Suppression of a Squamous Cell Carcinoma (SCC)-related Serpin, SCC Antigen, Inhibits Tumor Growth with Increased Intratumor Infiltration of Natural Killer Cells

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Abstract

Squamous cell carcinoma (SCC) antigen (SCCA), a member of the ovalbumin serine protease inhibitor family, serves as a circulating marker of squamous cell carcinoma (SCC). One of the SCCAs, SCCA1, has been suggested to play a role in the attenuation of apoptosis in vitro and in the augmentation of tumor growth in vivo. In the present study, the infection of a SCC cell line (SRG IIa) with recombinant retrovirus that expressed the antisense SCCA mRNA suppressed expression of SCCA in vitro. Local administration of this retrovirus into tumors by inoculation in nude mice suppressed tumor growth. Treatment of tumor tissue in vivo is also associated with increased numbers of apoptotic tumor cells and large mononuclear cells in the tumor. To test the possible role of SCCA in the infiltration of large mononuclear cells, we analyzed the effect of SCCA1 on migration of natural killer (NK) cells induced by monocyte-chemoattractant protein-1 in vitro. SCCA1 suppression of migration of NK cells completely, and this inhibitory effect was lost by mutation of the reactive site loop of SCCA1. These results suggest that antisense SCCA may suppress the growth of SCC in vivo not only by the augmentation of intracellular apoptosis but also by the increased infiltration of NK cells into the tumor.

Introduction

SCCA3 was isolated from human cervical SCC tissue, and serum levels of SCCA have been used as a tumor marker for SCC of various organs (1–3). The serum level of SCCA is correlated with the clinical stage of the disease, and measurement of SCCA after treatment is useful for monitoring the effect of therapy and for predicting the recurrence of disease. Column electrofocusing and two-dimensional electrophoresis have shown that SCCA consists of several fractions (4, 5). cDNA and genomic cloning analyses have revealed that SCCA is encoded by two genes, SCCA1 and SCCA2, and that SCCA1 and SCCA2 are 92% identical and belong to the ov-serpin family (6, 7). Culture medium of SCCA1-transduced cells contains considerable amounts of SCCA1, suggesting that at least some fractions of SCCA1 are secreted actively, although some part may be released by collapsing tumor cells. Therefore, the biological role of secreted SCCA is of particular interest. In vitro analyses indicate that SCCA1 can inhibit the protease activity of chymotrypsin and that SCCA2 can inhibit the activity of cathepsin G and chymase (8, 9). Furthermore, SCCA1 can also inhibit cysteine proteases such as cathepsin L and papain as a cross-class inhibitor (10). Although the target proteases are different, transduction experiments indicate that both SCCA1 and SCCA2 inhibit apoptosis. SCCA1 attenuates apoptosis induced by activated NK cells, tumor necrosis factor α, or anticancer drugs in vitro (11), and SCCA2 attenuates apoptosis induced by irradiation or anticancer drugs in vitro.4 Furthermore, tumor cells that are transduced by SCCA1 grow faster in nude mice than do control cells (11), although the role of SCCA2 in tumor growth has not been analyzed. These data suggest that inhibition of either SCCA1 or SCCA2 production may suppress the growth of SCCA. In this study, we introduced an antisense construct of SCCA into tumors in nude mice to examine the effect on tumor growth.

Materials and Methods

Cells and Reagents. The SKG IIa cell line (11) was a kind gift from Dr. Shiro Nozawa of Keio University (Tokyo, Japan). This cell line produces predominately SCCA1 and a small amount of SCCA2. NIH 3T3 cells were purchased from American Type Culture Collection (Rockville, MD). RNA-PCR kit was purchased from Applied Biosystems (Tokyo, Japan).

Antisense SCCA Construct. An antisense construct of SCCA was made to suppress the expression of SCCA. Total RNA was purified from SKG IIa cells, and the coding region of SCCA2 cDNA was amplified by reverse transcription-PCR with the RNA-PCR kit: sense primer (5'-TCACCATT-GAAATCCTCAG-3') and antisense primer (5'-TCTATGGGGAT-GGAATCTT-3'). The reverse transcription reaction was carried out at 42°C for 30 min before heat inactivation of the enzyme at 99°C for 5 min. PCR was performed with 30 cycles of 94°C, 55°C, and 72°C (each for 1 min). The reverse transcription-PCR product was ligated into a TA-cloning vector (pCRII; Invitrogen, Carlsbad, CA). The ligated product was digested with BamHI and NcoI, and this digested fragment, which begins at the translation initiation codon, was 97% homologous in total region (0.9 kb) and 100% homologous in the first 41 bp to the SCCA1 cDNA. This fragment was then ligated inversely into the Ncol-BamHI site of retroviral vector MFG (11). The product, MFG-SCC-AS, was digested with BamHI and ligated with a 1.4-kb BamHI fragment of MFG-SCC-A1 (11), which included the internal ribosomal entry site and Neo gene. The final product, MFG-SCC-AS-Neo, was used for transfection of the retrovirus-packaging cell line CRIP (11), and the transfected cells were selected in 750 µg/ml G418 (Geneticin; Life Technologies Inc., Gaithersburg, MD). The control vector, DFG-LacZ-Neo, which was derived from the MFG vector, was used to transfect CRIP cells and to make a control cell line (CRIP-LacZ; Ref. 12). Virus-containing medium from selected clones

