Potential role of SC1, a cell adhesion molecule, in mammary gland tumors

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Abstract. SC1, an immunoglobulin superfamily cell adhesion molecule, is expressed in embryonic tissues and plays an important role in development through its cell adhesive activity. SC1 is also found in a variety of tumors and its expression is associated with a poor prognosis. The expressional patterns of SC1 were examined in sporadic cases of canine mammary gland tumors and it was found that this molecule is enriched in adenocarcinomas and is weaker in benign mixed tumors. SC1 might therefore be involved in the malignancy and progression of canine mammary gland tumors. To confirm this paradigm, the mammary gland cell line JYG-B was used as the recipient of SC1 cDNA. The resulting SC1-transfected cells were subsequently analyzed using a convenient *in vitro* model system. The self-aggregation activity of SC1-transfected cells was significantly increased and was blocked by an anti-SC1 antibody generated by hyper-immunized ostrich yolk. In addition, cell locomotion assays revealed an enhanced migration activity of SC1-transfected cells on SC1-coated transwell chambers. The *in vivo* activities of the cells were examined by subcutaneous implantation into nude mice. Tumor growth was significantly promoted in the mice after implantation with SC1-transfected cells, in comparison to parental- and mock-transfectants. This growth was inhibited by oral administration of gold-ion water. The invasion of SC1-transfectants into the surrounding muscular and adipose tissues was rigorously enhanced. These findings suggest that SC1 might promote the progression of mammary gland tumor cells by increasing cell adhesion.

Introduction

Cell adhesion is an essential property in multicellular organisms for the construction of tissues and organs, as well as for the establishment and maintenance of cell-to-cell interactions (1,2). Cell adhesion molecules (CAMs) play a major role in a variety of developmental phenomena, including cell growth, death, migration, differentiation, polarization, neurite extension and epithelialization, through their cell-to-cell and/or cell-to-extracellular matrix (ECM) interactions. In addition, cell-to-cell binding has been shown to play a critical role in tumor progression, including cell dissociation from primary sites, invasion into surrounding tissues, intravasation, cell embolism onto the vascular endothelium and extravasation.

SC1 is an immunoglobulin superfamily CAM that can be purified from chick spinal cord tissue as a neurite promotion factor (3). This molecule is identical to BEN and DM-GRASP, which were isolated separately by several research groups (4,5). SC1 is distinguished by an extracellular domain consisting of two V-type motifs following three C-2 type II Ig-like loops. It has a homophilic adhesive activity (SC1-to-SC1) and also binds to Ng-CAM and CD6 in a heterophilic manner (3,6,7). These adhesive properties of the SC1 protein are regulated by Ig-like loops modified by N-glycosylation in the extracellular domains. SC1 is transiently expressed during avian embryogenesis by a variety of cell types, and its expression is developmentally regulated in several cell types of the nervous and hematopoietic systems, as well as in certain epithelial cells (4,7,8).

A recent study demonstrated the potential role of SC1 in tumorigenesis by showing that SC1 is over-expressed in sporadic chicken nephroblastomas and promotes the aggregation of cancer cells (7). SC1 appears to be a potential effector of tumor progression; however, its functional role in tumor cells *in vivo* is unclear. To confirm this paradigm, a model of tumor progression was developed by transfecting the SC1 gene into SC1-negative cells, and subsequently analyzing their tumorigenic, invasive and metastatic abilities in animals.

Over 90% of human malignant neoplasia is of epithelial origin. Mammary gland carcinomas are the major tumors, and are most often found in women. This cancer is often malignant and frequently metastasizes to other organs via the blood or lymph vessels. Mammary gland tumors are also the most commonly-occurring neoplasm in female animals, including experimental rodents and dogs. Consequently, it is quite important to determine the invasive and metastatic mechanisms of mammary gland tumors in order to prevent their malignant progression. Canine mammary gland tumors have

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various heterogeneous components, including tubular and acinar structures, as well as connective tissue and myoepithelial proliferations that facilitate metastasis into cartilage, bone, and myxomatous tissues. Such complexities cause considerable difficulties in the attempt to determine their malignancy; therefore, it would be beneficial to discover a tracer for the distinct diagnosis of the level malignancy, including tumor growth, invasive qualities and metastatic aspects (9,10). Previously, the expression of SC1 has been investigated in canine mammary gland tumors. SC1 is present on the neoplastic tubular epithelia of a subset of adenocarcinomas, but is totally negative in benign tumors. These findings indicate that SC1 might be involved in the malignancy and progression of mammary gland tumors in dogs.

