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Four New Human Renal Cell Carcinoma Cell Lines Expressing Globo-Series Gangliosides

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Satoh, M., Nejad, F.M., Nakano, O., Ito, A., Kawamura, S., Ohyama, C., Saito, S. and Orikasa, S. *Four New Human Renal Cell Carcinoma Cell Lines Expressing Globo-Series Gangliosides.* Tohoku J. Exp. Med., 1999, **189** (2), 95-105 —— Clinicopathological studies revealed that monosialosyl galactosyl globoside (MSGG) and disialosyl galactosyl globoside (DSGG) expressed by renal cell carcinoma (RCC) are one of the biochemical indicator related to the metastatic potential. The present study examines the characteristics of four new human RCC cell lines and compares the expression of MSGG and DSGG among them using TLC immunostaining and flow cytometry. TOS-1 and TOS-2 were derived from metastatic subcutaneous tissues. TOS-3 and TOS-3LN were derived from the primary lesion and from metastatic lymph nodes respectively. Monolayer culture, light microscopy and electron microscopy of these cells showed that these cell lines were derived from RCC. TLC immunostaining and flow cytometric analysis revealed increased levels of MSGG in TOS-2 and TOS-3LN, and increased DSGG in TOS-1 and TOS-3LN. These cell lines would be useful for functional studies of globo-series ganglioside expressed by RCC. ———— renal cell carcinoma; established cell lines; globo-series ganglioside

Despite the use of novel therapeutic agents such as interleukin-2, interferon α, the survival of patients with metastatic renal cell carcinoma (RCC) remains poor. Therefore, the biological nature of RCC should be investigated to reveal the mechanism of metastasis relative to the expression of cell surface antigens. We have studied the features of glycolipid expression in tumors of the urogenital system (Fukushi et al. 1989; Ohyama et al. 1990; Satoh et al. 1990, 1992; Saito et al. 1991) and defined the key features of monosialosyl galactosyl globoside (MSGG) and disialosyl galactosyl globoside (DSGG) expression in metastatic RCC (Saito et al. 1991, 1997).

The present study describes the establishment of TOS-1, TOS-2, TOS-3 and

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TOS-3LN cell lines derived from human RCC tissues and examines the expression of globo-series gangliosides, which were thought to be associated with lung metastasis (Satoh et al. 1996).

Materals and Methods

Sources of cell line

TOS-1 was derived from a specimen of the soft tissue metastatic lesion obtained from a 62-year-old male who underwent surgery on September 6, 1990. The patient was diagnosed with tumors of the left kidney that had metastasized to the lung and soft tissues in the left dorsal lesion. Histopathological study of this metastatic lesion showed an alveolar, spindle predominant-mixed with clear cell, renal cell carcinoma, grade 2. TOS-2 was derived from a specimen of the axillary subcutaneous soft tissue metastatic lesion obtained from a 78-year-old male who underwent surgical extirpation on April 15, 1991. This metastatic lesion was alveolar, clear cell sub type renal cell carcinoma grade 2. TOS-3 and TOS-3LN were derived from a specimen of the primary and lymphnode metastatic lesions obtained from a 56 year-old male who underwent radical nephrectomy on September 11, 1995 and lymphnode dissection of the left subclavicular lesion on February 8, 1996. A histopathological study of this primary lesion showed that it was of the papillary and granular subtype, grade 2. The metastatic lymphnode lesion was consisted of papillary and granular subtype RCC metastasis, grade 2. The histopathological findings were assessed in accordance with General Rule for Clinical and Pathological Studies on Renal Cell Carcinoma, second edition (Japanese Urological Association 1992).

Cell culture

An aseptically resected tumor was incised into small pieces that were then incubated for 60 minutes at 37°C in 101 medium (Cosmo Bio Co., Ltd., Tokyo) containing 1000 PU/ml of dispase (Godo Shusei, Tokyo). The cell suspension was filtered through a 100 μm pore-size steel mesh filter. The cell was placed in MEM (Gibco BRL, Rockville, MD, USA) supplemented with 2 mM L-glutamine, penicillin (1000 units/ml), streptomycin (1000 μg/ml) and 10% heat-inactivated fetal bovine Serum (FBS) (Dainippon Pharmaceutical Co., Ltd., Osaka) and plated in T25 flasks (Greiner, Frickenhausen, Germany) under the condition with humidified 5% CO_2 atmosphere at 37°C. When confluent, the cells were dissociated with trypsin (0.5%) and EDTA (0.02%) and were sedimented by low-speed centrifugation. The supernatant was removed and the cell pellet was resuspended in the same culture medium.

