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Activated K-*ras* in Tumorigenic and Non-tumorigenic Cell Variants from a Rat Colon Adenocarcinoma, Induced by Dimethylhydrazine

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Using NIH3T3 cell transfection assay, activated c-K-*ras* was detected in two cell lines, TRb and TSb, obtained from a single colon adenocarcinoma induced in a rat by 1,2-dimethylhydrazine. TRb cells give rise to progressive and metastatic tumors in the syngeneic rats, whereas TSb cells only induce regressive tumors. Levels of K-*ras* transcripts in TRb and TSb cells were higher than that of NIH3T3 cells, but no difference was found between TRb and TSb cells. No significant difference was observed in expression levels of c-*myc* in these two cell lines. c-*fos* expression was, however, significantly lower in TRb than TSb cells.

Key words: Dimethylhydrazine — Rat colon adenocarcinoma — TSb/TRb variants — Oncogenes — K-*ras*

From a colon adenocarcinoma induced in a rat by 1,2-dimethylhydrazine (DMH), two cell lines were obtained.[1] These two cell variants display different tumorigenic capacities when inoculated into the syngeneic host: TRb cells (clone b of a trypsin-mediated detachment-resistant subline TR) induce progressive tumors and TSb cells (clone b of a trypsin-mediated detachment-sensitive subline TS) induce tumors which regress in less than one month. Both lines give progressive tumors when inoculated into nude mice.[1] These two cell lines interact with each other, inducing a different immunological response in the host.[2] Since it has been reported that oncogenic virus or activated c-*onc* modifies the expression of the major histocompatibility complex antigen,[3–6] we examined the presence of activated oncogenes in these two cell lines.

Materials and Methods

Cell Culture TRb and TSb cells and their cloned sublines were grown in Ham F10 medium supplemented with 10% fetal calf serum. NIH3T3 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% calf serum and 4m*M* glutamine.

DNA and RNA Preparations High-molecular-weight DNA was obtained from culture cells by digestion with proteinase K and RNase and extraction with phenol followed by ethanol precipitation.[7] Total cellular RNA was isolated from cultured cells according to the guanidinium/cesium chloride method. Briefly, the cell pellets were disrupted in 6*M* guanidinium isothiocyanate containing 0.1*M* mercaptoethanol, 0.5% sarkosyl and 5m*M* sodium citrate. RNA was pelleted by centrifugation through a 5.7*M* CsCl cushion, extracted with chloroform-butanol mixture and precipitated in ethanol.[8]

DNA Transfection Assay High-molecular-weight DNA was transfected into mouse NIH3T3 cells according to the calcium phosphate DNA transfection method.[7] Foci were scored 21 days after transfection.

Southern Blots High-molecular-weight genomic DNA was digested with restriction endonucleases. Digested DNA (10 μg/lane) was fractionated by electrophoresis in a 0.7% agarose gel.[9]

Northern Blots Denatured total RNA (10 μg) was size-fractionated by electrophoresis in 1.0% or 1.2% agarose formaldehyde gel.[4]

Preparation of Labeled DNA Probes The following oncogene fragments were used as probes: ID4[10] for rat repetitive sequence, BS9[11] for H-*ras*, p6a1 (*Sal*I/*Nco*I insert)[12] for N-*ras*, HiHi 380[13] for

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K-*ras*, pHSR-1[14] for c-*myc* and *Pst*I fragment of p*fos*-1 for *fos*.[15] The fragments were nick translated to give 10^9 cpm/μg DNA. The filters were hybridized at 42° for 18 to 48 hr in buffer containing 50% formamide, 0.65*M* sodium chloride, 0.1*M* sodium PIPES (pH 6.8), 5m*M* EDTA, 10% dextran sulfate, 5×Denhardt's solution, 0.1% SDS, 100 μg/ml of denatured salmon sperm DNA and 20 ng of ^{32}P-labeled probes. The filters were washed in 2×SSC, 0.2% sodium pyrophosphate and 0.1% SDS at 50°. They were exposed to Kodak XAR-5 films at −70° with Dupont intensifying screens.

