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Neoplastic Transformation of Rat Colon Epithelial Cells by Expression of Activated Human K-*ras*

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Somatic mutations of the K-*ras* oncogene play an important role in colorectal carcinogenesis. We determined whether rat colon epithelial cells could be transformed by introducing retroviruses carrying the activated human K-*ras* oncogene alone. Primary epithelial cells from the rat distal colon were infected with retroviruses carrying wild-type and two types of activated K-*ras* (asp and val at codon 12) cDNAs. Cells infected with the wild-type K-*ras* virus showed no change in morphology and died within 3 weeks, whereas the activated K-*ras* virus-infected cells underwent morphological changes within 3 days and continued to proliferate. From these cells, several cell lines were subsequently established. Epithelial cells transformed by activated K-*ras* formed colonies in soft agar culture and tumors in athymic nude mice. Multiple copies of human K-*ras* genes and large amounts of K-*ras* mRNAs and proteins were found in the transformed cells. These data suggest that overexpression of activated K-*ras* transforms rat colon epithelial cells.

Key words: Colon — Epithelial cell — Human K-*ras* — Transformation — Rat

Somatic mutations in the K-*ras* oncogene have been detected in various human tumors and there is a particularly high frequency of mutation in pancreatic and colorectal cancers. A frequency of K-*ras* mutation of more than 80% was found in pancreatic hyperplasias and carcinomas.[1)] Mutations of the K-*ras* gene are most frequently found in codon 12 or 13.[2, 3)] Overexpression of mutated K-*ras* gene was reported in some human colon carcinomas.[4, 5)] While approximately 50% of colorectal carcinomas harbor K-*ras* mutations,[2, 3)] the mutation occurs with a frequency of more than 80% in aberrant crypt foci (ACF), a putative precursor of human colorectal cancer.[6)] We previously suggested that some ACF showing hyperplasia might develop into adenomas,[6)] and that K-*ras* activation might be the critical event in the formation of certain colon cancers.

To know the effects of K-*ras* activation on the early steps of colon carcinogenesis, it is necessary to establish cell lines of colon epithelial cells carrying activated K-*ras* gene *in vitro*. Primary epithelial cells of rat colon were infected with retroviruses that harbor activated human K-*ras* gene. The results demonstrated that an activated human K-*ras* gene alone caused neoplastic transformation of these cells in culture.

MATERIALS AND METHODS

Construction of retrovirus carrying the human K-*ras* gene Wild-type and activated K-*ras* cDNAs containing full-length coding sequences were synthesized from the total RNAs of human placenta and pancreatic carcinoma cell lines PSN-1[7)] and KP-3[8)] by reverse transcription-polymerase chain reaction (RT-PCR), using primers (FX2 and RX2), which correspond to noncoding regions of the human K-*ras* gene [9)] (Fig. 1), and contain a *Xho*I site. The sequences of the primers were as follows: FX2, GGGGCTCGAGGCTCAGCGGCTCCCAGGTG; RX2, ATTCTCGAGTGCTAAAACAAATGCTAATA. Codon 12 of the K-*ras* gene in PSN-1 and KP-3 cells is known to encode aspartic acid and valine, respectively. The cDNAs were subcloned, sequenced, and integrated into the *Xho*I cloning site of a Molony murine leukemia virus vector (pLXSN), which was a generous gift from Dr. A. D. Miller.[10)] Stocks of ecotropic recombinant K-*ras* viruses were obtained through transformation into Psei-2 cells. The titration of virus was done at 10^6 pfu/ml by focus-forming assay in NIH 3T3 cells. The culture media containing K-*ras* viruses were filtered and then stored at –80°C. The absence of helper virus in the K-*ras* virus stocks was confirmed by the horizontal spread of the reporter virus in indicator cells (208F/LRT-GFP) carrying green-fluorescent protein (GFP) gene, which was a kind gift from Drs. T. Watsuji and M. Hagiwara.[11)]

Primary culture and virus-infection of rat colon epithelial cells Primary culture of rat epithelial cells was carried out by the method of Vidrich *et al.*[12)] A segment of distal colon (~2 cm) was resected from a 3.5-week-old female F344 rat, opened to expose the mucosal surface, and cut into small pieces, which were incubated with Dispase I (1.6 U/ml, Boehringer Mannheim, Germany) in Dulbecco's modified Eagle's essential medium (DMEM; Nissui Pharmaceutical, Co., Ltd., Tokyo) containing 5%

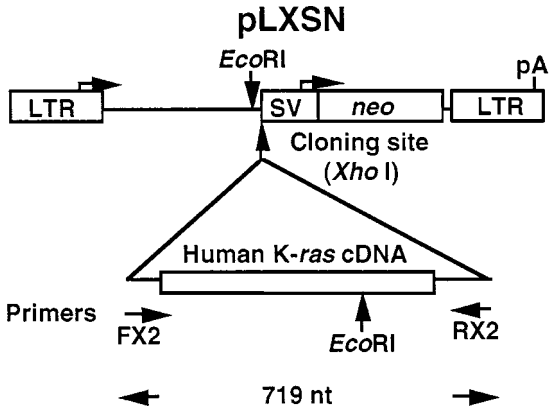

Fig. 1. Construction of recombinant retroviruses. Wild-type and activated human K-*ras* cDNAs were synthesized from RNA from normal placenta and pancreas cancer cell lines, respectively. Primers, FX2 and RX2, were used for the preparation of human K-*ras* cDNA. The cDNAs were inserted into the *Xho*I cloning site of pLXSN, Moloney murine leukemia virus expression vector, as described in "Materials and Methods."

fetal calf serum (Mitsubishi Kasei Corp., Tokyo) for 60 min at 37°C. After the incubation, the epithelial layers were removed from the submucosa with a scalpel. Crypts were isolated by pipetting and washing with the medium. Isolated crypts were cultured in DMEM supplemented with 5% fetal calf serum, epidermal growth factor (EGF; 10 ng/ml, Toyobo, Tokyo), hydrocortisone (0.5 μg/ml, Sigma, St. Louis, MO), selenium (5 ng/ml, Sigma), insulin and transferrin (each 5 μg/ml, Sigma) on collagen-coated dishes (Iwaki, Tokyo). After one week, epithelial-like cells showing a "cobblestone"-like morphology were trypsinized and plated onto new dishes. The next day, the solution containing K-*ras* viruses was added to the cells (multiplicity of infection, 10–50) with Polybrene (8 μg/ml, Sigma) in sub-confluent culture. The cells were cultured for 4 h. After 3 days, the cells were transferred to new dishes and continuously cultured with DMEM supplemented with the same materials.