Microscopic observation

RCC cells at various passages were directory examined using an inverse-phase microscope (Carl Zeiss, Jena, Germany). Cells were processed as follows for

electron microscopy. Cells removed from tissue culture flasks were pelleted by centrifugation, fixed in 2.5% glutaraldehyde in 0.1 M Na-cacodylate buffer, then post-fixed with OsO_4 (1%) in the same buffer. Samples were dehydrated in alcohol and embedded in an Epon mixture. The ultrathin sections of the specimen were counterstained with 1.5% uranyl acetate solution and lead citrate solution, then observed under an electron microscope (LEM1200EX, JEOL, Tokyo) observation.

Cell growth

Cell lines (5×10^4) suspended in 2 ml MEM with 10% FBS were plated in triplicate in 6 well plastic wells (Greiner, Austria), then cells were counted every other day. The doubling time for each of the cell line was determined by the growth curve during exponential phase of growth.

Xenotransplantation of cultured cells in nude mice

Renal cancer cells (5×10^6 cells/0.1 ml of PBS) were subcutaneously inoculated into 5 male nude mice (BALB/c origin nu/nu, 4 weeks old). Tumor formation was evaluated after 4 to 8 weeks. Tumors were removed as soon as they appeared, then fixed in 10% buffered formalin and stained with hematoxylin and eosin for histopathological study.

Chromosomal analysis

After dispersion and centrifugation of colocemid-treated confluent cells, they were treated with hypotonic KCl solution (0.075 M). Then, the cells were fixed with Carnoy's solution and treated by Giemsa-stain for chromosomal analysis.

Extraction of glycolipids

Cultured cells (500 mg of cells; 1×10^8 cells) were washed in physiological phosphate-buffered saline (PBS) 3 times and harvested by centrifugation. Glycolipids were extracted by the method previously described (Saito et al. 1991). Briefly, crude glycolipid was extracted from the cell pellets by homogenization and filtration with isopropanol-hexan-water (55 : 25 : 20) and chloroform methanol (2 : 1, 1 : 1, and 1 : 2 V/V). The specimens were separated into upper and lower phases by Folch's partitioning (Folch et al. 1957). The upper phase was dialyzed against distilled water for 2 days, and separated into upper neutral and acidic fractions by diethylaminoethanol Sephadex A25 (Pharmacia, Uppsala, Sweden) column chromatography (Leeden and Yu 1982). Acidic fractions containing the gangliosides extracted from the same wet weight of cells and standard gangliosides were placed on HPTLC plates (Baker, NJ, USA) using micro-syringe and separated using chloroform: methanol: 0.5% $CaCl_2$: 50 : 40 : 9 (V : V : V). Gangliosides were visualized using orcinol-H_2SO_4.

TLC immunostaining

HPTLC plates were immunostained according to a modified version of Magnani's procedure (Magnani et al. 1980). Total ganglioside fractions were applied on HPTLC plates using a solvent system of chloroform methanol water (CMW) 50 : 40 : 10 containing 0.05% $CaCl_2$. Plates were air-dried and immersed in 0.5%-poly (isobutyl-metracrylate) (high molecular weight; Aldrich Chemical Co., Milwaukee, WI, USA) in ether for 1 minute, blocked with 5% bobine serum albumin (BSA) in PBS for 1 hour at room temp, and then reacted overnight with monoclonal antibody (mAb) RM1 (anti MSGG) or RM2 (anti DSGG) and nonimmune mouse IgM at 4°C. Plates were washed, incubated with biotinylated secondary antibody for 1 hour, incubated with vector avidin-biotin solution for 30 minutes, and stained with 3′, 3-diaminobenzidine.