Results

Activated K-*ras* in TRb and TSb Cells High-molecular-weight DNAs obtained from both TRb and TSb cells induced transformed foci in NIH3T3 by transfection assay. Thirty micrograms of DNA was used to transfect 5×10^5 NIH3T3 cells, and transformation frequencies of 0.1 focus/μg DNA for both cell variants were obtained. Two primary transformed foci derived from each tumor cell line were isolated and grown in culture and their DNAs were isolated and analyzed by Southern blotting for the presence of a rat repetitive sequence (ID4) as described previously.[10] DNAs from these 4 primary transformants were shown to possess rat repetitive sequences (data not shown) and were used in a second round of transfection. The transformation frequencies in the second round were also 0.1 foci/μg DNA for both cell variants.

Two primary and two secondary transformants of each cell line were subjected to Southern blot analysis using probes for K-*ras*, N-*ras* and H-*ras* oncogenes. *Bam*HI-digested

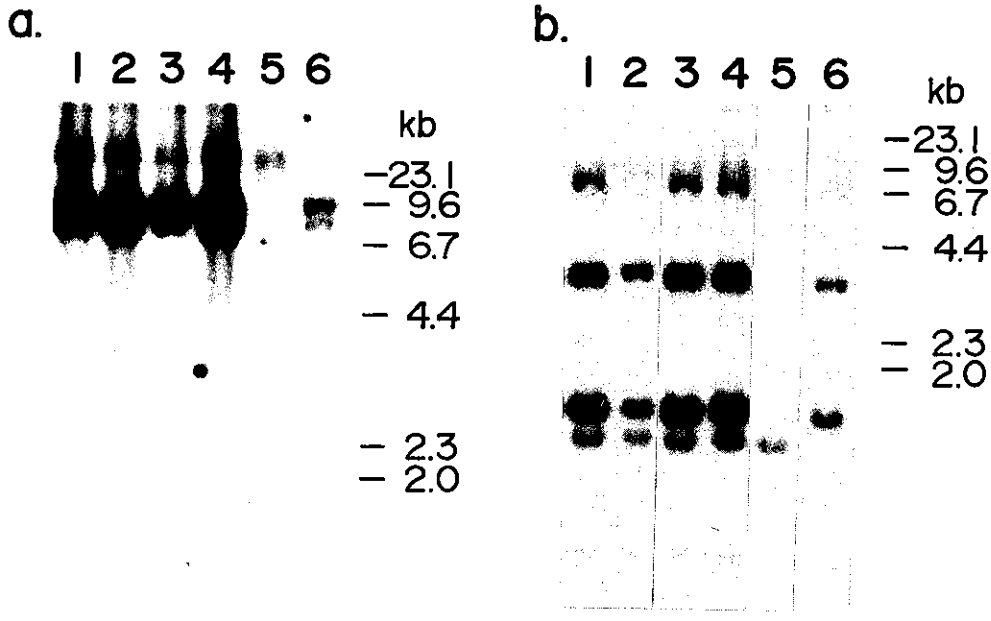

Fig. 1. Identification of rat K-*ras* sequences in primary and secondary transformants induced by DNA from TRb and TSb cells. Ten micrograms of each DNA obtained from transformants was cleaved with (a) *Bam*HI or (b) *Eco*RI restriction enzyme, fractionated by agarose gel electrophoresis and blotted onto nitrocellulose paper. The paper was hybridized with nick-translated ^{32}P-labeled HiHi 380.[13] The positions of molecular weight markers (*Hin*dIII digest of λcI865) are shown on the right. a) Lanes 1 and 2, primary transformants obtained by TRb cells; lanes 3 and 4, primary transformants by TSb cells; lane 5, NIH3T3 cells; lane 6, normal F344 rat liver. b) Lanes 1 and 2, secondary transformants obtained from different primary transformants of TRb cells; lanes 3 and 4, secondary transformants of TSb cells; lane 5, NIH3T3 cells: lane 6, normal F344 rat liver.