Immunological staining of epithelial cell markers Primary and K-*ras* virus-infected cells were washed with phosphate-buffered saline (PBS) and fixed with methanol at –20°C for 10 min. The cells were then washed with PBS and treated with 1% horse serum for 30 min at room temperature. Then they were incubated with a monoclonal antibody against an epithelial cell marker; mouse monoclonal antibody against human cytokeratins (AE1/AE3; Boehringer Mannheim) or bovine desmoplakin 1 (Progen Biotechnik, Germany), or rat antibody against mouse E-cadherin (Takara Biochemicals, Tokyo), at 37°C for 1 h. The cells were washed with PBS, treated with FITC-labeled antibody against anti-mouse or anti-rat IgG (Vector Labs., Burlingame, CA), washed again with PBS, then mounted in PermaFluor aqueous mountant (Lipshaw Immunon, Pittsburgh, PA), and observed under a fluorescence microscope.

Colony formation in soft agar Basal agar was made to a final concentration of 0.53% low melting temperature agarose (SeaPlaque; FMC BioProducts, Rockland, ME) with DMEM, supplemented with 5% fetal calf serum, insulin, transferrin, selenium, hydrocortisone and EGF. Untreated and virus-infected cells (10,000 cells) were mixed with the top agar layer of 0.3% agarose in DMEM with the same supplements. The number of colonies was counted under a phase-contrast microscope after 10 days of incubation.

Tumorigenicity in athymic nude mice Epithelial cells, 1×10^6, were suspended in 0.1 ml of Hanks solution and injected subcutaneously into the flanks of 5-week-old female athymic nude mice (ICR *nu*/*nu*). Each cell type was examined in three or five mice. The mice were monitored for 2 months, unless tumors appeared.

Southern blot analysis of K-*ras* gene integration in K-*ras* virus-infected cells Genomic DNAs of primary and virus-infected epithelial cells were isolated using a DNA extraction kit (DNA Extractor WB kit; Wako Chemicals, Osaka). Ten micrograms of DNA was digested with *Eco*RI, and the digest was subjected to 1% agarose gel electrophoresis, then transferred to a Hybond-N^+ nylon membrane (Amersham, Buckinghamshire, UK). Hybridization was performed with ^{32}P-labeled human K-*ras* cDNA in 50% formamide, 0.65 *M* NaCl, 5 m*M* EDTA, 0.1 *M* piperazine-1,4-bis(2-ethanesulfonic acid) (PIPES) buffer (pH 6.8), 0.1% sodium dodecyl sulfate (SDS), denatured salmon sperm DNA (250 μg/ml), 10% dextran sulfate and 5× Denhardt's solution for 18–20 h at 42°C. Finally, the membrane was washed with 0.2× SSPE containing 0.1% SDS at 50°C. Radioactive signals were detected by the use of an image analyzer (BAS2000; Fuji Photo Film Co., Ltd., Tokyo).

Northern blot analysis of K-*ras* mRNA in K-*ras* virus-infected cells Total RNA was extracted from primary and virus-infected epithelial cells by the acid guanidine-phenol-chloroform (AGPC) method.[13] Fifteen micrograms of total RNA was electrophoresed through 6% formaldehyde-1% agarose gels and transferred to a Hybond-N nylon membrane (Amersham). The same probe of K-*ras* as in Southern hybridization was used. For detection of c-*myc* mRNA, the probe of K-*ras* was removed from the membrane after the hybridization of K-*ras*, and the membrane was hybridized with ^{32}P-labeled rat c-*myc* plasmid, pSmBH, which was a generous gift from Dr. K. Hayashi.[14] Hybridization was carried out under the same conditions as Southern blot hybridization. Finally the membrane was washed with 0.1× SSPE containing 0.1% SDS at 50°C.

Western blot analysis of K-ras protein Equivalent amounts of cellular protein were subjected to 12% polyacrylamide gel electrophoresis, and then transferred to a membrane (Immobilon; Millipore Ltd., Bedford, MA). The blots were treated with 20% bovine serum, followed by incubation with mouse monoclonal antibody to K-ras (Oncogene Science, Cambridge, MA) diluted 1:20 in Tris-buffered saline containing 0.05% Tween 20. The K-ras protein in the blots was visualized using a biotin-labeled second antibody and biotin/avidin-conjugated glucose oxidase system (Vector Labs.).

RESULTS

Construction of K-*ras* expression vectors We constructed three human K-*ras* cDNA expression vectors, using a replication-defective retrovirus (Fig. 1). The transcription of the human K-*ras* genes was driven by a long terminal repeat (LTR) of the Moloney murine leukemia virus. The two mutant vectors differed at a glycine residue at codon 12 from wild type (K-*ras*^{WT}), containing activated K-*ras* with an aspartic acid (K-*ras*^{D12}) or a valine (K-*ras*^{V12}) at this position. These two activated K-*ras* genes are the major types of mutation in precancerous lesions and carcinomas of the colorectum.[1–3)]

Primary culture of rat colon epithelial cells Single or aggregated crypts were isolated from pieces of rat colon by treatment with Dispase I alone. After culture for 6 days in collagen-coated dishes, colonies of epithelial-like cells grew almost to confluence. They showed typical "cobblestone" morphology (Fig. 2a). Primary epithelial cells were grown in medium supplemented with 5% fetal calf serum, EGF, insulin, transferrin, hydrocortisone and selenium. This medium enhanced the growth of epithelial-like cells and prevented the growth of other cell types. The epithelial nature of the cultured cells was confirmed by immunological staining for cytokeratin. The primary colonies of epithelial-like cells were trypsinized and replated on type II collagen-coated dishes. Since three passages of the cells resulted in the appearance of larger, multinucleated cells, characterization and infection of the cells with recombinant virus were carried out within two passages.

