EXPRESSION OF ETS-1 TRANSCRIPTION FACTOR IN HUMAN HEAD AND NECK SQUAMOUS CELL CARCINOMA AND EFFECT OF HISTAMINE ON METASTATIC POTENTIAL OF INVASIVE TUMOR THROUGH THE REGULATION OF EXPRESSION OF ETS-1 AND MATRIX METALLOPROTEINASE-3

Barnabás Horváth, MD,1 Hargita Hegyesi, MD,2 Pál Nagy, MD,4 András Falus, MD,2 Zsuzsa Schaff, MD3

1 Department of Ear, Nose and Throat Diseases, National Medical Center, 1135, Szabolcs u. 35, Budapest, Hungary. E-mail: bhorvath@ogyik.hu
2 Department of Genetics, Cell and Immunobiology, Semmelweis University, Budapest, Hungary
3 2nd Department of Pathology, Semmelweis University, Budapest, Hungary
4 Department of Pathology, National Medical Center, Budapest, Hungary

Accepted 4 January 2005
Published online 10 May 2005 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/hed.20188

Abstract: Background. Ets-1 controls the expression of critical genes involved in matrix remodeling. The matrix metalloproteinase-3 (MMP-3) and urokinase type plasminogen activator (uPA) are typical Ets-1 responsive genes. Recent studies have shown an increase in histamine synthesis and content in various human neoplasias. We hypothesized that the increased local histamine overproduction contributed to activation of matrix remodeling through the activation of MMP-3 expression of peritumoral fibroblasts by means of ets-1 regulation in head and neck squamous cell carcinomas (HNSCCs).

Methods. Paraffin-embedded sections of 30 HNSCCs were immunostained for ets-1. The presence of ets-1 and MMP-3 mRNA in tumor samples was confirmed by reverse transcriptase–polymerase chain reaction (RT-PCR). To simulate stromal reaction in vitro, cultured human mucosal fibroblast was used. The level of ets-1 and MMP-3 mRNA was compared by use of RT-PCR, as was their protein with flow-cytometry, in the presence or absence of basic fibroblast growth factor (bFGF) (10 ng/mL) and histamine (1 μM).

Results. Correlation between ets-1 expression and clinicopathologic background was not significant. In all cases, expression of ets-1 was seen in the stroma. In in vitro study, histamine upregulates production of ets-1 and MMP-3 in cultured fibroblast, and bFGF can stimulate histamine expression in fibroblast. Immunofluorescence staining supported the results of RT-PCR and flow cytometry.

Conclusions. Ets-1 expression in HNSCCs has no prognostic value; however, ets-1 plays an important role in tumor–host interaction. Histamine may accelerate the spread of HNSCC through an ets-1–related mechanism. © 2005 Wiley Periodicals, Inc. Head Neck 27: 585–596, 2005

Keywords: head and neck cancer; metastasis; fibroblast; histamine; ets-1; MMP-3

A central role in tumor cell invasion and metastatic cascade has been dedicated to proteolytic enzymes, including the urokinase-type plasminogen activator (uPA); cathepsin B, L, and D; and the matrix metalloproteinases (MMPs).1 MMP-3 is a collagenase-related metalloproteinase whose
substrates include proteoglycans, fibronectin, gelatins, and types IV and V collagen.2,3

Ets-1 was originally characterized as the v-ets retroviral gene, one of the two oncogenes (v-myb and v-ets) in the avian leukemia retrovirus, E26.4 Ets-1 plays a role in the regulation of physiologic processes such as cell proliferation and differentiation. Ets-1 is also associated with invasive processes in the stromal tissues of human carcinomas.5,6 Ets-1 protein interacts with the uPA gene enhancer and with the promoters of the MMP-3 and MMP-1 gene. It is, therefore, suggested to regulate increased tumor invasion by activating the expression of uPA, MMP-3, and MMP-1.7 The expression of ets-1 transcription factor has been reported in various human tumors, including carcinoma of the stomach,5 lung,8 prostate,9 pancreas,10 esophagus,11 liver,12 angiosarcoma of the skin,13 oral squamous cell carcinoma,7 extrahepatic bile duct,14 and meningioma.15 Ets-1 is the first transcriptional factor to be detected during the host reaction to invasive tumor.5

