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Overexpression of *ras* in Mucus-secreting Human Colon Carcinoma Cells of Low Tumorigenicity[1]

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ABSTRACT

We have investigated the expression of the protooncogenes of the *myc* and *ras* family in HT29 cells and in three differentiated clonal cell lines derived from this colon carcinoma cell line. In contrast to the decrease in *myc* expression seen when leukemia cells are induced to differentiate, we have found no changes in expression of the *myc* gene family in differentiated colon carcinoma cells. However, a greater than 5-fold increase in expression of sequences which hybridize to Ha-*ras* was observed in cells which secrete mucin, with a smaller increase seen in expression of Ki-*ras* in the same cells. This increase was not seen in cells which exhibit vectorial transport of water and ions, and which are not mucus-secreting. All differentiated lines were less tumorigenic in nude mice than the parental HT29 cells, irrespective of the level of *ras* expression. These results are consistent with the reports that *ras* expression is highest in the most differentiated cells of the colon and is substantially decreased in metastatic human colon tumors as compared to primary colon tumors. The data also suggest that a high level of *ras* gene expression is a marker for a particular differentiated state in colon cells rather than being directly equated with transformation or tumorigenicity. Hence, the results may reflect on some of the discrepancies concerning *ras* gene expression in human colon and other tumors which appear in the literature.

INTRODUCTION

The induction of differentiation of cancer cells is of interest for two reasons. (*a*) The potential exists to use such induction therapeutically to revert cancer cells to a less malignant phenotype. Such treatment may cause the tumor to become a reduced threat to the host, or to become more responsive to traditional chemo- or radiation therapy (1, 2). (*b*) The induction of differentiation provides model systems for investigating the progression of cellular and molecular changes which accompany the development of the fully malignant phenotype. For example, induction of differentiation of mouse erythroleukemia cells (3) and the human promyelocytic leukemia cell line HL60 results in a marked decrease in expression (4) and transcription (5) of the c-*myc* gene. Further, these decreases are related to the induced differentiation rather than to effects on cell proliferation (3, 4). A decreased expression of N-*myc* has also been shown to accompany retinoic acid-induced differentiation of neuroblastoma cells (6).

Human colon cancer cells can also undergo differentiation in culture during exposure to polar solvents (7, 8) or by changing their nutritional status (9). Further, a reduction in tumorigenicity concomitant with maturational events induced by dimethylformamide has been demonstrated by Dexter and Hager (10). These effects, however, have been shown to be reversible upon removal of the cells from dimethylformamide. A second approach to investigate the changes associated with differentiation in colon cancer would be to compare cell populations of the same origin engaged in different lineages of colon cell differentiation. In such a model system, the expression of the differentiated phenotypes would be independent of the continuous presence of inducer. This model now exists since several populations have been isolated from a human colon carcinoma cell line (HT29) which stably differ from the parental cells with respect to their differentiated phenotype (11). Hence, it is possible to compare the tumorigenic potential as well as the expression of gene sequences in colon carcinoma cells which are either undifferentiated (HT29 cells) or which stably express morphological as well as functional properties of colon cell differentiation in standard culture conditions.