All the epithelial-like cells stained positive for antibodies against cytokeratin (Fig. 3a), but the intensity of staining was different among cells. Cytokeratin was detected in perinuclear regions or the entire cytoplasm. Desmoplakin and E-cadherin were also found in all the cells by immunostaining (Fig. 3, d and g).

Morphological changes and cytokeratin expression in epithelial cells infected with activated K-*ras* virus Rat colon epithelial cells grown for one week were transferred to new dishes, and on the following day, were infected with K-*ras* virus for 4 h. Two days after infection, most of the cells infected with activated K-*ras* showed morphological changes. On Day 3, the majority of these epithelial cells displayed polygonal shape (Fig.2, c and d), but flattened cells with processes, and giant cells were also evident. Such morphology was similar to that of *ras*-transformed epithelial cell lines.[15)] Morphological transformation was confirmed five times with repeat experiments using different batches of epithelial cells. Even a low titer of K-*ras* virus (multiplicity of infection, 2) completely transformed the epithelial cells (data not shown). Morphologically transformed cells grew for more than 40 passages. In contrast, the wild-type K-*ras* did not induce any

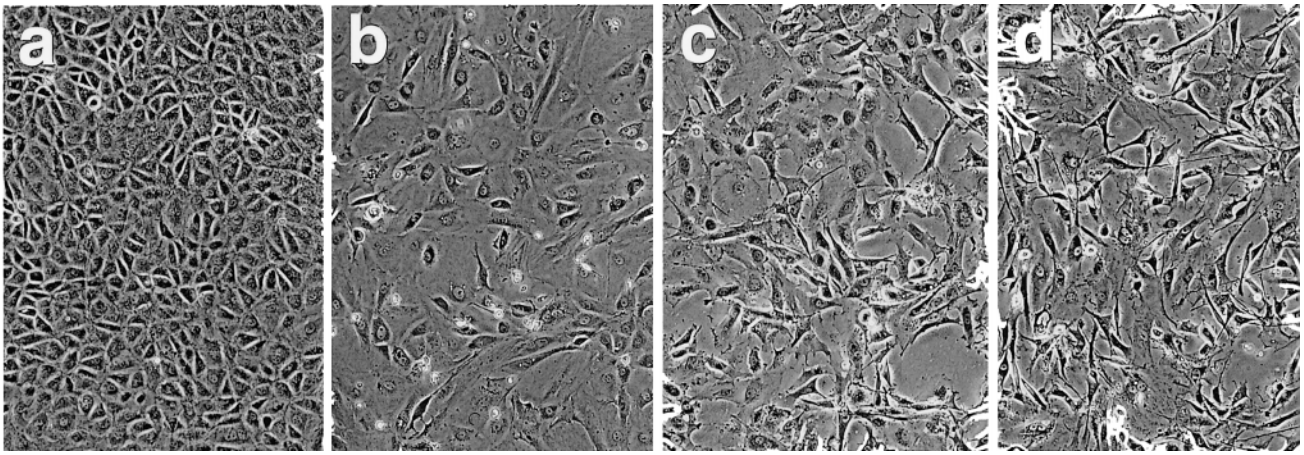

Fig. 2. Morphological changes in K-*ras*-transformed epithelial cells. Primary epithelial cells grown in culture (a) were infected with recombinant retroviruses carrying human K-*ras* cDNAs. Phase-contrast micrographs show the morphologies of K-*ras*^{WT} (b), K-*ras*^{D12}(c) and K-*ras*^{V12}(d) virus-infected epithelial cells 3 days after infection. Original magnification ×200.