Interactions between the tumor cells and their surrounding stroma play an important role in tumor growth and metastasis.16 The fibroblast, the predominant cell in the stroma, is responsible for the elaboration of most of the components of connective tissue and is the major source of many proteases associated with tumor invasion.17–19 Overexpression of growth factors and their receptors has been implicated in the genesis and progression of a variety of human neoplasms. The basic fibroblast growth factor (bFGF) is one of the best-characterized angiogenic signal molecules and has been commonly expressed by numerous tumor cells.20 In addition, Dellacono et al21 demonstrated increased expression of bFGF and bFGF receptor in head and neck squamous cell carcinomas (HNSCCs). Recent studies revealed that bFGF can stimulate the expression of ets-1 mRNA in human fibroblasts.6

The role of histamine in tumorigenesis and metastasis remains to be clarified. Histamine is a widely occurring chemical mediator that serves as a neurotransmitter and a modulator of gastrointestinal and smooth muscle functions; it is also involved in other physiologic effects: allergic reaction, inflammation, and cell proliferation. Histamine has also been reported as an angiogenic factor.22,23 Zenmyo et al24 reported that histamine stimulated MMP-1 expression by human rheumatoid synovial fibroblasts through histamine H1-receptor. Overexpression of histidine decarboxylase (HDC) has been detected in a wide range of tumors, including leukemia, as well as breast, stomach, melanoma, and lung cancers.25,26 Reynolds et al27 reported that there is a sufficiently high level of histamine in breast cancer to suppress lymphocyte activation. Falus et al28 demonstrated that the presence and effect of histamine seems to be relevant both by directly stimulating or suppressing growth of the malignant melanoma (depending on the local histamine receptor balance) and by indirectly shifting the local T-cell polarization toward a predominance of Th-2 cells. The central role of histamine in various physiologic and pathologic processes and its influence on tumorigenesis lead us to suppose that histamine may act on dissemination of malignant neoplasms.29

To explain the involvement of ets-1 in human HNSCC, we performed immunohistochemical analysis on tissue from 30 cases of HNSCC and its own metastasis. Reverse transcriptase–polymerase chain reaction (RT-PCR) analysis also confirmed the presence of ets-1 and MMP-3 mRNA in HNSCC. We proposed that histamine-induced production of MMP from fibroblasts may represent a paracrine mechanism of regulation of extracellular matrix (ECM) remodeling in various physiologic and pathologic situations associated with elevated extracellular concentration of histamine. To our knowledge, no studies have dealt with the influence of histamine on metastatic ability.

To test the hypothesis according to which histamine would be implicated in increasing the metastatic potential of invasive tumor promoting the proteolytic enzyme production of the neighboring connective tissue, we used an in vitro cell culture system. We looked for a potential relationship between exogenously added histamine and expression of ets-1 transcription factor and metalloproteinase MMP-3 in cultured human mucosal fibroblasts.

PATIENTS AND METHODS

Patients and Tumor Samples. We examined 30 patients (24 male and six female) with HNSCC at the Department of Ear, Nose and Throat Diseases of the National Medical Center, Hungary. All tumors were completely removed surgically between 1998 and 2000. Tumor origin was classified as mesopharyngeal, laryngeal, and hypopharyngeal according to the fourth edition of Union Interationale Contre le Cancer (UICC) TNM Classification of Malignant Tumors
(1987). Histologic grade was assigned as follows: grade 1, well-differentiated squamous cell carcinoma (SCC); grade 2, moderately differentiated SCC; grade 3, poorly differentiated SCC. Histopathologic diagnosis was made routinely at the Department of Pathology of National Medical Center. Tumor size was classified from pT1 to pT4 according to the TNM classification (see earlier). Cervical lymph node metastasis was classified from pN0 to pN3, also according to the TNM classification. The largest and deepest invasive site was selected for immunohistochemistry of ets-1.

