Antisense Ets-1 Transfection Restrains Oral Cancer Invasion by Reducing Matrix Metalloproteinase Activities

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Abstract: Ets-1 is an Ets family transcription factor, can up-regulate the transcription of matrix metalloproteinase (MMP) genes and confers an invasive phenotype on human cancer cells. HSC3 is an oral squamous cell carcinoma-derived cell line, and it manifests high levels of Ets-1 and MMP-9 gene expression that are associated with invasive potential. In this study, we investigated the effect of Ets-1 on the invasive properties of oral cancer from a molecular biological perspective. We constructed an Ets-1 antisense (AS) expression vector, transfected HSC3 cells with the vector, and obtained HSC3AS cells that express Ets-1 AS RNA. The expression of Ets-1 and MMP-9 was analyzed with RT-PCR. The invasive ability of the HSC3AS cells was determined using a matrigel invasion assay and MMP-9 production was measured using gelatin zymography. The amount of Ets-1 mRNA was significantly reduced in HSC3AS cells compared with parental HSC3 cells and the control transfected with empty vector. Matrigel invasion assay revealed that the HSC3AS cells had lower invasive ability. Gelatin zymography demonstrated that HSC3AS MMP-9 productions were decreased compared with those of parental HSC3 cells and the control. These results imply that transfection of AS Ets-1 inhibits oral cancer invasion by down-regulating MMP-9 genes.

Key words: antisense Ets-1, transcription factor, oral cancer, squamous cell carcinoma, MMP-9

Introduction

Squamous cell carcinoma (SCC) is the most frequent malignant tumor in the oral and maxillofacial region, and its metastatic and invasive ability results in a poor prognosis. Recent advances in molecular biology have revealed that these abilities are assisted by multiple proteinases that degrade the extracellular components of stromal and vascular tissues. Matrix metalloproteinases (MMPs) play a particularly important role in tumor progression, and the expression levels of MMPs are correlated with cancer invasion and metastasis. It has been reported that MMP genes are regulated by AP-1 and ets-related transcription factors that bind to promoter regions of MMPs.

The Ets transcription factors are large family implicated in the control of cellular proliferation, differentiation and tumorigenesis, and over-expression of various Ets proteins can lead to the neoplastic transformation of cells in culture and cause tumors in nude mice. Many studies showed that Ets-1 controls the expression of a number of genes involved in extracellular matrix remodeling and might play a role in cell migration and tumor invasion.

In this study, we investigated the direct inhibitory effects of Ets-1 caused by transfection with a vector expressing antisense (AS) Ets-1 mRNA and analyzed AS Ets-1 transfected HSC3 cells, comparing them with the...
highly invasive parental HSC3 cells, especially with regard to MMP9 expression and invasive ability.

Materials and Methods

1. Construction of the AS Ets-1 expression vector

The AS Ets-1 expression vector was constructed as follows. The Ets-1 cDNA was amplified by RT-PCR using total RNA extracted from the HSC3 cells. PCR was performed to generate amplified fragments including the Ets-1 open reading frames. Amplified cDNA was digested with BamHI and cloned into pGEM-4Z. The orientation of the inserts was determined by a restriction map and further confirmed by sequencing. The AS Ets-1 cDNAs corresponding to the open reading frames were subcloned into the BamHI site of pSG5.

2. Cell culture and transfection of the AS Ets-1

The human oral SCC derived cell line HSC3 (JCRB, Osaka, Japan) was used for the transfection of an AS Ets-1 expression vector. It was maintained in Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal bovine serum (FBS). One microgram each of AS Ets-1 expression vector or control pSG5 vector (without the inserted Ets-1 open reading frames) was transfected into the HSC3 with lipofectamine plus reagent® (Life Technologies, NY, USA) according to the manufacturer’s protocol.

After incubation for 24 hours at 37°C, FBS was added and cells were incubated. Experimental groups were divided into 3 groups of AS Ets-1 expression vector, control pSG5 vector and normal HSC3 cells. In each group, 1 × 10⁵ cells were cultured and seeded into 35 mm dishes.

