Effect of Suramin on Differentiation of Human Stomach Cancer Cell Lines

This study was designed to demonstrate that differentiation of stomach cancer cells can be modified by microenvironmental change and to look for a method inducing or promoting tumor cell differentiation. To evaluate the biomorphological characterization of tumor cell differentiation in suramin-containing *in vitro* culture of human stomach cancer cell lines, inverted phase-contrast microscopic examination, analysis of growth curves and BrdU-positive S-phase fraction, immunocytochemical study, radioimmunoassay for CEA, transmission electron microscopic examination, DNA flow cytometry, and heterotransplantation in SCID mice were performed. Suramin inhibited tumor cell growth. Development of intracytoplasmic lumina and intercellular lumina was noted in suramin-containing culture with formation of numerous microvilli and frequent desmosomes. The amount of CEA released by a cell was increased in suramin-containing culture. Suramin inhibited heterotransplantation, and a transplant from suramin-containing culture revealed a much higher degree of differentiation than that from suramin-absent culture. Suramin induced no change in DNA ploidy pattern. Elimination of suramin from the culture medium did not reverse the tumor cell differentiation. Each stomach cancer cell line showed a different degree of responsiveness to suramin. In conclusion, this study shows that suramin inhibits growth of SNU-5 and SNU-16 cells and that suramin induces differentiation of SNU-16 cells. ***(JKMS 1997; 12 : 433~42)***

Key Words : *Suramin, Stomach neoplasms, Tumor cells, Cultured, Cell differentiation*

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Received : May 29, 1997
Accepted : August 28, 1997

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* This study was in part supported by the Seoul National University Hospital Research Fund (04-93-029) and the Seoul City Boramae Hospital Research Fund (1997).

INTRODUCTION

Differentiation refers to the extent to which parenchymal cells of neoplasms resemble comparable normal cells, both morphologically and functionally (1). Well-differentiated tumor cells are thus composed of cells resembling the mature normal cells of the tissue of origin of the neoplasm. In general, benign tumors are well differentiated; malignant neoplasms, in contrast, range from well differentiated to undifferentiated. Indeed, lack of differentiation is considered a hallmark of malignant transformation. Cancers arise from stem cells present in all specialized tissue, and the well-differentiated cancer evolves from maturation or specialization of undifferentiated cells as they proliferate, whereas the undifferentiated malignant tumor derives from proliferation without maturation of the transformed cells (1).

We reviewed 400 cases of surgically resected gastric carcinoma using histo-topographic analysis, and observed changes in histologic type and degree of differentiation according to gastric wall invasion (2). Histologic type was homogeneous regardless of invasion in 44.7%; however, in 28%, degree of differentiation was changed according to tumor invasion, and 27.3% showed mixed histologic types both in the mucosa and in the invasive component. With the above observations, we hypothesized that the histologic type or differentiation of gastric carcinoma could be changed with tumor invasion and it might be influenced by the microenvironment around the carcinoma cells. Therefore, we attempted to use an *in vitro* culture model of human gastric carcinoma cell lines, and looked for factors inducing or promoting tumor cell differentiation.

As factors inducing differentiation in cultured cell lines, addition of retinoic acid (3), addition of suramin (4~8), and removal of glucose and its replacement by galactose (9) in the culture medium have been reported. These inducers of differentiation have been examined in the kidney epithelial cell line (3) or colon cancer cell lines (4~9); however, no study using stomach cancer cell lines has been reported according to induction of differentiation.

There has been a renewal of interest in suramin, a drug used in the therapy of trypanosomiasis and onchocerciasis, following recent discoveries : a) the observation that suramin exhibits an inhibitory effect on reverse transcriptase activity in vitro provided the basis for clinical trials of suramin in the treatment of acquired immunodeficiency syndrome (10, 11), b) suramin has been shown to be a potent anticancer agent and has been used successfully for the treatment of a variety of malignancies, such as metastatic prostate cancer, progressive nodular lymphoma, metastatic renal cell or adrenal gland carcinoma (12), and c) suramin has been shown to block specific receptor binding of epidermal growth factor, transforming growth factor-β, platelet-derived growth factor and insulin-like growth factor-1, and also to interfere with adrenocorticotropic hormone-stimulated adrenal cortical cell function. Furthermore, suramin is also active at various intracellular sites inasmuch as it is able to inhibit inositol 1,4,5-triphosphate-mediated Ca^{++} release from intracellular stores, protein kinase C as well as cyclic AMP-dependent protein kinase activity (12).

The effect of suramin on growth factor functions held our attention, and we investigated the action of suramin on the growth and differentiation of human stomach cancer cell lines.

MATERIALS AND METHODS

Materials

Human stomach cancer cell lines, SNU-1, SNU-5 and SNU-16, were obtained from the Korea Cell Line Bank (KCLB), Cancer Research Institute, Seoul National University College of Medicine. The cell lines are already characterized (13), and the basic characteristics are summarized in Table 1.

Thirty female 6 week-old SCID (severe combined immunodeficiency) mice were obtained from the Asan Life Science Research Institute, and they were kept in the specific pathogen free (SPF) observation room.

Suramin was obtained from CB Chemicals (Woodbury, CT, USA). It was prepared as a sterile stock solution of 100 mg/ml in 0.9% NaCl and stored at -20℃.

Cell culture

Three stomach cancer cell lines, SNU-1, SNU-5 and SNU-16, were cultured in RPMI-1640 medium (Gibco, #430-1800) containing 12 mM glucose and 10% fetal calf serum in a humidified atmosphere of 95% air, 5% CO_2. Suramin was added in the medium to the concentration of 100 μg/ml. The RPMI-1640 medium and the suramin-containing medium were respectively changed every 2 days, and the cells were grown for ten serial passages. The cells were harvested with 0.05% trypsin in 0.53 M ethylenediaminetetraacetate (EDTA).