MATERIALS AND METHODS

HT29 cells and derived cell lines were grown in Dulbecco's modified Eagle's medium (Gibco) containing 10% heat-inactivated fetal bovine serum, as previously described (11). HT29 cells do not contain a transforming *ras* gene as assayed by transfection into NIH 3T3 cells (Ref. 12; Footnote 3). For growth of tumors in nude mice, outbred athymic male *nu/nu* mice were obtained from Iffa Credo (domaine des Oncins, L'Arbresle, France). Mice were isolated in sanitary, but not germ-free conditions. At 8 wk of age, 2×10^7 cells in 0.5 ml of Dulbecco's medium-10% fetal bovine serum were inoculated s.c. into the right flank of mice midway between the arm and leg. Tumor development was monitored weekly by palpation. When tumors became visible, tumor growth was followed by caliper measurements in two dimensions (length, *L*; width, *W*), and the volume (*V*) was calculated by the formula for a prolate ellipsoid $V = [(L \times W) \times 3.14]/6$ according to the method of Stragand *et al.* (13). For analysis of protooncogene expression, total RNA was isolated from stationary cell cultures by guanidine thiocyanate solubilization of cells and centrifugation over a CsCl cushion according to the method of Glison *et al.* (14), with modifications as reported by Raymondjean *et al.* (15). RNA was denatured by glyoxylation, and dot-blots and Northern blots were prepared on nitrocellulose, as previously described (16). Probes were prepared by nick-translation, and hybridization was done in 50% formamide at 42° C. again, as described (16). The probes consisted of the following cloned sequences: c-*myc*, pMC41-5pp (17); N-*myc*, pBE (2)-c-59 (18); c-*myb*, pBR322/HAX4 (19); v-Ha-*ras*, BS-9 (20); v-Ki-*ras*, HiHi-380 (20); N-*ras*, p52C+ (21); human c-Ha-*ras* (22); human c-Ki-*ras* (23).

RESULTS AND DISCUSSION

The human colon carcinoma cell line HT29 is undifferentiated in standard culture conditions (Dulbecco's minimal essential medium-10% fetal bovine serum). The cells grow as multilayers, they are not polarized, and they do not secrete mucins. The isolation and characterization of differentiated clonal variants of the HT29 cell line have been already described (11). Briefly, the HT29 cells were treated long term (23 days) with 5 mM sodium butyrate and then returned to a standard culture medium. Cell populations with constitutive phenotypic features of differentiation emerged following this treatment. These populations which are stably differentiated in standard culture media have been cloned and characterized. Three clonal cell lines, 16E, 13G, and 19A, have been selected for this study.

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[3] L. H. Augenlicht, unpublished observations.

Table 1 *Comparison of the properties of the four cell lines*

HT29 cells were obtained from J. Fogh (35). The human colonic adenocarcinoma cell line HT29 and clones 16E, 13G, and 19A were cultured in Dulbecco's modified Eagle's medium-10% fetal bovine serum, and the differentiation parameters were scored under these culture conditions.

Cell line	Phenotypic characters of differentiation		
	Cell polarization[a]	% of mucus-secreting cells[b]	Dome formation
HT29	–	<1	–
c116E	+	60–80	–
c113G	+	15–30	+
c119A	+	<3	+

[a] As determined by electron microscopy of cells.
[b] The percentage of mucus-secreting cells was assessed by a metachromatic staining on cytocentrifuge preparations of postconfluent cells.

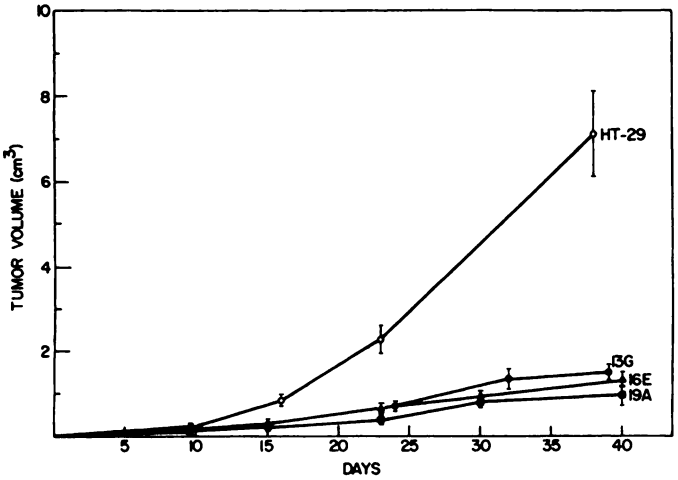

Fig. 1. Growth of tumors in nude mice. Outbred athymic male *nu/nu* mice were inoculated with 2×10^7 cells. Tumor development was monitored weekly by palpation, and the volume (V) calculated as described in "Materials and Methods." *Points*, mean of 6 animals; *bars*, *S.E.*