The present study is an investigation of the functional roles of SC1 in the malignant phenotypes of mammary gland tumor cells. The results are based on observations of the behavior of SC1-transfected JYG-B cells - a transplantable cell line derived from a mammary gland tumor - following *in vitro* analyses and implantation into nude mice.

Materials and methods

Samples of canine mammary gland tumors. Sporadic canine mammary gland tumors were provided by animal hospitals in Osaka Prefecture, Japan, and frozen for Western blot analysis or fixed in Zamboni's solution for immunohistochemistry. The specimens diagnosed as adenocarcinomas (4 cases) and benign mixed tumors (7 cases) were then used for further study.

Generation of antibodies against SC1-protein. An experimental rabbit (Japanese-White) was immunized subcutaneously in the lumbar region at multiple sites with SC1 chimeric protein, including the extracellular domain of human SC1 combined with the Fc fraction of rabbit IgG (50 μg/body) emulsified in Freund's complete adjuvant (FCA). The boosters were administered every other week with same antigen in Freund's incomplete adjuvant (FIA). At the hyper-immunized stage, confirmed with an ELISA, the serum was collected and the IgG was purified with protein G as a routine procedure.

In addition, an ostrich was used to obtain anti-SC1 antibodies. Female birds were immunized subcutaneously in the lumbar region at multiple sites with SC1 chimeric protein (50 μg/body) emulsified in FCA. The boosters were given on every other week with same antigen in FIA. The yolk was separated from the albumin of eggs from hyper-immunized birds and diluted to 6-fold with 0.01 M citrate buffer (pH 5.5). The supernatant containing the IgY was collected by centrifugation (10,000 x g at 4˚C for 15 min) and precipitated with 60% saturated ammonium sulfate. The solution was centrifuged again at 10,000 x g at 4˚C for 15 min. The precipitate was then re-dissolved in 0.05 M phosphate buffer (pH 8.0) and dialyzed against water.

Establishment of SC1-expressing JYG-B cells by gene induction. The JYG-B cells were cultured in dishes with Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS) at 37˚C. The cells at semi-confluent stages were transfected with pcDNA 3.1 plasmid vector (Invitrogen) with an intact SC1 gene. As a mock transfection, cells were transfected with an empty plasmid lacking SC1 cDNA. These transfected cells were selected in DMEM containing 10 μg of G418 (Gibco). G418 resistant colonies were picked up and 5 clones were established. SC1 expression was examined in each clone by immunocytochemistry and Western blot analysis. A stable clone with the strongest SC1 expression was used for further experiments.

In vitro cell-aggregation assay. Monolayer cultures of SC1-transfected cells and mock-transfected cells were digested with 0.05% trypsin and 0.53 M EDTA, and collected by centrifugation at 800 x g. They were then re-suspended in DMEM at $3x10^6$ cells/ml. Anti-SC1 rabbit polyclonal IgG generated by hyper-immunized rabbit serum or anti-SC1 immunoglobulin yolk (IgY) from hyper-immunized ostrich eggs were added at a final concentration of 20 ng/ml before starting the aggregation assay (11). To quantify the aggregation, a 1-ml aliquot of cell suspension was transferred to a 50-ml polypropylene tube coated with FCS. The assay was carried out by incubating the cell suspension at 37˚C without agitation. A small aliquot (10 μl) was withdrawn at 60 min, and the total particle number was counted by a hemocytometer. The degree of cell aggregation was indicated by the index N_{60}/N_0, where N_0 is the total cell number at the initial incubation time (*0 min*) and N_{60} is the total cell number after 60 min of incubation time (12). The experiment was performed four times, the scores were averaged, and the standard deviations were calculated.

In vitro cell migration assay. Polyvinylpyrrolidone-free polycarbonate filters (5-μm pore size) were coated with 1 μg SC1 protein and placed in modified Boyden-chambers (Gibco). A 100-μl aliquot of mock-transfected or SC1-transfected cells ($3x10^6$ cells/ml) was placed in the upper compartment of the Boyden-chambers. DMEM containing 20 ng/ml of pre-immune rabbit IgG or anti-SC1 IgG, pre-immune ostrich IgY or anti-SC1 IgY was placed in the lower compartment. After incubation for 18 h at 37˚C, the cells that had migrated into the lower compartment were counted under a microscope. All experiments were performed five times, the scores were averaged, and standard deviations were calculated.