Immunocytochemistry

Harvested cells were attached to the glass slides using a cytocentrifuge in the number of 2×10^4 per slide. They were fixed in 4℃ cold acetone, and were immunocytochemically stained with monoclonal anti-CEA (DAKO, Carpinteria, CA) using the avidin-biotin-peroxidase complex method (ABC Elite kit, Vector Laboratories, Burlingame, CA).

To evaluate S-phase fraction, 10 μM of bromodeoxyuridine (BrdU) was added to the 25 cm^2 culture plate containing 1×10^6 cells. After incubation for 30 minutes, the cells were washed with phosphate-buffered saline (PBS). They were attached to the glass slides and fixed as described above. The slides were incubated in 37℃, 1 M HCl for 30 minutes to denature DNA, and then, they were immunocytochemically stained with anti-BrdU monoclonal antibody as described above.

Radioimmunoassay (RIA)

To evaluate the levels of CEA released by cells, radioimmunoassay was performed. 1×10^5 cells from each cell line were cultured in 4 ml of medium (RPMI-1640 or suramin-containing medium, respectively) for three days. After centrifugation, the amount of CEA was measured in 1 ml of supernatant medium. The level of CEA determined with RIA was corrected by the number of viable

Table 1. Profile of original gastric carcinoma cell lines

Cell line	Date of initiation	Age	Sex	Country of origin	Blood type	Prior therapy	Tumor site[a]	Original tumor differentiation
SNU-1	4/84	44	M	Korea	O+	None	Stomach	Poor
SNU-5	6/87	33	F	Korea	O+	FAM[b]	Ascites	Poor
SNU-16	7/87	33	F	Korea	A+	None	Ascites	Poor

a : Tumor site used for cell culture
b : 5-fluorouracil, doxorubicin, and methotrexate

cells.

Electron microscopy

The cells were fixed in situ with 2.5% glutaraldehyde in 0.2 M sodium cacodylate buffer, pH 7.4, for two hours, washed overnight in the same buffer with 7.5% saccharose, post-fixed in 1% osmium tetroxide in 0.2 M sodium cacodylate buffer for one hour, then dehydrated in ethanol, and embedded in Epon. Thin sections were stained with lead citrate and uranyl acetate, and examined under a Jeol JEM-1200 ExII electron microscope.

DNA flow cytometry

DNA ploidy pattern was examined by flow cytometry as has been described in detail before (14,15). Briefly, the harvested cells were filtered with nylon mesh and fixed in ice-cold 70% ethanol for 30 minutes. After treatment with trypsin for 30 minutes, RNase treatment for 10 minutes and staining with propidium iodide for 10 minutes, DNA-content per cell was measured in a flow cytometer (EPICS, Coulter).

Heterotransplantation

To evaluate the capacity for tumorigenecity (heterotransplantation), each cell line was subcutaneously injected to five female 6 week-old SCID mice. $5x10^5$ tumor cells were suspended in 0.3 ml of RPMI-1640, and the suspension fluid was contained in a 1 ml syringe. SCID mice were punctured at the paravertebral flank with a 23 gauge needle, and the needle was subcutaneously advanced toward the head. Tumor cells were injected in the infra-scapular region. All the SCID mice were sacrificed at 10 weeks after inoculation and the size of tumor, if formed, was measured. The "Guiding Principles in the Care and Use of Animals" approved by the American Physiological Society were observed.

RESULTS

Inverted microscopic observation

SNU-1 cells grown in RPMI medium were round or oval, and the size was variable. They grew as a singly scattered floating individual without cohesive cluster or substrate attachment. SNU-1 cells grown in suramin-containing medium were similar to those grown in RPMI medium (Fig. 1). Long-term culture for more than three months revealed no specific morphologic change except degenerative change.

SNU-5 cells grown in RPMI medium were singly scattered or formed loosely attached clusters with partial substrate attachment (Fig. 2-A). In suramin-containing culture, round intracellular structures with distinct phase contrast in the margin were noted in the cytoplasm of SNU-5 cells. The intracytoplasmic structures were variable in size. Larger ones occupied nearly all the cytoplasm, and the nucleus was crescent and displaced by the structure to the periphery (Fig. 2-B). Smaller ones were found singly or in multiples in a cell. Compare to the electron microscopic findings, these intracytoplasmic structures are considered to be intracytoplasmic lumina (ICL). In long-term culture for more than three months, the intracytoplasmic structures persisted.

SNU-16 cells grown in RPMI medium formed loosely attached clusters with partial substrate attachment (Fig. 3-A). In suramin-containing culture, SNU-16 cells changed to round, singly scattered, floating cells in 24 hours (Fig. 3-B). The cells progressively formed cohesive clusters with partial substrate attachment. Large cohesive clusters were formed in long-term culture for more than three months(Fig. 3-C). The intracytoplasmic structure present in SNU-5 cells was not noted by inverted microscopic observation.

To evaluate whether the morphologic change by suramin was reversible or not, suramin-containing medium was replaced by RPMI-1640 medium after 4 week culture in suramin-containing medium. No additional morphologic change was induced in each cell line. There was no change in size and shape of cohesive clusters and

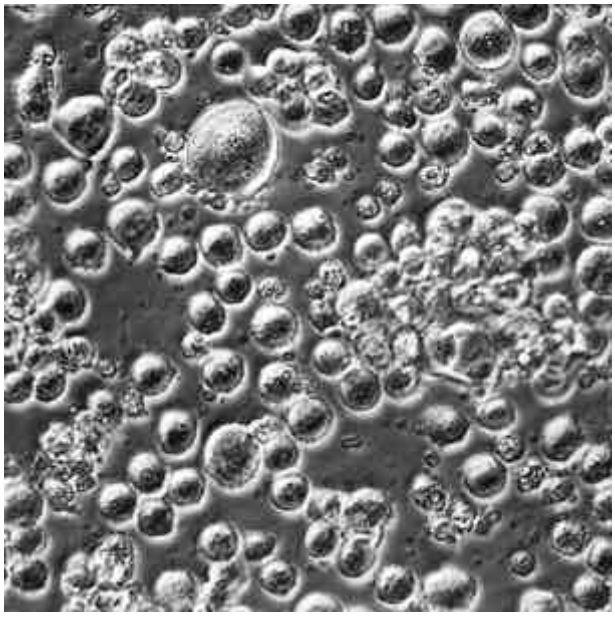

Fig. 1. Phase contrast micrograph of SNU-1 cells cultured in suramin-containing medium.