Flowcytometry

TOS-1, -2, -3, -3LN cells, (1×10^6 each) were incubated with RM1 and RM2 (10 μg/ml) in PBSSA (PBS containing 1% BSA and 0.1% sodium azide). Cells were washed 3 times with PBSSA and then incubated with a 1 : 200 solution for fluolescein conjugated rabbit anti-mouse IgM (DAKO, Tokyo) for 30 minutes on ice. After 3 washes with PBSSA, cells were suspended in Isoton (300 μl) (Becton Dickinson, Sunnyvale, CA, USA) and cell-surface immunofluorescence was detected using a FACScan (Becton Dickinson). Positivity (%) is expressed relative to non-immune mouse IgM as the negative control.

Results

Morphology

The four RCC lines were cultured as epithelial cell monolayers (Figs. 1 and 2). Microscopic examination of TOS-1 cells in culture revealed a spindle appearance with large nuclei containing one to two nucleoli. The cells were partially piled up, indicating a lack of contact inhibition. TOS-2 cells consisted morphologically of large polygonal cells with large nucleoli. These cells exhibited contact inhibition. TOS-3 and TOS-3LN were small and compact, with large nuclei and 1 or 2 prominent nucleoli. Loosely attached cells were spherical, and clusters of these cells frequently overlaid attached cells. During the early growth phase, all four cell lines were contaminated with fibroblasts, which later disappeared. All cell lines thrived for over 100 passages, representing a period of 24 months after initial culture.

Ultrastructural characteristics suggested their epithelial nature and their renal cancer origin (Fig. 3).

TOS-1 and TOS-2 contained abundant cytoplasmic glycogen and lipid droplets in the cytoplasm, and intercellular organelles were not well developed. TOS-3 and TOS-3LN contained numerous mitochondria and dense bodies, as well

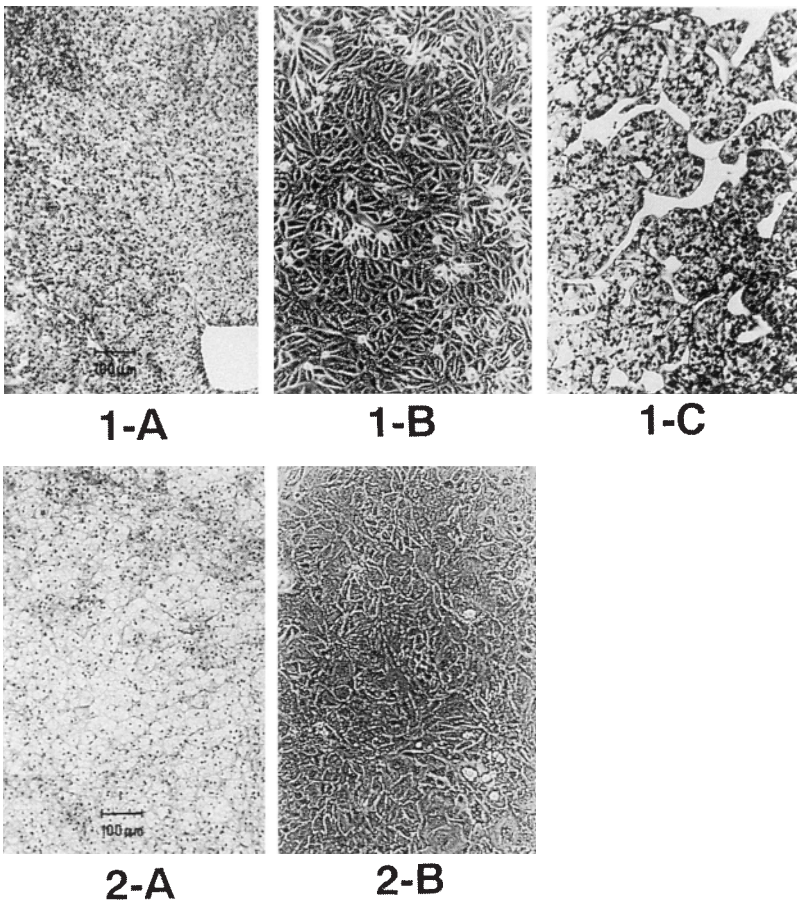

Fig. 1. Microscopic features of TOS-1 and TOS-2. 1, TOS-1; 2, TOS-2.
A: Histopathological features of the original tumor (H & E, ×100). B: Morphological features in the monolayer culture (×100). C: Histology of the tumor by xenotransplantation of the cell line (H & E, ×100).

as Golgi apparatus and endoplasmic reticulum. Microvilli were abundant on the border of all four cell lines.