Implantation of SC1-transfected cells into nude mice. Parental, mock- and SC1-transfected cells were suspended at $2.8x10^6$ cells in 1 ml DMEM and injected subcutaneously into the cervical back of female nude mice (6 mice for each cell). Commercial water containing 5-ppm gold-ion was given *ad libitum* to confirm the effect of SC1-transfectants on tumor growth by an anti-tumor material. Tumor volume was monitored at 1 and 2 weeks after implantation. The volume was determined by the following formula: tumor volume = length x width x thickness in their greatest dimensions. At 2 weeks, the mice were sacrificed under deep anesthesia with pentobarbital solution. The subcutaneous tumors, lungs and liver were fixed with 10% buffered formalin solution and then examined histopathologically to elucidate the invasive and metastatic activities of the cells.

Protein extraction and Western blotting. The mock- and SC1-transfectants and tumor tissue specimens were homogenized in PBS and centrifuged at 5000 x g for 15 min. The pellet was

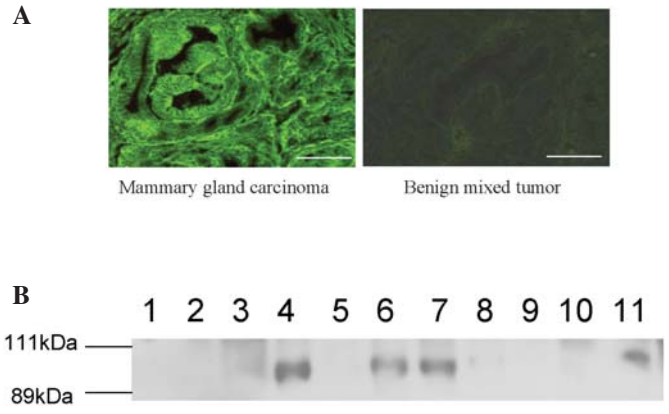

Figure 1. Expression of SC1 in sporadic cases of canine mammary gland tumors. (A) Immunohistochemistry for SC1 in canine mammary gland tumors. Strong immunoreactivity for SC1 is found in the neoplastic epithelial cells of adenocarcinoma, but not in benign mixed tumor. Bar, 50 μm. (B) Western blot analysis of SC1 expression in canine mammary gland tumors. Each lane is loaded with 20 μg membrane fractions from adenocarcinomas (lanes 4, 6, 7 and 11) and benign mixed tumors (lanes 1, 2, 3, 5, 8, 9 and 10). Found are strong positive signals in all lanes of adenocarcinoma, but only slight signals in benign mixed tumors.

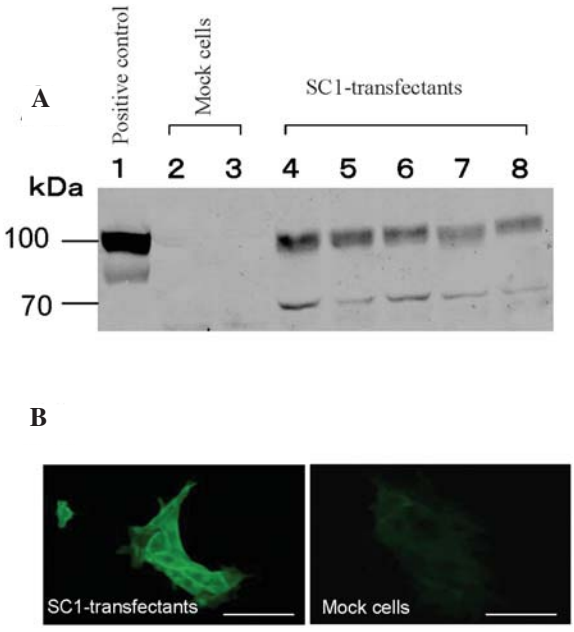

Figure 2. Expression of SC1 protein in the SC1 transfectants. (A) Western blot analysis of SC1 protein in the membrane fractions from lung cancer A549 cells as a positive control (lane 1), mock cells (lanes 2 and 3) and 5 SC1-transfectants (lanes 4-8). SC1 is expressed in all SC1-transfectants, but not in mock cells. (B) Immunohistochemistry for SC1 in mock cells (right) and SC1-transfectants (left). SC1 is expressed on the cell surface of SC1-transfectants, but not in mock cells. Bars, 200 μm.