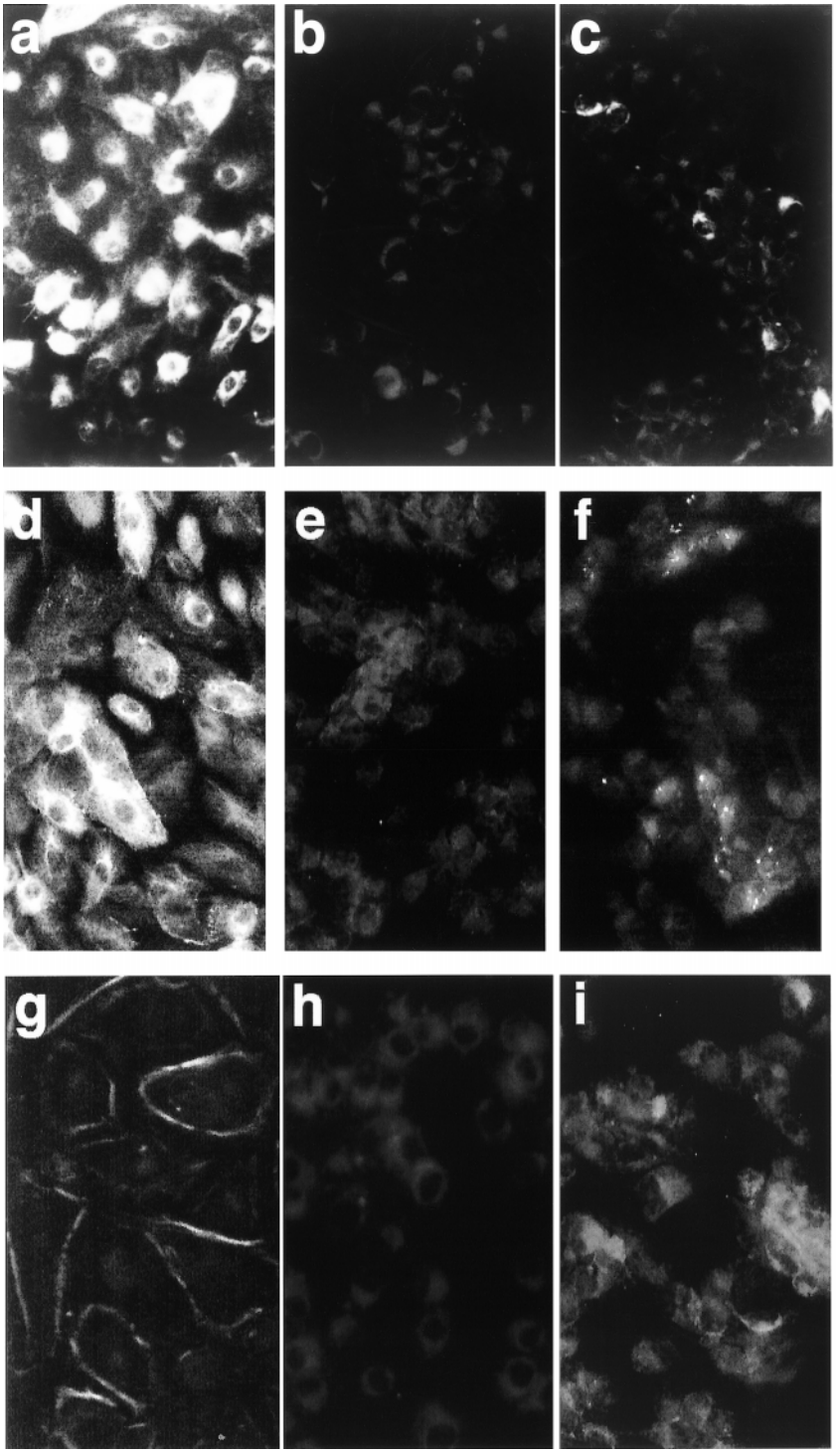

Fig. 3. Immunological staining of epithelial cell markers in primary and transformed cells. Intracellular cytokeratin (a, b and c), desmoplakin (d, e and f) and E-cadherin (g, h and i) were visualized by serial treatment with monoclonal antibodies to each proteins and FITC-labeled antibody against mouse or rat IgG. a, d and g, primary epithelial cells (passage 1); b, e and h, K-*ras*^{D12} virus-infected epithelial cells (passage 11); c, f and i, K-*ras*^{V12} virus-infected epithelial cells (passage 10). Original magnification ×400.

remarkable morphological changes. Although some spindle or large cells appeared, most cells remained flat and round (Fig. 2b). In addition, K-*ras*^{WT} cells could only be successfully passaged twice. These findings indicate that the morphological changes and immortalization were dependent on the activated K-*ras* gene.

Cytokeratin of the morphologically transformed cells was stained with a monoclonal antibody against human cytokeratin (AE1/AE3). No cytokeratin was detected in the K-*ras*^{D12} cells (Fig. 3b). A few K-*ras*^{V12} cells were positive to cytokeratin (Fig. 3c). Cytokeratin was also immunologically stained in all of the cells infected with wild-type K-*ras* virus (data not shown). Other marker proteins such as desmoplakin and E-cadherin were detected only in a few of the K-*ras*^{V12} cells (Fig. 3, f and i), but not in the K-*ras*^{D12} cells (Fig. 3, e and h).

Anchorage-independent growth and tumorigenicity in transformed epithelial cells Neoplastic transformation *in vitro* is judged on the basis of three criteria: morphological transformation, colony formation in soft agar, and tumorigenicity in athymic nude mice. The epithelial cells infected with three K-*ras* viruses (10^4 cells) were cultured in 0.3% agarose. Large colonies of K-*ras*^{V12} cells (passage 8) appeared at a frequency of 6.7% (Table I). The frequency of colony formation in K-*ras*^{D12} cells (passage 4) was 1.2% (Table I). In contrast, the K-*ras*^{WT} cells did not form any colonies within 2 weeks.

To examine tumorigenicity, 10^6 K-*ras*^{WT}, K-*ras*^{D12} or K-*ras*^{V12} cells were injected subcutaneously into athymic nude mice (ICR *nu*/*nu*). Two weeks after injection, tumors of an average size of 1200 mm^3 were produced by K-*ras*^{D12} (passage 13) and K-*ras*^{V12} cells (passage 11) (Table I). No tumors were formed by uninfected epithelial cells or K-*ras*^{WT} cells. All tumors were determined to be undifferentiated carcinomas by pathological examination (Fig. 4, a and b). Immunohistochemistry failed to detect cytokeratin in the cancer cells of nude mice (data not shown). However, when the transformed cells were injected into the colons of syngeneic rats, cytokeratin was immunohistochemically found in a very small number of the K-*ras*^{V12} cancer cells (Fig. 4 c). Desmoplakin and E-cadherin were also detected in a very small number of the K-*ras*^{V12} cancer cells (Fig. 4, d and e). A similar pattern of immunostaining of E-cadherin was found in the K-*ras*^{D12} cancer cells (Fig. 4f).

Establishment and characterization of transformed cell lines We established several cell lines of K-*ras*^{D12} and K-*ras*^{V12} transformed cells from colonies in soft agar or by ring cloning on plastic culture dishes. The cell lines Ta and TB2 from K-*ras*^{V12} cells, and AA3 and AA5 from K-*ras*^{D12} cells were isolated from colonies in soft agar culture. Another two cell lines, AG1 and AG5, were isolated from K-*ras*^{D12} cells by ring cloning.

These cell lines had different morphology (Fig. 5). The Ta cell line had long processes, whereas the AA5 cell line showed a round morphology and retained cell-to-cell contact, which is characteristic of epithelial cells. The AG1 and AG5 cell lines grew surrounding giant cells, which showed extensive cytoplasm and were sometimes multinuclear (Fig. 5). All the cell lines grew as compact and discrete colonies. These properties were similar to those of immortalized rat colon epithelial cells.[15] However, indirect immunofluorescence staining of cytokeratin produced weak staining in all of the cell lines (data not shown), which was similar to that of K-*ras*^{D12} cells (Fig. 3b).