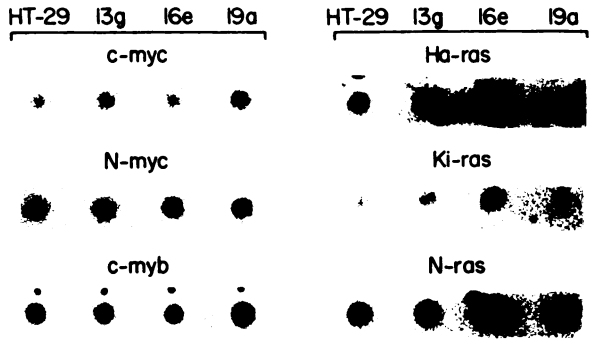

Fig. 2. Analysis of protooncogene expression. Total RNA was denatured by glyoxylation, and dot-blots prepared on nitrocellulose, each dot containing 10 μg of total cell RNA. Probes were prepared by nick-translation, and hybridization was done as described in "Materials and Methods."

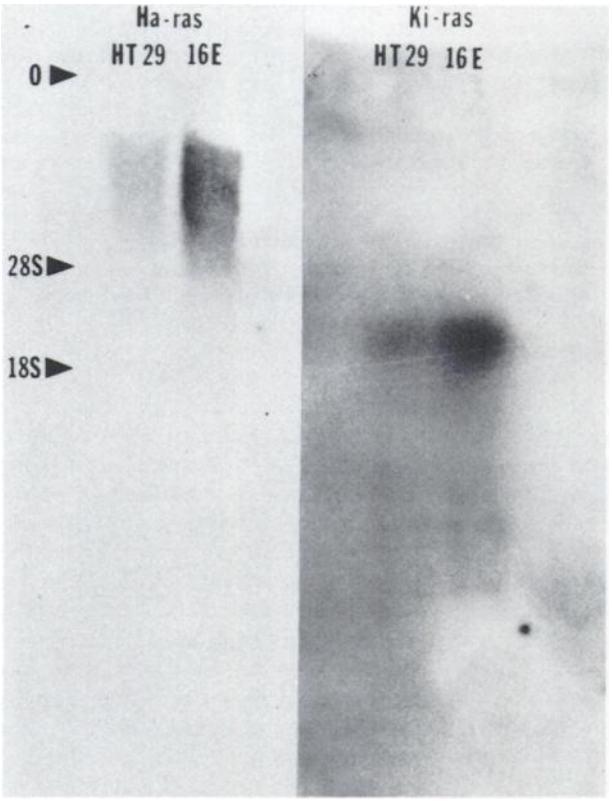

Fig. 3. Northern blot analysis of RNA. Glyoxylated RNA of HT29 and clone 16E cells was fractionated by electrophoresis in 2% agarose gels, blotted to nitrocellulose, and hybridized to either the human c-Ha-*ras* probe or c-KI-*ras* probe, as described in "Materials and Methods."

Table 1 summarizes the phenotypic characteristics of the parental HT29 cells and of the stably differentiated lines. The cells of the three clones grow as monolayers, they are morphologically polarized, and they exhibit an apical membrane which is separated from the basolateral membrane by typical junctional complexes. In addition, these clonal lines display functional characteristics of colon cell differentiation, namely, mucin secretion and vectorial transport of solutes. The mucins produced by these clonal lines have been characterized by histochemistry, electron microscopy (11), and immunohistochemistry by using antibodies specific for human gastrointestinal mucins.[4]

Fig. 1 shows that each of these three differentiated clones is less tumorigenic in nude mice as compared to the parental HT-29 cells. Upon sacrifice, none of the mice shows gross metastases. Survival of the mice is dependent on tumor bulk, which eventually interferes with feeding and drinking. Hence, the faster growing tumors (HT29 cells) will generally kill the animals sooner than the more slowly growing tumors, but this is an indirect effect.