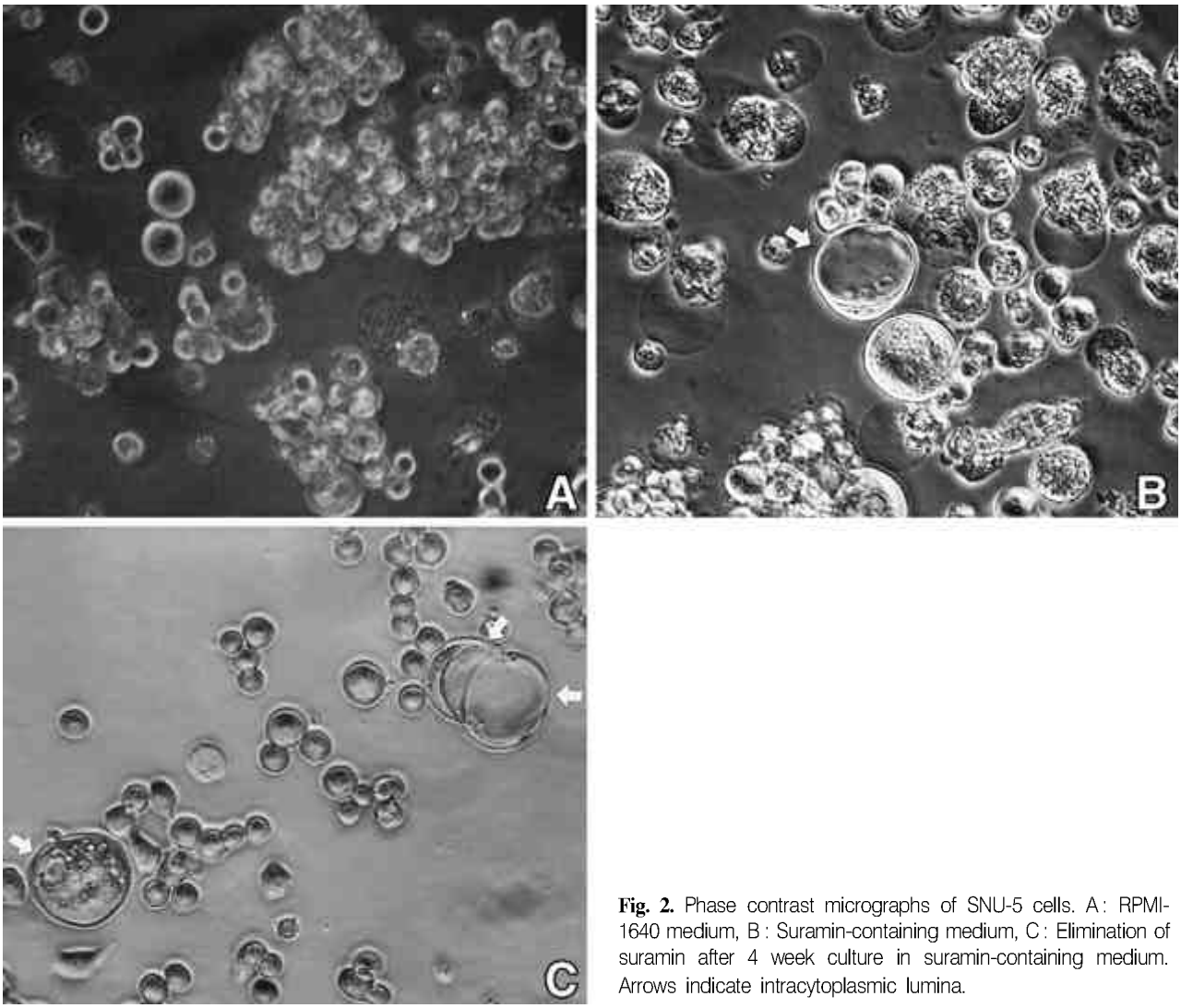

Fig. 2. Phase contrast micrographs of SNU-5 cells. A: RPMI-1640 medium, B: Suramin-containing medium, C: Elimination of suramin after 4 week culture in suramin-containing medium. Arrows indicate intracytoplasmic lumina.

partial substrate attachment. The intracytoplasmic structure representing ICL persisted in SNU-5 cells (Fig. 2-C), and the effect of suramin was considered to be irreversible after culture for 4 weeks.

Growth curve and cell cycle analysis

Suramin inhibited growth of SNU-5 cells and SNU-16 cells (Fig. 4). Growth inhibition by suramin was minimal in SNU-1 cells.

BrdU-positive S-phase fractions measured immunocytochemically in each cell line are listed in Table 2. The result of cell cycle analysis is considered to correlate well with growth curves.

Immunocytochemistry and RIA for CEA

SNU-1 cells and SNU-5 cells expressed no CEA in the cytoplasm or cell membrane, immunocytochemically. SNU-16 cells immunocytochemically expressed CEA diffusely and strongly both in the cytoplasm and in the cell membrane. The CEA immunoreactivity of SNU-16 cells grown in the presence of suramin was as strong as that of SNU-16 cells grown in the absence of suramin. Therefore, no immunocytochemical difference could be detected.

