Expression of c-myc and Other Cell Cycle-dependent Genes in Human Colon Neoplasia

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ABSTRACT

We have investigated the expression of certain cell cycle-dependent genes in total RNA isolated from normal and neoplastic cells of human epithelial colon tissue. The genes studied had been previously identified as cell cycle dependent in rodent and human fibroblasts. Levels of expression of G1 genes were compared to the level of expression of the S-phase-specific gene H3 in normal and adjacent neoplastic epithelial cells of six different individuals.

We have found that the increase in the expression of c-myc gene detected in colon tumor cells is accompanied by a parallel increase in the expression of two G1-specific genes (p2A9 and ornithine decarboxylase) and the S-phase-specific gene histone H3. An important conclusion that can be drawn from these findings is that the increased level of a cell cycle-specific RNA in a tumor may not indicate overexpression of that gene but simply reflect the increased fraction of cycling cells, unless the ratio of expression between G1 genes and G1-S-phase genes is altered.

INTRODUCTION

A feature of human neoplasia is the proliferative advantage of the neoplastic clone(s) (1). This proliferative advantage should be dependent upon an impairment in the control of cell proliferation whose molecular mechanisms remain at present largely elusive. A way to analyze the mechanisms of cell proliferation impairment that occurs in human cancers is by studying the expression of specific genes that are precisely regulated during the cell cycle of normal human cells. It is legitimate to predict that the expression of certain cell cycle genes might be altered in human neoplasia.

A number of genes and cDNA3 sequences (including at least 4 oncogenes) are known to be expressed in a cell cycle-dependent manner; i.e., the levels of specific mRNAs vary with the phases of the cell cycle. These include histone genes (2, 3), c-myc (4), c-ras (5), c-fos (6, 7), p53 (8), thymidine kinase (9), calmodulin (10), actin (5), and ornithine decarboxylase (11). Other cell cycle-dependent genes have been identified as cDNA clones (12-14) by differential screening of cDNA libraries.

The fact that certain genes are expressed in a cell cycle-dependent manner does not mean that they regulate the cell cycle progression, although evidence is accumulating that at least some oncogenes, whose expression is cell cycle dependent in vitro and in vivo (4, 5, 15, 16), may play an important role in the control of cell proliferation (17-19). We have reasoned that

the expression of cell cycle-dependent genes, defined here as genes that are preferentially expressed in a specific phase of the cell cycle, should be coordinated during the cell cycle of normal cells.

For instance, the expression of early G1-, late G1-, and S-phase-specific genes may vary from one tissue to another, reflecting the fraction of cycling cells, but their ratio should remain constant. This ratio could be altered in cancer cells if the coordinated pattern of cell cycle genes' expression is lost in association with the neoplastic phenotype. The comparison of G1- and S-phase genes' expression gives information on whether a cell cycle gene is truly overexpressed in neoplastic cells. The question we have asked here is whether the G1-S-phase ratio as measured by the level of expression of G1- and S-phase genes is altered in colon tumor cells in comparison to normal adjacent tissue.

MATERIALS AND METHODS

Tissue Samples for RNA Extraction. Six colon specimens were obtained immediately after surgical resection and processed on ice. They were opened, and the contents were rinsed off with cold phosphate-buffered saline. Tissue samples were taken from the tumor as well as the normal areas. Ulcerated and necrotic tissue was dissected off the tumor tissue, and the submucosa and muscularis were removed from the normal tissue. Thus only the intact tumor and normal mucosa were used for RNA extraction. Samples were taken from six carcinomas, one adenoma, six normal colons, and one ileum. They were immediately stored in liquid nitrogen until use in about 1 wk.

Tissue Samples for Microscopy. Whole thickness tissue slices were taken from the tumor as well as normal areas for microscopy. They were fixed in buffered formalin, embedded in paraffin, and routinely stained with hematoxylin and eosin for histological diagnosis.

RNA Isolation. Total cellular RNA was purified from colon tissues according to Frazier et al. (20). Briefly, the tissue was homogenized in a Waring blender in the extraction buffer (75 mM NaCl:20 mM EDTA:10 mM Tris-HCl (pH 8.0):0.2% SDS) mixed 1:1 with buffer-saturated phenol. The aqueous phase was recovered by centrifugation, reextracted with an equal volume of phenol and chloroform isoamyl alcohol (25:24:1), and once again with chloroform:isoamyl alcohol (24:1). The nucleic acids were precipitated with ethanol, and DNA was removed by treatment with DNase I and precipitation with 3 M sodium acetate (pH 5.5). The integrity of RNA samples was monitored by ethidium bromide staining of agarose formaldehyde gels.