Proliferation

Cell growth followed the curves shown in Fig. 4. When 5×10^4 cells/well were initially seeded. The doubling times of TOS-1, TOS-2, TOS-3, and TOS-3LN were 32, 37, 32, 33 hours, respectively (Table 1).

Ploidy study and karyotype analysis

The typical modal numbers of TOS-1, TOS-2, TOS-3 and TOS-3LN were 75, 88, 42, 45, respectively (Table 1).

Y-chromosomes were deleted in all four cell lines.

Rearrangement of TOS-1 involved chromosomes 1, 2, 3, 8, 15 and 17, chromosomes 3, 7 and 8 in TOS-2, chromosomes 4, 11, 13 and 19 in TOS-3, chromosome 4, 7, 8, 11 and 19 in TOS-3LN.

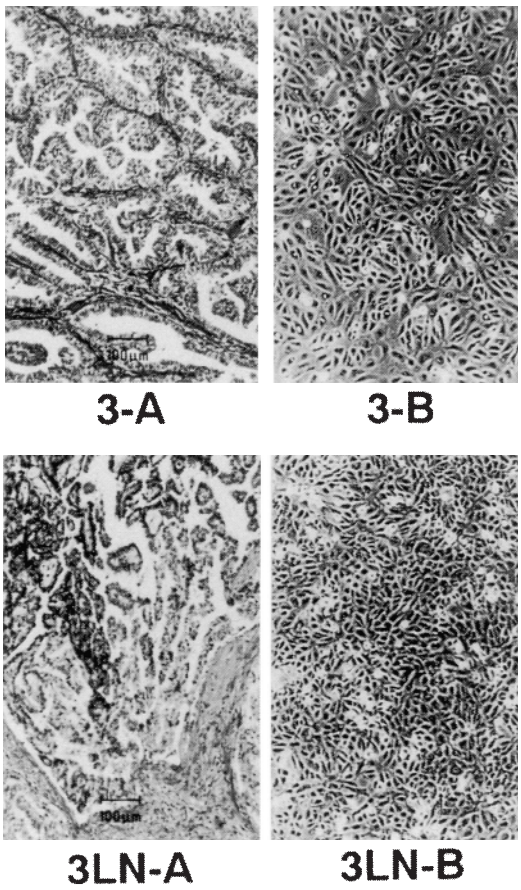

Fig. 2. Microscopic features of TOS-3 and TOS-3LN. 3, TOS-3; 3LN, TOS-3LN. A: Histopathological features of the original tumor (H & E, ×100). B: Morphological features in the monolayer culture (×100).

Tumorigenicity

Nude mice (4 groups of 5 each) were injected S.C. with 5×10^6 TOS 1, TOS-2, TOS-3 or TOS-3LN cells. Local tumors were appeared only in the group injected

Table 1. *Characterization of 4 RCC cell lines*

Cell line	Original tissue	Doubling time (hours)	Karyotype modal number	Growth in athymic nude mice
TOS-1	62y, male, Lt RCC back meta.	32	75 (68P)	+
TOS-2	78y, male, Lt RCC axilla meta.	37	88 (62P)	−
TOS-3	56y, male, Lt RCC primary site.	32	42 (102P)	−
TOS-3LN	56y, male, Lt RCC subclavicular LN meta.	33	45 (90P)	−

modal number = typical modal number.
P, passages; Lt, left; RCC, renal cell carcinoma; LN meta, lymphnode metastasis.