CEA levels measured by RIA in the supernatant medium of each cell line are listed in Table 3 with CEA

Table 2. Proportion of BrdU-positive cells in stomach cancer cell lines

Culture medium	SNU-1 (%)	SNU-5 (%)	SNU-16 (%)
RPMI-1640	66	47	72
Suramin + RPMI-1640	60	40	46

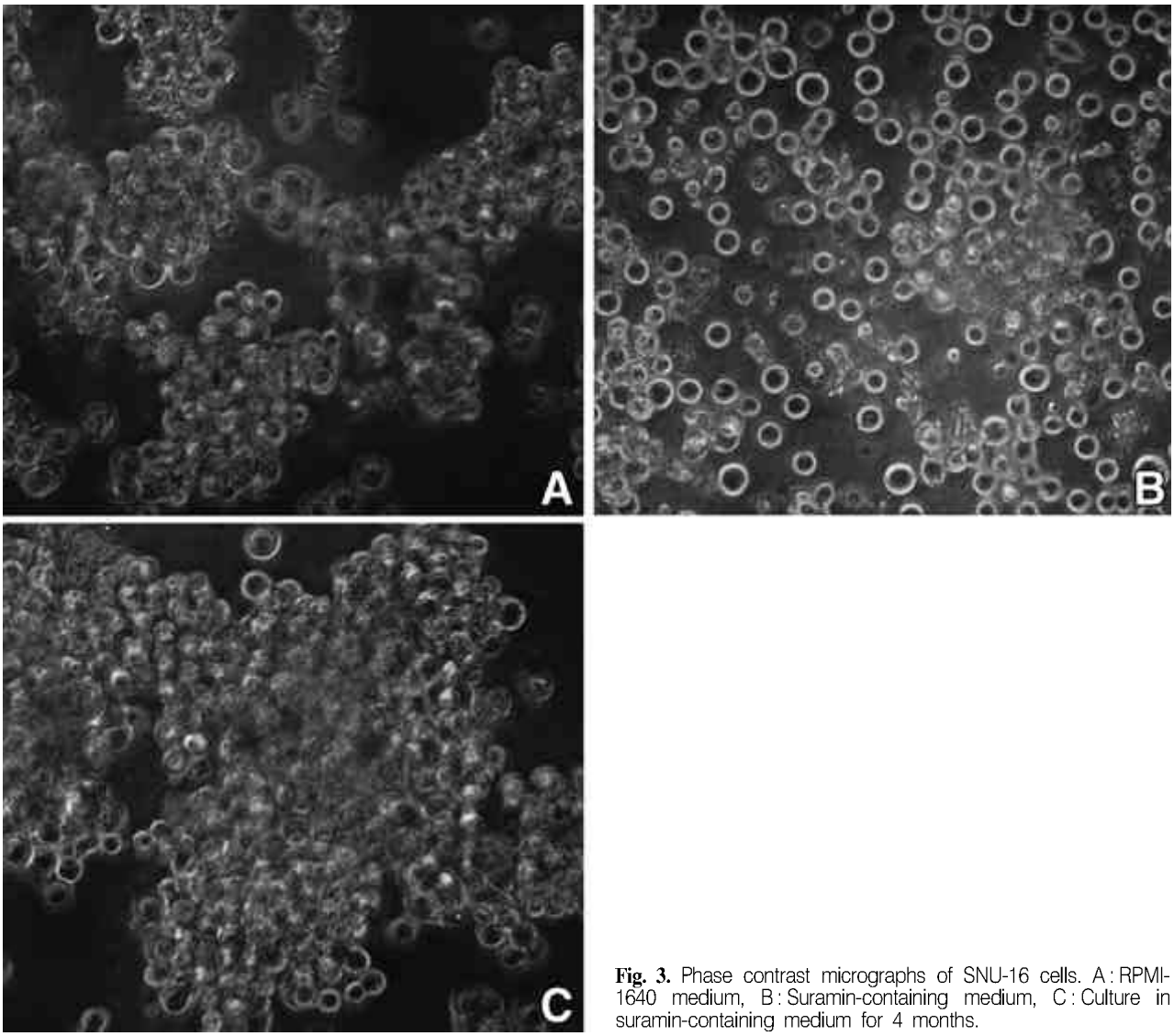

Fig. 3. Phase contrast micrographs of SNU-16 cells. A : RPMI-1640 medium, B : Suramin-containing medium, C : Culture in suramin-containing medium for 4 months.

levels corrected by viable cell number. The level of CEA released in the culture medium was higher in SNU-16 cells grown in the presence of suramin than in SNU-16 cells grown in the absence of suramin.

Electron microscopic observation

SNU-1 cells had a large irregular nucleus and prominent nucleoli with many mitochondria, rough endoplasmic reticulum and ribosomes in the cytoplasm. Microvilli were barely seen in the cytoplasmic membrane (Fig. 5), and therefore, SNU-1 cells were evaluated to be poorly differentiated (worst differentiation among three cell lines). SNU-1 cells grown in the presence of suramin frequently revealed karyolysis and many secondary lysosomes (Fig. 6). However, suramin induced no change in SNU-1 cells according to differentiation.

SNU-5 cells had a large irregular nucleus and prom-

Table 3. Measurement of CEA released in the culture medium of each cell line by RIA

Culture medium	SNU-1 (ng/ml)	SNU-5 (ng/ml)	SNU-16 (ng/ml)	Cell number of SNU-16	Corrected CEA level per 10^5 SNU-16 cells
RPMI-1640	3.3	2.9	84.4	4.0×10^5	21.1
Suramin + RPMI-1640	2.7	2.3	136.0	2.8×10^5	48.5

* CEA level lower than 6 ng/ml is insignificant

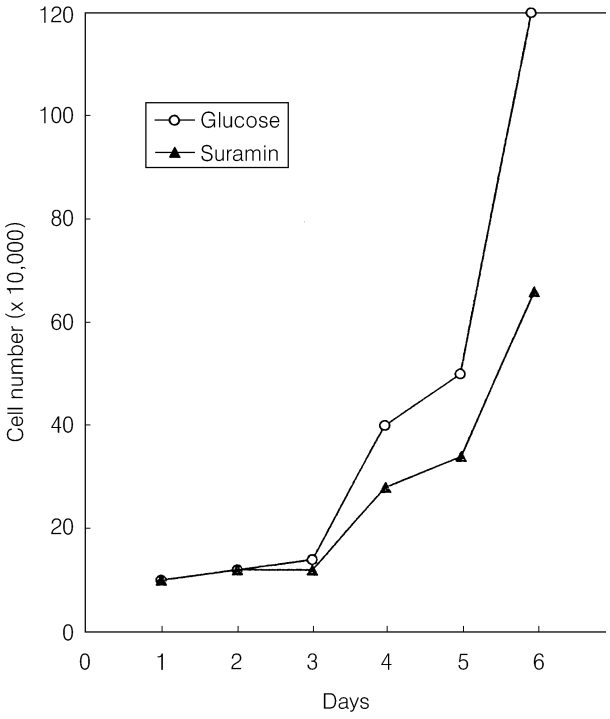

Fig. 4. Growth curves of SNU-16 cells. Note inhibition of tumor cell growth by suramin.