The frequency of colony formation in the four cell lines, Ta, TB2, AA3 and AA5, was higher than that in the original K-*ras*^{D12} and K-*ras*^{V12} cells (Table I). On the other hand, the frequency of colony formation in the other two cell lines (AG1 and AG5) was very low (0.12 and 1.4%, respectively). These values are similar to that of original K-*ras*^{D12} cells.

Tumorigenicity in these cell lines was examined in nude mice. The two cell lines (AG1 and AG5) produced tumors in nude mice with the same incidence as with the other four cell lines (Table I). Tumors of three of the cell lines (TB2, AA3 and AA5) developed to 1200–1600 mm^3 in size by 2 weeks, which was similar to those of the

Table I. Colony Formation and Tumorigenicity of *Ras*-transformed Epithelial Cells

Gene	Colony in soft agar[a] (%)	Tumorigenicity[b] incidence	size (mm^3)
None	0	0/5	
K-*ras*^{WT}	0	0/6	
K-*ras*^{D12}	1.2	5/5	1210±430
K-*ras*^{V12}	6.7	5/5	1260±360
K-*ras*^{D12} cell lines:			
AA3	39.8	3/3	1220±350
AA5	49.8	3/3	1330±620
AG1	0.12	3/3	73±44
AG5	1.4	3/3	620±126
K-*ras*^{V12} cell lines:			
Ta	24.7	3/3	490±10
TB2	49.2	3/3	1610±360

a) Epithelial cells (10,000 cells) were plated in 0.3% agarose in a 5-cm dish. In cell lines, AA3, AA5, Ta and TB2, 1000 cells were plated on a soft agar dish. After 10 days, colonies of each cell type were counted in two dishes. Frequency of colony formation indicates an average percentage of cells that formed colonies in two dishes.

b) Epithelial cells (10^6) were subcutaneously injected into the flanks of female athymic nude mice (ICR *nu*/*nu*). Incidence indicates number of mice bearing tumor/number of transplanted mice.

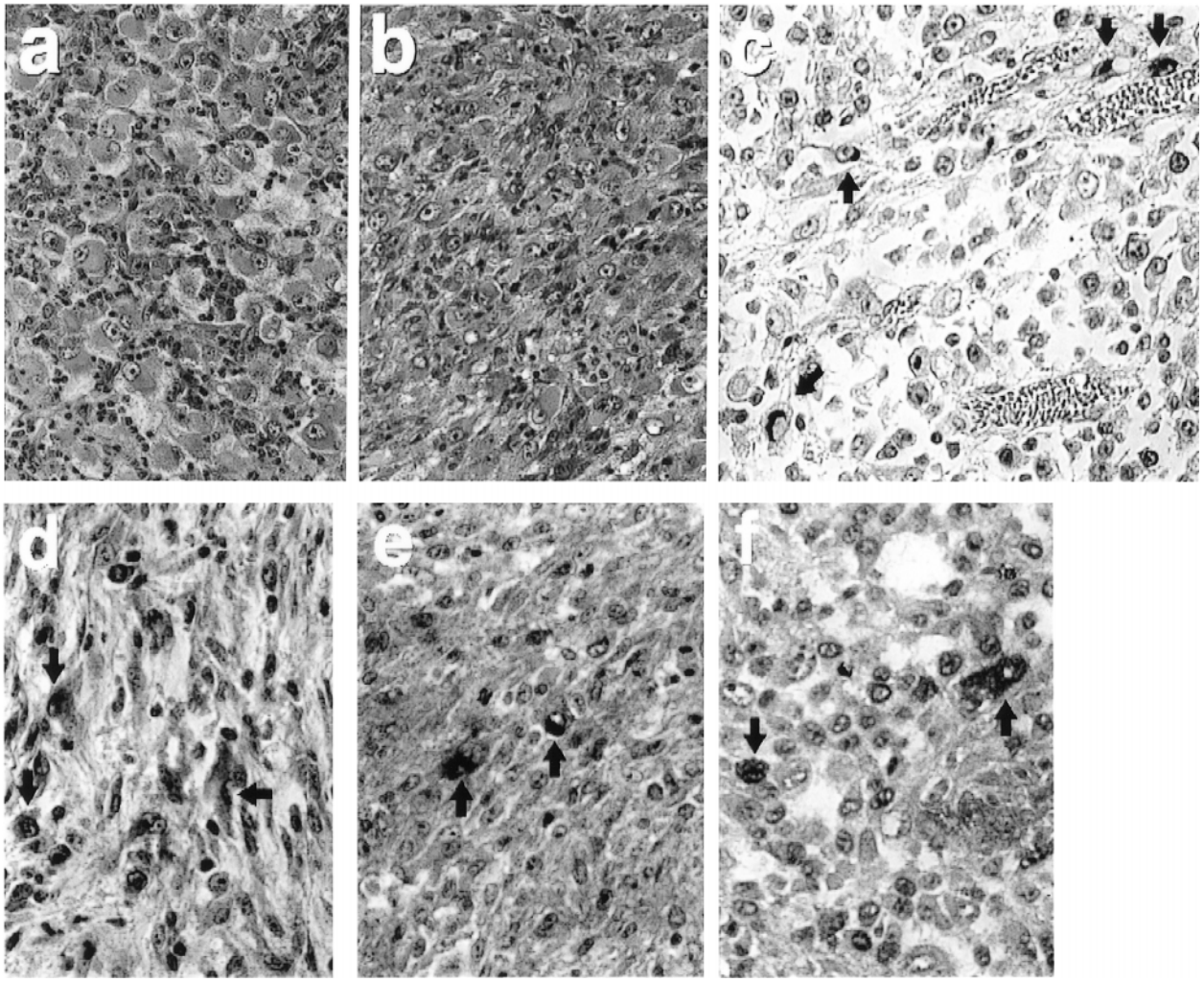

Fig. 4. Tumorigenicity of transformed epithelial cells in athymic nude mice. Epithelial cells infected with K-*ras* retroviruses (10^6) were subcutaneously injected into the flanks of athymic nude mice (ICR *nu*/*nu*). Tumors were removed 14 days after the injection. Sections of K-*ras*^{D12} (a) and K-*ras*^{V12} (b) tumors were stained with hematoxylin-eosin, and diagnosed pathologically. K-*ras*^{V12} (c, d and e) and K-*ras*^{D12} (f) transformed cells (10^4) were injected into the colon of syngeneic rats. One week after the transplantation, the colon were fixed with acetone, and sections of the colon were stained with mouse monoclonal antibodies to human cytokeratins (c) and bovine desmoplakin (d), and rat monoclonal antibody to mouse E-cadherin (e and f) using an avidin-biotin peroxidase system (Vector Labs.). Arrows indicate cells positive to each protein. Original magnification ×400.

original K-*ras*^{D12} and K-*ras*^{V12} cells, whereas the Ta cell line gave only small tumors with an average size of 490 mm^3. The average sizes of tumors of AG1 and AG5 cells were 73 and 620 mm^3, respectively.