**Cell Culture.** Primary culture of human mucosal fibroblasts from passage 7-14 was cultivated in RPMI medium (Sigma, St. Louis, MO) supplemented with 10% fetal calf serum and antibiotics. When subconfluence was reached, the cells were washed and incubated in RPMI medium in the presence or absence of the following factors: 10 ng/mL recombinant human bFGF (Sigma) and 1 μM histamine. Six hours later, the cells were processed for RNA extraction. The other part of bFGF or histamine-treated cultured fibroblasts was prepared 20 hours later for flow cytometry. Four hours before finishing incubation, 10 μg/mL Brefeldin A (Sigma) was added to the cell cultures to inhibit protein secretion.

**Immunohistochemical Staining and Evaluation.** Three-micron-thick paraffin-embedded sections were used. Endogenous peroxidase was inhibited with 3% hydrogen peroxide and nonspecific binding blocked with normal horse serum. Sections were incubated with a rabbit monoclonal antibody to ets-1 (c-20, Santa Cruz Biotech, Inc.) at a dilution of 1:20,Santa Cruz Biotech, Inc.) at a dilution of 1:20, Santa Cruz Biotech, Inc.) at a dilution of 1:20, Santa Cruz Biotech, Inc.) at a dilution of 1:20, Santa Cruz Biotech, Inc.) at a dilution of 1:20 . After incubation with the antibodies for 30 minutes, the cells were washed with phosphate-buffered saline (PBS), incubated with fluorescein isothiocyanate-conjugated (FITC) (Sigma, St. Louis, MO) anti-rabbit or anti-goat IgG for 30 minutes at a dilution of 1:500, and then observed under confocal microscopy.

**Immunofluorescence Staining.** Human mucosal fibroblast was cultured as described previously, incubated for a further 20 hours in the presence of 1 μM histamine, and fixed in 4% paraformaldehyde. Indirect immunofluorescence staining was performed with rabbit antibody against human ets-1 (c-20, Santa Cruz Biotech, Inc.) and goat antibody against human MMP-3 (Santa Cruz Biotech, Inc.) at a dilution of 1:300. After incubation with the antibodies for 30 minutes, the cells were washed with phosphate-buffered saline (PBS), incubated with fluorescein isothiocyanate-conjugated (FITC) (Sigma, St. Louis, MO) anti-rabbit or anti-goat IgG for 30 minutes at a dilution of 1:500, and then observed under confocal microscopy.

**RNA Isolation and RT-PCR.** Total RNA was extracted from cancer tissues by use of TRI reagent (Sigma) according to the protocol. RT-PCR was carried out on a Pharmacia Ataq Gene Controller. For the RT, the reaction mixture (20 μL) consisted of Mg2+ (25 mM) 4 μL, 10 × buffer 2 μL, dNTPs (4 × 10 mM) 2 μL, RNase inhibitor 1 μL (20 units) (Promega Corp, Madison WI), oligo-dT primer 1 μL (Promega Corp), Mu-LV reverse transcriptase 0.5 μL (25 units), DEPC-distilled water and 1 μg of total RNA. RNA samples (l μg) were reverse transcribed at 42°C for 30 minutes, incubated at 99°C for 5 minutes, and frozen. The PCR reaction mixture contained 10 × buffer, 1 mM MgCl2, dNTPs, Taq DNA polymerase (Promega Corp), 25 to 50 pmol sense and antisense primers, and cDNA in 50 μL of final volume. The sequences of oligonucleotide primers used in RT-PCR for each gene and the expected sizes of their RT-PCR products are as follows: (1) the human ets-1 5’-GGGTGACGACT-TCTTTG-3’ (sense primer) and 5’-GTTAATGGA-GTCAACCAGC-3’ (antisense primer), 247 bp; (2) mors were regarded as ets-1 positive if any of the tumor cells showed cytoplasmic and nuclear immunoreactivity. Cells positive for ets-1 were counted by monitoring at least 500 cells from at least three randomly selected fields. Ets-1 expression was classified into four categories depending on the percentage of cells stained: --, negative; +, 0% to 10% positive; ++, 10% to 40% positive; ++++, >40% positive tumor cells. The chi-square test was used to detect the differences between the groups. A p value <.05 was considered statistically significant.
MMP-3 5'-GCTGCAAGGGTGAGGACAC-3' (sense primer) and 5'-GATGCCAGGAAGGT-TCTGAAGTG-3' (antisense primer), 222 bp; (3) glyceraldehyde-3-phosphate dehydrogenase (GAPDH) 5'-ACCACTCTGCTGGAATC-3' (sense primer) and 5'-TCCACAATGTCTGTC-3' (antisense primer), 453 bp. Samples were subjected to 28 cycles of PCR amplification using a thermal cycler. Each cycle included denaturation at 94°C for 1 minute, annealing at 57°C for 1 minute, and primer extension at 72°C for 1 minute; 15 μL of each amplification mixture was subjected to electrophoresis on a 2.0% agarose gel, and DNA was visualized by ethidium bromide staining.

