Jun Activation Domain-binding Protein 1 Antisense (p27^{Kip1}) Induces Apoptosis of an Oral Tongue Cancer Cell

SUPRIATNO
Department of Oral Medicine, Faculty of Dentistry, Gadjah Mada University, Yogyakarta, Indonesia


ABSTRACT
The Jun activation domain-binding protein 1 (Jab1), aside from being an activator protein 1 coactivator, is involved in degradation of the cyclin-dependent kinase inhibitor p27^{Kip1}, a dosage-dependent tumor suppressor protein. The antisense effect was confirmed in an oral tongue cancer cell line Supri’s clone-1 (Sp-C1). In this study, the antisense-mediated apoptosis of Sp-C1 derived cultures was analyzed. Jab1-antisense (Jab1-AS) treatment induced apoptosis characterized by an increase in the early apoptosis (33.5%) and late apoptosis (17.6%). Also, the proteolitic activation of caspase-3 and caspase-9 was increased. These data suggest that Jab1 AS could induce apoptosis in oral tongue cancer cell (Sp-C1). Targeting this molecule could represent a promising new therapeutic approach for this type of cancer.

Key words: Jab1, apoptosis, antisense, oral tongue cancer (Sp-C1).

INTRODUCTION
Human oral tongue squamous cell carcinoma (OTSCC) accounts for the majority of malignancies that arise in head and neck region. OTSCC is characterized by a high degree of local invasion and a high rate of metastases to the cervical lymph nodes. Moreover, human oral tongue cancer cells frequently shows local recurrence after initial treatment, probably due to microinvasion and/or micrometastasis of the tumor cells at the primary site. Therefore, the ratio of mortality/incidence in 1980 and 1990 was 0.48 and 0.47, respectively, and the prognosis has not changed during the past 10 years.
The human Jab1 was originally identified as a coactivator of the gene-regulatory AP-1 proteins (Jun/Fos proto-oncogenes) involved in the control of cell proliferation. Jab1 is also known as COP9 signalosome subunit 5 (CSN5), which is a component of the COP9 signalosome regulatory complex. Recently, decreased expression of p27Kip1 has been demonstrated that Jab1 can specifically interact with the cyclin-dependent kinase (CDK) inhibitor protein p27Kip1. Transient coexpression of Jab1 with p27Kip1 accelerated the degradation of p27Kip1 in mammalian cells by translating p27Kip1 from the nucleus to the cytosol, in which degradation could occur. p27Kip1 is a universal CDK inhibitor that directly inhibits the enzymatic activity of cyclin-CDK complexes, resulting in cell cycle arrest at G1. Recently, decreased expression of p27Kip1 has been frequently detected in human cancer, including head and neck carcinoma. These studies indicated that p27Kip1 protein level may be associated with the development of human cancers and appear as an important marker of cancer progression. In the latter study, Jab1 overexpression was inversely associated with p27Kip1 levels. However, to the best of our knowledge, the status of Jab1 expression in human oral tongue cancer has not been examined.

In the present study, the mechanism of the observed growth inhibition induced by Jab1 AS was explored. Antisense oligonucleotides into human oral tongue cancer cell lines (Sp-C1) that had exhibited amplification and consequent overexpression of the gene was transfected.

**MATERIALS AND METHODS**

**Cell and Cell Culture**

Oral tongue squamous cell carcinoma cell lines (Sp-C1) was used in this study. Sp-C1 cell line was established in our laboratory. The cell line was grown in Dulbecco’s modified Eagle medium (DMEM, Sigma, St Louis, MO, USA) supplemented with 10% fetal calf serum (FCS, Moregate BioTech, Bulimba, Australia), and 100 μg/ml streptomycin, 100 units/ml penicillin (Invitrogen Corp., Carlsbad, CA, USA). Sp-C1 cell cultures were incubated in humidified atmosphere of 95% air and 5% CO2 at 37°C.

**Antisense Experiments**

Antisense experiments were performed as described previously. Briefly, two oligonucleotides containing phosphorothioate backbones were synthesized (Fasmac, Japan) as follows: AS, 5’-CGGACGGCGCCTGCAGCGAAGT-3’ (the antisense direction of human Jab1 cDNA nucleotides 15 to 24), Sense (S), 5’-CTCTTACCTCAGTACAATTTA-3’ (the sense direction of human Jab1 cDNA nucleotides 15 to 25). The oligonucleotides were transfected into Sp-C1 cells using Tris-EDTA reagent according to the manufacturer’s instructions.

**Flow Cytometry**

Cells were seeded on 100 mm dish (Falcon, USA) at 2x10^5 cells/well in DMEM containing 10% FCS. After 72 hours, the stuck and floating cells were collected in conical tube (Falcon, USA). Then, the cells were incubated with 5μM FITC and propidium iodine (PI) in 500 μl binding buffer (Annexin-V-FITC, Bio Vision Inc, CA). Flow cytometry was analysed by a digital flow cytometry system EPICS (Coulter, Miami, FL, USA).

