SPANDIDOS PUBLICATIONS

Expression and adhesive activity of SC1, an Ig superfamily cell adhesion molecule, in sporadic nephroblastomas of chicken

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Abstract. SC1, an immunoglobulin superfamily cell adhesion molecule, is transiently expressed during avian embryogenesis by a variety of cell types. This molecule has a homophilic binding activity with SC1 itself and promotes neurite projection from embryonic neurons. However, the potential role of this molecule in pathologic tissue specimens from chickens has yet to be elucidated. In this study, we examined the expression and functional role of SC1 in the sporadic nephroblastomas of chickens. Western blot analysis showed SC1 to be recognized as approximately 100 kDa and enriched in embryonic metanephros with a lower level in the adult kidney, while it was overexpressed in nephroblastomas. Immunohistochemically, SC1 was abundantly found in the tubular epithelia and blastemal cells of embryonic metanephros. In contrast, it had almost completely disappeared in the adult kidney; parts of the distal convoluted and intermediated tubules, collecting ducts, and Bowman's capsule slightly expressed SC1. In all 32 cases of nephroblastoma, SC1 was overexpressed in most characteristic components in tumors such as neoplastic epithelia with various types of differentiation, blastemal cell condensations, and glomeruloid bodies. Primary culture cells from a nephroblastoma expressed SC1 on the cell surface, whereas cells from the adult kidney showed only weak expression. A cell aggregation assay revealed that the dissociated cells from a nephroblastoma have strong aggregation activity, which was inhibited by anti-SC1 antibody. In contrast, the self-aggregation of adult chicken kidney cells was weaker than that of the tumor and not inhibited by the antibody. These findings suggest that the expression of SC1 might play a potential role in both the structural formation of nephroblastomas, based on its adhesive activity, and normal renal development.

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Introduction

SC1 is a membrane integral cell adhesion protein of the immunoglobulin (Ig) superfamily that has been discovered independently by different researchers: SC1 (1,2), BEN (3), and DM-GRASP (4). This molecule is characterized by an intracellular domain carrying two V-type motifs following three C2-type Ig-like motifs. SC1 has been shown to have homophilic properties as demonstrated by cell adhesion experiments on substrates and *in vitro* self-aggregation assays (2,5-7). However, heterophilic interactions have also been described, notably with Ng-CAM during the extension of neurites from sympathetic neurons and with CD6 in the hemopoietic system (8,9). Homologs of SC1 have now been identified in zebrafish, mice, rats and humans (10). SC1 is transiently expressed during chick embryogenesis, particularly by cells of the nervous and hemopoietic systems and certain epithelia (3,6). This molecule is also involved in various physiological processes including hematopoiesis, thymus development, immune response, neurite extension, neural cell migration, and osteogenesis. The ALCAM/CD166/MEMD, a human homolog of SC1, is expressed in malignant melanoma cells and represents a new molecular marker for the progression of melanoma with possible prognostic value (11). However, there have been no reports concerning the expression and functional role of SC1 in sporadic tumors of chickens.

A nephroblastoma, also called 'embryonic nephroma,' is thought to originate from the metanephros while demonstrating various stages of metanephrous development (12,13). In addition, these tumors are commonly found in young broiler chickens and can be easily sampled at poultry slaughterhouses (14). Previously, we performed an *in vitro* cell aggregation assay using primary cultures from chicken nephroblastoma, and thus confirmed the cell-cell interactions by gicerin, another cell adhesion molecule (14). Accordingly, the chick kidney and nephroblastomas are considered to be good models to study the possible role of cell adhesion molecules

