Growth-dependent Expression of Human M, 53,000 Tumor Antigen Messenger RNA in Normal and Neoplastic Cells

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ABSTRACT

We have investigated the expression of M, 53,000 protein (p53) in total RNA isolated from human peripheral blood mononuclear cells stimulated by phytohemagglutinin, in serum-stimulated human diploid fibroblasts, and in normal and tumor cells of human epithelial colon tissue. We have found that the expression of p53 messenger RNA is growth regulated in human cells following kinetics similar to that previously shown in mouse 3T3 cells, and is increased in the large majority of colon adenocarcinomas in comparison to adjacent normal mucosa and adenoma. This increased expression of p53 is accompanied by a nearly proportional increase in the expression of histone H3. As the expression of histone H3 is restricted to the S phase of the cell cycle and therefore measures the growth fraction of a given population, we suggest that the increased expression of p53 observed in the large majority of colon tumors simply reflects the increased number of cycling cells frequently found in a neoplastic tissue. At variance with these findings a true overexpression of p53 was detected in one SV40-transformed human fibroblasts cell line.

INTRODUCTION

The term p531 is used to identify a family of proteins ranging in molecular weights from 48,000 to 55,000 that are found in high amount in mammalian cells transformed by a variety of agents such as DNA tumor viruses (1, 2), RNA tumor viruses (3), and chemical agents (2-4). It was also shown that the p53 protein level was higher in transformed cell lines derived from human tumors than in nontransformed cell lines (5). More recent studies have suggested that p53 may play a direct role in the process of neoplastic transformation (6-8). Of particular importance, in this regard, is the observation that cotransfection of a gene encoding the mouse p53 with an activated human ras gene yields transformed foci of primary embryonic cells (6-8).

A second direction of research on p53 function deals with the role that this protein might have in the control of normal cell proliferation. It has been shown that an increase of p53 protein and mRNA precedes the synthesis of DNA in mouse 3T3 fibroblasts moving from a growth-arrested state to an actively dividing state (9), and that there is an increase of p53 gene yields transformed foci of primary embryonic cells (6-8).

In three significant functional studies it was shown that microinjection of monoclonal antibodies against p53 protein inhibited the entry into S phase of serum-stimulated Swiss 3T3 fibroblasts (12, 13), and that microinjection of active p53 encoding sequences stimulated DNA synthesis in quiescent mouse 3T3 cells (14).

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3 The abbreviations used are: p53, M, 53,000 protein; PHA, phytohemagglutinin; PBM cells, peripheral blood mononuclear cells.

These two distinct and yet intermingling facets of p53 (role in normal cell proliferation and involvement in neoplastic transformation), made this gene a valuable molecular tool to investigate the impairment of proliferative control that is a key factor in the origin and the progression of human neoplasia (15). A legitimate theory of neoplastic transformation is that an increased activity of some key genes is actively involved in transforming a normal cell into a neoplastic one (16). Therefore, we have chosen to compare the expression of p53 in normal and in adjacent tumor colon tissue of the same patients.

Normally, cell division in the colonic mucosa is restricted to the deeper two-thirds of the crypt. Neoplastic transformation is accompanied by the loss of such restriction in cell growth. This change is visualized by the ability of the cells along the entire length of the crypt, including the surface cells, to synthesize DNA and divide (17).

We reasoned that the correct assessment of the level of expression of a growth-regulated gene like p53 in normal and tumor tissues requires the evaluation of the growth fraction of both cell populations. The expression of histone H3 gene, like the other core histones, is finely regulated (18, 19) and is limited to the S phase of the cell cycle. Therefore, it is a good indicator of the growth fraction (20) of different cell populations.

By comparing the expression of a growth-regulated gene to a S-phase gene and by measuring this ratio in normal and tumor cells, we can determine whether a growth-regulated gene is truly overexpressed in neoplastic cells.

Before studying the expression of p53 in human colon neoplasia, we wanted to be sure that the mRNA level of p53 is truly growth regulated in human cells. For this purpose human peripheral blood mononuclear cells and diploid fibroblast cell strains were used. In addition, cell lines derived by transformation with SV40 virus were included for comparison in the studies reported here.