**Caspase-3 and -9 Activity**

Caspase-3 and -9 activity were measured using the colorimetric assay kit (BioVision, caspase-3/CPP32 and caspase-9/Mch6 colorimetric assay kit, CA, USA) according to the manufacturer’s directions. This test is based on the addition of a caspase-specific peptide conjugated to a color reporter molecule p-nitroanilide. The cleavage of the peptide by caspase releases the chromophore p-NA, which is quantitated spectrophotometrically at 405 nm. Briefly, equal amounts of cell extracts prepared from Sp-C1 cells treated with AS or S were incubated with the substrate (DVED-pNA and LEHD-pNA) in the assay buffer for 2 hours at room temperature. Absorbance was measured at 405 nm using a microplate reader (Bio-Rad Laboratories, Hercules, CA). Each determination was performed in triplicate.

**Statistical Analysis**

Statistical differences between the means for the different groups were evaluated with Stat View 4.5 (version 5.0J, SAS Institute Inc, Cary, NC, USA) using one-way ANOVA and t-test. The significance level was set at 5% for each analysis.

**RESULTS**

**Flow Cytometry Analysis**

To determine whether down regulation of Jab1 protein can induce apoptosis, flow cytometry analysis was performed on each transfectant. Flow cytometry analysis demonstrated that high percentage of early apoptosis was detected in Sp-C1 Jab1 AS (33.5%) compared with that of Sp-C1 Jab1 S (18.8%). Furthermore, percentage of late apoptosis was found in Sp-C1 Jab1 AS (17.6%) compared with of Sp-C1 Jab1 S (17.6%) (Fig. 1).

**Kip 1 Induces Apoptosis by Activation of Caspases**

The activity of caspase-3 and -9 in Sp-C1 cancer cells treated with or without oligonucleotide (AS and S) for 72 h was examined. As seen in Fig. 2, Sp-C1 Jab1 AS showed increased caspase-3 (2.4 fold) and -9 (1.9 fold) proteolytic activity compared with the respective neo-transfectant or
Discussion

The orderly transit of cells through the cell cycle requires a delicate balance between positive and negative regulatory factors. Any alteration in this balance can result in abnormal cell proliferation, which may contribute to cancer. Jab1 was initially described as a coactivator of AP1 transcription factor and is a subunit of a large protein complex (called the COP9 signalosome). Recent study found that an increasing level of Jab1 causes an increasing breakdown of p27Kip1 and indicated that Jab1 controls the activity of p27Kip1 by facilitating its degradation. This finding suggested that Jab1 can act as a negative regulator of important cell cycle control proteins by targeting them for degradation.7

The study of Claret et al. by immunofluorescence analysis indicated that Jab1 is a nuclear protein.4 Through selective interaction with the Jun proteins, Jab1 can increase the specificity of target-gene activation by this large family of related transcription factors. However, to date, the detailed biological functions of Jab1 in mammalian cells have not been identified.

In the present study, an antisense strategy to investigate the effect of Jab1 on growth of head and neck cancer cells that were overexpressing this gene was employed. Transfection of an antisense oligonucleotide
(AS) into cultured Sp-C1 cells induced early and late apoptosis (Fig. 1), followed by increasing proteolytic activity of caspase-3 (2.4 fold) and -9 (1.9 fold) (Fig. 2). These circumstances, together with observations of cell death in the Jab1-antisense-treated cells, prompted us to investigate possible involvement of apoptotic mechanism in the inhibition of cell growth following antisense treatment. An increase in early and late apoptosis per centation and activation of caspase-3 and -9 in AS-treated cells strongly suggested that apoptosis had occurred in those cultures.

Several investigators had already detected a relationship between Jab1 and apoptosis in their experiments. Jab1 -/- embryonic cells, which lacked other CSN components, expressed higher levels of p27, p53 and cyclin E, resulting in impaired proliferation and accelerated apoptosis. Mutant Jab1 down-regulates the UPR signaling pathway through tight binding with IRE1α. These result suggested that Jab1 may act as a key molecule in selecting the UPR or cell death by association and dissociation with IRE1α. However, the actual role of Jab1 in apoptosis remains unclear. In our study found that down regulation of Jab1 led to apoptosis. This result may be compatible with observation that overexpression of p27Kip1 was able to induce apoptosis in several cancer cell lines, because expression of Jab1 is inversely correlated with that of p27Kip1 in oral squamous cell carcinomas.

Since components of apoptotic programs represent promising targets for anticancer therapy, down-regulation of Jab1 by the antisense approach could be a useful apoptosis-modulating strategy for treatment of head and neck cancers. Further studies are needed to clarify the apoptotic mechanism revealed here, and to determine whether Jab1 down-regulation also induces apoptosis in cells derived from tumors of other types.

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