plasmin in SCC cells, resulting in the enhancement of the cell aggregation and the suppression of the cell motility. Moreover, α2-AP also reduced the ability of SCC cells to invade type I collagen gel, and suppressed tumorigenicity *in vivo*. These results suggested that downregulation of the plasminogen activator/plasmin system by α2-AP might be a potent therapeutic approach to prevent the progression of oral SCC.

Introduction

One of the lethal properties of malignant tumors is the ability to invade surrounding tissues and form metastatic foci at distant sites. Dissociation of cancer cells in the primary lesion, which requires overcoming their cell-cell adhesions, is an initial step in tumor invasion and metastasis (1,2). E-cadherin is the prototypic member of the classical cadherin family, and plays a critical role in epithelial cell-cell adhesion (3,4). Several studies have shown that loss of the expression of E-cadherin is a frequent event in many types of malignant epithelial tumors (5-8). Loss of the expression and function of E-cadherin is caused by several different mechanisms including deletion or mutational inactivation of E-cadherin (9,10).

Tumor invasion and metastasis are largely mediated by proteolytic enzymes that degrade extracellular matrix (ECM) proteins (11-14). The plasminogen activator/plasmin system is one of the main proteinase systems involved in tumor invasion and metastasis (15,16). A variety of cancer cells including squamous cell carcinoma (SCC) cells produce urokinase-type plasminogen activator (u-PA) (16-20). u-PA binds to u-PA receptor (u-PAR), and activates plasminogen to plasmin on the cell surface. Plasmin, which is a broad spectrum proteinase and an efficient activator of several latent enzymes such as collagenase, stromelysin and MMP-9, acts both directly and indirectly to degrade ECM proteins surrounding cancer cells (19,21,22). Our previous study has demonstrated that the plasminogen activator/plasmin system regulates the processing of E-cadherin of oral SCC cells. The plasminogen activator/plasmin system has been shown to facilitate the dissemination of SCC cells through the downregulation of E-cadherin-mediated cell-cell adhesion (23). Therefore, the plasminogen activator/plasmin system could serve as a therapeutic target to prevent tumor invasion and metastasis. The downregulation of the plasminogen activator/plasmin system might suppress the progression of SCCs by inhibiting ECM degradation and cell dissemination.

The plasminogen activator/plasmin system is downregulated at two different levels, which are the suppression of the formation of plasmin by plasminogen activator inhibitors (PAIs) and the inactivation of plasmin by α2-antiplasmin (α2-AP) (24,25). Plasminogen activator inhibitor-1 (PAI-1), a member of the serine proteinase inhibitor (SERPIN) family, is synthesized by a variety of cells including endothelial cells, hepatocytes, smooth muscle cells and tumor cells. PAI-1

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binds to the uPA-uPAR complex on the cell surface and forms an enzymatically inactive trimeric complex. The enzymatically inactive trimeric complex is internalized by the cells and is rapidly degraded, resulting in the inhibition of plasminogen activation (26,27). However, several clinical studies have shown that the level of PAI-1 is significantly increased in several tumors compared to the corresponding normal tissues (28-31). Moreover, the high tumor level of PAI-1 is associated with poor prognosis for survival in several human cancers (12,32-34). PAI-1 also mediates protease-independent effects on cell motility and angiogenesis. Recent studies have shown that PAI-1 stimulates cell migration and dissemination in several tumor cells such as fibrosarcoma cells and breast cancer cells (35,36) and induces VEGF synthesis in glioma cells (37). These results suggest that PAI-1 might stimulate tumor progression by the enhancement of cell motility and angiogenesis despite the inhibition of ECM degradation.

α2-AP, a single chain glycoprotein with a molecular weight of ~70,000, is a member of the SERPIN family and is mainly synthesized in liver. α2-AP rapidly inhibits plasmin activity by forming an inactive stoichiometric complex with plasmin. In addition, α2-AP covalently binds to fibrin, and inhibits the binding of plasminogen to fibrin (38,39). Although these functions of α2-AP significantly contribute to the inhibition of fibrinolysis, there is little information about the role of α2-AP in cellular behaviors such as organogenesis, differentiation and tumor progression, which are regulated by pericellular proteolysis.