MATERIALS AND METHODS

Cells and Culture Conditions. The human diploid fibroblast cell strain WI-38 and an SV40-transformed cell line VA13-2RA derived from WI-38 were both kindly provided by Dr. R. Baserga (Temple University School of Medicine). The human diploid cell strains IMR-90 and GM1142, and SV40-transformed derivatives SV1MR-90 and SVGM1142 were kindly provided by Dr. W. Nichols (Merck, Sharp and Dohme). They were grown in Eagle's minimum essential medium with vitamins, antibiotics, in the presence of 10% (v/v) fetal calf serum. Peripheral blood mononuclear cells were isolated by Ficoll-Hypaque gradients from venous blood collected from healthy volunteers, and cultured as described by Maizel et al. (21). The cells were washed with Hanks' solution and then cultured in plastic Falcon tissue cultures flasks for the desired periods of time at a concentration of 10⁵ cells/ml of RPMI 1640 supplemented with penicillin, streptomycin, l-glutamine, 5% heat-inactivated fetal calf serum, and PHA (Sigma Chemical Co.) at 10 μg/ml. Each culture was monitored for the level of stimulation by determining autoradiographically the percentage of cells that incorporate [³H]thymidine during 66-72 h in culture. After culture, cells were harvested by centrifugation, washed twice in 0.9% NaCl...
solution, and total cellular RNA was then extracted. Human fibroblasts WI-38 (22) were cultured in plastic Petri dishes in 10% fetal calf serum for 14 days until the cells become quiescent and were then used for the experiments. Fibroblasts were either stimulated with fresh medium for 1, 6, 16, and 24 h, or were replaced at dilution 1:20 and after 3 days synchronized in S phase with 2.5 mM hydroxyurea. After overnight exposure to hydroxyurea the drug was removed by extensive washing and cells were next harvested 4, 9, 13, 18, and 23 h later. Total cytoplasmic RNA was extracted (23) from quiescent as well as from stimulated and hydroxyurea block-released cells.

**Tissue Samples for RNA Extraction.** Eleven 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 from the normal areas. Ulcerated and necrotic tissue was dissected off the tumor tissue, and the submucosa and muscularis were removed from normal tissue. Thus only the intact tumor and normal mucosa were used for RNA extraction. Samples were taken from 11 carcinomas, 4 adenomas, and 11 normal colons. They were immediately stored in liquid nitrogen until use in about 1 week.

**Tissue Samples for Microscopy.** Whole thickness tissue slices were taken from the tumor as well as from 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 culture cells and colon tissue according to Frazier et al. (24). 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% sodium dodecyl sulfate] 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 (24:2:1), 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 mM sodium acetate (pH 5.5). The integrity and the correct amount of the electrophoresed RNA samples were monitored with ethidium bromide staining of agarose gels.

**Northern Blot Analysis.** Total cellular RNA was denatured with 6.3% formaldehyde and 50% formamide and then size fractioned on a 1.2% agarose gel containing 6.6% formaldehyde. Blotting of RNA to nitrocellulose was done as described by Thomas (25). Nick translation (26, 27) of the plasmid DNA at high specific activity was performed as described by Lai et al. (28). Prehybridization, hybridization, and posthybridization washes were as essential as described by Wahl et al. (29). Filters were exposed to Kodak X-ray films using intensifying screen at −70°C. Densitometer readings of the films were performed with the aid of a Zeineth soft laser densitometer (Biomed Instruments, Inc., Fullerton, CA).

**Plasmids.** Plasmids carrying the gene probes used in these studies were: php53c-1 kindly provided by Dr. D. Givol (The Weizmann Institute of Science) containing a full-length human p53 cDNA described by Zakut-Houri et al. (30), and pFo 422 carrying a histone H3 gene (kind gift of Dr. G. Stein, University of Florida).

