Establishment and Characterization of a SV40 T-Antigen Immortalized Epithelial-Like Cell Line Derived from the Newborn Rat Colorectum and Its Malignant Transformation by the ras Oncogene

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ABSTRACT. Epithelial-like cells from the colorectum of one-day-old newborn rats were immortalized by transfection with the simian virus 40 (SV40) T-antigen gene, and a cell line OUMS-25 was established. The cells were positive for the SV40 T-antigen, and immunoreactive to a colonic epithelial cell monoclonal antibody and a keratin-18 monoclonal antibody. Ultrastructural studies revealed the presence of microvilli on the cell surface and desmosomes between the adjacent cells. Karyotypic analysis showed that OUMS-25 cells were aneuploid. Cloning efficiency of the cells was 0.01% in soft agar. However, the cells were not tumorigenic in the syngeneic newborn rats. The cells were further transformed by transfection with the cloned activated c-Ha-ras oncogene containing a point mutation within codon 61. Characteristics of the activated-c-Ha-ras transfected cells (OUMS-25/RAS) were different in some respects from those of the parent cells (OUMS-25). OUMS-25/RAS cells demonstrated more malignant morphology, elevated cloning efficiency in soft agar, and tumorigenicity. This is the first report on the immortalization and malignant transformation of colorectal epithelial-like cells by transfection with a combination of SV40 T-antigen gene and cloned activated c-Ha-ras oncogene.

In recent years the increased mortality from colorectal cancer is predicted to exceed that from gastric cancer, presently the most deadly form of cancer in Japan, by the year 2000 (30). Recent studies have revealed that the combination of several genetic changes is associated with colorectal carcinogenesis (13). Cell lines derived from the normal colorectal epithelium will be useful for analyzing these genetic changes in the process of multistep carcinogenesis of the colorectum.

Although several cell lines have been established from colon cancer (11, 33, 34), they are of limited use for studies on analysis of multistep carcinogenesis of the colorectum. In attempts to culture normal colonic mucosa, two different explant culture methodologies have been applied: 1) a stationary method, in which colonic tissue from a hamster embryo is maintained for up to 3 weeks on human fibrin foam as a matrix (28); and 2) a rocking culture method, in which rat colonic tissue is maintained for at least 28 days (2). These cultures, however, resulted only in short-term maintenance.

Primary monolayer cultures of colonic epithelial cells have increasingly become successful owing to marked improvement of cell culture techniques (9, 10, 31, 35, 36). Several attempts have been done to immortalize colonic epithelial cells by using viruses, oncogenic plasmids, or chemical carcinogens. Moyer et al. (19, 20) reported that normal human colon mucosal cells showed increased longevity by infection with simian virus 40 (SV40), or by treatment with the chemical carcinogen azoxymethane. Berry et al. (4) also reported that the life span of human fetal colonic epithelial cells were considerably extended after transfection with the SV40 T-antigen gene. However, their attempts to establish cell lines by cloning colonies in soft agar or by continuous subculturing were unsuccessful. Recently, Pories et al. (23, 24) established cell lines from normal rat colonic epithelium by the combined introduction of oncogenes such as myc/ras and myc/src.

In the present study, we tried to obtain colorectal epithelial cell lines from normal newborn rats by transfection with a recombinant plasmid containing the SV40 T-antigen gene. We established a new immortalized cell line, OUMS-25, from the newborn rat colorectum. The cell line showed some characteristics of colorectal epithelium. Furthermore, OUMS-25 cells was transformed into the malignant ones by transfection with the cloned activated c-Ha-ras oncogene.
MATERIALS AND METHODS