In the present study, to clarify the role of α2-AP in the behavior of oral SCC, the oral SCC cell line, SCCKN was stably transfected with α2-AP cDNA. We examined the effect of α2-AP expression on E-cadherin processing mediated by the plasminogen activator/plasmin system and the motility in oral SCC cells. Moreover, the influence of α2-AP on the tumorigenicity of oral SCC cells was investigated.

Materials and methods

Cells and culture. The human oral squamous cell carcinoma cell line, SCCKN was grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) in 5% CO_2 at 37˚C (18). Treatment with plasminogen (Sigma, St. Louis, MO) was performed as follows; cells were washed twice with DMEM and incubated in DMEM containing 5 μg/ml plasminogen for 12 h.

Plasmids and transfection. First-strand cDNA was synthesized from the human liver total RNA (Origene Technologies, Rockville, MD) using random hexamers (Applied Biosystems, Foster City, CA), Moloney murine leukemia virus reverse transcriptase (Applied Biosystems) and RNase inhibitor (Applied Biosystems). Human α2-AP cDNA was amplified from the human liver cDNA by PCR using the sense primer (5'-ccgctcgagaacatggcgctgctctgggggctcctg-3') containing a *Xho*I site (underlined) and the antisense primer (5'-gctctaga tcacttggggctgccaaactgggggtaatc-3') containing a *Xba*I site (underlined) under the following conditions: 94˚C for 1 min, 68˚C for 1 min and 72˚C for 1 min 40 sec. The PCR fragments were double-digested with *Xho*I (New England Biolabs, Beverly, MA) and *Xba*I (New England Biolabs), and then ligated into mammalian expression vector, pCI-neo (Promega, Madison, WI) digested with *Xho*I and *Xba*I. The inserted cDNA sequences were all verified by DNA sequence analysis. The resultant plasmid was termed as pCI-neo/α2-AP, and pCI-neo without insert was used as negative control vector. SCCKN cells were transfected with 5 μg of pCI-neo/α2-AP or pCI-neo using TransFast™ transfection reagent (Promega) according to the manufacturer's instructions. Selection was initiated 48 h after transfection by adding 600 μg/ml G418 (Geneticin; Invitrogen, San Diego, CA) to the culture medium. The selection medium was changed every 4 days for 2 weeks until all nontransfected cells died. Resistant cell clones were isolated. Cell clones transfected with pCI-neo/α2-AP or pCI-neo were termed as $KN_{\alpha2\text{-}AP}$ or KN_{mock}, respectively.

Reverse transcription-polymerase chain reaction. Total RNA of SCCKN, KN_{mock} or $KN_{\alpha2\text{-}AP}$ in confluent cultures was isolated using EASYPrep RNA (Takara Bio, Shiga, Japan) according to the manufacturer's instructions. Samples of total RNA (2 μg) were reverse-transcribed with random hexamers, Moloney murine leukemia virus reverse transcriptase and RNase inhibitor. Subsequent PCR amplification was performed for 25 cycles in the presence of 10 pmol primers [α2-AP, 5'-aggtgctcagaaccacacgttgcag-3' and 5'-aggctcgggtcaaacttgttc ctcc-3'; glyceraldehyde 3 phosphate dehydrogenase (GAPDH), 5'-tgatgacatcaagaaggtggtgaag-3' and 5'-tccttggaggccatgtgggcc at-3']. Each PCR cycle included a denature step at 94˚C for 1 min, a primer annealing step at 63˚C for α2-AP or 59˚C for GAPDH for 1 min, and an extension step at 72˚C for 1 min. The PCR products were analyzed with 1% agarose gel and visualized by ethidium bromide staining.