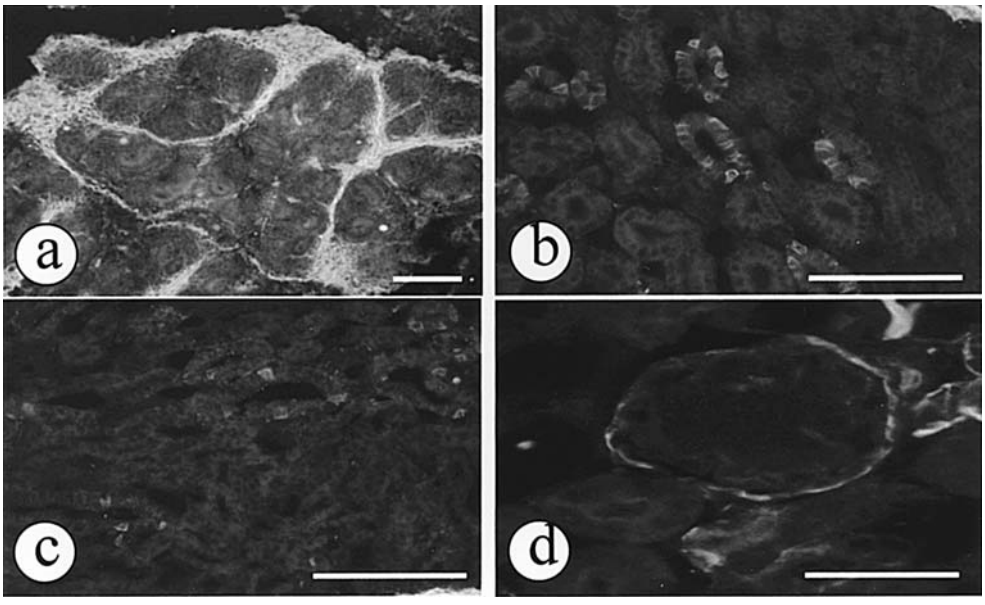

Figure 1. The expression of SC1 in the embryonic and adult chicken kidney. In the metanephros of an embryo at day 11, SC1 is abundantly expressed in the condensing blastemal and tubular epithelial cells (a). In contrast, SC1 is slightly restricted in the subset of epithelial cells of the distal convoluted tubules (b), collecting ducts (c) and epithelial cells of Bowmann's capsule (d) in the adult chicken kidney. Bars, 100 μm.

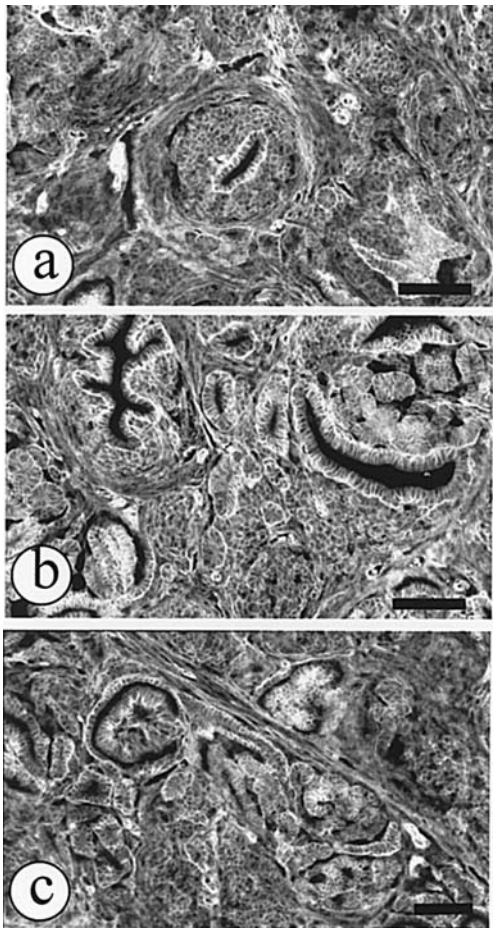

Figure 2. The expression of SC1 in the sporadic nephroblastomas of chickens. SC1 is abundantly expressed in the various components of nephroblastomas: the epithelial cells of immature tubular structure, condensing blastemal cell fibrous stroma, are intensively stained in classical triphasic nephroblastoma (a); most epithelial cells in either immature or well-formed tubular structures (b) and glomerular-like invasinations (c) are also strongly positive for SC1. Bars, 100 μm.