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3 The abbreviations used are: SCCA, squamous cell carcinoma antigen; ov-serpin, ovalbumin serine protease inhibitor; NK cells, natural killer cells; IL-2, interleukin 2; GST, glutathione S-transferase; MCP-1, monocyte-chemoattractant protein-1; RSL, reactive site loop.


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(CRIP-AS7 and CRIP-AS14) was used for experiments. Medium from CRIP-LacZ was used as a control.

**In Vitro Suppression of SCCA by Antisense Construct.** SKG IIIa cells (10^6) were seeded on 100-mm tissue culture dishes (Becton Dickinson, Franklin Lakes, NJ) 1 day before infection. The medium was removed, and cells were rinsed once with PBS. Cells were incubated with each viral supernatant for 3 h with 8 μg/ml of polybrene. The viral supernatant was changed to conditioned medium, and the cells were incubated at 37°C for 48 h. Cells were trypsinized and sonicated. After centrifugation at 15,000 rpm for 5 min, the supernatant was analyzed for SCCA concentrations with an automated assay kit (Imx; Dainabot Co., Ltd., Tokyo, Japan) as described previously (11). The IMx system measures the total SCCA level, which includes SCCA1 and SCCA2.

**In Vivo Treatment of Tumors with Antisense Construct.** SKG IIIa cells (5 × 10^6) were inoculated s.c. into male BALB/c nude mice 8 weeks of age, and from day 2 after inoculation, 0.1 ml of viral supernatant was injected into the inoculation site every day (n = 4 in each group). Tumor size was measured with calipers, and tumor volume was calculated according to the formula V = 0.52 × a^2 × b (with a being the smallest superficial diameter and b the largest superficial diameter). Two weeks after inoculation, the tumors were removed, embedded in OCT compound, sectioned, and stained with H&E to observe the infiltration of large mononuclear cells. To confirm the effect of antisense of SCCA on the infiltration of large mononuclear cells into the tumor, nude mice were inoculated s.c. with 5 × 10^6 antisense-transfected cells (SKG IIIa-AS-1) and mock cells (SKG IIIa-CEP; Ref. 11). Tumor sections from these mice were also stained with polyclonal antibody against SCCA (ATR15; Ref. 13) or monoclonal antibody against mouse macrophages (Mac-3; PharMingen, San Diego, CA). The study was approved by the Committee for the Ethics on Animal Experiment of Yamaguchi University under the Law (No. 105) and Notification (No. 6) of the Government of Japan.

**Detection of Apoptotic Cells.** Apoptotic cells in the tumor were detected with the In Situ Cell Death Detection Kit (Boehringer Mannheim, Mannheim, Germany) following the manufacturer’s protocol.

**Chemotaxis Assay.** With the approval of the Institutional Review Board of Yamaguchi University Hospital, we purified NK cells from peripheral blood mononuclear cells as CD3^+ CD56^- lymphocytes from healthy donors who gave informed consent (14). The purity of purified NK cells was >95% as assessed by flow cytometry analysis. SCCA1-transfected cells (K562-SCC) and control cells (K562-NEO) were prepared as described previously (11). NK cell migration was analyzed in vitro with a modified Boyden chamber. Purified NK cells (2 × 10^5) were suspended in 200 μl of RPMI 1640 containing 1% FCS and placed in the chemotaxel chamber (upper chamber) with a 3-μm-pore size membrane (Kurarbo, Osaka, Japan). This chamber was submerged in a 24-well plate (lower chamber) containing 600 μl of supernatant from K562-SCC or control cells (K562-NEO). The supernatant used in the lower chamber was diluted serially with RPMI 1640 containing 10% FCS, IL-2 (Chiron, Emeryville, CA; final concentration, 500 units/ml) and human MCP-1 (Pepro Tech, London, England; final concentration, 50 ng/ml). The chambers were incubated for 3 h at 37°C in a 5% CO2 incubator. RPMI 1640 containing 10% FCS, IL-2, and human MCP-1 in a 24-well plate was used as a positive control, and RPMI 1640 containing 10% FCS alone was used as a negative control. Mutant forms of SCCA1 were prepared by creating individual amino acid substitutions in the RSL as a GST fusion protein (AlaP14Arg: GST-SCCA1-A341R; PheP3Ala: GST-SCCA1-F352A; Ref. 15). These mutants were added to RPMI 1640 containing 1% FCS, IL-2, and human MCP-1 and placed in the lower chamber. The negative and positive controls were the same as those described above, except that 1% FCS was used instead of 10% FCS. NK cells that infiltrated into the membrane were stained with Giemsa and counted in 10 high-magnification fields in duplicate.