Western blot analysis. To detect α2-AP protein, SCCkN, $KN_{\alpha2\text{-}AP}$ and KN_{mock} cells were lysed with lysis buffer (10 mM Tris HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 1% NP-40). The samples containing 20 μg of total protein were electrophoresed on 10% SDS-polyacrylamide gel under reducing conditions and transferred to polyvinylidene difluoride (PVDF) membrane filters (Millipore, Bedford, MA). The filters were blocked in PBS containing 5% skim milk for 1 h at room temperature and then incubated with rabbit anti-α2-AP polyclonal antibody (American Research Product, Belmont, MA), followed with incubation with horseradish peroxidase-conjugated secondary antibody (Bio-Rad Laboratories, Hercules, CA). Protein bands were visualized by enhanced chemiluminescence detection (ECL Plus System; GE Healthcare, Piscataway, NJ).

To evaluate the processing of E-cadherin, E-cadherin protein in the membrane fractions and the culture supernatants was analyzed with Western blotting. The membrane fraction was prepared as described previously (23). SCCKN, $KN_{\alpha2\text{-}AP}$ or KN_{mock} cells treated with 5 μg/ml plasminogen for 12 h were rinsed and scraped with Ca^{2+} - Mg^{2+}-free phosphate buffer saline (PBS, pH 7.3). The cells were suspended in buffer A (20 mM Tris-HCl pH 7.5, 250 mM sucrose, 10 mM EGTA, 2 mM EDTA) and lysed by passing through a 27-gauge needle. The cell lysates were centrifuged at 500 x g for 15 min at 4˚C, and then the supernatants were centrifuged at 120,000 x g for 45 min at 4˚C. The pellet was collected as crude membrane. The crude membrane fraction was washed

with buffer A, and sonicated in buffer A containing 1% Triton X-100. After incubation for 30 min at 4˚C, the crude membrane fraction was centrifuged at 120,000 x g for 45 min at 4˚C, and the supernatant was isolated as cell membrane fraction. The cell membrane fractions (20 μg of protein) were electrophoresed on 10% SDS-polyacrylamide gel under reducing conditions and transferred onto PVDF membrane filters. After blocking in PBS containing 5% skim milk, the membranes were incubated with monoclonal antibody recognizing the extracellular domain of human E-cadherin (HECD-1; Takara Bio), followed by the incubation with horseradish peroxidase-conjugated secondary antibody (Bio-Rad Laboratories). For detection of soluble E-cadherin, the culture supernatant from equal numbers of the cells was concentrated with Centricon tube (Millipore) and subjected to Western blot analysis as described above.

Cell aggregation assay. The cells treated with 5 μg/ml plasminogen for 12 h were dissociated into single cell suspensions by treatment with 5 mM EDTA in PBS for 5 min at 37˚C and by 2 passes through a 27-gauge needle. After washing with Puck's saline (5 mM KCl, 140 mM NaCl, 8 mM $NaHCO_2$, pH 7.4) three times, the cells ($3x10^5$ cells/ml) were suspended in Puck's saline containing 0.8% FBS, 50 U/ml DNase I (Roche, Indianapolis, IN) and 5 mM Ca^{2+}. The cells were placed in each well of a 24-well culture plate and incubated for 120 min at 37˚C on a gyratory shaker rotating at 80 rpm. The rate of aggregation was calculated as the percentage of the cells that formed aggregates using the formula $[(N_0-N_{120})/N_0]x100$, where N_0 and N_{120} indicate the number of isolated cells at the initial time and the number of isolated cells after 120 min of aggregation as measured by a hemocytometer, respectively. The experiment was repeated three times.

Cell motility assay. Cell motility was analyzed with modified Boyden chamber assay using a Transwell insert (6.5 mm diameter) with 8-μm pores (Coaster, Cambridge, MA) as described previously (23,40,41). The filters were coated with 100 μg/ml gelatin to enhance cell attachment. The cells were harvested with 0.01% trypsin in the presence of 2.5 mM Ca^{2+} to protect E-cadherin, and suspended in DMEM containing 10% FBS for 1 h to recover. The cells were incubated in DMEM containing 5 μg/ml plasmin (Sigma) for 1 h and rinsed with DMEM three times. The cells ($5x10^5$) resuspended in DMEM containing 0.1% BSA were added to the upper compartment of each Transwell insert. After incubation for 24 h at 37˚C, Transwells were fixed with methanol and stained with Diff Quick (Dade Behringen, Duedingen, Switzerland). Cells on the upper surface of the filter were removed with a cotton swab. The number of cells on the lower surface of the filter was counted by light microscopy under a high power field (x100). Eight fields were counted in each of three different experiments. Results were expressed as mean number of migrating cells/mm^2 ± SD.