**RESULTS**

**Growth-dependent Expression of p53 RNA in Human Cells.** Previous work from our laboratory has shown that the p53 protein recognized by monoclonal antibody PAb122 is not detectable in resting (G₀) human peripheral blood mononuclear cells; however, following PHA stimulation it is detectable at 18–24 h and reaches a maximum level of synthesis between 42 and 48 h after stimulation (10). We wanted to establish that the increased p53 protein synthesis observed in PHA-stimulated PBM cells is paralleled by an increase in the steady-state levels of p53 mRNA. Fig. 1A shows a Northern blot of RNA isolated from PBM cells at different times after PHA stimulation, hybridized with a human p53 cDNA probe. The results presented in Fig. 1A show that p53 mRNA levels increase at 18 h (Fig. 1A, Lane b), reach the highest level at 40 h (Fig. 1A, Lane c), and remain at essentially the same high level until 66 h (Fig. 1A, Lane d) following PHA stimulation. Under our culture conditions, 60% of the cells enter the S phase between 40 and 66 h of PHA stimulation. Therefore, the highest level of p53 RNA (and protein) expression in normal PBM cells occurs when the majority of the cells are in late G₀ or S phase.

We also examined the expression of p53 RNA in synchronized cultures of the human diploid fibroblasts cell strain, WI-38 (22). Histone H3 was used as a control probe because the expression of this gene is preferentially limited to the S phase of the cell cycle. In this study, we wanted to compare directly the steady-state level of p53 RNA with the level of histone H3 RNA in serum-stimulated fibroblasts during the G₀-G₁-S-phase transition, and also during the S-G₂-M-phase transition. For this purpose WI-38 fibroblasts were first made quiescent by serum deprivation and subsequently were stimulated with serum to reenter the cell cycle, or exponentially growing cells were first blocked at the G₁-S-phase boundary by hydroxyurea treatment and subsequently were released from the block by removing the hydroxyurea-containing medium and replacing it with fresh medium.

![Fig. 1A](image-url)
Fig. 1B shows the levels of p53 mRNA expression in serum-stimulated WI-38 cells (Fig. 1B, Lanes a-e) and in cells released from the G1-S-phase block (Fig. 1B, Lanes b-j). The results show that p53 mRNA levels increase very early after serum addition in WI-38 cells and continue to increase, reaching the highest level of expression after 16–24 h. The kinetics of p53 expression during the G0-G1-S-phase transition in serum-stimulated fibroblasts is similar to that of PHA-stimulated lymphocytes with the highest levels occurring in all cell types in late G1-early S phase. In WI-38 cells released from a G1-S-phase hydroxyurea block during the S-G2-M transition p53 RNA expression remains relatively constant (Fig. 1B, Lanes f-j) and is considerably higher than in quiescent (G0) cells (Fig. 1B, Lane a). The expression of histone H3 mRNA corresponds well with the entry into S phase of the cells following serum stimulation from quiescence (Fig. 1B, Lane e) and in cells released from the G1-S-phase hydroxyurea block (Fig. 1B, Lanes f and j). It is noteworthy to mention that in the latter system, at 4 h after hydroxyurea release (Fig. 1B, Lane f), a large proportion of the cells are still in S phase and express histone H3 mRNA. Moreover, a second peak of histone H3 mRNA expression occurs at 23 h after hydroxyurea release (Fig. 1B, Lane j), which corresponds to the S phase of the second cell cycle.

Expression of p53 and Histone H3 RNA in Normal and SV40-transformed Fibroblasts. We next ask whether the expression of p53 RNA is altered in SV40-transformed established human fibroblast cell lines. The human diploid fibroblast strains (WI-38, IMR-90, and GM1142) and their SV40-transformed counterparts (VA13-2RA, SVIMR-90, and SVGM1142) were examined and the level of p53 mRNA expression was compared to the levels of histone H3 mRNA expression. Because the expression of histone H3 is limited to the S phase of the cell cycle (18, 19), this approach allowed us to evaluate the dependence of the levels of expression of a growth-regulated gene (p53) on the growth fraction of each matched set of cells analyzed.