Cell preparation from the colorectum. Segments of the large bowel (cecum-rectum) were resected from one-day-old newborn Donryu rats, opened to expose the mucosal surface, and the epithelial sheets were carefully peeled off from the underlying submucosa with a pair of forceps under a dissecting microscope. The epithelial sheets were pooled, cut into small pieces, and incubated in a 0.05% collagenase (Type I, Sigma Chemical Co., St. Louis, MO) solution containing antibiotics (1 mg/ml penicillin, 500 µg/ml kanamycin and 1 µg/ml Fungizone) for 1.5 h at room temperature. Crypts were isolated by pipetting, and washed twice with a basal medium of 1 : 1 mixture of Ham’s medium F12 (Nissui Pharmaceutical Co., Tokyo, Japan) and Dulbecco’s modified Eagle medium (Nissui), which was supplemented with 6% fetal bovine serum (FBS). The crypts were suspended in a basal medium containing 5% FBS, 30% Hep G2 [a human hepatoblastoma cell line (1)]-conditioned medium and the antibiotics described above, and then inoculated into collagen-coated 60-mm plastic dishes (Corning glass works, Corning, NY). The cultures were maintained at 37°C in a humidified atmosphere of 5% CO2 in air. The culture medium was renewed twice a week.

Recombinant plasmids. The plasmid used for transfection, pSV3neo, is a pBR322 recombinant containing the SV40 early T antigen-coding region genes including the early region promoter, and the bacterial neomycin phosphotransferase gene which confers resistance to the antibiotic geneticin (G418, a derivative of neomycin) (32). The plasmid, pras-neo, is also a pBR322 recombinant containing the 6-kilobase Eco RI fragment of the activated c-Ha-ras oncogene cloned from human lung carcinoma, which has a point mutation (conversion of CAG into CTG) at codon 61 in the second exon (38), and the bacterial neomycin phosphotransferase gene. The inserted Eco RI fragment includes the c-Ha-ras oncogene promoter.

Transfection and isolation of transformed cells. Colorectal epithelial-like cells (1 × 10⁶ cells/60-mm dish) were transfected by the calcium phosphate procedure with the plasmid pSV3neo (20 µg DNA/dish) one day after the initiation of culture (7). The cells were incubated with calcium phosphate/DNA coprecipitate for 24 h at 37°C and 3% CO2. The cells were then washed once with the culture medium, and continued to be cultured. Four days after transfection, G418 (100 µg/ml; Gibco Laboratories, Grand Island, NY) was added to the cultures for 25 h to select neomycin-resistant cells. On day 118, surviving cell colonies were washed with Ca2+- and Mg2+- free phosphate-buffered saline (PBS), covered with pieces of filter paper saturated with PBS containing 0.1% trypsin (1 : 250, Difco Laboratories, Detroit, MI) and 0.02% EDTA (Sigma), and incubated at 37°C for 5 min. The trypsinized cells were transferred with the filter paper into a collagen-coated 24-well-plate, each well of which contained 0.5 ml of culture medium, and cultured under the same conditions described above. Thereafter, the cells were serially subcultured by trypsinization. On day 93, semiconfluent cells were cotransfected with the plasmid pras-neo (20 µg DNA/60-mm dish) and the Blasticidin S resistant gene-containing plasmid pSV2brs (2 µg DNA/60-mm dish, Funakoshi Co., Ltd., Tokyo, Japan) by the calcium phosphate procedure (7). The cells were incubated with the calcium phosphate/DNA coprecipitate for 20 h under the same conditions as described above. Eleven days after transfection (on day 104), Blasticidin S (1.5 to 2.0 µg/ml; Funakoshi) was added to the cultures for 92 h to select the antibiotic-resistant cells. On day 118, surviving cell colonies were picked up as described above.

Immunofluorescent analysis. Cells grown on glass coverslips were rinsed with PBS and then fixed with acetone for 15 min at room temperature (for detection of SV40 T-antigen and colonic epithelial antigen), or with methanol/acetone (3/1, v/v) for 20 min at −20°C (for cytokeratins). In all cases, the fixed specimens were reacted with the first antibodies for 1 h at 37°C, and washed three times with PBS. The specimens were then incubated with fluorescein isothiocyanate (FITC)-conjugated secondary antibodies for 30 min at room temperature, and washed three times with PBS. In all cases, cells were mounted in glycerol/PBS (10/1, V/V), and observed under a fluorescence microscope.