In order to determine whether the expression of members of the *myc* and *ras* gene families was altered in relation to the differentiated phenotype or tumorigenicity of the cells, replicate dot blots of RNA from the HT29 cells and the three clonal isolates were hybridized to probes of c-*myc*, N-*myc*, c-*myb* (whose gene product is related to the *myc* family (24), v-Ha-*ras*, v-Ki-*ras*, and N-*ras*. The results are shown in Fig. 2. Surprisingly, the expression of Ha-*ras* was increased over 5-fold in clone 16E, the clone which displays the highest percentage of mucus-secreting cells, and 3-fold in 13G, the clone which displays a lower percentage of mucus-secreting cells and which transports water and ions. Ki-*ras* expression was also increased, but to a lower extent. To confirm these data, Northern blot analysis of RNA prepared from the parental HT29 cells and the mucin-secreting differentiated clone 16E cells is shown in Fig. 3. Using the corresponding human probes, Ha-*ras* is clearly increased in the 16E cells, and a smaller increase, consistent with the dot blots, was seen for Ki-*ras*. Clone 19A, the cells which do not secrete mucins but which display the function of transport, showed no change in *ras* expression (Fig. 2). No alterations in expression of the other sequences were seen, or in the expression of *sis* or *mos* (not shown). Since RNA was always prepared from stationary cells, the results presented cannot be attributed to differences in the growth state of the

[4] J. Bara, personal communication.

cultures. Whether the expression of these sequences is related to cell growth in these cells is not known.

It should be noted that the N-*myc* probe represents the 3'-end of the N-*myc* mRNA and does not cross-hybridize with c-*myc* (18). Although there was no change in the expression of c-*myc* or N-*myc* with differentiation, Northern blots clearly confirmed that both genes were expressed in HT29 cells as well as in SW480 colon carcinoma cells (not shown). This is of interest since it has been reported that N-*myc* expression has been reported to be restricted to neuroectodermal tissue (25).

In work similar to that presented here, Dexter has also found that Ki-*ras* as well as Ha-*ras* expression was increased in DLD-1 colon carcinoma cell lines during *N*-methylformamide- or butyrate-induced differentiation.[5] Thus, increased expression of these protoonc sequences correlates with decreased tumorigenicity of colon carcinoma cells, and in the system studied here, it is a marker of one specific lineage of colon cell differentiation, *i.e.*, mucin secretion. *ras* expression has recently been found to be highest in the most differentiated cells at the top of the colonic crypt (26). *ras* is also markedly decreased in late stage and metastatic colon tumor tissue as compared to earlier (Duke's B stage) primary tumor (27). While tumorigenicity and metastatic potential are certainly not equivalent, it is reasonable to assume that, as the tumor progresses and metastasizes, the cells are in general less well differentiated than in the early primary tumor (28). Thus, *in vivo*, *ras* expression may again be higher in the more differentiated colon tumor tissue which precedes the most malignant phenotype.

It is important to emphasize that our results demonstrate a marked difference in Ha-*ras* expression in cells along different lineages of phenotypic differentiation. A recent report has demonstrated that microinjection of the T24 *ras* protein induces morphological differentiation of PC12 cells into neuron-like cells (29). *ras* expression may therefore be related to various lineages of differentiation in different cell types.

The *ras* gene product is related to the G-binding protein of the membrane adenylate cyclase system (30, 31). Although its normal function in eukaryotic cells is not known, our data demonstrate that the normal activity of members of this family is regulated, like many other genes, in relation to the overall differentiated state of the colon cell unrelated to cell tumorigenicity. In contrast to colon cancer, the difference between *ras* expression in the primary and metastatic tumor tissue is not seen in breast and other tumors (27). Hence, *ras* expression cannot be considered independent of the expression of many other genes which in concert determine the cell phenotype. From a practical point of view, it is therefore not surprising that levels of *ras* expression vary so widely in different tumors (32) and, given the heterogeneity present in human tumors (33), even among cells of the same tumor (34).