3. RNA preparation and RT-PCR

After 48 hours from transfection, total RNA was isolated using TRIZOL Reagent® (Life Technologies, Inc., NY, USA) according to the manufacturer’s protocol. Ten microliters of reverse transcription buffer (3 mM MgCl₂, 10 mM Tris-HCl, pH 8.3, 75 mM KCl, 1 mM BSA) containing 1 μg of RNA, 0.2 μg oligo-dT primers, 0.5 mM dNTP, 5 U of RNasin, and 100 U of MMLV-RT (Becton Research Laboratories Life Technologies, Inc.) was incubated at 37°C for 60 min, and a portion of each RT product was amplified by PCR.

Ets-1, MMP9 and β-actin primer sequences were as follows: 5’-GATAGCAAGTAGTGATCTGC-3’ and 5’-TCAAGTTAATGGAGTCAACCCA-3’ for Ets-1 mRNA, 5’-TGGCAGAAATAGGCTTTCTCT-3’ and 5’-GGAACACATCGCTGTATTTGTCTCA-3’ for MMP9 mRNA, and 5’-GAAAATCTGGCACCACACCTT-3’ and 5’-TTGAAGGTAGTGGGCATGATCC-3’ for β-actin.

The reactions consisted of 40 cycles of heat denaturation at 94°C for 1 min, annealing the primers to the DNA at 52°C for 2 min, and chain extension with Taq polymerase at 72°C for 1 min, followed by a final extension at 72°C for 20 min. After amplification, the PCR reaction mixture was analyzed by 2% agarose gel electrophoresis and stained with ethidium bromide.

4. Matrigel invasion assay

The assays were performed according to the manufacturer’s instructions. Briefly, cells (1 × 10⁵/well) were seeded in a 6-well BioCoat MATRIGEL Invasion Chamber® (Becton Dickinson, Bedford, MA, USA) in DMEM containing 10% (v/v) heat inactivated fetal calf serum. After 48 hours of incubation, the noninvading cells were removed from the upper surface of the membrane by scrubbing, and the membrane was stained with a Diff-Quick stain kit. Subsequently, invading cells were counted using a microscope and the invasion index (cells migrating through Matrigel coated membrane/ cells migrating through control insert membrane) was...
5. Zymogram

SDS-substrate polyacrylamide gel electrophoresis (SDS-PAGE) was performed with gelatin (0.1%) and 12% polyacrylamide in the gels. After incubation in serum-free medium for 48 hours, conditioned medium from AS Ets-1, control and normal HSC3 cells were subjected to zymography. Electrophoresis was carried out at 60 mA for 2 hours, and then samples were incubated overnight in 0.05 mol/L Tris/HC1 (pH 7.5) buffer containing 10 mol/L CaCl. Gels were stained with Coomassie Blue (0.25%) and destained in methanolacetic acid water (50: 10: 40).

Results

1. Effect of AS Ets-1 RNA expression on Ets-1 mRNA

To determine whether AS Ets-1 reduced the level of Ets-1 mRNA, total RNA from AS Ets-1 transfectants was analyzed by RT-PCR. The amount of Ets-1 mRNA was significantly reduced in the HSC3AS cells compared with those of the parental HSC3 cells and the control (Fig. 2).

2. In vitro invasion activities of AS Ets-1 transfectants

The invasive activity of transfectants was assayed in vitro by a matrigel invasion chamber. Both parental HSC3 cells and control transfectants with the empty vector were invasive. The invasiveness of AS Ets-1 transfectants was lower compared to the parental cells and the control (Fig. 3).