Fig. 2, left, shows a Northern blot of RNA from normal IMR-90 and GM1142 cell strains and their SV40-transformed established cell lines SVIMR-90 and SVGM1142, hybridized first with histone H3 and next with the p53 probes. We measured the levels of hybridization by soft laser densitometry of the autoradiograph of this blot and found very little, if any, difference in the levels of p53 RNA expression relative to histone H3 expression in these normal and SV40-transformed cells. Fig. 2, right, is a composite picture of a Northern blot of RNA from the normal cell strain WI-38 and its SV40-transformed derivative VA13-2RA. It is rather obvious that there is a modest difference in the intensity of the H3 band between WI38 and VA13-2RA cells; whereas, the difference in intensity of the p53 band is much more pronounced. We found that there is at most a 2-fold difference for H3, but more than a 10-fold difference for p53. Therefore, the increase in the level of p53 mRNA expression in VA13-2RA in comparison to the parental cell strain WI38 is not merely a reflection of the difference in the growth fraction.

Expression of p53 in Normal and Neoplastic Colon Mucosa. We next decided to extend the same type of analysis to spontaneously occurring human neoplasia. We have selected the adenocarcinoma of the colon because this neoplasm is rather common in Western countries and exhibits a pattern of growth that is a clear subversion of the regular pattern found in the normal mucosa. The specimens from which total cellular RNA was isolated were examined macroscopically and microscopically. The histological diagnosis and the location of the lesions are listed in Table 1. The normal mucosa had a similar histology in all cases. Tubulovillous adenoma was present in 4 cases and in 2 it was the seat of cancer origin (cases 1 and 7). The carcinoma was moderately differentiated in 8 cases, poorly differentiated in 2, well differentiated in one (Table 1). As described below, the degree of tumor differentiation had no apparent effect on the degree of expression of the tested genes.

Fig. 3A is a composite picture of autoradiograms of a filter hybridized first to p53 and subsequently to histone H3. The increased expression of p53 found in most cases in tumor RNA (Fig. 3A, Lanes c, f, h, and l) is paralleled by an increase in the mRNA level of histone H3. In Fig. 3A, Lanes i and j, corresponding to normal and neoplastic tissue of patient 4 the expression of p53 is essentially equal, although in the tumor RNA there is a higher H3 expression.

Fig. 3B shows the same type of analysis extended to six additional patients. Once again, the filter was hybridized to p53 and next to histone H3. It is evident that the increased expression of p53 found in most cases in tumor RNAs (Fig. 3B, Lanes c, f, l, and n) is paralleled by an increase in the mRNA level of histone H3. It is noteworthy that at variance with the prevalent pattern of p53 expression, there are two patients in whom the

### Table 1 Location and histological diagnosis of colonic neoplasms

<table>
<thead>
<tr>
<th>Case</th>
<th>Location</th>
<th>Histological diagnosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>1*</td>
<td>Cecum</td>
<td>Moderately differentiated adenocarcinoma, tubulovillous adenoma</td>
</tr>
<tr>
<td>2</td>
<td>Ascending colon</td>
<td>Well differentiated adenocarcinoma, tubulovillous adenoma</td>
</tr>
<tr>
<td>3</td>
<td>Sigmoid colon</td>
<td>Poorly differentiated adenocarcinoma</td>
</tr>
<tr>
<td>4</td>
<td>Ascending colon</td>
<td>Moderately differentiated adenocarcinoma</td>
</tr>
<tr>
<td>5</td>
<td>Sigmoid colon</td>
<td>Moderately differentiated adenocarcinoma</td>
</tr>
<tr>
<td>6</td>
<td>Ascending colon</td>
<td>Moderately differentiated adenocarcinoma, tubulovillous adenoma</td>
</tr>
<tr>
<td>7*</td>
<td>Cecum</td>
<td>Moderately differentiated adenocarcinoma, tubulovillous adenoma</td>
</tr>
<tr>
<td>8</td>
<td>Cecum</td>
<td>Moderately differentiated adenocarcinoma</td>
</tr>
<tr>
<td>9</td>
<td>Sigmoid colon</td>
<td>Moderately differentiated adenocarcinoma</td>
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<tr>
<td>10</td>
<td>Ascending colon</td>
<td>Moderately differentiated adenocarcinoma</td>
</tr>
<tr>
<td>11</td>
<td>Sigmoid colon</td>
<td>Poorly differentiated adenocarcinoma</td>
</tr>
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* In cases 1 and 7 the carcinomas arose in the tubulovillous adenomas.
level of p53 is essentially the same in normal mucosa and tumor (Fig. 3B, Lanes g, h, i, and j). In one of these two patients (Fig. 3B, Lanes i and j), the expression of H3 is higher in tumor RNA than in normal mucosa. To quantitate our findings, we have performed densitometer scanning of the autoradiographs of Fig. 3, A and B, and we have found that the increase in the expression of p53 in most tumor RNAs is accompanied by a proportional increase in H3 expression.