Detection of Changes in Ets-1, MMP-3 Content by Flow Cytometry. Polyclonal rabbit anti-human ets-1 antibody (c-20, Santa Cruz Biotech, Inc.) and polyclonal goat anti-human MMP-3 antibody (Santa Cruz Biotech, Inc.), as well as indirect labeling, were used for detecting intracellular ets-1 and MMP-3. After fixation in 4% paraformaldehyde and permeabilization with 0.1% saponin in PBS, the cells were labeled by anti-human Ets-1 or MMP-3 antibody at the dilution of 1:100, followed by FITC-conjugated rabbit and goat IgG 1:1000 (Sigma). For the measurement of intracellular histamine content, the cells were fixed by 4% 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDAC) (Sigma), permeabilized with 0.1% saponin in PBS, and incubated with antihistamine rabbit antibody 1:100 (Sigma) followed by FITC-conjugated anti-rabbit antibody 1:100 (Promega Corp). Analysis was performed by CellQuest 3.2 software. Data were evaluated for statistical significance using the Student's t test, *p < .05, as compared with untreated

<table>
<thead>
<tr>
<th>Case no.</th>
<th>Ets-1 expression</th>
<th>Tumor cell</th>
<th>Stromal fibroblast</th>
<th>Case no.</th>
<th>Ets-1 expression</th>
<th>Tumor cell</th>
<th>Stromal fibroblast</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Primary</td>
<td>+</td>
<td>Positive</td>
<td>16</td>
<td>Primary</td>
<td>–</td>
<td>Positive</td>
</tr>
<tr>
<td>2</td>
<td>Primary</td>
<td>+</td>
<td>Positive</td>
<td>17</td>
<td>Primary</td>
<td>–</td>
<td>Positive</td>
</tr>
<tr>
<td>3</td>
<td>Primary</td>
<td>+</td>
<td>Positive</td>
<td>18</td>
<td>Primary</td>
<td>–</td>
<td>Positive</td>
</tr>
<tr>
<td>4</td>
<td>Primary</td>
<td>–</td>
<td>Positive</td>
<td>19</td>
<td>Primary</td>
<td>+</td>
<td>Positive</td>
</tr>
<tr>
<td>5</td>
<td>Primary</td>
<td>+</td>
<td>Positive</td>
<td>20</td>
<td>Primary</td>
<td>–</td>
<td>Positive</td>
</tr>
<tr>
<td>6</td>
<td>Primary</td>
<td>+</td>
<td>Positive</td>
<td>21</td>
<td>Primary</td>
<td>++</td>
<td>Positive</td>
</tr>
<tr>
<td>7</td>
<td>Primary</td>
<td>–</td>
<td>Positive</td>
<td>22</td>
<td>Primary</td>
<td>–</td>
<td>Positive</td>
</tr>
<tr>
<td>8</td>
<td>Primary</td>
<td>–</td>
<td>Positive</td>
<td>23</td>
<td>Primary</td>
<td>+</td>
<td>Positive</td>
</tr>
<tr>
<td>9</td>
<td>Primary</td>
<td>++</td>
<td>Positive</td>
<td>24</td>
<td>Primary</td>
<td>–</td>
<td>Positive</td>
</tr>
<tr>
<td>10</td>
<td>Primary</td>
<td>+</td>
<td>Positive</td>
<td>25</td>
<td>Primary</td>
<td>–</td>
<td>Positive</td>
</tr>
<tr>
<td>11</td>
<td>Primary</td>
<td>–</td>
<td>Positive</td>
<td>26</td>
<td>Primary</td>
<td>+</td>
<td>Positive</td>
</tr>
<tr>
<td>12</td>
<td>Primary</td>
<td>++</td>
<td>Positive</td>
<td>27</td>
<td>Primary</td>
<td>++</td>
<td>Positive</td>
</tr>
<tr>
<td>13</td>
<td>Primary</td>
<td>+</td>
<td>Positive</td>
<td>28</td>
<td>Primary</td>
<td>–</td>
<td>Positive</td>
</tr>
<tr>
<td>14</td>
<td>Primary</td>
<td>+</td>
<td>Positive</td>
<td>29</td>
<td>Primary</td>
<td>++</td>
<td>Positive</td>
</tr>
<tr>
<td>15</td>
<td>Primary</td>
<td>–</td>
<td>Positive</td>
<td>30</td>
<td>Primary</td>
<td>+</td>
<td>Positive</td>
</tr>
</tbody>
</table>