Antibodies. To detect SV40 T-antigen, serum from Syrian golden hamsters bearing SV40-induced tumor (22) was used. The anti-SV40 T-antigen hamster serum was kindly supplied by Professor Shuji Seki (Department of Molecular Biology, Institute of Molecular and Cellular Biology, Okayama University Medical School, Okayama 700, Japan). For detection of the colonic epithelial antigen (25), mouse monoclonal antibody (PR4D2) against colonic mucus, epithelial cells and cell membranes was purchased from Serotec (England). For cytokeratins, the following anti-keratin mouse monoclonal antibodies were used: KL-1, an antibody raised against keratin polypeptide of 55,000-57,000 Da (Dianova, Germany); CK-5, an antibody specific for keratin polypeptide 18 (Sigma); CK-7, an antibody specific for keratin polypeptide 7 (Amersham International PLC, England). FITC-conjugated antibodies were used as follows: for SV40 T-antigen stain, rabbit antihamster immunoglobulin (a gift from Professor Shuji Seki); for the colonic epithelial antigen, sheep anti-mouse IgG3 (Serotec); for cytokeratins, sheep anti-mouse IgG1 (Serotec).

Morphological characterization. For scanning electron microscopy, cells were cultured on glass coverslips for 2 days; fixed with 2.5% glutaraldehyde for 2 h; postfixed with 1% osmium tetroxide for 1 h; dehydrated in a graded ethanol series; replaced in isoamylacetate; and dried by the critical-point method using a critical-point dryer (HPC-1, Hitachi, Tokyo, Japan). They were then coated with Pt-Pd using an Eiko Ion Coater (model LB-3) and observed under a scanning electron microscope (Hitachi S-2300). For transmission electron microscopy, cells were postfixed with osmium tetroxide, dehydrated with ethanol and embedded in Epon 812. Ultrathin sections were stained with uranyl acetate and lead citrate, and observed under a transmission electron microscope (JEM, 1988).
Collagen gel culture. Collagen solution (0.3%) prepared from rat tails (16) were mixed with 10-times concentrated Ham's medium F12 and reconstitution buffer (0.26 M \( \text{NaHCO}_3 \) in 0.14 M \( \text{NaCl} \)) at a ratio of \( 8:1:1 \) (v/v/v), and the mixture was kept on ice, according to the method of Enami et al. (12). One ml aliquots of the mixed solution were placed on 35-mm dishes, and immediately gelled by incubating at 37°C. The above-mentioned mixed solution (2 ml) containing cells were then overlayed and gelled in the same way. Finally, the cells were fed with 1 ml of culture medium, and incubated at 37°C in 5% \( \text{CO}_2 \). The culture medium was renewed twice a week.

Measurement of alkaline phosphatase activity (ALP). ALP activity was determined by the method of Lowry et al. (15) using \( \text{p-nitro phenylphosphate} \) as a substrate. Cells were scraped into 0.2% Nonidet p-40 (Sigma) by a rubber policeman after being washed three times with PBS, and disrupted by ultrasonication. Newborn rat colorectum tissues were also disrupted by ultrasonication in 0.2% Nonidet p-40 after being thoroughly washed with PBS. The cell and tissue homogenates were treated with \( \text{n-butanol} \) at a ratio of \( 1:1 \) (v/v) to remove lipoproteins binding to ALP (18). Aqueous layers were separated by centrifugation at \( 2,500 \times g \) and \( 4^\circ C \) for 45 min, and used for the enzyme assay.

Determination of cell growth and cloning efficiency in soft agar. For determination of cell growth rate, cells were seeded at a density of \( 1 \times 10^5 \) cells/35-mm collagen-coated dish (Corning) in 2 ml of basal medium containing 0.1% FBS. On days 1, 2, 4 and 7 of culture, the cells were detached by treatment with 0.1% trypsin and 0.02% EDTA, and the viable cell number was determined by trypan blue exclusion test in a hemocytometer. Each point represents the average of two dishes. Population doubling time was calculated from the log-phase growth curve.

