Urokinase-Type Plasminogen Activator Expression and Proliferation Stimulation in Head and Neck Squamous Cell Carcinoma In Vitro and In Situ

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**Background:** Stimulation of proliferative activity by urokinase-type plasminogen activator (uPA) has been demonstrated in vitro for cultured primary and carcinoma cells.

**Objective:** To examine the effect of uPA stimulation on cultured squamous cell carcinoma cell lines of the head and neck in vitro and to compare the results with the situation in tumor tissue specimens.

**Design:** The uPA-mediated growth stimulation of 2 head and neck squamous cell carcinoma cell lines after suppression of endogenous uPA production was monitored by measuring 3H-thymidine uptake into cellular DNA. Alternatively, applications of antibodies against the uPA-binding domain of the urokinase receptor were used to suppress autostimulation. To analyze the situation in situ we performed Western blot and zymographic studies on tissue homogenates of 25 squamous cell carcinoma specimens. We tested the expression of proliferating cell nuclear antigen (PCNA), a marker for proliferative activity, and uPA in tissue lysates and correlated uPA and PCNA expression by regression analysis.

**Results:** High-molecular-weight urokinase had a proliferation stimulative effect on both cell lines in vitro. The uPA autostimulation was decreased by blocking the uPA-binding domain of urokinase receptor with antibodies. Regression analysis of zymographic and Western blot data of tumor tissue lysates revealed no significant coherency between PCNA and uPA expression. Immunohistochemical stainings frequently showed different sublocalization of uPA and PCNA within tumors.

**Conclusion:** In vitro uPA-mediated growth stimulation is not necessarily transferable to the in situ situation.


**UROKINASE-TYPE plasminogen activator (uPA) is a multidomain serine protease secreted by a variety of cell types. Proteolytic cleavage of extracellular matrix proteins occurs via proteolytic cleavage of plasminogen into plasmin. Urokinase-type PA itself is secreted as a single-chain proenzyme, which has to become activated by proteolytic cleavage to the 2-chain form of uPA. Activation is catalyzed by plasmin and other proteinases (for a review see Andreasen et al). Active uPA can also occur cleaved in the form of the low-molecular-weight uPA, which possesses the complete catalytic domain. The amino-terminal part of uPA contains a growth factor domain, which binds to the cell surface urokinase receptor. In 1987, Kirchheimer et al reported an in vitro proliferation stimulation by uPA in human epidermal tumor cells and in primary and malignant renal cells in vitro. During the past decade, increasing evidence for growth stimulation caused by urokinase has accumulated. The growth factor–like activity of urokinase has been shown to be independent of a plasmin-mediated process. Concerning head and neck cancer, uPA has been demonstrated to be increased in carcinoma tissue compared with normal mucosa.

The aim of this study was to examine the effect of exogenous urokinase stimulation on cultured squamous cell carcinoma cell lines of the head and neck in vitro. Furthermore, we tried to relate the in vitro results to the situation in situ and examined expression of uPA and the proliferation marker proliferating cell nuclear antigen (PCNA) in head and neck tumor tissue extracts using Western blot, zymography, or immunohistochemical analysis.

**RESULTS**

**IN VITRO STIMULATION OF HEAD AND NECK CARCINOMA CELL LINES**

The head and neck carcinoma cell lines HLaC79 and HSmC78 were tested for secretion of urokinase and expression of its receptor using fibrin zymography and
Northern blot, respectively. Urokinase and its receptor were detected in both cell lines (Figure 1) in different amounts. To suppress endogenous urokinase production, we incubated the cell lines before 3H-thymidine incorporation experiments with cycloheximide14 and afterwards with increasing concentrations of urokinase or equivalent amounts of cell culture medium as controls. Incubation with cycloheximide decreased 3H-thymidine uptake considerably and decreased uPA secretion in vitro (data not shown). Increasing concentrations of high-molecular-weight uPA were able to partially reverse the growth inhibitory effect of cycloheximide (Figure 2).

In addition, we carried out cycloheximide treatment and subsequent addition of urokinase (2.8 nmol/L) and tested PCNA expression in cell extracts using Western blot. Applying 30 µg of total cell protein to Western blots, we observed an up-regulation of PCNA in the cell lines (Figure 2). In a second series of experiments, we compared untreated cells under serum-free conditions with cells incubated with an antibody against the uPA-binding site of the urokinase receptor, preventing uPA receptor binding.9 Cells incubated with the antibody reacted with a decrease in 3H-thymidine uptake, which probably represents the elimination of the growth-stimulating effect of endogenously produced urokinase. HLaC79 and HSmC78 reacted to antibody treatment with average decreases of 64.98% and 3.78%, respectively. The weaker growth inhibition in HSmC78 might be explained by the much higher expression of uPA receptor in these cells.

QUANTITATIVE EXPRESSION ANALYSIS OF uPA AND PCNA IN TISSUE HOMOGENATES

Expression of the proliferation marker PCNA and urokinase was quantitatively determined by Western blot and fibrin zymography, respectively. Figure 3 shows the results of fibrin zymography and Western blot for 11 tumor samples. Densitometric evaluation and statistical regression analysis revealed no trend toward increasing PCNA levels in tumors with high uPA contents. Figure 3 shows the regression analysis of uPA level vs PCNA expression. Although not statistically significant, there was a trend toward lower PCNA expression in tumor tissue with higher uPA contents.