Abbreviation: RLNM, regional lymph node metastasis.

Note. Staining intensity was classified as follows: –, negative; +, 0% to 10% positive tumor cells; ++, 10% to 40% positive tumor cells; ++++, >40% positive tumor cells.
controls. The results were reproducible for four independent experiments.

RESULTS

Immunopositivity of Ets-1 in HNSCCs. Table 1 shows the immunohistochemical distribution of ets-1 in primary tumor and regional lymph node metastasis. In addition, in 17 cases (56%) we clearly observed ets-1 protein located in carcinoma cells. Strong staining of ets-1 (++++) was observed in one tumor sample only (3%), medium (++) staining in five cases (17%), and weak staining (+) in 11 cases (36%). Figure 1 shows a representative example of immunohistochemical ets-1 staining in normal pharyngeal mucosa and HNSCCs. Normal pharyngeal mucosa obtained from sites contralateral to the tumor of a patient with cancer did not show ets-1 immunoreactivity. Ets-1 antigen was expressed heterogeneously in carcinomas. Overexpression of ets-1 was observed in the cytoplasm and the nucleus in carcinomas. The invasive front of the primary tumor was intensely stained compared with the superficial and central parts of the tumor in almost all cases. Weak staining of ets-1 in regional lymph node metastasis was detected in 11 (36%) of the 30 cases. The regional lymph node metastasis showed ets-1 staining in one case only, and its primary tumor was found negative.

The correlation between Ets-1 expression at the deepest invasive site and the clinicopathologic features is shown in Table 2. When comparing the clinicopathologic background, no significant differences were found with regard to sex, histologic grade, TNM stage, or primary tumor localization.

The expression of ets-1 in primary tumor and regional lymph node metastasis is shown in Figure 2. The ets-1 expression tended to be enhanced in the primary tumor, but this differ-

FIGURE 1. (A) Representative section of HNSCC showing immunohistochemical expression of ets-1. Ets-1 protein overexpression was found in the nuclei of tumor cells (arrows); normal hypopharyngeal mucosa was not found to be ets-1 immunopositive (magnification ×300). (B) Representative section of stromal staining adjacent to the tumor cells (arrows). (magnification ×300). (C) HNSCC (T) showing predominantly cytoplasmic staining. (D) Expression of ets-1 within the fibroblastic stroma of invasive human HNSCC (T). Positive signals were seen directly adjacent to invasion formation, in the fibroblast (F), endothelial cell (V) (magnification ×200).
ence from regional lymph node metastasis was not significant.