These findings suggested that the frequency of colony formation was not always directly related to the growth rate of the tumors in nude mice.

Southern blot analysis of human K-*ras* gene in transformed cells To determine whether the activated human K-*ras* DNA was really integrated in the epithelial cells, Southern blot analysis was performed using a full-length cDNA of the human K-*ras* gene as a probe. The endogenous rat K-*ras* gene was detected as 1.7 and 4.1 kbp bands in primary epithelial, K-*ras*^{D12} and K-*ras*^{V12} cells upon digestion with *Eco*RI, as expected (Fig. 6). Both types of transformed cells showed additional bands. A 480 bp band indicated the 5′ two-thirds of the human K-*ras* cDNA (Fig. 1). Another band of more than 3 kbp contained the 3′ third of the cDNA, flanked with cellular DNA. Eight and five copies of human activated K-*ras* cDNAs were integrated into the K-*ras*^{D12} and K-*ras*^{V12} cell DNAs, respectively.

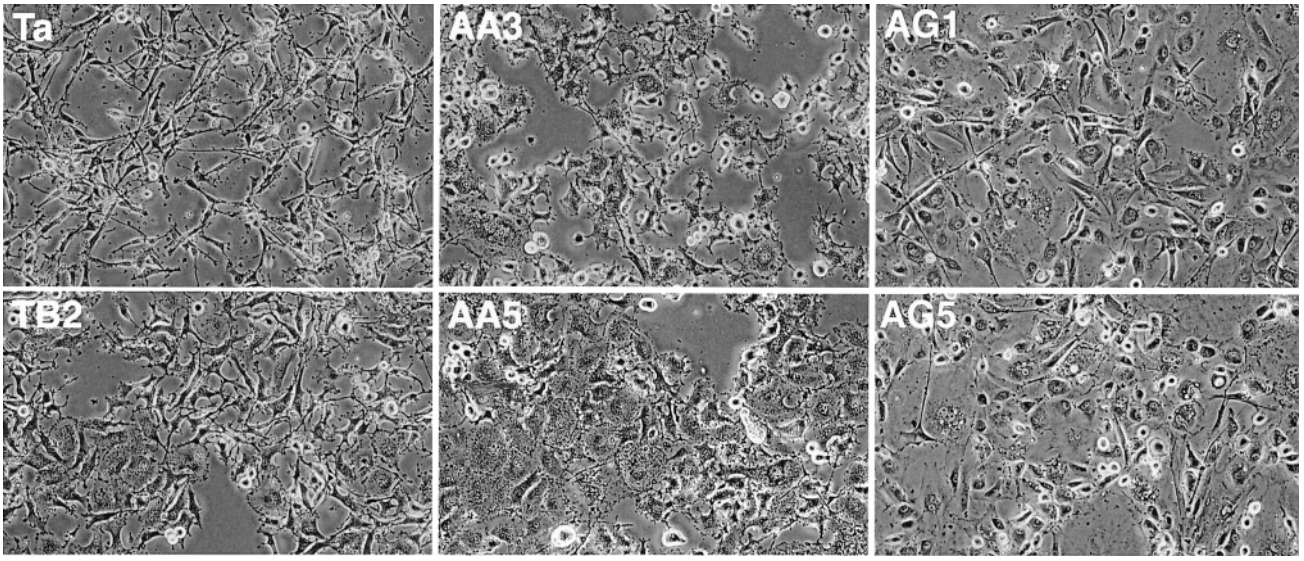

Fig. 5. Morphology of activated K-*ras*-transformed cell lines. Lines Ta and TB2 of K-*ras*^{V12} cells, and AA3 and AA5 of K-*ras*^{D12} cells were isolated from colonies in soft agar culture. Two other cell lines, AG1 and AG5, were isolated from K-*ras*^{D12} cells by ring cloning. Photographs show the morphology of the cell lines under a phase contrast microscope. Original magnification ×200.

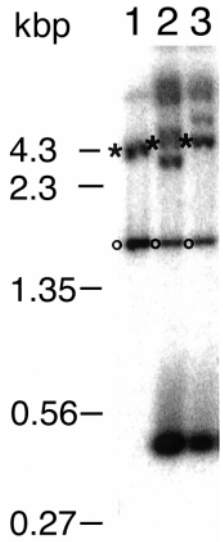

Fig. 6. Southern blot analysis of genomic DNA in normal and K-*ras* virus-infected epithelial cells. Genomic DNA of primary epithelial (1) and K-*ras*^{D12} (2) and K-*ras*^{V12} (3) transformed cells was digested with *Eco*RI, and then subjected to Southern blot hybridization using a ^{32}P-labeled human K-*ras* cDNA as a probe. Symbols ∗ and ○ indicate the 4.1 and 1.7 kbp bands of endogenous K-*ras* gene, respectively.

Wild-type and mutated human K-*ras* cDNAs could be distinguished by PCR-RFLP (restriction fragment length polymorphism) of genomic DNA.[16] Since the genomic PCR product of K-*ras*^{WT} cells was digested with *Mva*I, the wild-type sequence of K-*ras* was proved to be integrated in the K-*ras*^{WT} cells (data not shown).