Collagen gel culture. Five hundred microliter of 0.21% type I collagen gel in DMEM neutralized with reconstitution buffer (50 mM NaOH, 260 mM Na_2CO_3, 200 mM HEPES) was pipetted into each well of a 24-well culture plate, and gelled with the incubation at 37˚C for 1 h as a basal layer of the gel. Thereafter, 500 μl collagen solution containing cells ($2.5x10^4$) was poured onto the basal layer, and gelled, and received 1 ml DMEM containing 1% FBS. The cells in the gels were cultured in the presence of 5 μg/ml plasminogen for 12 days. The colonies in the gel were fixed with phosphate-buffered 10% formalin. Sections were prepared and stained with hematoxylin and eosin (H&E).

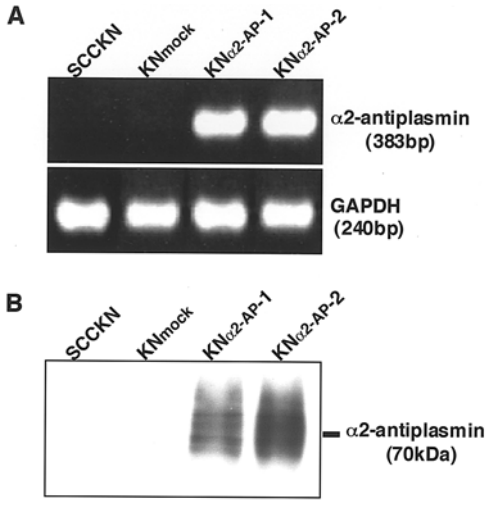

Figure 1. Expression of α2-AP in SCCKN and α2-AP transfectants. (A) RT-PCR analysis of the expression level of α2-AP mRNA in SCCKN, KN_{mock}, and $KN_{α2\text{-}AP}$ cells. GAPDH was used as an internal control. RT-PCR for the α2-AP or GAPDH gene was performed as described in Materials and methods. Amplified products were analyzed by electrophoresis on a 1% agarose gel. (B) Western blot analysis of the expression level of α2-AP protein in SCCKN, KN_{mock}, and $KN_{α2\text{-}AP}$ cells. The cell lysates were electrophoresed on a 10% SDS polyacrylamide gel and transferred to a PVDF membrane. The expression of α2-AP protein was detected by anti-α2-AP antibodies, followed by enhanced chemiluminescence detection reagents as described in Materials and methods.

Nude mouse tumorigenicity assay. The cells ($5x10^6$) suspended in 100 μl of DMEM were subcutaneously inoculated into the right flanks of 4-week-old male nude mice (BALB/c-nu/nu; CLEA Japan, Tokyo, Japan). The tumor size was measured twice a week for 4 weeks with a microcaliper. The tumor volume was calculated using the following formula: V = L x W^2 x 1/2, where V is the volume, L is the length and W is the width of a xenograft. The values are represented as mean ± SD of 5 xenografts per group. Animal studies were performed in accordance with the guidelines of Hiroshima University.

Results

Effect of α2-AP expression on E-cadherin processing by the plasminogen activator/plasmin system in SCC cells. Expression of α2-AP mRNA was examined with RT-PCR. No signal of α2-AP mRNA was detected in both SCCKN and KN_{mock} cells. Transfection with α2-AP cDNA led to the marked expression of α2-AP mRNA (Fig. 1A). Moreover, Western blotting showed that SCCKN and KN_{mock} cells synthesized no α2-AP protein and α2-AP transfectants, $KN_{α2\text{-}AP}$ cells synthesized a large amount of α2-AP (Fig. 1B). To examine the effect of α2-AP on E-cadherin processing by the plasminogen activator/