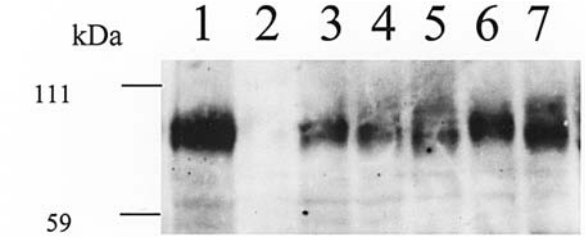

Figure 3. A Western blot analysis of SC1 expression in the embryonic and adult kidney and sporadic nephroblastomas. Each lane is loaded with 30 μg of membrane fractions of the embryonic kidney at day 11 (lane 1), adult kidney (lane 2), and five cases of nephroblastomas (lanes 3-7). Numbers on the left side of the panel indicate molecular size of the marker in kDa.

in normal development and tumor formation. Here, we describe the expression and cell adhesive activity of SC1 in the chicken nephroblastoma.

Materials and methods

Specimens and immunohistochemistry for SC1. The 32 sporadic cases of nephroblastomas found in young broiler chickens were provided from a poultry slaughterhouse in Japan. The whole embryonic body at day 11 (E11), adult chicken kidney and portions of nephroblastomas were fixed in Zamboni's fixative solution [0.21% picric acid, 2% paraformaldehyde, and 130 mM phosphate buffer (pH 7.4)] at 4˚C for immunohistochemistry. They were allowed to equilibrate overnight in 30% sucrose in phosphate-buffered saline (PBS) at 4˚C, and then frozen sections were created using a cryostat. After drying, the sections were incubated with polyclonal antibody against SC1 protein (1:1000) overnight at 4˚C (2). This was followed by washing twice with 0.2% Tween-20 in PBS, then sections were incubated with an FITC-conjugated swine secondary antibody against rabbit Igs (1:40) (Dako Japan) for

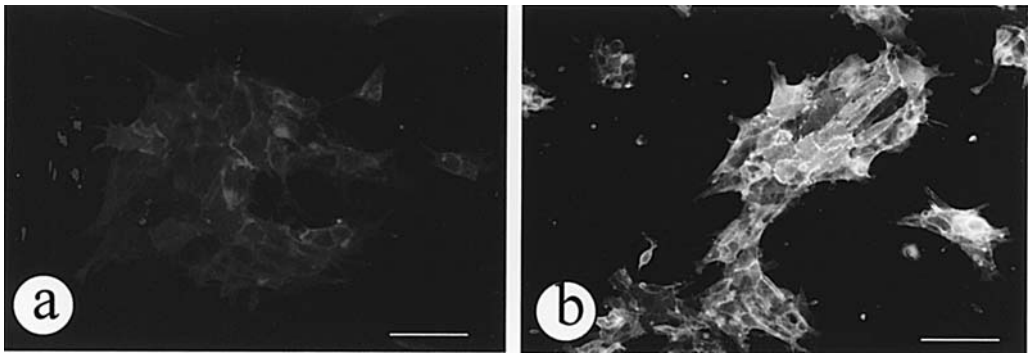

Figure 4. The expression of SC1 on the cell surface of primary culture cells from an adult kidney and a nephroblastoma. The dissociated cells from the kidney and nephroblastoma were cultured in a Petri dish, stained with the anti-SC1 antibody, then incubated for 2 h and stained with an anti-SC1 antibody after fixation. SC1 was only weakly found on the cell membrane of a small number of adult chicken kidney cells (a), whereas intense expression was observed in most nephroblastoma cells (b). Bars, 100 μm.

1 h at 37˚C, washed with 0.2% Tween-20 in PBS and examined under fluorescent microscopy. In addition, primary cultures from the adult kidney and a fresh nephroblastoma were also grown on the culture dish (see below) and fixed in Zamboni's solution at 4˚C for immunostaining as mentioned above.