**Statistical Analyses.** Data were examined by Student’s t-test or Duncan’s new multiple range test. Differences were considered to be significant at P < 0.05.

**Results**

**In Vitro Suppression of SCCA by Antisense Construct.** Because SCCA1 and SCCA2 are highly homologous at the nucleotide level (95%), it is difficult to suppress expression of each SCCA independently. To suppress the expression of both SCCAs, we ligated the 5’-region of the SCCA2 cDNA (97% homologous to that of SCCA1 cDNA) into a retroviral vector in the antisense direction. Retrovirus-producing cells that carry the antisense construct (CRIP-AS7 and CRIP-AS14) were analyzed for viral titer. The titer of each clone and the negative control cells (CRIP-LacZ) were approximately 5 × 10^5 CFU/ml. Supernatants of the culture medium of these retrovirus-producing cells were used to infect the SCCA-producing cell line SKG IIIa, and the expression of SCCA protein was analyzed in vitro. The culture medium of CRIP-LacZ cells did not affect levels of SCCA, but those of CRIP-AS7 and CRIP-AS14 reduced SCCA levels significantly (Fig. 1).

**Suppression of Tumor Growth by Transduction of Antisense SCCA.** Because the antisense construct of SCCA inhibited the expression of SCCA in vitro, we analyzed the effect of this construct on tumor growth in nude mice. Nude mice were inoculated s.c. with SKG IIIa cells, and, starting on the following day, the supernatant of
retrovirus-producing cells was injected every day for 2 weeks into the inoculation site. After 2 weeks, the tumor size was significantly smaller in the CRIP-AS7 and CRIP-AS14 groups than in the CRIP-LacZ and medium-alone groups (Fig. 2). Immunohistochemical studies with a polyclonal antibody against SCCA revealed that tumors in CRIP-AS7 and CRIP-AS14 groups showed weaker staining of SCCA compared with that of the CRIP-LacZ group (Fig. 3A).

Effect of Antisense SCCA on Infiltration of Large Mononuclear Cells into Tumor. In the tumors, the numbers of apoptotic cells (data not shown) and large mononuclear cells were increased significantly in the antisense-infected group (CRIP-AS7) compared with the control group (CRIP-LacZ; Fig. 3B, a and b). To confirm these data, we used a stable transfecant of antisense SCCA. When SKG IIIa cells were transfected stably with the antisense construct before inoculation into nude mice, the cells showed reduced levels of SCCA, and the tumors from these cells showed increased numbers of large mononuclear cells (Fig. 3B, c and d).

Inhibition of NK Cell Migration by SCCA1. Although we could not determine the origin of the large mononuclear cells in the tumors, they appeared morphologically to be NK cells. Immunohistochemistry with an antibody against macrophages indicated that macrophages were present only around the tumor (data not shown). We hypothesized that SCCA would inhibit infiltration of NK cells. Then we analyzed the effect of SCCA on chemotaxis of NK cells. Using MCP-1 as the chemoattractant and IL-2 as the activator of NK cells, we monitored the infiltration of NK cells with a modified Boyden chamber. When SCCA1 from K562-SCC cells was added to the medium in the lower chamber, infiltration of NK cells was inhibited significantly in a dose-dependent manner (Fig. 4A). Furthermore, a GST fusion protein of SCCA1 (GST-SCCA1) inhibited chemotaxis of NK cells completely (Fig. 4B), and this was blocked when a mutation was introduced into the hinge region or variable region of the RSL of SCCA1. These mutants also lost the ability to inhibit proteinase (15). Because the antisense construct of SCCA inhibited expression of both SCCA1 and SCCA2, we also analyzed the effect of SCCA2 on the inhibition of NK cell migration. However, SCCA2 (GST-SCCA2) had little effect on NK cell migration (Fig. 4B).

Discussion

The escape of cancer cells from apoptosis contributes to tumor progression. Physiological inhibitors of apoptosis, including members...