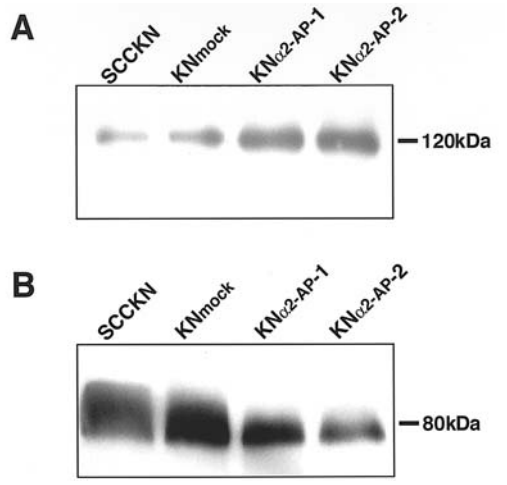

Figure 2. Proteolytic processing of E-cadherin by the plasminogen/plasminogen system in SCCKN cells and α2-AP transfectants. Confluent monolayers of SCCKN, KN$_{mock}$ and KN$_{α2\text{-}AP}$ cells were cultured in the presence of 5 μg/ml plasminogen. After incubation for 12 h, E-cadherin of cell membrane fractions (A) and culture supernatants (B) were analyzed with Western blotting using anti-E-cadherin monoclonal antibody.

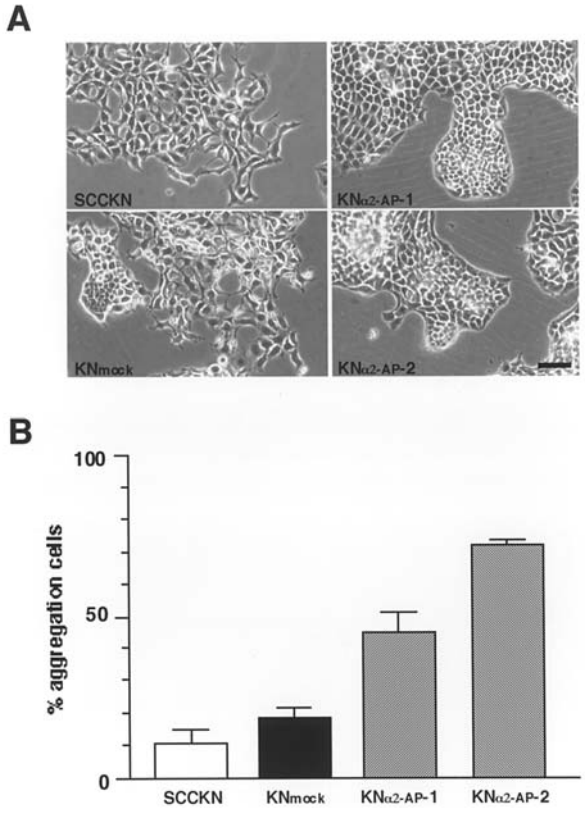

Figure 3. Effect of α2-AP expression on the cell-cell adhesion of SCC cells modulated by the plasminogen activator/plasmin system. (A) Morphology of SCCKN, KN$_{mock}$ and KN$_{α2\text{-}AP}$ cells cultured in the presence of plasminogen. Subconfluent monolayers of SCCKN, KN$_{mock}$ and KN$_{α2\text{-}AP}$ cells were cultured in the presence of 5 μg/ml plasminogen for 12 h and observed with a phase-contrast microscope at the same magnification. Scale bar, 30 μm. (B) Cell aggregation of SCCKN, KN$_{mock}$ and KN$_{α2\text{-}AP}$ cells treated with plasminogen. The cells were treated with 5 μg/ml plasminogen for 12 h, and then dissociated into single cell suspensions. The cells ($3x10^5$ cells/ml) suspended in Puck's saline containing 0.8% FBS and 50 U/ml DNase I (Roche) with 5 mM Ca^{2+} were placed in each well of a 24-well culture plate, and incubated for 120 min at 37˚C under agitating at 80 rpm with a gyratory shaker. The results represent the percentage of cells forming aggregates, and are the means ± SD of triplicate determinations.