Western blot analysis. Kidneys from E11 and adult chickens and five cases of nephroblastomas were homogenized in PBS with a Polytron homogenizer. The homogenates were centrifuged at 18000 x g for 10 min, and the pellets were solubilized in 10 mM Tris/Acetate (pH 8.0), 1 mM EDTA, 0.5% Nonidet P-40 (Sigma), then centrifuged at 40,000 x g for 90 min after incubating at 4˚C on a rotating shaker for 90 min. The resulting supernatants were used for immunoblotting. Equal volumes (30 μg) of proteins were separated by 8% SDS-PAGE and electrotransferred to PVDF membranes (Bio-Rad). The blots were incubated with polyclonal anti-SC1 antibody diluted in 10 mM Tris-HCl buffer (pH 8.0) containing 150 mM NaCl (TS buffer) for 1 h at room temperature after blocking with 2% skim milk in TS buffer. After washing with TS buffer containing 0.05% Tween-20 (TST buffer), the blots were incubated with HRP-conjugated secondary antibody against rabbit Igs (Dako Japan) diluted in TS buffer (1:1000) for 1 h at room temperature. After washing 4 times with TST buffer and twice with TS buffer, the X-ray film was developed with ECL solution (Pharmacia).

Cell aggregation assay. The adult chicken kidney and a fresh nephroblastoma were then dissected into small pieces and dispersed into single- to small-cell clusters with 0.05% trypsin and 0.53 mM EDTA. After centrifugation and suspension in DMEM containing 20% fetal calf serum (FCS), $9x10^6$ cells were seeded onto 9-cm diameter Petri dishes. At 4 h post-seeding, the attaching cells were re-dispersed into single cells with 0.01% trypsin and 0.53 mM EDTA, then suspended in Ca^{2+}-free Hank's solution at $2x10^6$/ml of cells. The anti-SC1 polyclonal antibody or control rabbit IgG was added at a concentration of 0.2 mg/ml and kept on ice for 30 min before starting the aggregation assay. To quantify aggregation, a 2-ml aliquot of the cell suspension was transferred into a 50-ml polypropylene tube pre-coated with FCS. The assay was carried out by cell incubation at 37˚C without agitation. A small aliquot was withdrawn after 2 h incubation and gentle mixing, and the particle number was counted with a hemocytometer. The degree of cell aggregation was indicated by the index *Nt/N0* following conventional methods (2,15,16). The index *N0* was the total particle number at the initial incubation time (0 min), and the index *Nt* was the total particle number at the incubation time *t*. Experiments were performed 4 times, and the scores were averaged with SD.

Results

Immunohistochemistry. SC1 was abundantly found in the membranes of the tubular epithelial cells and condensing blastemal cells in embryonic metanephros (Fig. 1a). In the adult kidney, SC1 expression dramatically decreased and was also weakly restricted to parts of the distal convoluted and intermediated tubules, collecting ducts, and epithelium of Bowman's capsule (Fig. 1b-d).

Histopathologically, all examined nephroblastoma specimens contained characteristic major components such as tubuli in different developmental stages, including a focal and dense accumulation of blastemal cells, and immature or well-formed tubuli and areas of fibrous stroma, although the degree of these components varied among specimens. Some tubuli tended to produce glomerulus-like invaginations. Sections from all nephroblastomas were stained with anti-SC1 antibody. SC1 expression was detected in various tumor elements and found to be up-regulated in nephroblastomas: the blastemal cell condensation, atypical epithelial components and glomeruloid body gave an intensely positive reaction for SC1 (Fig. 2a-c). In addition, the fibrous stroma was positive for SC1.

Western blot analysis. Membrane fractions of kidneys from E11 and adult chickens and nephroblastomas showed an immunoreactive band of approximately 100 kDa (Fig. 3). The amount of SC1 protein was dramatically decreased in the adult chicken kidney in comparison to the embryonic kidney. However, all nephroblastomas abundantly expressed SC1, which was consistent with the results of immunohistochemistry.

Cell aggregation assay. SC1 was only slightly expressed on the cell membrane of a small number of primary cells from