Expression of human K-*ras* mRNA and protein in transformed cells To determine whether multiple copies of activated human K-*ras* cDNA resulted in overexpression of K-*ras* mRNA and protein in transformed cells, northern and western blotting was performed. In northern blots of total RNA, endogenous K-*ras* mRNA was not detectable in primary epithelial and transformed cells (Fig. 7A). A large amount of human K-*ras* mRNA was detected in the K-*ras*^{D12} and K-*ras*^{V12} cells as a 3.4 kbp band (Fig. 7A, lanes 2 and 3). Human K-*ras* mRNA in six cell lines was also analyzed. The four cell lines (Ta, TB2, AA3 and AA5), which showed a high efficiency of colony formation, expressed large amounts of K-*ras* mRNA (lanes 4–7). In addition, the other two cell lines, which showed lower colony formation (AG1 and AG5), also expressed equivalent amounts of mRNA to the original K-*ras*^{D12} cells and the other four cell lines (Fig. 7A, lanes 8 and 9). In a previous report,[15] overexpression of activated H-*ras* and c-*myc* transformed rat colon epithelial cells, so northern blot analysis of c-*myc* mRNA was performed. Expression of the c-*myc* gene was not enhanced in any of the four cell lines compared to primary epithelial cells (Fig. 7B).

In western blot analysis using antibody against human K-ras protein, the three types of K-*ras* virus-infected epithelial cells expressed large amounts of K-ras proteins,

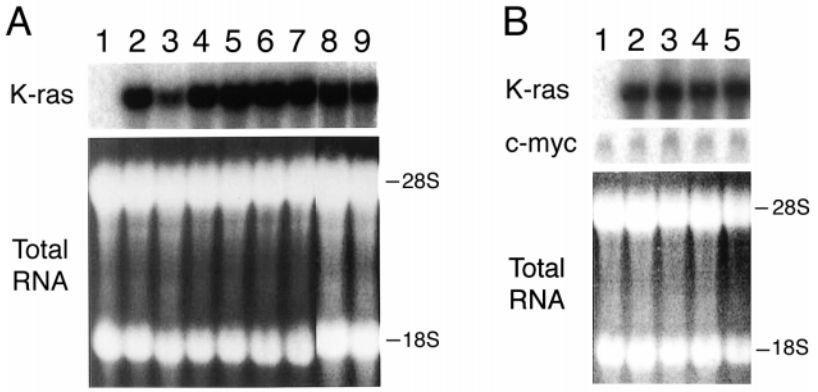

Fig. 7. Expression of K-*ras* and c-*myc* mRNAs in normal and transformed epithelial cells. K-*ras* mRNA was detected by northern blot hybridization using the ^{32}P-labeled K-*ras* cDNA probe (A). Lane 1, primary epithelial cells; lane 2, K-*ras*^{D12} virus-infected cells; lane 3, K-*ras*^{V12} virus-infected cells; lanes 4 and 5, cell lines (Ta and TB2) of K-*ras*^{V12} virus-infected cells; lanes 6 to 9, cell lines (AA3, AA5, AG1 and AG5) of K-*ras*^{D12} virus-infected cells. c-*Myc* mRNA was analyzed using pSmBH plasmid DNA as a probe (B). Lane 1, primary epithelial cells; lanes 2 to 5, transformed cell lines (Ta, TB2, AA3 and AA5, respectively). Patterns of ethidium-bromide stainings in the corresponded gels appear to confirm that similar amounts of total RNA had been loaded in each lane.

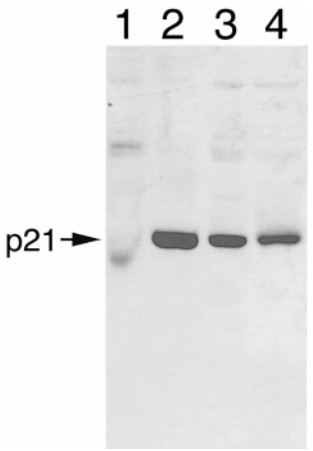

Fig. 8. Western blot analysis of K-*ras* proteins. Equal amounts of cellular proteins were subjected to 12% SDS-polyacrylamide gel electrophoresis, and then analyzed by western blotting using a monoclonal antibody to human K-*ras* protein. 1, primary epithelial cells; 2, K-*ras*^{WT} cells; 3, K-*ras*^{D12} cells; 4, K-*ras*^{V12} cells.

compared to little endogenous K-ras protein in the uninfected epithelial cells (Fig. 8).

These findings suggested that transformation of rat colon epithelial cells occurred as a result of overexpression of activated human K-*ras* cDNA owing to the presence of multiple copies of the genes and the viral LTR, and that differences in the frequency of colony formation among cell lines were not necessarily related to the amount of activated human K-*ras* mRNA.

DISCUSSION

It is widely accepted that neoplastic transformation is at least a two-stage process. However, we have demonstrated here that the activated K-*ras* gene alone is able to cause neoplastic transformation in primary rat colon epithelial cells *in vitro*. The epithelial cells transformed by activated K-*ras* genes displayed morphological changes, anchorage-independent growth, and tumorigenicity in athymic nude mice. Several cell lines were established from colonies in soft agar culture or by ring cloning. Although we cannot rule out the possibility that a second mutation of some other oncogene or tumor suppressor gene might have occurred during the culture of K-*ras* virus-infected epithelial cells, five lines of evidence support the idea that the transformation was achieved by activated K-*ras* alone: (1) morphological transformation occurred diffusely without any focus formation; (2) these changes were observed within 2 days after infection; (3) transformed cells were tumorigenic after a small number of passages; (4) c-*myc* was not overexpressed in transformed cells; and (5) the recombinant activated K-*ras* cDNA retroviruses did not induce any morphological changes in rat lung fibroblasts (to be published elsewhere).