Northern Blot Analysis. Total cellular RNA was denatured with 6.3% formaldehyde and 50% formamide and then size fractionated on a 1.2% agarose gel containing 6.6% formaldehyde and 50% formamide and then size fractionated on a 1.2% agarose gel containing 6.6% formaldehyde. Blotting of RNA to nitrocellulose was done as described by Thomas (21). Nick translation (22) of the plasmid DNA at high specific activity was performed as described by Lai et al. (23). Prehybridization, hybridization, and posthybridization washes were essentially as described by Wahl et al. (24). Filters were exposed to Kodak X-ray films using intensifying screens at −70°C. Densitometer readings of the films were performed with the aid of a Zeinit soft-laser densitometer (Biomed Instruments, Inc., Fullerton, CA).

Received 5/3/85; revised 8/14/85; accepted 8/19/85.

1 This work was supported by Grant BRSG 507-RR05417 (B. C.).
2 Special Fellow of the Leukemia Society of America. To whom requests for reprints should be addressed.
3 The abbreviations used are: cDNA, complementary DNA; ODC, ornithine decarboxylase.
Plasmids. Plasmids carrying the gene probes used in these studies were described in detail elsewhere. Plasmids were: p2A9 by Hirschhorn et al. (14); pMC415 (carrying the c-myc gene probe, a kind gift of Dr. G. Franchini and Dr. F. Wong-Staal) by Dalla Pavera et al. (25); PODC934 carrying a mouse cDNA clone of ornithine decarboxylase by Berger et al. (26); and pF0422 carrying a histone H3 gene probe, a kind gift of Dr. G. Stein.

RESULTS

Histology of Colon tissues. The specimens were examined macroscopically and microscopically. This histological diagnosis and the location of the lesions are listed in Table 1. Fig. 1A shows normal colonic mucosa; Fig. 1B shows a tubulovillous adenoma detected in adjacent colon tissue, and Fig. 1C shows the adenocarcinoma in a near area in the colon of the same patient, exhibiting moderate glandular and cellular differentiation. Fig. 1D is the carcinoma from Specimen 2, showing solid sheets of undifferentiated cells and a few small lumens. The adenocarcinoma from the other specimens has a histological appearance similar to Specimen 1.

Selection of Cell Cycle-specific cDNA Clones. We have selected for these experiments the following cell cycle-specific cDNA clones: the oncogene c-myc, whose expression is cell cycle dependent, with a peak in early to mid-G1 (4, 15); a cDNA clone isolated from a Syrian hamster cDNA library, p2A9, also G1 specific, as it is induced early after serum stimulation (14); a mouse cDNA clone of the ornithine decarboxylase gene, PODC934, which increases early in G1 and remains at a high level throughout G1 (11); and the histone H3 clone, whose expression is limited to the S phase of the cell cycle (2, 3). These four genes are expressed in a cell cycle-dependent manner in serum-stimulated Syrian hamster fibroblasts (14), in serum-stimulated mouse 3T3 cells, and in diploid human fibroblasts induced to proliferate by the addition of 10% fetal calf serum (27).

Expression of Cell Cycle-dependent Genes in Normal and Tumor Epithelial Colon Tissue. The purpose of these studies was to compare the levels of expression of four cell cycle-dependent genes (c-myc, ODC, 2A9, and H3) in normal and neoplastic epithelial colon tissue. To obtain accurate data, in some cases the same Northern blots were hybridized concurrently to two different probes or rehybridized with all the probes used in these experiments. A typical example is shown in Fig. 2.}

<table>
<thead>
<tr>
<th>Case</th>
<th>Location</th>
<th>Histological diagnosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Ascending colon</td>
<td>Moderately differentiated adenocarcinoma</td>
</tr>
<tr>
<td>2</td>
<td>Transverse colon</td>
<td>Tubulovillous adenoma</td>
</tr>
<tr>
<td>3</td>
<td>Cecum</td>
<td>Poorly differentiated adenocarcinoma</td>
</tr>
<tr>
<td>4</td>
<td>Sigmoid colon</td>
<td>Moderately differentiated adenocarcinoma</td>
</tr>
<tr>
<td>5</td>
<td>Descending colon</td>
<td>Moderately differentiated adenocarcinoma</td>
</tr>
<tr>
<td>6</td>
<td>Transverse colon</td>
<td>Moderately differentiated adenocarcinoma</td>
</tr>
</tbody>
</table>