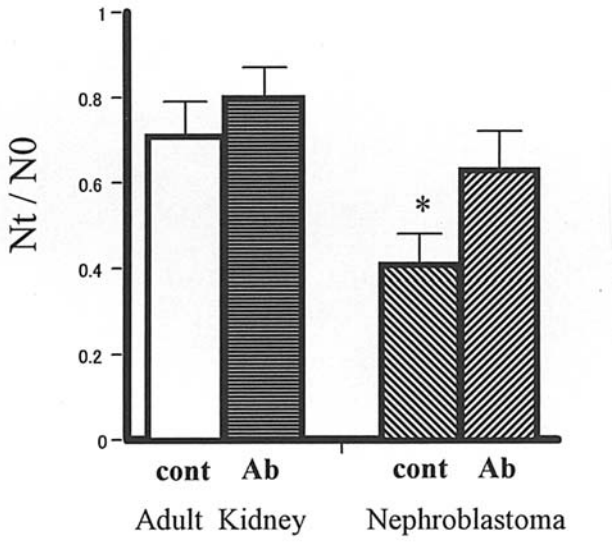

Figure 5. Cell aggregation activity of the dissociated cells from an adult kidney and a nephroblastoma. The degree of aggregation (2 h incubation in Ca^{2+}-free medium) was estimated by the index Nt/N0. Cont, incubation with control rabbit IgG; Ab, incubation with anti-SC1 polyclonal antibody *$p<0.01$ compared with the data of adult kidney cells with control rabbit IgG (Student's t-test).

the adult chicken kidney, whereas intense expression was found on the primary nephroblastoma cells (Fig. 4). We next performed an *in vitro* cell aggregation assay to check whether SC1 of the normal kidney and nephroblastoma had functional binding ability. After dissociation with a low concentration of trypsin-EDTA solution, the cells were incubated with a control rabbit IgG or polyclonal anti-SC1 antibody in a Ca^{2+}-free medium. The cell aggregation activity is shown in Fig. 5. Cells from the nephroblastoma showed strong aggregation with control IgG, but these aggregations were strongly inhibited with anti-SC1 antibody. In contrast, adult kidney cells showed weak aggregation, which was not inhibited by the antibody.

Discussion

In this study, we examined the expression and functional activity of SC1 in the normal chicken kidney and sporadic nephroblastomas to show the possible involvement of SC1 in renal tissue formation and oncogenesis. One novel and important finding is that SC1 was overexpressed in sporadic nephroblastomas. A Western blot analysis showed that SC1 is abundantly expressed in the embryonic kidney and nephroblastomas, while it is only weakly expressed in the mature adult kidney. Immunohistochemically, SC1 is highly expressed on various components of nephroblastomas, such as blastemal cells, glomeruloid bodies and tubular structures. It has been reported that cell-cell interaction is involved in the epithelial cell connection and blastemal condensation during kidney development (17-19). We therefore thought that the adhesive activity of SC1 might participate in cell-cell interactions, such as the connection between adjacent epithelial cells and condensation of blastemal cells in both the developing kidney and nephroblastomas.

To elucidate these paradigms, we carried out an *in vitro* cell aggregation assay using dissociated cells from the kidney and nephroblastomas to examine the cell binding ability of SC1. Cells from nephroblastomas showed strong aggregation activity, while those from the adult kidney only showed weak aggregation. Furthermore, an anti-SC1 polyclonal antibody blocked the strong aggregation of tumor cells, while adult kidney cells were not affected. These novel findings indicate that the adhesion ability of SC1 might thus play a role in cell-cell interactions in tumor cells. Accordingly, we propose that the binding activity of SC1 is considered to play an important role in the development of nephroblastomas. However, further study is needed to elucidate whether the clustering of nephroblastoma cells occurs through homophilic (SC1-SC1) or heterophilic adhesion (SC1-other ligand such as CD6, Ng-CAM and unknown molecules).

We also found weak expression of SC1 in parts of the mature adult kidney such as the distal convoluted and intermediated tubules, collecting ducts, and epithelium of Bowman's capsule. One possible hypothesis is that even a weak expression of SC1 might be sufficient to maintain the stabilization of these structures after maturation. However, the functional binding ability of SC1 in these portions of adult chickens remains to be investigated.

Cell adhesion molecules such as cadherins, N-CAM and gicerin have been reported to participate in the invasive or metastatic potential of a wide range of tumors (20-23). In addition, CD166/ALCAM, an ortholog of SC1 in human, is also present in some neoplasias including malignant melanoma, and its expression is also involved in tumor progression, which has been confirmed by the implantation of transfectants into animals (10,24). In this study, no metastasis to any distant organs was observed. In the future, the relationship between SC1 expression and the invasive or metastatic property of tumor cells in chickens should be elucidated.

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