Fig. 3. Increase in the number of tumor-infiltrating mononuclear cells by suppression of SCCA. A, immunostaining of tumor with polyclonal antibody that recognizes both SCCA1 and SCCA2. Tumor infected with viral supernatant of CRIP-LacZ cells (a), CRIP-AS7 cells (b), and negative control (c) are shown (original magnification, ×100). B, suppression of SCCA increases the number of mononuclear cells in the tumor. H&E staining of tumor infected with viral supernatant of CRIP-AS7 cells (a) and CRIP-LacZ cells (b). H&E staining of tumor derived from antisense-transfected culture cells expressing a reduced level of SCCA (SKG IIIa-AS-1, c) and mock cells (SKG IIIa-CEP4, d). Arrowsheads indicate large mononuclear cells (original magnification, ×400).
of the Bcl-2 family, IAPs and ov-serpins, contribute to the regulation of apoptosis. Our previous data indicated that SCCA1 (11) and SCCA2 inhibited apoptosis induced by several kinds of stimuli, and that SCCA1 promoted the growth of tumor in vivo. These prior data suggest that inhibition of expression of either SCCA1 or SCCA2 production may suppress growth of SCC. The present study indicates that in vivo retroviral infection of tumor cells with the antisense construct suppresses expression of SCCA, and that tumor growth is inhibited significantly with increased numbers of apoptotic cells.

In this study, we used an SCCA2 antisense construct to suppress both SCCAs because SCCA1 and SCCA2 are highly homologous at the nucleotide level, and because it is difficult to suppress expression of each SCCA independently. As both SCCAs have inhibitory activity against apoptosis in vitro, we thought that the inhibition of either might cause the same antitumor effect as a result of the suppression of apoptosis. Furthermore, expression of SCCA2 mRNA, not SCCA1 mRNA, is increased in cancer tissue (16), and we therefore considered that it was preferable to use antisense SCCA2 to suppress expression of both proteins. However two-dimensional electrophoresis with subsequent Western blotting indicated that the spot of SCCA2 was detectable neither in the tumor tissue sample with antisense treatment nor in the sample without antisense treatment, although the spot of SCCA1 was diminished by the treatment with antisense construct (data not shown). Therefore, inhibitory effect of antisense construct may be attributable to suppression of SCCA1, although the function of SCCA2 to tumor growth remains unclear.

The present results also indicate that the number of infiltrating large mononuclear cells was increased by suppression of SCCA. This data were confirmed with the use of a stable transfectant of the antisense SCCA construct. Furthermore, when tumor cells were transduced with SCCA1 cDNA, the number of large mononuclear cells in the tumor decreased (data not shown). Therefore, infiltration of immune cells into the tumor site may be blocked by extracellular SCCA1. Because chemotaxis of NK cells can be inhibited by SCCA1 in a dose-dependent manner, SCCA1 probably has an inhibitory function against migration of NK cells. This hypothesis agrees with the in vitro result that, among various apoptotic stimuli, the stimulus from activated NK cells was most effectively suppressed by SCCA1 (11). Because there is a possibility that the antisense construct also suppressed SCCA2, the increase of large mononuclear cells into the tumor may be attributable to the suppression of SCCA2. However, in the chemotaxis assay using MCP-1 as a chemoattractant, the inhibition of chemotaxis by SCCA2 was not as dramatic as that by SCCA1. This result does not exclude the contribution of SCCA2 to NK cell migration; SCCA2 may inhibit chemotaxis induced by a different chemoattractant.

Although some members of the ov-serpin family have been reported to inhibit apoptosis (17–19), to the best of our knowledge, this is the first report that an ov-serpin may regulate migration of immune cells to the tumor site. This observation is clinically important because elevated serum levels of SCCA may indicate attenuated immune function, which could contribute to the escape of SCC from host control. Indeed, in stages Ib and Ila cervical cancers, an elevated serum level of SCCA before treatment is a risk factor for poor prognosis, independent of tumor size or lymph node metastasis (20). The mechanism of the antichemotactic effect of secreted SCCA1 is still unclear. Migration of NK cells may be blocked by the binding of SCCA1 to NK cells, but we did not observe direct binding of isotope-labeled SCCA1 to NK cells (data not shown). Because antichemotactic activity was lost with mutation of the hinge region (GST-SCCA1-A341R) or variable region (GST-SCCA1-F352A) of the RSL, the RSL appears to be important for the antichemotactic effect. Because the RSL is the region where serpins react with target proteases, secreted SCCA1 may inactivate the protease that is involved in the migration of NK cells.

In summary, the present results suggest that secreted SCCA1 inhibits the infiltration of NK cells into tumor tissue. This function, together with the inhibitory role against apoptosis, may contribute to the escape of squamous cell carcinoma from the host immune system. Therefore, the antisense construct of SCCA may be a useful therapeutic tool for the treatment of SCC.
References


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