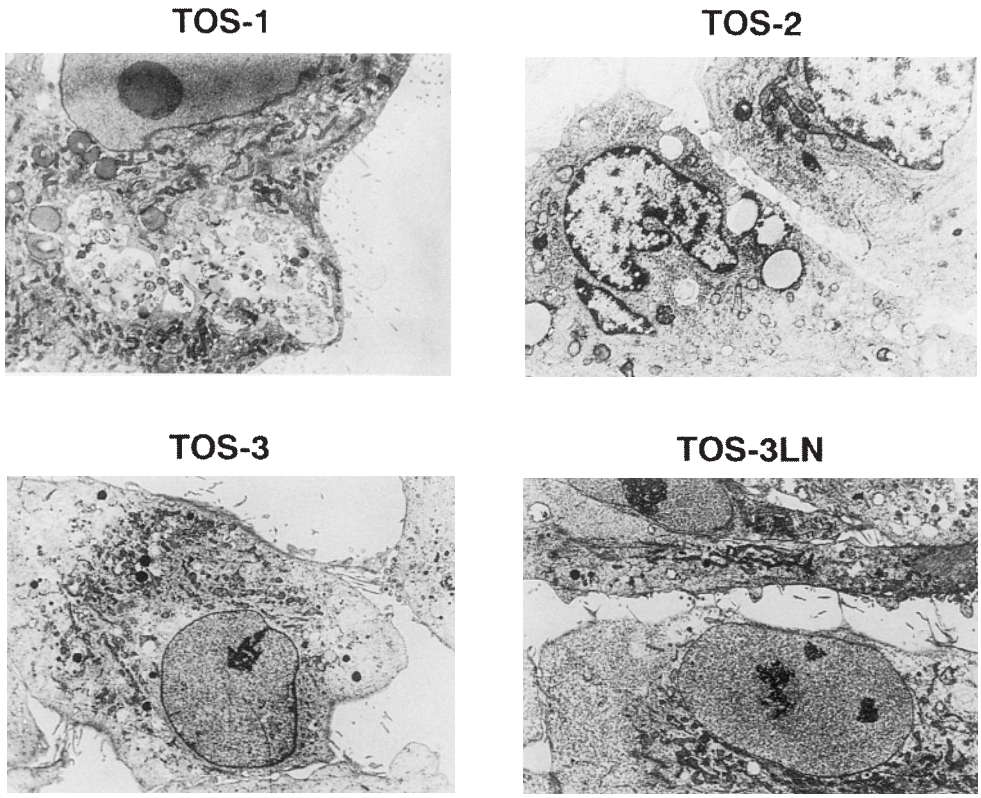

Fig. 3. Electron microscopic features of TOS-1, TOS-2, TOS-3 and TOS-3LN. TOS-1, ×12 000; TOS-2, ×11 040; TOS-3, ×4140; TOS-3LN, ×4140.

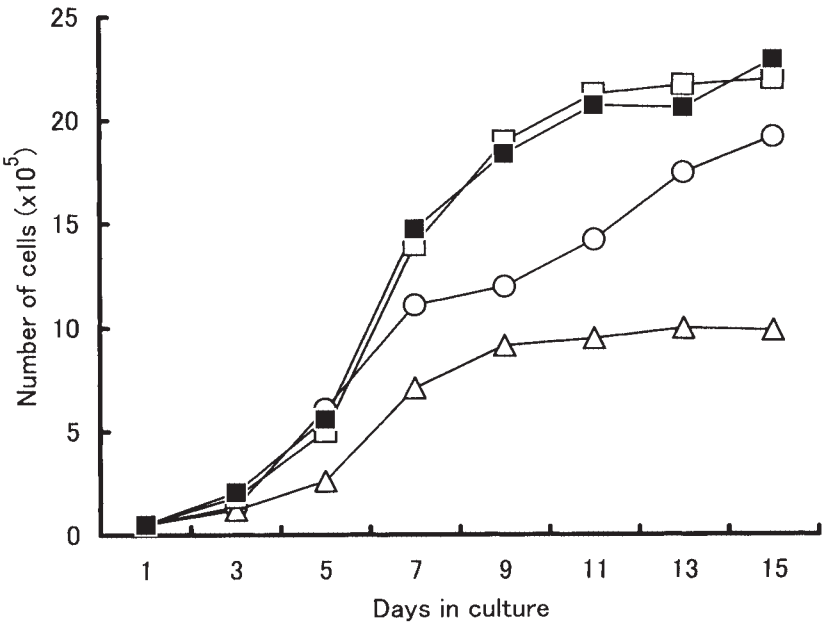

Fig. 4. Growth curves of TOS-1 (○) at passage 134, TOS-2 (△) at passage 116, TOS-3 (□) at passage 105 and TOS-3LN (■) at passage 98.

with TOS-1 (Table 1).