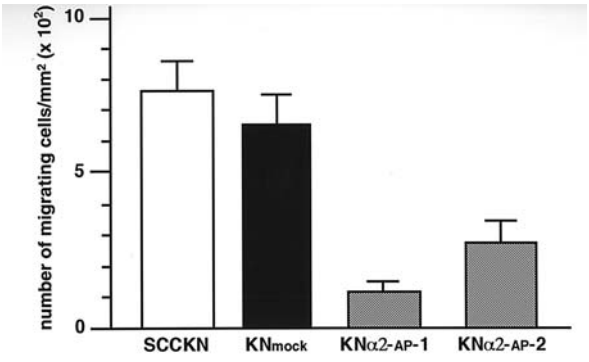

Figure 4. Effect of α2-AP expression on plasmin-mediated motility of SCC cells. SCCKN, KN$_{mock}$ and KN$_{α2\text{-}AP}$ cells treated with 5 μg/ml plasmin for 1 h were suspended in DMEM containing 0.1% BSA and then added to the upper compartment of Transwells. After incubation for 24 h at 37˚C, the number of cells migrating to the lower surface of the filter was counted. The results represent the mean number of migrating cells/mm^2 ± SD of triplicate determinations.

plasmin system, E-cadherin protein in the membrane fractions and culture supernatants of the cells treated with 5 μg/ml plasminogen were analyzed with Western blotting. SCCKN and KN$_{mock}$ cells treated with plasminogen expressed a small amount of full-length E-cadherin at 120 kDa in the membrane fractions, and released a large amount of soluble E-cadherin at 80 kDa in the culture supernatants. In contrast, the membrane fraction of KN$_{α2\text{-}AP}$ cells contained a large amount of full-length E-cadherin, and soluble E-cadherin in the culture supernatant was decreased compared to SCCKN and KN$_{mock}$ cells (Fig. 2).

Effect of α2-AP expression on cell-cell adhesion in SCC cells. SCCKN and KN$_{mock}$ cells treated with 5 μg/ml plasminogen displayed mesenchymal cell-like morphology with loose intercellular interactions. In contrast, KN$_{α2\text{-}AP}$ cells formed a polygonal epithelial monolayer with tight intercellular interactions in the presence of plasminogen (Fig. 3A). We next examined the effect of α2-AP on cell-cell adhesion of SCC cells treated with plasminogen. Our previous study has shown that treatment with plasminogen leads to a decrease in Ca^{2+}-dependent cell aggregation in SCC cells (23). Only 11% of SCCKN cells treated with plasminogen cells aggregated after 2-h incubation in the presence of 5 mM Ca^{2+}. Induction of α2-AP led to a marked increase in Ca^{2+}-dependent cell aggregation in the presence of plasminogen, and 44% of KNα2-AP-1 cells and 72% of KN$_{α2\text{-}AP}$-2 cells aggregated after 2-h incubation (Fig. 3B).

Effect of α2-AP expression on motility of SCC cells. Our previous study has shown that the plasminogen/plasmin system facilitates the motility of SCC cells by downregulation of the intercellular adhesion mediated by E-cadherin (23). We examined the effect of the inhibition of E-cadherin processing by α2-AP on the motility of SCC cells. The migration of SCCKN, KN$_{mock}$ and KN$_{α2\text{-}AP}$ cells pretreated with plasmin was estimated with modified Boyden chamber method. KN$_{α2\text{-}AP}$ cells exhibited decreased cell migration compared to SCCKN and KN$_{mock}$ cells. The number of migrating cells of KN$_{α2\text{-}AP}$-1 and KN$_{α2\text{-}AP}$-2 was 15% and 36%, respectively, of that of SCCKN (Fig. 4).