inent nucleoli with increased nuclear/cytoplasmic ratio (Fig. 7). Endoplasmic reticulum and mitochondria were well developed in the cytoplasm. SNU-5 cells disclosed more differentiated microvilli than SNU-1 cells, but less differentiated microvilli than SNU-16 cells. In SNU-5

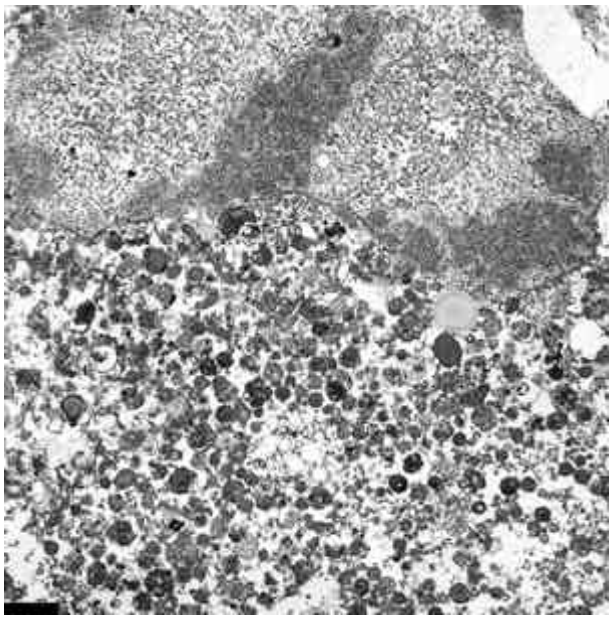

Fig. 6. Transmission electron micrograph of SNU-1 cells grown in suramin-containing medium. Note numerous secondary lysosomes and myelin figures in the cytoplasm (bar=1 μm).

cells grown in the presence of suramin, a specific intracellular structure, "intracytoplasmic lumen (ICL)", was noted (Fig. 8). The intracytoplasmic lumen was an intracytoplasmic cavity lined by microvilli, which displaced the nucleus and cytoplasmic organelles to one side. No communication was noted between the ICL and the cell exterior. Round intracellular structures with distinct

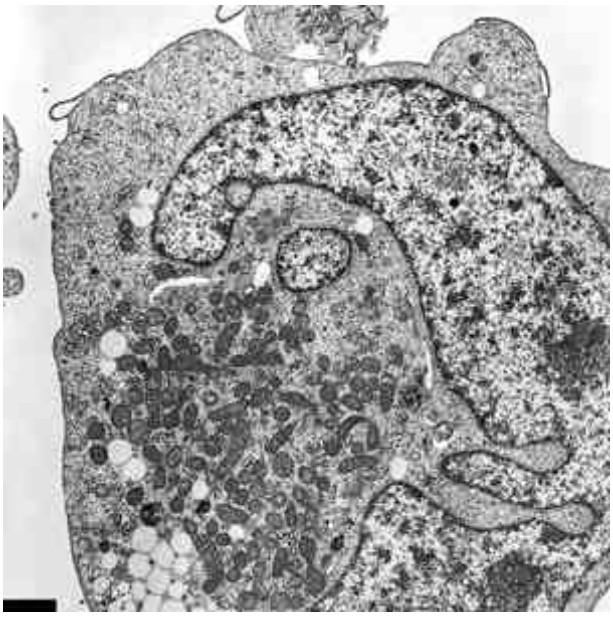

Fig. 5. Transmission electron micrograph of SNU-1 cells. Note poorly developed microvilli (bar=1 μm).

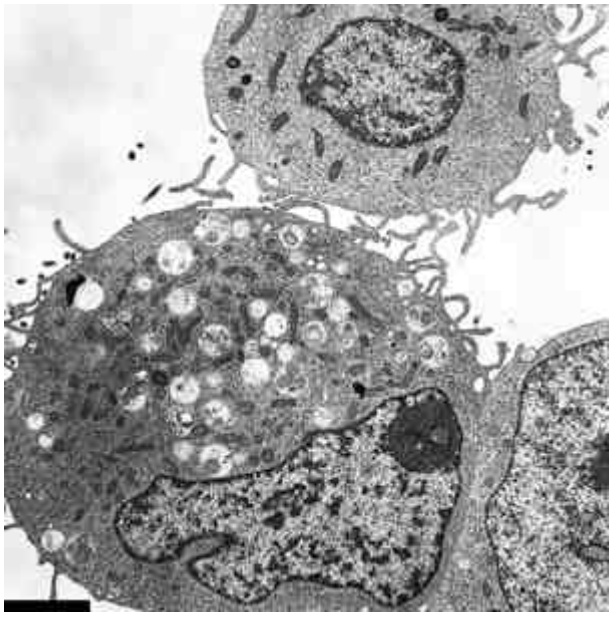

Fig. 7. Transmission electron micrograph of SNU-5 cells. Note moderate degree of microvilli development (bar=2 μm).