solubilized by incubation in 10 mM Tris/acetate (pH 8.0), 1 mM EDTA, 0.5% NP-40 at room temperature for 1.5 h on a rotating shaker, and then centrifuged at 5000 g for 30 min. The resulting supernatants were used as membrane fractions for Western blot analysis. An equal volume of membrane fractions was separated on a 7.5% SDS-PAGE and electro-transferred to polyvinyl difluoride membrane (Bio-Rad). The blots were blocked with 2% skim milk in phosphate-buffered saline (PBS) containing 0.05% Tween-20 (PBST) for 1 h at 37˚C and then incubated with a primary antibody against SC1 diluted in PBST with 2% skim milk for 1 h at 37˚C. After being washed with PBST three times, they were incubated with the secondary antibody in PBST with 2% skim milk for 1 h at 37˚C. The samples were washed three times in PBST and then in PBS, and the blots were visualized on X-ray film with ECL solution (Gibco) (11).

Immunocytochemistry and immunohistochemistry. The transfectants grown on coverslips were fixed with Zamboni's fixative solution [0.21% picric acid, 2% paraformaldehyde, and 130 ml phosphate buffer (pH 7.4)]. The canine tumor tissue samples were also fixed with Zamboni's solution and equalized with 20% sucrose solution, and cryosections were cut. The cells and sections were washed twice with PBS and incubated with anti-SC1 polyclonal IgG for 1 h at 37˚C (13). After being washed twice with PBS, they were incubated with fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulins (Dako) for 1 h at 37˚C. They were then washed with PBS and examined under fluorescent microscopy.

Results

Expression of SC1 in sporadic cases of canine mammary gland tumors. Immunohistochemistry showed the SC1 proteins to be highly expressed in the neoplastic epithelial cells in all adenocarcinomas, but only slightly expressed in benign mixed tumors (Fig. 1A). A Western blot analysis using membrane fractions from tumor specimens showed that strong expression of SC1 proteins, a single band around 100 kDa, was seen in all cases of adenocarcinomas, while the level in all benign mixed tumors was lower (Fig. 1B). These findings show the expression of SC1 to be enhanced in adenocarcinomas.

Establishment of stable SC1-transfectants by SC1 gene induction. Five neomycin-resistant stable clones as SC1-transfectants and two clones as mock cells were identified and confirmed by Western blot analysis (Fig. 2A). An immunocytochemical analysis showed that SC1 protein was expressed on the membrane of all SC1 transfectants, but not in any of the mock cells (Fig. 2B).

Cell aggregation and locomotion activities of SC1-transfected cells. Mock- and SC1-transfected cells were incubated in DMEM, and the numbers of cell clusters were counted. Fig. 3 shows the results of the cell-aggregation assay. The SC1-transfectants displayed enhanced self-aggregation activity, while the mock cells aggregated at a lower level. Notably, only anti-SC1 IgY generated by ostrich yolk could inhibit the enhanced cell aggregation of SC1-transfectants.

In the *in vitro* cell migration assay, a higher numbers of SC1-transfectants than mock cells migrated through the pores of the filters coated with SC1 protein (Fig. 4A). The cell migration activity of SC1-transfectants was partially inhibited by the anti-SC1 IgY (Fig. 4B).

Activities of SC1-transfected cells in nude mice. No difference was observed in the doubling times for cell growth between the mock- and SC1-transfectants and parental cells under the same culture conditions. The tumor volume was monitored at