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REFERENCES

1. Spermuli, E. N., and Dexter, D. L. Polar solvents: a novel class of antineoplastic agents. J. Clin. Oncol., *2:* 227–241, 1984.
2. Schwartz, E. L., and Wiernik, P. H. Differentiation of leukemia cells by chemotherapeutic agents. Leuk. Rev. Int., *3:* in press, 1987.
3. Lachman, H. M., and Skoultchi, A. I. Expression of c-*myc* changes during differentiation of mouse erythroleukemia cells. Nature (Lond.), *310:* 592–594, 1984.
4. Filmus, J., and Buick, R. N. Relationship of c-*myc* expression to differentiation and proliferation of HL-60 cells. Cancer Res., *45:* 822–825, 1985.
5. Grosso, L. E., and Pitot, H. C. Transcriptional regulation of c-*myc* during chemically induced differentiation of HL-60 cultures. Cancer Res., *45:* 847–850, 1985.
6. Thiele, C. J., Reynolds, C. P., and Israel, M. A. Decreased expression of N-*myc* precedes retinoic acid-induced differentiation of human neuroblastoma. Nature (Lond.), *313:* 404–406, 1985.
7. Hager, J. C., Gold, D. V., Barbosa, J. A., Fligiel, Z., Miller, F., and Dexter, D. *N,N*-dimethylformamide-induced modulation of organ- and tumor-associated markers in cultured human colon carcinoma cells. J. Natl. Cancer Inst., *64:* 439–446, 1980.
8. Kim, Y. S., Tsao, D., Bader, S., Whitehead, J. S., Arnstein, P., Bennett, J., and Hicks, J. Effects of sodium butyrate and dimethyl sulfoxide on biochemical properties of human colon cancer cells. Cancer Res., *45:* 1185–1192, 1980.
9. Pinto, M., Appay, M-D., Simon-Assmann, P., Chevalier, G., Dracopli, N., Fogh, J., and Zweibaum, A. Enterocytic differentiation of cultured human colon cancer cells by replacement of glucose by galactose in the medium. Biol. Cell, *44:* 193–196, 1982.
10. Dexter, D. L., and Hager, J. C. Maturation-induction of tumor cells using a human colon carcinoma model. Cancer (Phila.), *45:* 1178–1184, 1980.
11. Augeron, C., and Laboisse, C. L. Emergence of permanently differentiated cell clones in a human colonic cancer cell line in culture after treatment with sodium butyrate. Cancer Res., *44:* 3961–3969, 1984.
12. Perucho, M., Goldfarb, M., Shimizu, K., Lama, C., Fogh, J., and Wigler, M. Human-tumor-derived cell lines contain common and different transforming genes. Cell, *27:* 467–476, 1981.
13. Stragand, J. J., Barlogie, B., White, R. A., and Drewinko, B. Biological properties of the human colonic adenocarcinoma cell line SW 620 grown as a xenograft in the athymic mouse. Cancer Res., *41:* 3364–3369, 1981.
14. Glisin, V., Crkvenjakov, R., and Byus, C. Biochemistry, *13:* 2633–2637, 1974.
15. Raymondjean, M., Kneip, B., and Schapira, G. Preparation and characterization of mRNAs from rat heart muscle. Biochimie, *65:* 65–70, 1983.
16. Augenlicht, L. H., Kobrin, D. E., Pavlovec, A., and Royston, M. E. Elevated expression of an endogenous retroviral long terminal repeat in a mouse colon tumor. J. Biol. Chem., *259:* 1842–1847, 1984.
17. Dalla Favera, R., Gelmann, E. P., Martinotti, S., Franchini, G., Papas, T. S., Gallo, R. C., and Wong-Staal, F. Cloning and characterization of different human sequences related to the onc gene (v-*myc*) of avian myelocytomatosis virus (MC-29). Proc. Natl. Acad. Sci. USA, *79:* 6497–6501, 1982.