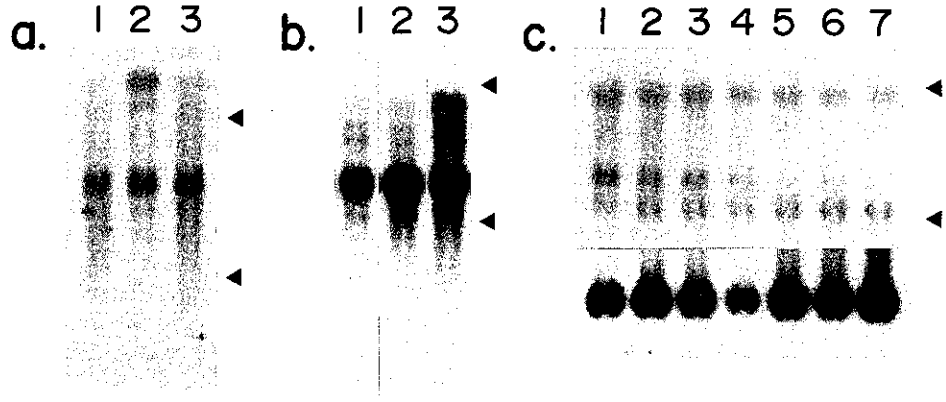

Fig. 2. Northern blot analysis for (a) c-K-*ras*, (b) c-*myc* and (c) c-*fos* expression. Ten micrograms of total RNAs prepared from cultured cells was used. (a) Lane 1, BMSR6; lane 2, TSb cells; lane 3, TRb cells. (b) Lane 1, NIH3T3 cells; Lane 2, TSb cells; lane 3, TRb cells. (c) Lane 1, BMSR6; lane 2, TSb cells; lane 3, TSc cells; lane 4, TSd cells; lane 5, TRb cells; lane 6, TRc cells; lane 7, TRd cells. p*fos*-1 probe hybridized with 2.2 kb mRNA and also with 28S and 18S rRNAs. β-Actin probe was used for internal reference (the lowest bands in c). Triangles indicate the positions of 28S and 18S rRNA.

DNAs of all four primary transformants gave strongly hybridized bands at 12.6 and 8.5 kb in addition to mouse sequences with K-*ras* probe (Fig. 1a). The presence of activated rat c-K-*ras* sequence was confirmed by analyses of secondary transformant DNAs: after *Eco*RI digestion of secondary transformant DNAs, rat specific hybridizing sequences were detected at 11, 3.9 and 1.7 kb (Fig. 1b). Both TRb and TSb cells thus had the activated form of c-K-*ras* gene. No hybridizing sequences besides mouse sequences were detected with probes for H-*ras* and N-*ras*.

K-*ras* gene dosages in these two cell lines were examined. DNAs from TRb and TSb cells and norml rat kidney were digested by *Eco*RI, and the Southern blot was hybridized with K-*ras* probe. K-*ras* was neither amplified nor reduced to a single allele in either tumor cell DNA as in normal rat kidney DNA, and no significant difference could be seen between the two cell lines (data not shown).

Expression Levels of K-*ras*, *myc* and *fos* The levels of K-*ras* transcripts in TRb and TSb cells were examined. Total RNAs were extracted from TRb and TSb cells and hybridized on Northern blots with a probe for K-*ras*. The concentrations of the two transcripts, 5.0 and 3.7 kb, were increased in the tumor cells compared with NIH3T3 cells and were almost the same as in fibroblasts (BMSR6) obtained from a 6-day-old newborn rat. No difference in the expression level was observed between TRb and TSb cells (Fig. 2a).

c-*myc* expression was not significantly different in the two cell lines (Fig. 2b). c-*fos* was expressed in both cell lines and the transcripts were 2 to 3 times higher in TSb cells than in TRb cells. To confirm the significance of this difference, c-*fos* expression in cloned sublines, TRc, TRd, TSc and TSd was examined. TRc and TRd also showed decreased expression of c-*fos* compared to TS sublines (Fig. 2c).