For estimation of cloning efficiency in soft agar, \( 1 \times 10^5 \) cells were suspended in 2 ml of 0.33% agar (Difco) in basal medium and seeded on 4 ml of 0.5% agar medium in 55-mm glass petri dishes, and incubated at 37°C in 5% \( \text{CO}_2 \). Two weeks after inoculation, colonies were counted, and the cloning efficiency was determined as the percentage of the inoculated cells which formed colonies. Each test was done in duplicate.

Northern blot analysis. Total cellular RNA was isolated from cell monolayers by lysis in guanidine thiocyanate (8). Denatured RNA samples (10 \( \mu \)g) were size-fractionated by electrophoresis on 1% agarose gel containing 2.2 M formaldehyde, transferred to a Hybond-N+ nylon membrane (Amer sham International PLC, Buckinghamshire, England), and hybridized to the \( ^{32} \text{P}-\text{labeled} \) c-Ha-ras probe. The membrane was then exposed to X-ray film between two intensifying screens at \(-80^\circ C\).

Southern blot analysis. Genomic DNA was isolated by the standard methods, cleaved with EcoRI (Toyobo, Tokyo, Japan), resolved by electrophoresis through 0.8% agarose gel, transferred to the nylon membrane, and hybridized to the \( ^{32} \text{P}-\text{labeled} \) c-Ha-ras probe in the presence of 50% formamide at 42°C for 12 h (27). After washing thoroughly, the membrane was exposed to X-ray film between two intensifying screens for seven days at \(-80^\circ C\).

Reverse transcriptase-polymerase chain reaction (RT-PCR). Randomly primed cDNAs were prepared from 1 \( \mu \)g of total RNA by reverse transcriptase (Toyobo) following PCR amplification by thermal cycler (37). To amplify the sequence of c-Ha-ras exon 2 region including codon 61, the fol-
lowing two primers were used: 5'-AGATCTACCGGAAGCAGGTGG-3' and 5'-AGATCCCCGGTGCGCATGTAC-3' (17). The amplified DNA samples were electrophoresed on 4% agarose gel [3% NuSieve (FMC, Rockland, ME) and 1% SeaKem GTG agarose (FMC)] and stained with ethidium bromide.

**Cytogenetic studies.** Colcemide (0.1 µg/ml) was added 4 h before cell harvest. The cells were fixed with a 3:1 mixture of methanol and acetic acid. For banding, the chromosome preparations were stained with 2% Giemsa after trypsin treatment according to the standard procedure (29). In each case, about 100 metaphases were counted.

**Test for tumorigenicity.** To examine tumorigenicity, 1 x 10⁷ cells were injected subcutaneously into the syngeneic newborn rats. All animals were observed for up to 3 months.

**RESULTS**

Epithelial-like cells from the colorectum of newborn rats were transfected with the plasmid pSV3neo containing SV40 T-antigen gene and neomycin-resistant gene one day after initiation of primary culture. From four

![Fig. 2](image.png)

**Fig. 2.** Phase-contrast micrographs of rat colorectal epithelial-like cells on day 92. A: OUMS-25 cells. B: OUMS-25/RAS (human activated c-Ha-ras oncogene-transfected) cells. Bar indicates 100 µm.
days after transfection, cells were treated with G418 (a derivative of neomycin) at 100 μg/ml for 24 h. We decided upon these conditions, because the rat colorectal cells were very sensitive to cytotoxic effects of the drug at more than 100 μg/ml concentrations. Twenty-one days after G418 treatment, an epithelial-like cell strain, OUMS-25, was obtained by cloning a colony. OUMS-25 cells grew well, but untransfected colorectal cells did not grow. Under any conditions tested, the survival of primary colorectal cultures never exceeded 2 weeks.

On day 93, OUMS-25 cells were further cotransfected with the plasmid pras-neo containing the cloned activated c-Ha-ras oncogene and the plasmid pSV2bsr containing Blasticidin S-resistant gene. From 11 days after cotransfection, the cells were treated with Blasticidin S at 1.5–2.0 μg/ml for 96 hr. On day 118, Blasticidin S-resistant cells (OUMS-25/RAS) were obtained by cloning a colony. As shown in Fig. 1, both OUMS-25 and OUMS-25/RAS cells grew well without crisis and reached over 100 population doublings at the present time. Thus, both cell lines are considered to have been immortally transformed.