Histology of the tumor generated by xenotransplantation of TOS-1 revealed a similarity to clear cell carcinoma (Fig. 1).

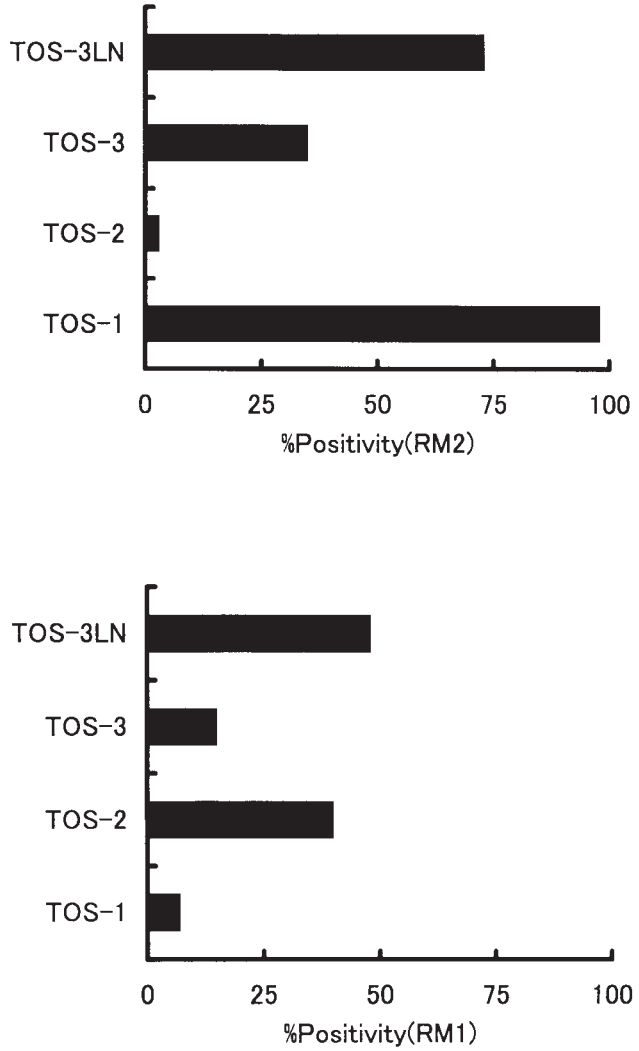

Fig. 5. Reactivities of RCC lines with mAbs directed to DSGG, MSGG. The percentage of positivity was expressed relative to negative control.
RM1, anti MSGG mAb; RM2, anti DSGG mAb.

Flowcytometry

TOS-2 and TOS-3LN reacted against anti-MSGG RM1 and TOS-3 reacted weakly against this antibody.

TOS-1 and TOS-3LN reacted significantly against anti-DSGG RM2, whereas TOS-3 was about 40% reactive against this antibody (Fig. 5).

TLC immunostaining

TLC immunostaining with RM1 revealed increased levels of MSGG in TOS-2 and TOS-3LN. Immunostaining with RM2 revealed increased levels of DSGG in TOS-1 and TOS-3LN (Fig. 6).

TLC immunostaining with nonimmune mouse IgM revealed no bands (data not shown).