Discussion

Cancer development is the result of multistep genetic and/or epigenetic changes. Using two cell variants, TRb and TSb, and their cloned sublines derived from a single rat colon carcinoma, the roles of oncogenes in the development of progressive tumors were studied.

Both TRb and TSb DNAs had the same transforming activity on NIH3T3 cells, and the transforming genes were identified as activated c-K-*ras*. *ras* oncogene activations have

been demonstrated in human colorectal carcinomas[16–18] and this is the first report on activation of a *ras* gene in a carcinogen-induced rat colon cancer.

Northern blot analysis showed that the expression levels of K-*ras* in TRb and TSb cells were the same. From the results of Southern blot analysis, copy numbers of c-K-*ras* gene were not changed in TRb and TSb cells, and activation of at least one of two alleles was proved by transfection assay.

If expression levels of mutated K-*ras* are the same in both cell lines, activated K-*ras* is not sufficient to induce tumorigenic progression of TRb cells in the adult syngeneic host. Activation of *ras* genes in benign lesions has been reported.[19] Papillomas as well as fibrosarcomas induced by 7,12-dimethylbenz[*a*]-anthracene and 12-O-tetradecanoylphorbol-13-acetate contained activated H-*ras* oncogene.[20] Activated H-*ras* oncogene was also found in spontaneous benign and malignant hepatocellular tumors of the mouse.[21] Furthermore, expression levels of H-*ras* were similar in both benign and malignant skin lesions.[20]

The difference in tumorigenicity between TRb and TSb could be due to a difference in expression of other oncogenes cooperating with K-*ras* oncogene.[22, 23] Nuclear protein-encoding oncogenes, such as c-*myc* and c-*fos* may act in cooperation with K-*ras* oncogene. Furthermore, Yander *et al.* have recently reported amplification and over-expression of c-*myc* gene in a DMH-induced transplantable colon tumor of the mouse.[24] Expression of *myc* oncogene was not changed between TRb and TSb cells, though two- to three-fold decreases of c-*fos* expression in TRb were observed. The decrease of c-*fos* expression was also confirmed in two other cloned sublines producing progressive tumors. In contrast, elevated expression of c-*fos* in highly metastatic clones of a spontaneous rat mammary tumor was reported.[25] In the case of myeloid leukemic cells, high expression of c-*fos* was observed at the stage of differentiation into macrophages.[26, 27] TS cells grow as less differentiated tumors than TR cells.[2] Implication of different c-*fos* expression levels in the different characteristics of these TS and TR variants remains to be elucidated.

Meanwhile, factors associated with immunogenic or immunosuppressive activity may explain the difference in tumorigenicity between TR and TS cell clones. First, TSb cells have been shown to be more sensitive than TRb cells to natural killer (NK) cells.[28] Furthermore, a single injection of anti asialo GM1 serum, a known inhibitor of NK activity, prior to TSb cell challenge, leads to the outgrowth of TSb cells as progressive tumors,[29] demonstrating the essential role of NK cells in the regression of TSb tumors. TSb cells also produce progressive tumors when inoculated into athymic nude mice.[1] This suggests that immune T lymphocytes cooperate with NK cells to effect the regression of TSb tumors. TRb cells are able to induce an immunosuppression leading to the progressive growth of TRb tumors, and also TSb tumors.[2] The present study suggests that the difference in tumorigenicity and immunogenicity between TRb and TSb cells is due to genetic and/or epigenetic changes induced after the activation of c-K-*ras*.

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