IMMUNOHISTOLOGICAL LOCALIZATION OF PCNA AND uPA

To investigate sublocalization of urokinase and the proliferation marker PCNA in tumor tissue, we performed immunohistological staining on frozen sections of tu-
mor specimens. Immunohistological localization showed that in many cases uPA and PCNA were sublocalized in distinct tumor compartments. Figure 4 shows a sample with uPA and PCNA expression in the same tumor specimen but in different subareas.

**COMMENT**

High-molecular-weight urokinase has been shown to stimulate cell proliferation in many in vitro cell systems. In 1989, Kirchheimer et al tested the in vitro proliferation stimulatory effect of uPA on a melanoma cell line. Because the cell line secreted uPA itself, the authors carried out antibody inhibition experiments and also treated the cells with cycloheximide before exogenous application of uPA. In their experiments, uPA secretion decreased and the number of uPA-occupied uPA receptors decreased, but the total number of uPA receptors remained almost the same. Using cycloheximide inhibition experiments and blocking of uPA-uPA receptor binding by specific antibodies, we demonstrated that head and neck carcinoma cell lines also react to urokinase stimulation in vitro. However, there are only a few speculations about the significance of these uPA-associated properties in situ or in vivo. The first evidence for an in vivo-relevant importance of urokinase expression was provided by Jensen and Lavker, who showed decreased proliferation of epidermal cells in mice with targeted uPA deletion. However, in complex cellular systems such as tumor tissue, there is still a lack of understanding concerning the functions of uPA. Urokinase has been shown to be overexpressed in head and neck cancer, although the significance of uPA overexpression for metastatic and invasive behavior of head and neck squamous cell carcinoma is still controversial. Gohring et al found a significant correlation between uPA and PCNA expression in primary breast cancer specimens. Volm et al analyzed 137 non–small cell lung carcinoma specimens immunohistochemically for expression of urokinase and further determined the proliferative activity of the tumors using a flow cytometric approach. The authors detected uPA expression in 77% of the tumors. However, they did not show any relationship between urokinase expression and cell cycle phase distribution (proliferative activity). This is in agreement with our observations in head and neck carcinoma specimens. Immunohistochemical analysis with PCNA and uPA antibodies revealed distinct sublocalization of uPA-positive and highly proliferative areas in many tumor specimens. Therefore, it seems likely that in a highly deregulated system such as tumor tissue, urokinase-mediated autostimulation does not necessarily take place. There are several possible explanations for the lack of endogenous urokinase proliferation stimulation.

Probably a certain balance of uPA production and secretion of plasminogen activator inhibitors (PAIs) can affect the behavior and proliferation of cells. In vitro uPA cleaves the amino-terminal fragment of urokinase in an autocatalytic process. The release of aminoterminal fragment (aminoterminal fragment of uPA with proliferating activity) by uPA cleavage has been shown to be inhibitable by PAIs. Thus, in hepatocellular carcinoma cells it has been demonstrated that PAI-1 can decrease proliferation and invasiveness, and Hibino et al eliminated the growth stimulatory effect of uPA in keratinocytes by PAI-2. Furthermore, overexpression of PAI-1 (together with uPA) in head and neck cancer has been demonstrated. Plasminogen activator inhibitor–1 might at least in part abolish the growth stimulatory effect of urokinase.

Another question concerns sublocalization of urokinase and its receptor in carcinoma tissue. In a previous study, we investigated uPA and uPA receptor expression in head and neck carcinomas. We did not show...
a relationship between uPA and uPA receptor content in tumor homogenates or a compelling colocalization within the tumors. The different sublocalization might prevent signal transmission by uPA receptor.

Furthermore, distinct components of uPA-mediated signaling cascades might be missing because of chromosomal deletions occurring frequently in cancer cells. As long as the uPA-mediated signaling pathway has not been identified in detail, a failure of signal transmission in certain cell types might explain the lack of autostimulation in tumors and the lacking proliferation stimulation by uPA in certain cell lines (eg, U 937 lymphoma cells\(^{(22)}\)).

Previously, Schmidt and Hoppe\(^{(23)}\) showed that patients with head and neck carcinomas can be recognized by significantly increased levels of soluble uPA receptor in blood plasma, which suggests “shedding” of uPA receptor by tumor cells. Soluble uPA receptor within the tumor tissue might be a further suppressor of autostimulation by binding uPA and thus competing with cell-associated uPA receptor.

Fischer et al\(^{(2)}\) stimulated OV-MZ-6 ovarian cancer cells with high-molecular-weight urokinase. Considering the concentration-dependent growth stimulation in these cell cultures, it is conspicuous that maximal growth stimulation reached a peak at about 1-nmol/L uPA concentration and decreased again with increasing uPA concentrations. Certain effective uPA concentrations are probably also necessary in vivo.

In summary, we showed an in vitro growth stimulative effect of urokinase in head and neck carcinoma cell lines. We did not extend these observations to the situation in native tumor tissue comparing uPA and PCNA expression, neither quantitatively nor qualitatively.

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### REFERENCES