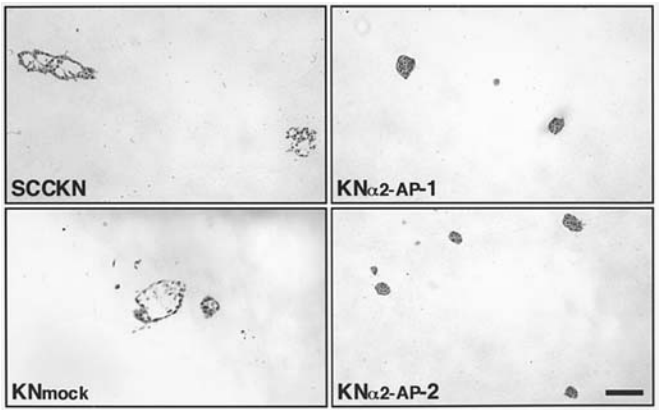

Figure 5. Morphology of SCCKN and α2-AP transfectants in type I collagen gel. The cells ($2.5x10^4$) embedded in type I collagen gel were cultured in the presence of 5 μg/ml plasminogen for 12 days. The cells were fixed in buffered formalin, embedded in paraffin and the sections stained with hematoxylin and eosin. Scale bar, 250 μm.

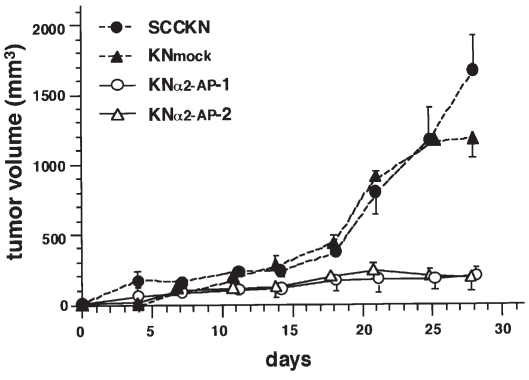

Figure 6. Effect of α2-AP expression on tumorigenicity of SCC cells. SCCKN, KN_{mock} and $KN_{\alpha2\text{-}AP}$ cells ($5x10^6$) were subcutaneously inoculated into the right flanks of 4-week-old male nude mice. The tumor size was measured twice a week for 4 weeks with a microcaliper. The tumor volume was calculated using the following formula: $V = L \times W^2 \times 1/2$, where V is the volume, L is the length and W is the width of a xenograft. The values are represented as mean ± SD of 5 xenografts per group.

Effect of α2-AP expression on morphology of colonies of SCC cells in three-dimensional collagen gels. The behaviors of SCCKN, KN_{mock} and $KN_{\alpha2\text{-}AP}$ cells were examined by three-dimensional culture using collagen gel. The cells embedded in type I collagen gel containing plasminogen were cultured for 12 days. SCCKN and KN_{mock} cells formed dilated colonies with irregular margin, and some cells migrated into the surrounding collagen gel. In contrast, $KN_{\alpha2\text{-}AP}$ cells formed small and spherical colonies, which consisted of tightly-packed cell aggregates (Fig. 5).

Effect of α2-AP expression on tumorigenesis of SCC cells. We evaluated the effect of α2-AP expression *in vivo* by comparing the growth of nude mouse xenografts from $KN_{\alpha2\text{-}AP}$ to the growth of xenografts from SCCKN and KN_{mock}. The xenografts from $KN_{\alpha2\text{-}AP}$ grew significantly slower than those from SCCKN and KN_{mock}. Four weeks after inoculation, the xenografts from SCCKN and KN_{mock} reached the average volume of 1,656 mm^3 and 1,173 mm^3, respectively. In contrast, the average volume of $KN_{\alpha2\text{-}AP}$-1 and $KN_{\alpha2\text{-}AP}$-1 xenografts was 183 and 193 mm^3, respectively (Fig. 6).

Discussion

Dissociation of cancer cells in the primary lesion is an initial step in tumor invasion and metastasis. Therefore, the suppression of the dissociation of cancer cells could inhibit invasion and metastasis. Our previous study has shown that the plasminogen activator/plasmin system downregulates cell-cell adhesion by the proteolytic processing of E-cadherin and facilitates the dissemination and motility of oral SCC cells (23).