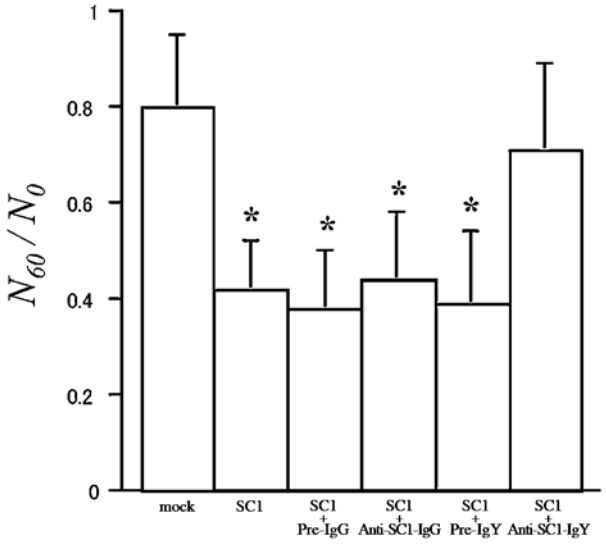

Figure 3. Self-aggregation activity of SC1-transfectants. An aliquot of mock- and SC1-transfectants was incubated at 37˚C, and the number of aggregates was counted in a hemocytometer after 60 min. The experiments were conducted in the presence of a pre-immune IgG (pre IgG), anti-SC1 IgG, pre-immune IgY (pre IgY) or anti-SC1 IgY. The degree of cell aggregation was estimated by the index N_{60}/N_0 (see Materials and methods). Note that the lower index in SC1 transfectants (SC1) shows higher cell aggregation activity than mock cells (mock). Only SC1-IgY inhibits the enhanced aggregation activity of SC1 transfectants. Each result represents an average ± SD of four independent experiments. *P<0.05, in comparison to data from mock cells.

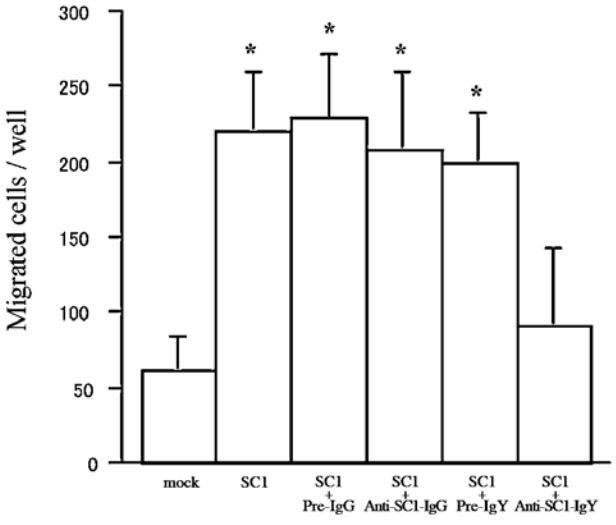

Figure 4. Migration activity of SC1 transfectants. Mock- or SC1-transfectants were cultured in the upper compartment of the transwell (Boyden) chambers. After incubation for 18 h, the cells which had migrated onto the lower compartment were counted under a microscope. Experiments were conducted in the presence of a pre-immune IgG (pre IgG), anti-SC1 IgG, pre-immune IgY (pre IgY) or anti-SC1 IgY. Data were expressed as the average number ± SD of cells on the lower surface of each of the five experiments. Note that the migration activity is enhanced in SC1 transfectants (SC1), unlike in mock cells (mock). Only SC1-IgY inhibits the enhanced aggregation activity of SC1 transfectants. *P<0.05 in comparison to the data from mock cells.

1 and 2 weeks after the subcutaneous injection of cells in nude mice. Fig. 5 shows that SC1-transfected cells formed larger tumors than the other cells at 2 weeks. Tumor growth of SC1-cells was partially inhibited by feeding mice water containing gold ions. Macroscopically, the subcutaneous tumors derived

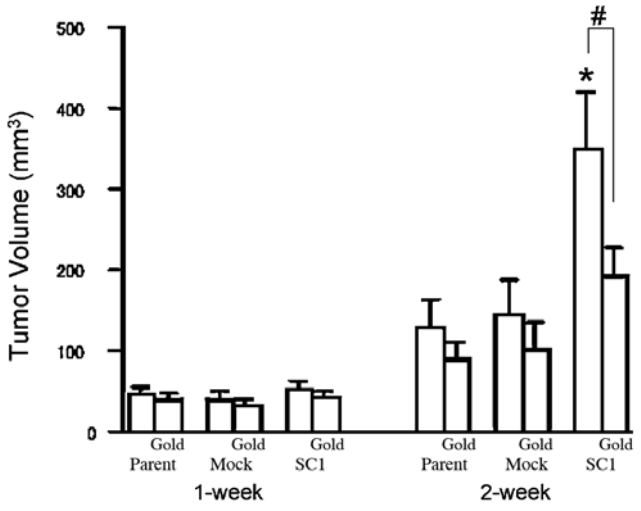

Figure 5. The tumor growth of SC1 transfectants after implantation into nude mice. Tumor volume was monitored 1-2 weeks after a subcutaneous injection of parental-, mock- and SC1-cells into nude mice. These mice were given 5-ppm gold ion containing water *ad libitum* from the start of implantation. Data is expressed as the average volume ± SD of six mice. Note that SC1-transfectants form the larger tumors in mice at 2 weeks, in comparison to parental and mock cells. Gold ion water inhibits the growth of tumors by SC1-transfectants at 2 weeks. *P<0.01, in comparison to data from the tumors of parental cells at 2 weeks. #P<0.05, in comparison to data from the tumors of SC1-cells at 2 weeks without gold ion water.