DISCUSSION

It has been already documented that, in colon epithelial tumors, the expression of c-myc is increased in comparison to the normal adjacent colon tissue (28). The discovery that the expression of c-myc does increase when quiescent mammalian cells are stimulated to proliferate in vitro and in vivo by a variety of mitogens (4, 5, 15, 16) makes it feasible to correlate more clearly the expression of certain oncogenes and cell cycle genes with the growth characteristics of neoplastic cells. Recently (29, 30) it has been claimed that the expression of c-myc might be constitutive and not cell cycle regulated in proliferating cells. These observations do not contradict the fact that several laboratories have found the expression of c-myc increased during the transition from G0 to G1 in vitro (4, 5, 15) and in vivo (16). Therefore, we may consider c-myc as a growth-dependent, G0-G1-specific gene. Perhaps, to avoid a semantic problem, we should add that we refer to genes induced by the transition G0 to G1 as G0 genes. In mixed cell populations of cycling and noncycling cells (G0 and G1), which is a Northern blot of total RNA from one normal ileum, one normal colon mucosa, one adenoma, one adenocarcinoma from the same individual (Lanes a to d), one normal colon mucosa, and one adenocarcinoma from a second patient (Lanes e and f). It is evident that the increase of c-myc mRNA in tumor RNA is paralleled by an increase in the messenger RNA levels of 2A9 and H3. The same filter was also hybridized with pODC with a similar result (not shown). It is worth noting that the ratio of expression between c-myc and the other cell cycle-dependent genes is higher in the first adenocarcinoma of Fig. 2 (Fig. 2, Lane d) than in the adenoma from the same patient (Fig. 2, Lane c). We have performed densitometer scanning of the autoradiograph of Fig. 2, and we have found that the H3:c-myc ratio is 3.7 in the adenoma (Fig. 2, Lane c), but it is only 2.1 in the adenocarcinoma (Fig. 2, Lane d). This difference in the ratio is entirely due to an increased expression of c-myc in the adenocarcinoma, because the expression of histone H3 is essentially the same in adenoma and adenocarcinoma. Fig. 3 shows the same type of analysis extended to one additional patient with moderately differentiated colon adenocarcinoma. The qualitative findings are similar to those shown in Fig. 2. Again the increased expression of c-myc in colon adenocarcinoma is accompanied by a parallel increase of ODC, 2A9, and histone H3. We always observed some degree of cross-hybridization of the pODC probe with rRNA. In fact, in Fig. 3, there is cross-hybridization to 18S RNA below the mRNA species, 2.2 kilobases in length, hybridizing to the pODC probe. Fig. 3 shows the hybridization of the four cell cycle-dependent genes in total RNA of normal and tumor colon tissue of two additional patients. The findings are in agreement with those of Figs. 2 and 3. We have extended our observation to a sixth patient (not shown), obtaining similar results.

Finally, we have hybridized the RNA filters described in this paper with another gene sequence, p2F1, identified as cell cycle dependent in rodent and human fibroblasts (14, 27) (not shown). We did not detect differences in the expression of this gene sequence between normal and tumor cells. This suggests that the expression of this sequence might not be growth related in epithelial cells and proves that the same amount of RNA was transferred in each lane.

DISCUSSION

It has been already documented that, in colon epithelial tumors, the expression of c-myc is increased in comparison to the normal adjacent colon tissue (28). The discovery that the expression of c-myc does increase when quiescent mammalian cells are stimulated to proliferate in vitro and in vivo by a variety of mitogens (4, 5, 15, 16) makes it feasible to correlate more clearly the expression of certain oncogenes and cell cycle genes with the growth characteristics of neoplastic cells. Recently (29, 30) it has been claimed that the expression of c-myc might be constitutive and not cell cycle regulated in proliferating cells. These observations do not contradict the fact that several laboratories have found the expression of c-myc increased during the transition from G0 to G1 in vitro (4, 5, 15) and in vivo (16). Therefore, we may consider c-myc as a growth-dependent, G0-G1-specific gene. Perhaps, to avoid a semantic problem, we should add that we refer to genes induced by the transition G0 to G1 as G0 genes. In mixed cell populations of cycling and noncycling cells (G0 and...
terminally differentiated cells), overexpression of a growth-dep-
dependent G1 gene like c-myc may simply be due to an increase in
the fraction of cycling cells, i.e., the growth fraction (31). We
have observed in all cases of colon adenocarcinomas studied
that, in comparison to normal colon epithelial tissues, the in-
creased expression of c-myc is paralleled by an increase in the
expression of two other G1 genes (2A9 and ODC) and a S-phase
gene (histone H3). Thus the expression of c-myc in colon aden-
ocarcinomas reflects to a considerable extent the increased
proliferative capability of these tumor cells as exemplified by the
concomitant high expression of two d genes (2A9 and ODC)
and a S-phase gene (histone H3). Interestingly, the highest levels
of expression of the four cell cycle genes were shown by
Specimen 2 (Fig. 2, Lane d). This finding is in good agreement
with the undifferentiated morphology of this tumor (Fig. 1D).
Based on these findings and on similar findings in human leukae-
mas,9 we suggest a more conservative assessment of the oc-
currence of “overexpression” of oncogenes and cell cycle-
dependent genes in human cancers.