3. Reduction of MMP-9 expression in AS Ets-1 cells

We examined the effects of AS Ets-1 on MMP-9 expression by RT-PCR. The amount of MMP-9 mRNA was significantly reduced in the HSC3AS cells compared with those of the parental HSC3 cells and the control (Fig. 4). After incubation in serum-free medium for 24 hours, conditioned medium from HSC3AS, HSC3 and the control cells were subjected to gelatin zymography. HSC3AS cells showed less 92-kDa type collagenolytic activity than parental HSC3 cells and the control on a zymogram (Fig. 5).
Discussion

Invasion into surrounding tissues and metastasis to distant organs are major characteristics of malignant tumor cells. Various matrix-degrading enzymes are involved in these processes, and MMPs have been shown to play a key role in helping cancer cells to invade through extracellular matrix and form metastatic lesions\(^\text{12}\). It has been reported that there is a close relationship between the level of MMP expression and cancer cell malignancy both in vitro\(^\text{13}\) and in vivo\(^\text{14}\). Especially MMP-1, -3, and -9 are thought to play important roles in cancer invasion and metastasis\(^\text{15-19}\).

The v-ets oncogene was originally discovered as a part of a fusion protein expressed by a transforming retrovirus (avian E26)\(^\text{20,21}\), and was later shown to be transduced from a cellular gene\(^\text{22}\). Approximately 30 related proteins have been found in various species that resemble the v-ets protein in the so-called Ets domain.

The Ets domain has been shown to be a DNA-binding domain that specifically interacts with sequences containing the common core nucleotide GGAA/T\(^\text{23}\). Furthermore, it is involved in protein-protein interactions with co-factors that help determine its biological activity. Many of the Ets-related proteins have been shown to be transcription activators, and it has been revealed that the ets-binding sequence is important for the active transcription of MMP genes\(^\text{4-6}\). The ets-binding sequences, as well as a phorbol-ester-responsive element, are required for the phorbol-ester- and oncogene-stimulated transcription of MMP-1 and MMP-3 (stromelysin) genes\(^\text{4,24}\).

In this study, we used HSC3, an invasive oral SCC-derived cell line, and investigated the inhibitory effects of Ets-1 on invasive HSC3 cells after transfection with an AS Ets-1 expression vector.

The signal intensity of endogenous Ets-1 mRNA in AS cells was significantly weaker than those of parental HSC3 cells and control.

The amount of MMP-9 mRNA was significantly reduced in the HSC3AS cells compared with the parental HSC3 cells and control.

Lane 1: HSC3 AS, Lane 2: control, Lane 3: HSC3.

Fig. 4 RT-PCR expression analyses of MMP-9

Fig. 5 Gelatin zymography

SDS-PAGE was performed with gelatin (0.1%) and 10% polyacrylamide in the gels. After incubation in serum-free medium for 48 hours, conditioned medium from AS Ets-1, control and normal HSC3 cells were subjected to zymography. Electrophoresis was carried out at 60 mA for 2 hours, and then samples were incubated overnight in 0.05 mol/L Tris/HCl (pH 7.5) buffer containing 10 mol/L CaCl. Gels were stained with Coomassie Blue (0.25%) and destained in methanol acetic acid water (50: 10: 40). HSC3AS cells showed less 92-kd type collagenolytic activity than the parental HSC3 cells and control.

Lane 1: HSC3 AS, Lane 2: control, Lane 3: HSC3.
cance of Ets-1 in the progression of oral cancer. Further study on this point is necessary.

Recently, gene-based therapy targeting genetic abnormalities has been attempted for cancer treatment in addition to conventional methods such as surgical excision, chemotherapy, and radiation. A number of oncogene targets have been identified, and investigators have used AS oligonucleotides and AS RNA expression vectors to study the inhibitory functions of targeting sequences. The precise mechanism by which AS RNA functions has not been clarified yet, and there are still some problems in applying it clinically. However, it is possible to target specific genes to reduce expression by AS RNA and interfere with one of the steps in a dramatic down-modulation of the invasive potential of tumor cells. This AS Ets-1 strategy can suppress oral cancer invasion by reducing the expression of several MMP genes, including at least MMP-9. Furthermore, this strategy is more stable than other methods such as antisense oligodeoxynucleotides (ODN), small interfering RNA (siRNA), ribozyme and decoy ODN. We therefore believe that it offers a new avenue for gene therapy development as an adjuvant treatment for human oral cancer.

References
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