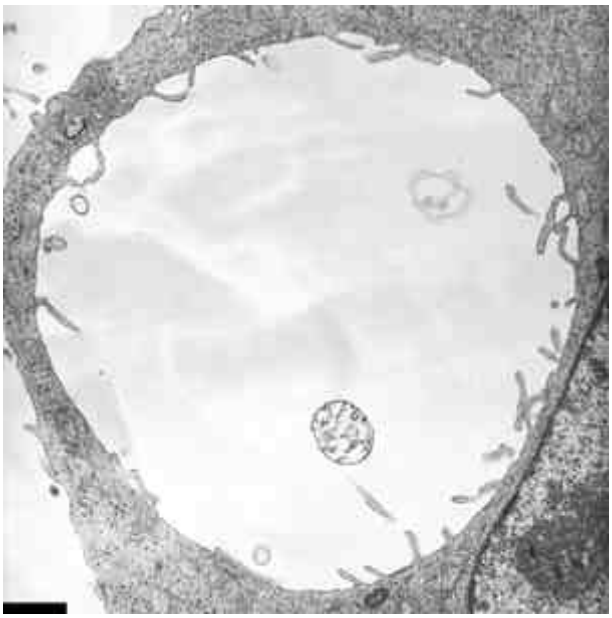

Fig. 8. Transmission electron micrograph of SNU-5 cells grown in suramin-containing medium. Note a intracytoplasmic lumen (ICL) lined by microvilli (bar=1 μm).

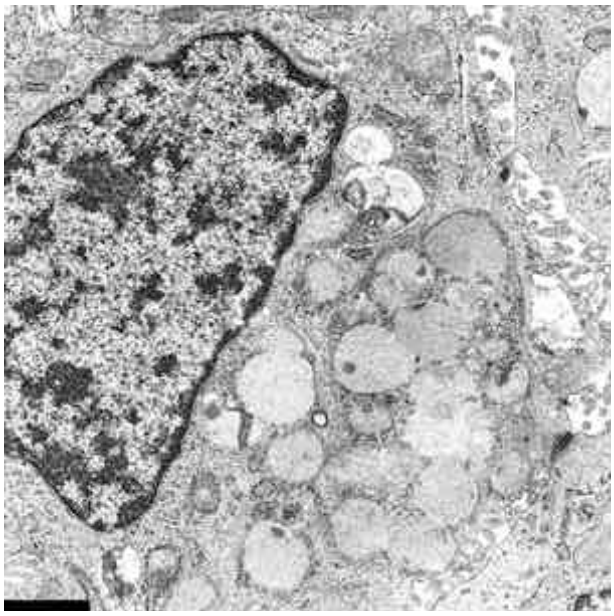

Fig. 9. Transmission electron micrograph of SNU-16 cells. Note abundant mucin granules in the cytoplasm with well developed microvilli and desmosomes (bar=1 μm).

phase contrast in the margin, which were observed by inverted microscope, were considered to be intracytoplasmic lumina.

SNU-16 cells had a large nucleus with irregular nuclear membrane and coarse heterochromatin. Mitochondria, endoplasmic reticulum and ribosomes were well developed and mucin granules were abundant in the cytoplasm. Many microvilli were present along the cytoplasmic membrane, and desmosomes were also frequently noted in the cell junctions (Fig. 9). SNU-16 cells were evaluated to be relatively well differentiated (best differentiation among three cell lines). SNU-16 cells grown in the presence of suramin disclosed numerous microvilli and more frequent desmosomes with formation of tightly cohesive clusters (Fig. 10). By inverted microscope, no ICL was observed in SNU-16 cells; however, ICL's were demonstrated by electron microscope in SNU-16 cells. In some cells, "intercellular lumen" was also noted (Fig. 11). The intercellular lumen lined by numerous microvilli was formed along the cell junctions including desmosomes, mimicking primitive gland formation composed of small numbers of tumor cells. Small ICL's were present in the vicinity of intercellular lumen in the same cell, which suggested a close relationship between intracytoplasmic lumen and intercellular lumen.

When suramin was eliminated from the culture medium after culture in suramin-containing medium for 4 weeks, no reversible change was induced. The degree of differentiation remained stationary in each cell line. The intracytoplasmic lumina were also observed in SNU-5 and SNU-16 cells.

DNA flow cytometry

By DNA histogram analysis, SNU-1 cells showed tetraploidy with a DNA index between 2.01 and 2.02.

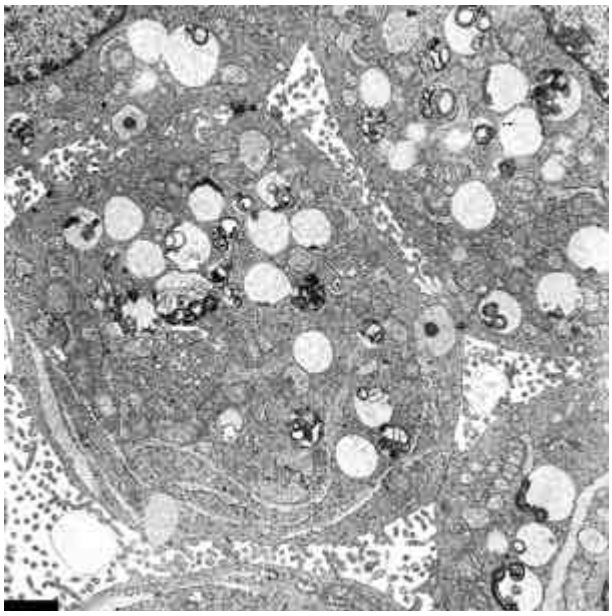

Fig. 10. Transmission electron micrograph of SNU-16 cells grown in suramin-containing medium. Note exuberant microvilli formation and more frequent desmosomes compared to Figure 9 (bar=1 μm).