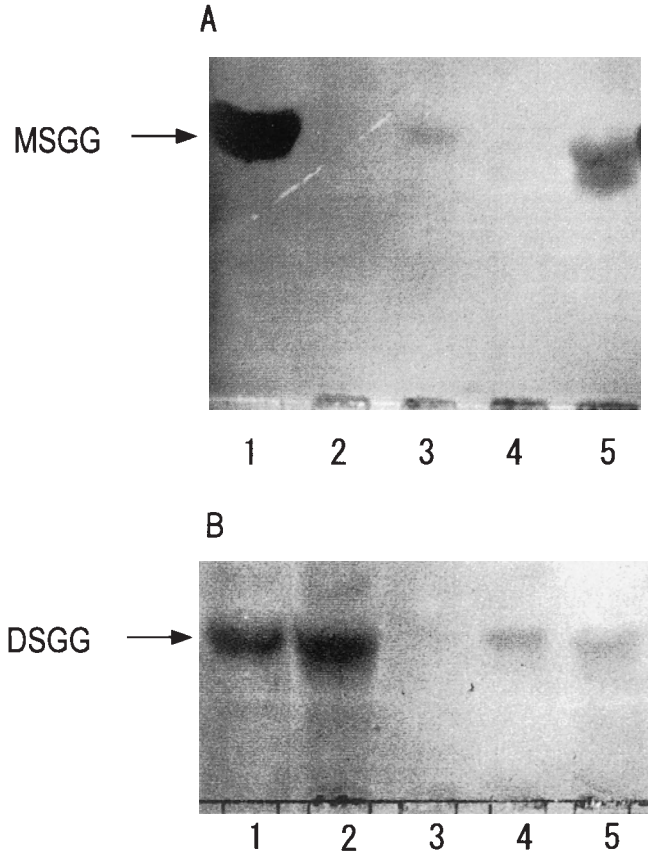

Fig. 6. TLC immunostaining.
A: TLC immunostaining by mAb RM1. Lane 1, Purified MSGG; Lane 2, TOS-1; Lane 3, TOS-2; Lane 4, TOS-3; Lane 5, TOS-3LN. B: TLC immunostaining by mAb RM2. Lane 1, Purified DSGG; Lane 2, TOS-1; Lane 3, TOS-2; Lane 4, TOS-3; Lane 5, TOS-3LN.

Discussion

The only effective treatment for RCC is surgical extirpation, since most chemotherapy and radiological strategies have proven ineffective, although immunotherapy using interferon or IL-2 has had limited success (Muss 1988). Moreover, 30% of patients present with progressive disease at the first medical examination. Among those that are radically operable due to localized RCC, 30%–50% will experience subsequent metastasis (Dekernion and Mukamel 1987; Ritchie and Chisholm 1987). It is therefore necessary to understand the biological nature of RCC, such as which glyco-chain antigens are expressed on cell surface. Our previous study on glycolipids associated with human RCC found that levels of slower-migrating gangliosides increase in metastatic deposits (Saito et al. 1991), that globo-series gangliosides are the major components and that disialosyl galactosyl globoside (DSGG) is an adhesion molecule (Satoh et al. 1996). Therefore, cells originating from RCC with characterized glycolipid antigens are thought to be useful as experimental models. However, glycolipid expression by cell lines derived from RCC has not been investigated in detail (Hoon et al. 1993; Honke et al. 1998).

We established four malignant RCC cell lines, designated TOS-1, TOS-2, TOS-3, TOS-3LN. Each of these cell lines can be classified as epithelial because of their morphologic appearance in monolayer cultures.

In addition, the ultrastructural features generally accepted as specific traits of epithelial cells were found in all four cell lines at various levels. In TOS-1 and TOS-2, the findings (particulate glycogen and fat, little endoplasmic reticulum, few Golgi apparatus, and few mitochondria) were clear cell-specific. TOS-3, TOS-3LN showed granular cells specific findings (numerous mitochondria and dense bodies, developed Golgi apparatus and endoplasmic reticulum) (Presti et al. 1991).

All four cell lines displayed chromosomes with structural abnormalities and some unidentified chromosomes.

TOS-2 had lost the chromosome arm 3p sequence, which is characteristically absent in clear cell RCC. The most frequently observed changes in papillary RCC (trisomies of chromosomes 7 and 17) were not found in TOS-3 and TOS3LN (Hughson et al. 1993).

The four cell lines expressed different levels of galactosyl globoside. TOS-2 and TOS-3LN were RM1 positive. The order of RM2 positivity was TOS-1 > TOS-3LN > TOS-3. Moreover, TOS-1, TOS-3, TOS-3LN adhered to human lung sections by Stumper-Woodruff assay (Satoh et al. 1996), but this property was totally inhibited by RM2 (data not shown).

The neoplastic nature of these four lines was confirmed. Therefore, these cell lines should represent new models with which to study the biology of RCC and help provide new insight into the function of galactosyl globoside expressed on renal cancer cells.

Acknowledgments

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