from the mock and parental cells were well-demarcated by their fibrous capsule and displayed no invasive growth. In contrast, some tumors formed by the SC1-transfected cells deeply invaded the underlying muscular tissue and could not be easily separated from the body. Histopathologically, the invasive behavior varied between the mock- and SC1-transfected cells (Fig. 6). Mock cells proliferated within the subcutaneous tissues, but had scarcely invaded into the surrounding tissue at 2 weeks post-implantation. However, the SC1-transfectants exhibited more infiltrative growth patterns in the subcutaneous tissues. These cells invaded the adipose tissue and dermis of the skin, and also destroyed the adjacent skeletal muscle (Fig. 6). Both the mock- and SCI-transfected cells showed no metastasis to the lung and liver from the cranial subcutaneous site.

Discussion

Cell adhesion molecules (CAMs) play an important role in the establishment and maintenance of tissue structure and functional integrity (1,14-16). CAMs are also of prime importance in histopathology, as well as in normal histology. Adhesive and non-adhesive interactions between tumor cells and neighboring components, such as the ECM-network and endothelium, determine the degree of invasion and metastasis. In a variety of tumors, the dysregulation and dysfunction of CAMs frequently occurs in association with tumor progression. For example, the loss of expression or the dysfunction of E-cadherin in epithelial cells can contribute to cell transformation (17). A putative CAM of the Ig-superfamily is often lost during the progression of a subset of colorectal tumors. The loss of these CAMs on the cell surface may facilitate the migration and escape of tumor cells from the primary lesion, thus resulting in invasion and

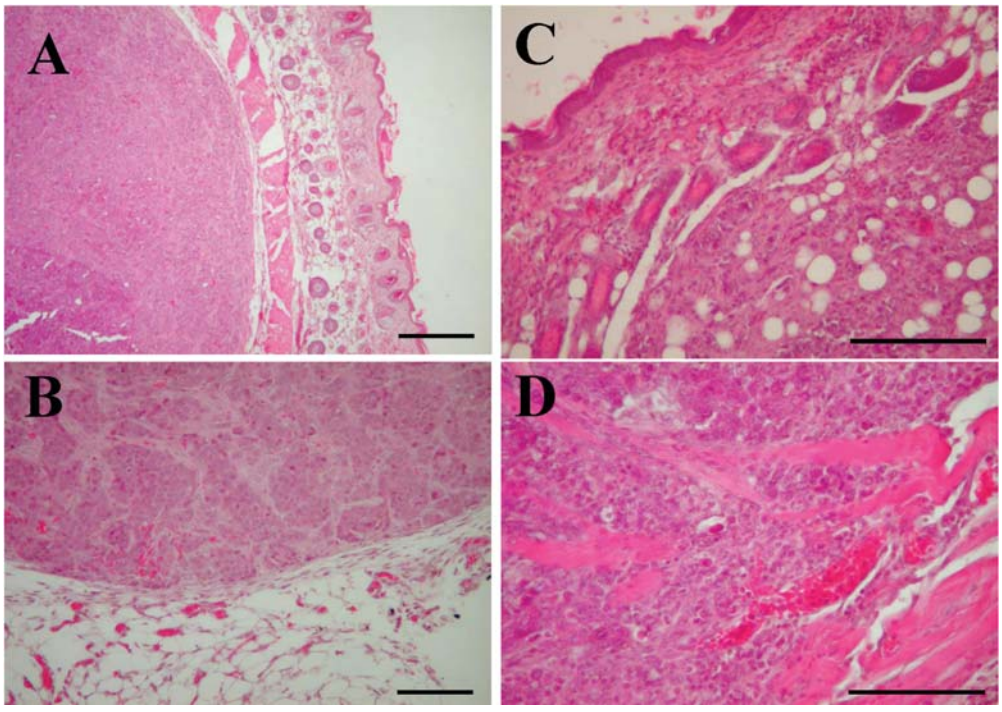

Figure 6. Histopathology of subcutaneous tumors from nude mice implanted with SC1 transfectants. Subcutaneous tumors of mock-transfectants are composed of adenocarcinoma cells, but scarcely invaded into adjacent tissues (A and B). In contrast, SC1-transfectants exhibited more infiltrative growth patterns: cells are invading into dermis of the skin and adipose tissues (C), and also destroyed the adjacent skeletal muscles (D). Bars, 100 μm.

disseminating metastasis. However, CAMs are over-expressed in a variety of tumors and may contribute to tumor cell invasion and metastasis through their adhesive activities (18).