Ras-associated transformation *in vitro* has mainly been examined in fibroblasts. Mutant *ras* genes transformed primary fibroblasts only when supplemented with immortalizing oncogenes such as *src* and *myc*.[17–19] Several attempts to transform colon epithelial cells *in vitro* have been reported. Rat fetal colon epithelial cells could be fully transformed with activated H-*ras* or K-*ras* genes

only when initially immortalized with an SV40 large T antigen.[20, 21] The combination of *myc* and activated H-*ras* or *src* genes produced cell lines of rat fetal colon epithelial cells.[15] v-Ha-*ras* gene-transformed colon epithelial cells possessed the *APC*^{min} gene.[22] These reports are consistent with the theory of multistep carcinogenesis in human colorectum.[23] However, there is evidence that conflicts with the idea of a multi-step process. A high level of activated H-*ras* alone can transform early passage rat embryo cells.[24] The SV40 large T or v-*src* induces neoplastic transformation in rat embryo fibroblasts.[25, 26] Although the efficiency of neoplastic transformation by a single oncogene was relatively low as compared with that in the case of multiple oncogenes, these findings suggest that a single oncogene can fully transform rodent cells under certain conditions, such as high levels of the oncogene products, specific culture conditions, or intrinsic heterogeneity of primary cultures. Tovoloni *et al*.[27] reported that inefficiency of neoplastic transformation by a single oncogene involved heterogeneity of primary cultures in rat embryo fibroblasts. In this study, primary epithelial cells were grown in a culture of crypts isolated from rat colon. Since epithelial cells capable of growth probably consist of heterogeneous populations in the crypts, the epithelial cells grown in our culture system might have factors making them susceptible to transformation by activated K-*ras* alone. The activated K-*ras* genes did not induce morphological change or focus-forming activity in rat lung fibroblasts as judged by the same method as used for colon epithelial cells. The level of expression of human activated K-*ras* gene in the fibroblasts was much less than that in the colon epithelial cells (unpublished data). It is possible that the level of expression of activated K-*ras* produced by the viral LTR might be responsible for differences in the transformation. Our preliminary study of cell fusion between colon epithelial cells and lung fibroblasts suggested that there are some dominant factor(s) that mediate transformation in colon epithelial cells.

It is not clear why colon epithelial cells were transformed by activated K-*ras* alone, in contrast to previous reports.[15, 20, 21] However, there are two important differences between our experiments and previously reported ones. Firstly, the plasmid-vectors carrying oncogenes were transfected into immortalized rat colon epithelial cells.[21] Thus, the efficiency of transformation was very low in that case. The efficiency of our system for the introduction of the gene is likely to be much higher. Secondly, the epithelial cells were separated from the stromal cells in the present work, but in the previous studies, retroviruses carrying oncogenes were applied to rat colon segments in culture.[15, 20] In this case, the conditions for transformation may be similar to those *in vivo*. Although it is not known whether a high concentration of EGF or serum factors is necessary for transformation, these culture conditions might explain why colonic epithelial cell transformation by K-*ras* alone was possible in the present work. It is also possible that the difference in the strain of rats might be responsible for the highly efficient promoter function of the retrovirus, and the number of retrovirus-receptors on the colonic epithelial cells might be different. We have successfully transformed rat colon epithelial cells from a second strain of rat, Brown Norway (unpublished data), indicating that the present result does not merely reflect some strain-specific characteristic. It is possible that continuous exposure of the colon epithelial cells to recombinant viruses carrying K-*ras* cDNA might enhance the transformation by K-*ras* gene. However, this possibility was excluded by the following evidence. No helper virus was detected in the K-*ras* virus stocks of psei-2 cells, and the conditioned media of transformed epithelial cells could not transform primary colon epithelial cells.

The frequency of colony formation of K-*ras*^{D12} and K-*ras*^{V12} cells in soft agar was low (Table I), raising the possibility that only a small fraction of transformed cells that undergo a second mutation might show anchorage-independent growth and tumorigenicity. We think that this is unlikely for following reasons. Although the AG5 cell line showed a very low frequency of colony formation in soft agar (0.49%), it showed a level of tumorigenicity in nude mice that was comparable to those of the other cell lines (Ta, TB2, AA3 and AA5) which showed a high frequency of colony formation in soft agar. Seshimo *et al*.[21] reported that the frequency of colony formation in rat epithelial cells transformed with SV40-large T and v-Ha-*ras* genes was 1.9%. This frequency is similar to that observed in our system. Thus, tumorigenicity may not always be directly related to the ability to form colonies in soft agar. Since primary cultures of rat colon epithelial cells contain a heterogeneous population of growing epithelial cells, the properties of the cells transformed by activated K-*ras* may differ.

The epithelial-like cells were derived from isolated crypts of rat colon during primary culture. All the primary cells stained positive with antibodies against cytokeratin, desmoplakin and E-cadherin, confirming that these cells were epithelial cells. However, the expression of these marker proteins in the transformed cells decreased in culture. The marker proteins could barely be detected in cancer cells in nude mice, and very few transplanted cells were positive to the marker proteins in the colon of syngeneic rats. Thus, we cannot exclude the possibility that the activated *ras*-transformed cells might have originated from another type of cell in the primary culture. As mentioned above, morphological transformation took place homogenously and within a few days, suggesting that the majority of the cells were transformed. In addition, the morphology of the transformed cells was similar to that of

transformed epithelial cells reported previously.[15] Therefore, the virtual absence of marker proteins for epithelial cells was probably due to the loss of differentiation properties in the activated K-*ras* transformed cells. Such a loss of differentiation characteristics in the presence of activated *ras* was reported in rat colon epithelial cells transformed with v-Ha-*ras* and *myc*,[15] and *ras*-transformed human bronchial epithelial cells.[28]

In the case of heterotopic and reconstituted organs *in vivo*, the combination of *myc* and *src* genes produced adenocarcinoma in heterotopic rat colon,[29] and carcinomas were induced by the combination of v-H-*ras* and *myc* in reconstituted prostate.[30] In both cases, single oncogenes such as the v-Ha-*ras* gene produced hyperplasia or dysplasia in these organs. Overexpression of oncogenic mutant c-H-*ras* produces hyperplasia and dysplasia in the liver of transgenic mice.[31] Next, to know whether there are intrinsic or environmental factors responsible for the neoplastic transformation, we need to examine the tumorigenic activity of K-*ras* epithelial cell lines by implantation of the cells into syngeneic rat colon.

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