It is of interest to note that in all cases, the expression of ets-1 protein was seen in the peritumoral fibroblast, the nuclei of endothelial cells, and tumor infiltrating inflammatory cells. Strong staining was detected within the fibroblast directly adjacent to the invasive tumor front, whereas no signal was shown in more distant areas (Figure 1D).

**Ets-1 and MMP-3 mRNA in Human HNSCC Tissue.**

The results of RT-PCR of ets-1 mRNA in normal pharyngeal mucosa, HNSCC tissue, and tonsil tissue as positive control are shown in Figure 3A. Compared with normal mucosa, which did not express ets-1 mRNA, all tumor tissues and tonsil tissues expressed ets-1 mRNA.

Representative expression of ets-1, MMP-3, and GAPDH mRNA detected by RT-PCR is shown in Figure 3B. Ets-1 mRNA was expressed in all tissues from patients undergoing surgery for head and neck tumors, although the expression was not significantly different between metastatic and nonmetastatic human HNSCC tissues. In metastatic tumor tissues, MMP-3 mRNA was significantly upregulated compared with nonmetastatic HNSCC tissues. The housekeeping gene GAPDH showed similar expression in all cases. Interestingly, as shown in Figure 3B, high-level expression of ets-1 mRNA was not accompanied in all cases by high-level expression of MMP-3 mRNA.

**In Vitro Studies.**

**Ets-1 and MMP-3 Protein on Histamine Treatment in Fibroblasts.**

Immunofluorescence staining showed that in vitro 1 μM histamine stimulated both ets-1 and MMP-3 production in cultured fibroblasts compared with untreated control cells (Figures 4A, 4B). In the untreated cells, ets-1 and MMP-3 signals were not observable at the protein level because of the undetectable amount of these proteins. These results were consistent with those of RT-PCR and flow cytometry analysis.

**Effect of Histamine on ets-1 and MMP-3 Expression in Primary Human Mucosal Fibroblast.**

Incubation of primary fibroblast cells for 6 hours in the presence of 1 μM histamine and 10 ng/mL bFGF increased the mRNA expression of ets-1 and MMP-3, respectively, compared with cells incubated with the vehicle. The results of the semiquantitative PCR are shown in Figure 3C. Histamine and bFGF significantly increased both ets-1 and MMP-3 mRNA levels in the fibroblast after a 6-hour incubation period compared with the untreated cells.

**Demonstration of ets-1 and MMP-3 in Fibroblasts by Flow Cytometry.**

Upregulation of ets-1 transcription factor and MMP-3 expression by flow cytometry is demonstrated in Figures 5A and 5B. The ets-1 and

---

**Table 2.** Clinicopathologic background of patients with head and neck squamous cell carcinoma, as related to Ets-1 expression in primary tumor (n = 30).

<table>
<thead>
<tr>
<th>Clinicopathologic details</th>
<th>Total no. of cases</th>
<th>Ets-1 expression</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Negative no. (%)</td>
<td>Positive no. (%)</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>24</td>
<td>11 (42)</td>
</tr>
<tr>
<td>Female</td>
<td>6</td>
<td>2 (33)</td>
</tr>
<tr>
<td>Squamous cell carcinoma histologic grade</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G1</td>
<td>16</td>
<td>7 (37)</td>
</tr>
<tr>
<td>G2</td>
<td>14</td>
<td>7 (43)</td>
</tr>
<tr>
<td>Tumor classification</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1/T2</td>
<td>13</td>
<td>6 (38)</td>
</tr>
<tr>
<td>T3/T4</td>
<td>17</td>
<td>7 (41)</td>
</tr>
<tr>
<td>Location</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Larynx</td>
<td>14</td>
<td>7 (50)</td>
</tr>
<tr>
<td>Hypopharynx</td>
<td>10</td>
<td>4 (40)</td>
</tr>
<tr>
<td>Mesopharynx</td>
<td>6</td>
<td>2 (33)</td>
</tr>
</tbody>
</table>

Abbreviation: NS, not significant. Chi-square test, p < .05.