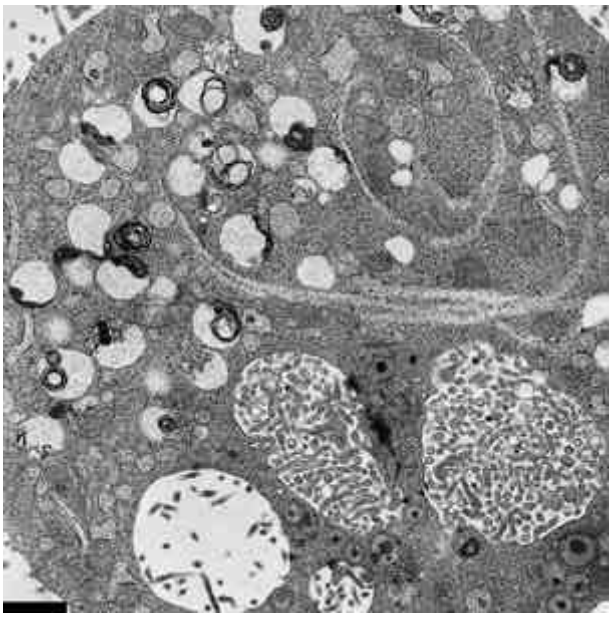

Fig. 11. Ultrastructural morphology of SNU-16 cells grown in suramin-containing medium. Note intercellular lumina lined by numerous tall microvilli and ICL's in a cell cluster. Desmosomes are present in the vicinity of intercellular lumina (bar=1 μm).

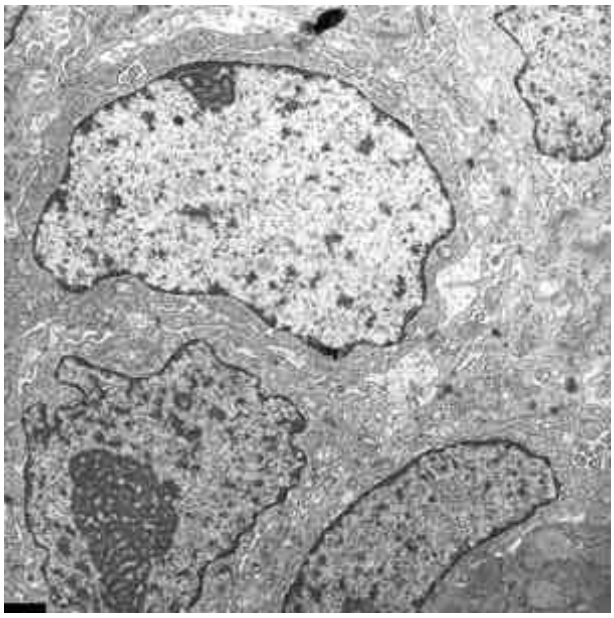

Fig. 12. Transmission electron micrograph of a heterotransplanted tumor originated from SNU-16 cells grown in the absence of suramin. Note a compact sheet of tumor cells with no gland formation. Nuclei have prominent nucleoli and a signet ring cell with abundant mucin granules is seen (bar=1 μm).

SNU-5 cells had a tetraploidy pattern with a DNA index between 1.97 and 2.02. SNU-16 cells revealed near-tetraploidy/tetraploidy with DNA indices ranging from 1.85 to 1.92. Suramin made no recognizable change on the DNA ploidy pattern in SNU-1, 5 and 16 cells.

Heterotransplantation

SNU-16 cells grown in the RPMI-1640 medium without suramin were tumorigenic in 3 out of 5 SCID mice. Sizes of the transplanted tumors were 7 mm, 5 mm and 5 mm in maximal diameter, respectively. SNU-16 cells grown in the presence of suramin were tumorigenic in only one out of 5 SCID mice. The size of the tumor was 3 mm in maximal diameter. The result could be interpreted as that suramin inhibited tumorigenecity of SNU-16 cells. No metastasis was detected in the internal organs of SCID mice. SNU-1 cells and SNU-5 cells were not tumorigenic with both culture media.

Electron microscopic examination was performed to compare the ultrastructure of transplanted tumors. The tumor originated from SNU-16 cells grown in the absence of suramin disclosed solid or compact sheets of tumor cells with rare gland formation. Signet ring cells, which had abundant cytoplasmic mucin granules and a peripherally displaced crescent nucleus, were also noted (Fig. 12). By contrast, the tumor originated from SNU-16 cells grown in the presence of suramin disclosed numerous well-developed gland formations, the lumina of which were lined by many microvilli and were filled with secretions (Fig. 13). Small intercellular lumina were also

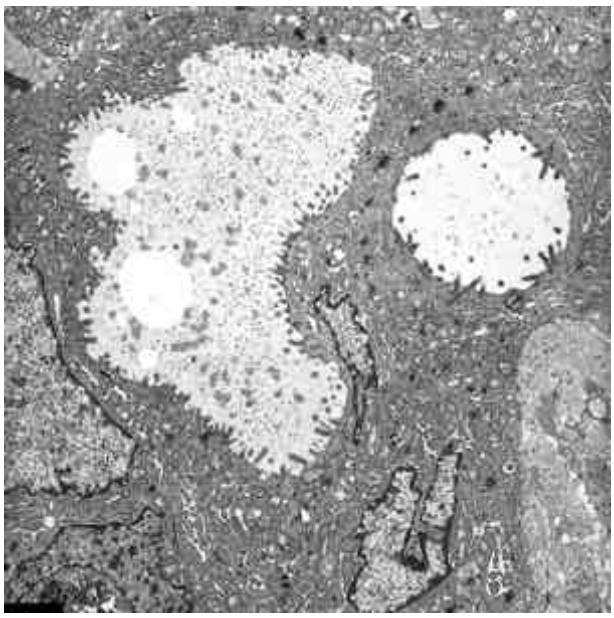

Fig. 13. Transmission electron micrograph of a heterotransplanted tumor originated from SNU-16 cells grown in the presence of suramin. Note well-developed intercellular lumina or glandular lumen formations. The lumina are lined by microvilli and are filled with secretions (bar=2 μm).