OUMS-25 cells exhibited compactly packed polygonal-cell monolayers (Fig. 2A). The cells did not exhibit criss-crossed or piled-up foci. On the other hand, OUMS-25/RAS cells were more closely packed, and tended to pile up and to detach from the surface of culture dishes (Fig. 2B). Scanning electron microscopy of OUMS-25 cells showed the presence of microvilli on cell surfaces and in intercellular spaces (Fig. 3A). OUMS-25/RAS cells were somewhat round in shape with numerous microvilli (Fig. 3B). Transmission electron microscopy of OUMS-25 (Fig. 4) and OUMS-25/RAS cells showed that these cells had microvilli on their apical surfaces and desmosomes between cells. In collagen gel culture, both OUMS-25 (Fig. 5) and OUMS-25/RAS cells formed a duct-like structure.

Expression of SV40 T-antigen was examined by immunofluorescent staining in OUMS-25 and OUMS-25/RAS cells. Through the entire culture period, SV40 T-antigen was observed in the nuclei of all the cells of both the cell lines (data not shown). Colorectal origin of OUMS-25 (Fig. 6) and OUMS-25/RAS cells was confirmed by immunofluorescent staining of the colonic epithelial cell antigen. This antigen was localized in the cytoplasm. Furthermore, keratin 18 was demonstrated in both OUMS-25 (Fig. 7) and OUMS-25/RAS cells by immunofluorescent staining with a monoclonal antibody CK-5, which reacts specifically with a variety of simple epithelia, e.g., intestine and liver, but does not react with stratified squamous epithelial or non-epithelial cells. In contrast to these findings, the cells did not react...
to monoclonal antibodies against epidermal keratinocytes (KL-1) and glandular epithelia (CK-7).

Alkaline phosphatase (ALP) activity was measured in OUMS-25 and OUMS-25/RAS cells at the different culture ages (Table I). On day 121, OUMS-25 cells had 50% of ALP activity in the colorectal mucosa of newborn rats. ALP activity in the cells decreased with time in culture. By day 231, both OUMS-25 and OUMS-25/RAS cells became negative for this enzyme.

As shown in Fig. 8, the number of chromosomes in OUMS-25 cells on day 51 was distributed broadly in the diploid to hypotetraploid region with a modal chromo-

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Fig. 5. Collagen gel culture of OUMS-25 cells. The cells on day 120 were inoculated into collagen gel and cultured for 14 days. The cells grew and formed a duct-like structure. Bar indicates 100 μm.

Fig. 6. Immunofluorescent staining for the colonic epithelial antigen. OUMS-25 cells on day 49 were reacted with mouse monoclonal antibody against colonic epithelial cells and then incubated with FITC-conjugated sheep antimouse immunoglobulin. The colonic epithelial antigen is visualized in cytoplasm of the cells. Bar indicates 100 μm.
Transformation of Normal Rat Colorectal Cells

Fig. 7. Immunofluorescent staining for cytokeratin. OUMS-25 cells on day 49 were reacted with an anti-keratin mouse monoclonal antibody (CK-5) and then incubated with FITC-conjugated sheep anti-mouse immunoglobulin. Keratin 18 is visualized in cytoplasm of OUMS-25 cells. Bar indicates 100 μm.

some number of 42 (23%). The number of cells distributed in the hyperdiploid to hypotriploid region increased in the culture of OUMS-25 cells during serial passage up to at least day 147. On the other hand, the chromosome number in OUMS-25/RAS cells on day 147 were distributed in the hypotriploid to hypotetraploidal region with a modal chromosome number of 52 (12%). Thus the numerical and structural aberrations of the chromosomes were profound both in OUMS-25 and OUMS-25/RAS cells, but no marker chromosomes were found in these cells. In addition, it was noticeable that introduction of the activated c-Ha-ras oncogene caused a remarkable change in the chromosome distribution.