By determining the ratio of expression of G1-S genes, we can
more properly ask whether the expression of certain cell cycle
genes is truly overexpressed. In this regard a careful look to Fig.
2 may support this notion. The ratio of expression between c-
mvc and H3 is higher in the adenocarcinoma (Fig. 2, Lane d)
than in the adenoma (Fig. 2, Lane c). This is also true for the
ratio of expression between c-myc and 2A9, as well as c-myc and
ODC (not shown). This finding is of interest because, al-
though the colon adenoma is considered a precancerous lesion
(32), the more malignant phenotype of the adenocarcinoma is
associated with a real overexpression of c-myc. This overexp-
ression could be due to a loss of temporal control; i.e., the G1 gene
is expressed independently of the position of a cell in the cell
cycle. In this respect, the observation by Campisi et al. (5), that
the cell cycle control by c-myc expression (but not of c-ras)
is lost following chemical transformation, clearly supports the
above hypothesis. Two important conclusions that can be drawn
from these findings are that (a) the increased level of G1-specific
mRNA in a tumor must be looked at in association with the
expression of other G1 genes and S-phase genes and (b) if the
ratio between G1 genes and G1-S-phase genes is altered, we
can legitimately assume that there is overexpression of a cell
cycle gene.

ACKNOWLEDGMENTS

We would like to express our gratitude to Renato Baserga for his constant
encouragement and support.
We thank also Dr. F. Berger, Dr. G. Franchini, and Dr. G. Stein for the gene
probes.

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Fig. 1. A, normal colonic mucosa from Specimen 1. H & E, × 150. B, adenoma from Specimen 1, showing goblet cell-lined glands. H & E, × 150. C, adenocarcinoma from Specimen 1, showing moderate glandular and cellular differentiation. The tumors in Specimen 3 have similar histological appearance. H & E, × 150. D, the carcinoma from Specimen 2, showing solid sheets of undifferentiated cells and a few small glandular lumens. H & E, × 150.
**Fig. 2.** Composite picture of autoradiographs from Northern blots of total RNA isolated from normal and neoplastic colon epithelium. Total RNA was isolated as described in “Materials and Methods,” and 15 μg per lane were electrophoresed on a 1.2% agarose-formaldehyde gel and subsequently transferred to nitrocellulose as described by Thomas (21). The first hybridization was carried out using c-myc (25) and H3 plasmids, nick translated to a specific activity of 4 to 5 × 10^6 cpm/μg (see “Materials and Methods”). The filter was subsequently washed in boiling water to remove the previous probes and rehybridized with the p2A9 probe (14). The size of the c-myc band is 2.4 kilobases; the size of the H3 band is ~0.5 kilobases; the size of the 2A9 band is ~0.6 kilobases. The sources of the RNAs in the various lanes are as follows: Lane a, normal ileum; Lane b, normal colon mucosa; Lane c, adenoma; Lane d, adenocarcinoma, from the first patient; Lane e, normal colon mucosa; Lane f, adenocarcinoma, from the second patient.

**Fig. 3.** Composite picture of autoradiographs from Northern blots of total RNA isolated from normal and neoplastic colon epithelium. Total RNA (15 μg/lane) was electrophoresed, blotted, and hybridized as described in “Materials and Methods.” The filter was concomitantly hybridized with c-myc (25) and H3 probes, and after washing the residual probes, it was rehybridized with pODC (26) and p2A9 (14). The size of the mRNA band hybridizing to pODC is 2.2 kilobases. The sources of the RNAs in the two lanes are as follows: Lane a, normal colon mucosa; Lane b, adenocarcinoma, from the third patient.

**Fig. 4.** Composite picture of autoradiographs from Northern blots of total RNA isolated from normal and neoplastic colon epithelium. Total RNA (15 μg/lane) was electrophoresed, blotted, and hybridized as described in “Materials and Methods.” The filter was concomitantly hybridized with c-myc (25) and H3 probes, and after washing, the residual probes were rehybridized with p2A9 (14) and finally with pODC (26). The sources of the RNAs in the various lanes are as follows: Lane a, normal colon mucosa; Lane b, adenocarcinoma, from the fourth patient; Lane c, normal colon mucosa; and Lane d, adenocarcinoma, from the fifth patient.
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