---
MMP-3 contents measured in 10 nm/mL bFGF and 1 μM histamine-treated cultured fibroblasts (after 20 hours) were compared with the untreated control cells, and a significantly increasing ets-1 and MMP-3 amount was detected. These data are consistent with the result obtained for RT-PCR.

Increased histamine content in bFGF-treated cultured fibroblasts is shown in Figure 5C. After 20 hours of bFGF (10 ng/mL) incubation, a significantly elevated amount of histamine was detected by flow cytometry.

**DISCUSSION**

Cell invasion and metastasis require the expression of proteases for degradation of the ECM. MMPs are frequently overexpressed in transformed cells and in tumors with metastatic potential. MMP-1, MMP-3, MMP-9, and uPA contain the ets binding motif in their cis regulatory elements, and the expression of these proteases has been shown to be induced by Ets family transcription factors. Wasylyk et al demonstrated that ets-1 is a component of the signaling network that links oncoproteins outside the nucleus, Ha-Ras, Src, and Mos, with transcription of a gene that has an important role in both pathologic and normal processes, such as stromelysin. The ets-1 transcription factor has been implicated in the metastatic process, but unifying concepts regarding its role are lacking.

Immunohistochemical analyses of various tumors have identified ets-1 expression in 35% to 83% of tumors examined. In our study, 56% (n = 17) of the specimens demonstrated specific staining of ets-1 protein. The expression of the ets-1 protein was also evidenced in a part of the tumor cells and in all cases of the stromal fibroblast adjacent to the tumor. These results suggest that the presence of expression of ets-1 transcription factor within the tumor...
cells of HNSCCs itself has no prognostic value (Table 2).

To ascertain whether the expression of ets-1 in cancer cells changes during the steps of lymph node metastasis, we compared the immunohistochemical expression of ets-1 between primary tumor and regional lymph node metastases. In metastatic lesion, ets-1 expression tended to be slightly suppressed (Figure 2). These data indicate that upregulation of ets-1 in cancer cells is not maintained through the end stage of metastasis.

By use of RT-PCR on tumor samples from patients who underwent radical surgery, we confirmed the presence of ets-1 transcription factor and MMP-3 (Figure 3A). In addition, the MMP-3 mRNA level of tumor samples with metastatic ability was higher than in samples without it (Figure 3B).

Gilles et al.\textsuperscript{33} demonstrated that the expression of ets-1 in epithelial cells is closely associated with an epithelial-to-mesenchymal transition (EMT) and that during cancer progression, ets-1 might be expressed in epithelial cancer cells that migrate and metastasize. This phenomenon may help to explain why only a relatively small fraction of the epithelial tumor cells in the primary tumor showed specific staining of ets-1 transcription factor. Thus, ets-1 expression may be a marker for a subclone of SCC cells that has a different metastatic behavior.