OUMS-25/RAS cells grew more rapidly than OUMS-25 cells. The average population doubling times of OUMS-25 and OUMS-25/RAS cells were 27.7 and 21.4 h in the basal medium containing 0.1% FBS, respectively. After reaching confluence, many floating cells were observed in OUMS-25/RAS culture. This indicated that OUMS-25/RAS cells continued to grow even after reaching confluence.

Both OUMS-25 and OUMS-25/RAS cells could grow in soft agar at cloning efficiencies of 0.01

<table>
<thead>
<tr>
<th>Tissue or cell line</th>
<th>ALP activity (IU/l)</th>
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<tr>
<td>New born rat colorectum mucosa</td>
<td>64</td>
</tr>
<tr>
<td>OUMS-25</td>
<td>31</td>
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<tr>
<td>OUMS-25/RAS</td>
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Table 1. Alkaline phosphatase (ALP) activity in rat colorectal epithelial-like cell lines, OUMS-25 and OUMS-25/RAS.
Fig. 9. Southern blot analysis of restriction enzyme EcoRI digested DNAs extracted from rat colorectal epithelial-like cell lines OUMS-25 and OUMS-25/RAS using the human c-Ha-ras probe. Lane 1: OUMS-25 cells on day 280. Lane 2: OUMS-25/RAS (activated c-Ha-ras oncogene-transfected) cells on day 280. A faint band (a) of 8.6-kbp corresponding to the endogeneous rat c-Ha-ras was detected in both lanes 1 and 2. However, two extra sharp bands (b, 10.8 and c, 4.0 kbp) corresponding to the exogeneous activated human c-Ha-ras was detected only in lane 2.

and 1.9%, respectively. Tumorigenicity of OUMS-25 and OUMS-25/RAS cells on day 208 was then examined by subcutaneous injection into the syngeneic newborn rats. As a result, OUMS-25 cells formed no tumors in the host animals, whereas OUMS-25/RAS cells formed tumors at a take rate of 3/5. There results suggested that introduction of activated c-Ha-ras oncogene caused malignant transformation of the immortal colorectal OUMS-25 cells.

To confirm the above mentioned possibility, an attempt was made to determine whether the transfected activated c-Ha-ras oncogene might really be integrated in the host genome and expressed. Southern blot analysis using the c-Ha-ras probe revealed two additional bands corresponding to the transfected c-Ha-ras in OUMS-25/RAS cells (Fig. 9). Expression of the integrated activated-c-Ha-ras oncogene was analyzed by Northern blot analysis. Constitutive expression of c-Ha-ras was observed as a band of 1.4 Kb in both OUMS-25 and OUMS-25/RAS cells (data not shown). Discrimination between the mRNA transcripts of the endogeneous and the transfected c-Ha-ras genes was impossible, because both transcripts were the same size (1.4 Kb), hence for the analysis of the expression of the integrated activated-c-Ha-ras mRNA, the RT-PCR method (37) was used. As a result, amplification of the sequence of c-Ha-ras exon 2 including codon 61 was detected as a band of 113 bp (arrow) only in OUMS-25/RAS cells (lane 2).

Fig. 10. Expression of integrated activated human c-Ha-ras mRNA in OUMS-25/RAS cells. Total RNA samples (1 μg) extracted from OUMS-25 and OUMS-25/RAS cells were analyzed by RT-PCR. PCR products were electrophoresed and stained with ethidium bromide. Lane 1: OUMS-25 cells on days 280, lane 2: OUMS-25/RAS (activated human c-Ha-ras oncogene-transfected) cells on day 280. Amplification of the sequence of activated c-Ha-ras exon 2 including codon 61 was detected as a band of 113 bp (arrow) only in OUMS-25/RAS cells (lane 2).

DISCUSSION

The main purpose of this work was to develop a culture system suitable for investigating the mechanisms of colorectal carcinogenesis. In this report, we described a novel and highly reproducible in vitro transformation system for epithelial-like cells from the colorectum of newborn rats by introduction of the SV40 T-antigen gene and the cloned activated c-Ha-ras oncogene.