18. Michitsch, R. W., Montgomery, K., and Melera, P. W. Expression of the amplified domain in human neuroblastoma cells. Mol. Cell Biol., *4:* 2370–2380, 1984.
19. Perbal, B., and Baluda, M. A. Avian myeloblastosis virus transforming gene is related to unique chicken DNA regions separated by at least one intervening sequence. J. Virol., *41:* 250–257, 1982.
20. Ellis, R. W., Lowy, D. R., and Scolnick, E. M. The viral and cellular p21 (*ras*) gene family. Adv. Viral Oncol., *1:* 107–126, 1982.
21. Murray, M. J., Cunningham, J. M., Parada, L. F., Dautry, F., Lebowitz, P., and Weinberg, R. A. The HL-60 transforming sequence: a *ras* oncogene coexisting with altered *myc* genes in hematopoietic tumors. Cell, *33:* 749–757, 1983.
22. Pulciani, S., Santos, E., Lauver, A. V., Long, L. K., and Barbacid, M. Transforming genes in human tumors. J. Cell Biochem., *20:* 51–61, 1982.
23. McCoy, M. S., Bargmann, C. I., and Weinberg, R. A. Human colon carcinoma Ki-*ras2* oncogene and its corresponding proto-oncogene, Mol. Cell Biol., *4:* 1577–1582, 1984.
24. Ralston, R., and Bishop, J. M. The protein products of the *myc* and *myb* oncogenes and adenovirus E1a are structurally related. Nature (Lond.), *306:* 803–806, 1983.
25. Kohl, N. E., Gee, C. E., and Alt, F. W. Activated expression of the N-*myc* gene in human neuroblastomas and related tumors. Science (Wash. DC), *226:* 1335–1337, 1984.
26. Garin Chesa, P., Rettig, W. J., Melamed, M. R., Old, L. J., and Niman, H. L. Expression of p21 *ras* in normal and malignant tissues: lack of association with proliferation and malignancy. Proc. Natl. Acad. Sci. USA, in press; 1987.
27. Gallick, G. E., Kurzrock, R., Kloetzer, W. S., Arlinghaus, R. B., and Gutterman, J. U. Expression of p21*ras* in fresh primary and metastatic human colorectal tumors. Proc. Natl. Acad. Sci. USA, *82:* 1795–1799, 1985.
28. Foulds, L. Multiple etiological factors in neoplastic development. Cancer Res., *25:* 1339–1347, 1965.
29. Bar-Sagi, D., and Feramisco, J. R. Microinjection of the *ras* oncogene protein into PC12 cells induces morphological differentiation. Cell, *42:* 841–848, 1985.
30. Hurley, J. B., Simon, M. I., Teplow, D. B., Robishaw, J. D., and Gilman, A. G. Homologies between signal transducing G proteins and *ras* gene products. Science (Wash. DC), *226:* 860–862, 1984.
31. Toda, T., Uno, I., Ishikawa, T., Powers, S., Kataoka, T., Broek, D., Cameron, S., Broach, J., Matsumoto, K., and Wigler, M. In yeast, *ras* proteins are controlling elements of adenylate cyclase. Cell, *40:* 27–36, 1985.
32. Spandidos, D. A., and Kerr, I. B. Elevated expression of the human *ras* oncogene family in premalignant and malignant tumors of the colorectum. Br. J. Cancer, *49:* 681–688, 1984.
33. Heppner, G. H. Tumor heterogeneity. Cancer Res., *44:* 2259–2265, 1984.
34. Thor, A., Hand, P. H., Wunderlich, D., Caruso, A., Muraro, R., and Schlom, J. Monoclonal antibodies define differential *ras* gene expression in malignant and benign colonic diseases. Nature (Lond.), *311:* 562–564, 1984.
35. Fogh, J., and Trempe, G. New human tumor cell lines. *In:* J. Fogh (ed.), Human Tumor Cells *In Vitro*, pp. 115–141. New York: Plenum Press, 1975.

[5] D. L. Dexter, personal communication.