Several studies have demonstrated an increased stromal staining adjacent to the tumor cells. In vitro studies have shown that ets-1 expression can be enhanced in fibroblast co-cultures with malignant keratinocytes,\textsuperscript{34} cultured in the presence of conditioned media from cancer cells,\textsuperscript{35} or stimulated by different cytokines or growth factors.\textsuperscript{36} In addition, recent studies have shown that ets-1 is induced in endothelial cells in response to angiogenic growth factors, including
FIGURE 5. (A) Upper panel, representative data of flow cytometric analysis of ets-1 expression in cultured fibroblast–treated basic fibroblast growth factor (bFGF) (10 ng/mL) and histamine (1 μM) for 20 hours. Lower panel, relative effects of bFGF (gray bar), histamine (black bar), and untreated control (open bar) on ets-1 protein expression. (B) Upper panel, representative data of flow cytometric analysis of MMP-3 expression in cultured fibroblast–treated bFGF (10 ng/mL) and histamine (1 μM) for 20 hours. Lower panel, relative effects of bFGF (gray bar), histamine (black bar), and untreated cells (open bar) on MMP-3 protein expression detected by flow cytometric analysis. (C) Upper panel, representative data of flow cytometric analysis of histamine expression in cultured fibroblast–treated bFGF (10 ng/mL) for 20 hours. Lower panel, relative effects of bFGF (black bar) and untreated cells (open bar) on histamine level in cultured human mucosal fibroblasts. Data are expressed in percentage of geometric mean value ± SEM compared with untreated cells (100%). *p < .05 compared with corresponding values of untreated cells (Student's t test). The results were reproducible for four independent experiments (solid line, second antibody, control; dotted line, bFGF treated; dashed line, histamine (HA) treated).
acidic fibroblast growth factor (FGF), bFGF, and vascular endothelial growth factor (VEGF). Our results of in situ examination performed on specimens from patients with HNSCC suggest that ets-1 has a relevant role in the host reaction to invasive tumor. Because each tumor sample can be considered a mixed population of varying content of tumor cells and histologically normal cells such as connective tissue elements, tumor-infiltrating lymphocytes, polymorphonuclear leukocytes, and mast cells, we carried out an in vivo study to ascertain whether upregulation of ets-1 in peritumoral fibroblast is due to growth factors expressed by tumor cells.

In this study, we tested the hypothesis that extracellular histamine can promote matrix remodeling by triggering the synthesis of MMP from fibroblasts. We compared the level of expression of ets-1 and MMP-3 mRNA by semi-quantitative RT-PCR and their proteins by flow cytometry in cultured human mucosal fibroblasts in the presence or absence of human bFGF and histamine. We found that mRNAs of ets-1 and MMP-3 were markedly augmented in both bFGF and histamine-treated cells (Figure 3C). In addition, the same results were confirmed by flow cytometry (Figures 5A, 5B). The bFGF is known to increase the expression of ets-1 in peritumoral fibroblast, but to our knowledge, this is the first report on the possible relevance of histamine on ets-1–mediated tumor invasion.

Our investigations clearly demonstrated that bFGF can stimulate histamine expression in fibroblasts (Figure 5C). In relation to our results, the presence of expression of the ets-1 transcription factor in a restricted fraction of epithelial tumor cell may be due to EMT; however, over-expression and abnormal distribution of the ets-1 protein occurring in the fibroblast adjacent to the tumor may be induced by growth factors produced by tumor cells or factors in connection with tumor formation. Histamine is thought to be such a factor, which can arise from tumor cells themselves or from mast cells invading tumor tissue, and from peritumoral fibroblasts.

There is evidence to suggest that histamine is present in sufficient amounts in cancer to play a significant role. Reynolds et al demonstrated that histamine content in tumor tissue was significantly higher than in adjacent breast tissue, and Garcia-Caballero et al have found that histidine decarboxylase activity of cancerous tissue was significantly higher than that registered in the healthy mammary gland tissue. In addition, mast cells have been reported in high numbers around the periphery of various solid tumors. Because our present in vitro study is not specific for HNSCC, we considered these results to be acceptable.

Obviously, the question arises as to whether ets-1 expression follows the change in histamine concentration. However, our aim was not to
investigate the pharmacologic profile of the effect of histamine on ets-1 expression but to register a possible connection. We used a histamine concentration (1 μM) that is thought to have an effect on fibroblast proliferation, and its effect is in all certainty receptor mediated.\textsuperscript{43–45}

However, the significance of the role of histamine in the complex phenomenon of tumorigenesis and metastasis remains to be clarified. Our results show a clear positive correlation between the effect of histamine and induced ets-1 and MMP-3 mRNA and protein expression. These results suggest that a high level of histamine in tumor tissue may accelerate invasiveness of SCC by inducing ets-1 transcription factor and, consequently, MMP-3 in peritumoral fibroblasts. Knowledge of the contributions of molecules involved in the metastasis cascade would be of importance in understanding the molecular nature of the metastatic process, as well as for providing reasonable targets for the development of antimitastatic therapy.

REFERENCES