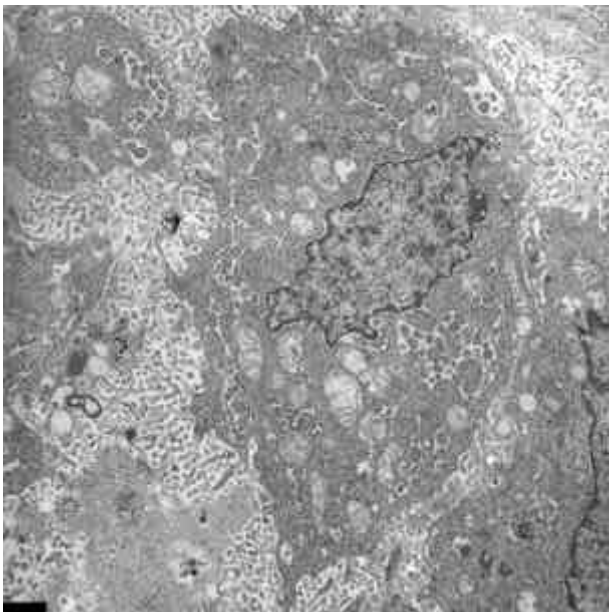

Fig. 14. Ultrastructural morphology of a heterotransplanted tumor originated from SNU-16 cells grown in the presence of suramin. Tumor cells reveal florid microvilli even in an area where tumor cells form a solid or diffuse sheet rather than gland formation (bar=1 μm).

present. Tumor cells revealed florid microvilli even in an area where tumor cells formed a solid or diffuse sheet rather than gland formation (Fig. 14). Ultrastructurally, the tumor originated from SNU-16 cells grown in the presence of suramin demonstrated a much higher degree of differentiation than that originated from SNU-16 cells grown in the absence of suramin.

DISCUSSION

This study shows that suramin inhibits growth of SNU-5 and SNU-16 cells and that suramin induces differentiation of SNU-16 cells. We found that BrdU-positive S-phase fraction was decreased by suramin in SNU-5 and SNU-16 cells (Table 2). Similarly, the growth curves (Fig. 4) demonstrated that the addition of suramin to the culture medium entailed a significant inhibition of cell growth. Several lines of evidence presented in this paper indicate that suramin induces differentiation of SNU-16 cells. First, SNU-16 cells grown in the presence of suramin disclosed numerous microvilli and more frequent desmosomes with formation of tightly cohesive clusters (Fig. 10). Second, SNU-16 cells grown in the presence of suramin disclosed intracytoplasmic lumina and intercellular lumina which are lined by numerous microvilli (Fig. 11). Third, suramin inhibited tumorigenecity of SNU-16 cells in SCID mice. Fourth, the transplanted tumor originated from SNU-16 cells grown in the presence of suramin demonstrated a much higher degree of differentiation than that originated from SNU-16 cells grown in the absence of suramin. Fifth, the level of CEA released in the culture medium was higher in SNU-16 cells grown in the presence of suramin than in SNU-16 cells grown in the absence of suramin.

Differentiation refers to the extent to which parenchymal cells of neoplasms resemble comparable normal cells, both morphologically and functionally (1). Well-differentiated tumor cells are thus composed of cells resembling the mature normal cells of the tissue of origin of the neoplasm. Therefore, in stomach cancer cells, development of microvilli and desmosomes, mucin production, cellular polarity and gland formation can be used as indicators to evaluate the degree of morphological differentiation. In our experiments, numerous microvilli and more frequent desmosomes with formation of tightly cohesive clusters were developed by addition of suramin in the culture medium of SNU-16 cells. Similarly, it can be confidently said that the transplanted tumor originated from SNU-16 cells grown in the presence of suramin showed a much higher degree of differentiation than that originated from SNU-16 cells grown in the absence of suramin. However, the significance of intercellular lumen and intracytoplasmic lumen is not yet established in relation to differentiation.

Intracytoplasmic lumen is described as a round cytoplasmic cavity which does not communicate with the cell exterior (16, 17). Intracytoplasmic lumina have been observed in various epithelial cancer cells in vivo and in vitro, for example in the breast, in the bladder, in the cervix, in the stomach and in the pleura. Some authors consider these structures to be characteristic of undifferentiated or poorly differentiated carcinomas. However, these structures are not characteristic of only carcinoma cells since they also appear in normal cells isolated for culture such as porcine thyroid cells, rat thyroid cells stimulated by thyroxin in vivo and fetal rat thyroid stimulated by thyrotropin in vitro (9). Nevalainen and Jaervi consider ICL as expression of a lost surface polarity, in other words, surface structure built inside the cytoplasm (16). Kim et al. consider that ICL reflects the ultrastructural expression of a compensatory unicellular secretory function by expansion of the surface area (18). Remy et al. consider that intracytoplasmic lumina represent both a compensatory mechanism for the loss of polarity and also an important step in the repolarization process (9). These observations support the view that the formation of intracytoplasmic lumen is an intracellular process closely related to differentiation.

Intercellular lumen is a more differentiated structure

than ICL. As found in our observations, the intercellular lumen consists of more than two cells which are tightly attached with well-developed desmosomes. Intercellular lumen is lined by numerous microvilli. Although intercellular lumen is relatively small, it mimics the lumen of gastrointestinal gland or tubule. It is an interesting finding that both intercellular lumen and ICL are present in the same cell (Fig. 11). This observation leads us to speculate that an intracytoplasmic lumen can differentiate to become an intercellular lumen, and then, to form a well-developed glanular lumen with terminal differentiation.

In our study, the level of CEA released in the culture medium was higher in SNU-16 cells grown in the presence of suramin than in SNU-16 cells grown in the absence of suramin. This finding is consistent with the observation of previous investigators that CEA release in the culture medium was correlated with cell differentiation (5).

In our study, when suramin was eliminated from the culture medium after culture in suramin-containing medium for 4 weeks, no reversible change was induced. This observation is contrary to that of an earlier study (6), in which the process of differentiation was fully reversible when suramin was removed from the culture medium. The discrepancy may be due to the duration of suramin treatment : 4 days in the earlier study vs. 4 weeks in our study.

In conclusion, this study shows that suramin inhibits the growth of SNU-5 and SNU-16 cells and that suramin induces differentiation of SNU-16 cells.

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