In the present study, SC1-expressing JYG-B cells were created by transfecting SC1 cDNA, and the resulting cell activity was observed. Cell aggregation assays revealed that the homophilic adhesive activity of SC1 was a dynamic force in tumor cell binding. In addition, cell migration assays showed the enhanced locomotion activity of JYG-B cells due to SC1 expression. It was notable that only ostrich IgY against SC1 protein inhibited the self-aggregation and migration of SC1 transfectants. These observations led to the speculation that the epitopes, which were masked by only ostrich IgY, are essential to the cell activities of SC1-expressing cells. Since the amino acid sequences are highly homologous among mammals (19), the antibodies that recognize the essential regions on SC1 protein for cell activities are generated by ostrich (avian species), not by rabbit (mammal). The avian antibody might be useful for the functional analysis of cell membrane proteins with strong homology among mammals.

In this study, the expression patterns of other ligands of the SC1 protein were not examined in JYG-B cells. Therefore, further research is required to elucidate whether the clustering of tumor cells occurs through homophilic adhesion (SC1-SC1) or heterophilic adhesion to other ligands, such as CD6, Ng-CAM and other unknown molecules.

One particularly interesting and potentially important finding from these results was the observation that SC1 could remarkably enhance the tumor growth and invasive ability of JYG-B cells. This is supported by the result of an *in vitro* locomotion assay, in which SC1-transfected cells displayed enhanced migratory activity. Tumor progression is a multi-step process characterized by a dynamic relationship between tumor cells and their micro-environment. Tumor cells must detach from the primary tumor mass and then migrate through the neighboring cells and ECM networks. Metastasis to other organs is achieved via intravasation, forming tumor embolisms in the vessels in other organs, extravasation and re-invasion into the surrounding tissues (16). For example, the loss of E-cadherin is thought to be instrumental in releasing cells from a primary mass by reducing contact-mediated regulation (17). On the other hand, over-expression of other CAMs encourages the migration of cells through the ECM (18). Therefore, the pathway of tumor cell invasion by SC1 seems to be quite complicated. These observations suggest that SC1-expressing tumor cells play a significant role in the early processes of tumor metastasis, including tumor growth, cells releasing from the primary site and subsequent invasion due to their cell adhesive properties. In both normal and neoplastic tissues, SC1 is enriched in adipocytes, muscular cells and endothelial cells, and slightly in mesenchymal cells. This distribution pattern suggests that SC1-expressing tumor cells can attach easily onto the surface of surrounding tissues by SC1-SC1 binding, and can consequently invade the tissues. In addition, the interaction between CAMs and cytoskeletal elements is involved in a number of cell functions, such as the ability to form junctions, cell mobility and the mediation of signal transduction (20). SC1 localization on the cell and its interaction with cytoskeletal molecules might enhance various cell actions, such as binding activity and cell locomotion, and could thus result in a high invasive potential.

Canine mammary gland tumors show a significant heterogeneity of cells (9). Some molecules, including neural CAMs, bone morphogenetic protein-6, and calponin, are possible markers that could be used to determine the malignancy and diagnosis of canine mammary gland tumors (9,10). SC1 was

enriched on a subset of malignant tumors, but was not expressed in any benign tumors in canine mammary glands. It may therefore play a role in indicating the malignancy of canine mammary glands. The present study indicated that the tumor growth of SC1-expressing cells, as well as other cells, was inhibited by the administration of gold ion water, thus implying a potential effect of gold ion on the growth of mammary gland tumors with a high degree of malignancy.

In humans, CD166/ALCAM, an ortholog of SC1, is also present in some tumors, including breast cancer and malignant melanoma, and its expression is also associated with tumor malignancy (19,21,22). Future studies should investigate SC1 expression in tumor samples and metastatic lesions to determine the potential role of this molecule in the labeling of tumor progression.

Acknowledgments

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