We succeeded in isolating colorectal epithelial-like cells by employing a combination of mechanical treatment and collagenase digestion. The mucosal layer was carefully peeled off from the underlying submucosa using a pair of forceps under a dissecting microscope. This procedure caused little trauma on the epithelial-
like cells and efficiently avoided contamination of fibroblasts. Most of the crypts were released from the mucosal layer by incubation with 0.05% collagenase for 1.5 h and by gentle pipetting. The isolated crypts were structurally intact and had high viability, thus they adhered easily to the collagen coated dishes. In addition, to further enhance spreading of colorectal epithelial cells, we used the conditioned medium obtained from Hep G2 human hepatoblastoma cell cultures, because these cells synthesized and secreted human serum spreading factor under the conventional culture conditions (3). In fact, this conditioned medium efficiently promoted attachment and spreading of newborn rat colorectal epithelial-like cells.

Since normal colorectal epithelial-like cells hardly grew and their survival did not exceed 2 weeks in primary culture, it was important to find a way of extending their replicative potential. Introduction of the SV40 T-antigen gene into rat colorectal epithelial-like cells by the standard calcium phosphate method was successful in extending the lifespan of the cells. The prolonged lifespan of the cells (OUMS-25) coincides with expression of the SV40 T-antigen in the cells, as revealed by intranuclear immunofluorescence. Human colorectal epithelial cells transfected with SV40 DNA also showed extended lifespans but senesced (4, 19, 20). However, in the present study, OUMS-25 cells have reached over 100 population doublings without culture crisis, and are considered to have been immortalized. The difference in immortalization frequency between human and rat colorectal cells may be dependent on their genetic stability. In fact, OUMS-25 cells showed remarkable aberrations of chromosomes as early as on day 51 in culture.

Ultrastructural studies showed that both the SV40 T-antigen immortalized OUMS-25 and the activated c-Ha-ras transfected OUMS-25/RAS cells had numerous microvilli on the cell surfaces and in intercellular spaces, and had desmosomes between adjacent cells. Both OUMS-25 and OUMS-25/RAS cells were stained for the epithelial antigen (25) and for cytokeratin 18. Furthermore, these cells formed a duct-like structure in collagen gel culture. However, neither OUMS-25 nor OUMS-25/RAS cells exhibited production of mucous, a typical characteristic of the colorectal epithelium (data not shown). In addition, ALP activity in OUMS-25 cells disappeared after day 121 in culture. These findings possibly indicate that OUMS-25 and OUMS-25/RAS cells arise from epithelial cells that dedifferentiated prior to immortalization. The undifferentiated phenotype in these cell lines may also be due to their derivation from immature colorectal tissue of newborn rats.

The development of human colon cancer is potentially supposed to be related to genetic alterations involving tumor suppressor genes and oncogenes, such as p53, MCC, APC, DCC (6, 13). Moreover, it has been reported that human colonic neoplasia involved mutational activation of the ras proto oncogenes (5, 14). In the present study, the activated-c-Ha-ras transfected OUMS-25/RAS cells acquired tumorigenicity, whereas the parent OUMS-25 cells did not. These findings indicate that immortalization alone by the SV40 T-antigen is insufficient to induce tumorigenicity, and that the activated c-Ha-ras oncogene may play an important role in the malignant transformation of immortalized colorectal epithelial-like cells. However, the v-Ha-ras oncogene alone did not immortalize normal rat colon epithelial cells (23, 24). Similarly normal human foreskin fibroblasts could not be immortalized or neoplastically transformed only by introduction of the EJ gene, a mutant from of c-Ha-ras proto oncogene (26). In addition, Newbold et al. (21) reported that immortalization was prerequisite for neoplastic transformation of hamster fibroblasts by the EJ c-Ha-ras oncogene. Taken together, it is likely that at least two processes, immortalization of cells and activation of the c-Ha-ras proto oncogene, are involved in neoplastic transformation of rat colorectal epithelial cells.

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