In this study, we examined the effect of α2-AP, a specific plasmin inhibitor on the proteolytic processing of E-cadherin by the plasminogen activator/plasmin system. α2-AP, a member of the SERPIN family is mainly synthesized in liver, and inhibits fibrinolysis by inactivating plasmin (38,39). However, there is little information about the role of α2-AP in the pericellular proteolysis of normal cells and cancer cells, which regulate morphogenesis, differentiation, and tumor invasion and metastasis. SCCKN cells express no mRNA and protein of α2-AP. Transfection with α2-AP cDNA led to a remarkable expression of α2-AP in SCCKN cells. SCCKN cells treated with plasminogen exhibited a mesenchymal cell-like morphology with loose intercellular interactions. In contrast, α2-AP transfectants formed a polygonal epithelial monolayer with tight intercellular interactions in the presence of plasminogen. SCCKN and KN_{mock} cells treated with plasminogen released a large amount of extracellular domain of E-cadherin at 80 kDa in the culture supernatants, and expressed a small amount of full-length E-cadherin at 120 kDa in the membrane fractions. Induction of α2-AP expression led to the inhibition of the plasminogen-stimulated release of soluble E-cadherin in association with the increased expression of full-length E-cadherin in the membrane fraction. However, there was no difference in the expression of E-cadherin mRNA among SCCKN cells and α2-AP transfectants (data not shown). These finding indicated that α2-AP does not regulate the synthesis of E-cadherin in SCC cells but inhibits the proteolytic processing of E-cadherin by the plasminogen activator/plasmin system.

Our previous study has shown that the plasminogen activator/plasmin system reduces the aggregation of SCC cells by the proteolysis of E-cadherin (23). We therefore examined the effect of α2-AP on the aggregation of SCC cells in the presence of plasminogen. Treatment with plasminogen led to a remarkable suppression of Ca^{2+}-dependent cell aggregation of SCCKN and KN_{mock} cells. In contrast, Ca^{2+}-dependent cell aggregation of α2-AP transfectants was enhanced in the presence of plasminogen. These findings indicated that α2-AP blocks the proteolysis of E-cadherin by the plasminogen activator/plasmin system, resulting in the enhancement of cell-cell adhesion of SCC cells. Cell-cell adhesion is known to play an essential role in regulating cellular behaviors including cell motility, and tumor invasion and metastasis. We investigated whether the enhancement of cell-cell adhesion by α2-AP could alter the motility of SCC cells. The motility of SCCKN and KN_{mock} cells was stimulated by plasmin as described previously (23). The induction of α2-AP expression

strongly suppressed plasmin-stimulated motility of SCC cells. We examined the effect of α2-AP on the behavior of SCC cells using the three-dimensional collagen gel culture system. SCCKN and KN_{mock} cells embedded in type I collagen gel containing plasminogen formed dilated colonies with loose intercellular adhesion, and some cells migrated into the surrounding type I collagen gel. In contrast, α2-AP transfectants in type I collagen gel containing plasminogen formed spherical colonies, which consisted of tightly-packed cell aggregates. These findings suggested that downregulation of the plasminogen activator/plasmin system by α2-AP decreases the ability of SCC cells to migrate by the enhancement of cell-cell adhesion and that α2-AP might suppress the invasiveness of SCC cells. To investigate the potential therapeutic utility of α2-AP in oral SCC, we examined the effect of α2-AP on the tumorigenesis of oral SCC by evaluating the growth rates of the xenografts from SCCKN, KN_{mock} or $KN_{\alpha2\text{-}AP}$. The growth of the xenografts from α2-AP transfectants was significantly reduced compared to that of the xenografts from SCCKN and KN_{mock}, indicating that α2-AP suppresses the tumorigenicity of SCC cells.

In conclusion, α2-AP inhibits E-cadherin processing mediated by the plasminogen activator/plasmin system, leading to the suppression of dissemination and invasiveness of oral SCC cells through the enhancement of cell-cell adhesion. Moreover, induction of α2-AP resulted in the strong inhibition of tumorigenicity of oral SCC cells. These findings suggest that the plasminogen activator/plasmin system could serve as a therapeutic target to prevent invasion and metastasis of oral SCC and that downregulation of this protease system by α2-AP might be a potent therapeutic strategy for oral SCC. Further *in vivo* studies will be required to establish the effective delivery system of the α2-AP gene into oral SCC tissue.

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