In conclusion, these findings suggest that, although the p53 mRNA level is in general higher in neoplastic compared to normal or adenomatous tissue, a true overexpression is not detected.

DISCUSSION

It was initially shown (in two cases of colon carcinomas) that the expression of four growth-regulated protooncogenes (c-myc, c-fos, c-Ha-ras, and c-Ki-ras) is higher in adenocarcinoma than in normal colon mucosa (33). Recently, Erisman et al. (34) analyzed the expression of c-myc in 29 cases of human colon cancer and found that in 72% of them the level of c-myc expression significantly exceeded that observed in adjacent nonneoplastic tissue. These investigators suggested the occurrence of frequent deregulation of c-myc expression in colon adenocarcinomas (34). More recently, however, we have suggested that the increased expression of c-myc that is found in most of colon adenocarcinomas might simply reflect the increased number of cycling cells in a tumor population (35).

Our suggestion was based on the comparison of the expression of c-myc and two other growth-regulated sequences (ornithine decarboxylase and p2A9) to histone H3. In those studies, we found a concomitant increased expression of c-myc, ODC, and p2A9 paralleled by an increase of H3 expression in colon adenocarcinoma in comparison to adjacent normal mucosa (35). In the present studies, we used the same approach to investigate the expression of the growth-regulated p53 gene in colon neoplasia. The human p53 cDNA clone (30) was only recently isolated and we are not aware of other studies of p53 expression in human neoplasia. The significant findings of our experiments are the following.

The expression of p53 is growth regulated in both human cellular systems we have studied (PHA-stimulated lymphocytes and serum-stimulated fibroblasts). The kinetics of induction of p53 is similar to that described in mouse 3T3 fibroblasts (9), with an early increase and the highest expression coincident with the entry of cells into S phase (Fig. 1, A and B). Furthermore, the expression of p53 does not change in the cell cycle of proliferating human diploid fibroblasts WI38 during the transition G2-M-G1-S. However, it is significantly higher in these cycling cells than in quiescent cells.

Although p53 mRNA levels reach a maximum in late G1-S phase during the G0-G1-S-phase transition, the finding that the p53 mRNA expression in normal human fibroblasts is relatively invariant throughout the phases of subsequent cell cycles is significant, in that it reinforces the idea that p53 expression is truly growth regulated. A similar conclusion was reached by Coulier et al. (36), who demonstrated by analyzing pure cell fractions representative of cells in various phases of the cell cycle in rat fibroblasts, that the p53 gene is continuously expressed along the cell cycle. On the basis of experiments involving an analysis of either the steady-state levels of p53 protein, or its possible functional role in established mouse 3T3 cells arrested by serum deprivation and subsequently restimulated to divide; we and others (9, 12, 13) have suggested that p53 may play a role in the initial transition of cells from a resting to a growing state. Our current results in the human systems we have studied supports this idea.

We observed that in proliferating VA13-2RA (a cell line derived from SV40-transformed WI-38 cells) there is a true overexpression of p53. However, in the other two SV40-transformed human cell lines, SVIMR-90 and SVGMI142 (derived from SV40-transformed IMR-90 and GM1142 cells, respectively), the p53 gene is not overexpressed even though these cell lines contain elevated p53 protein levels.

This conclusion is based on a comparison of the level of expression of p53 and histone H3 RNA levels.

In the VA13-2RA cell line there is at least a 10-fold increase of p53 expression relative to the levels observed in the parental clone 4B2, Lanes i and j), the expression of H3 is higher in tumor RNA than in normal mucosa. To quantitate our findings, we have performed densitometer scanning of the autoradiographs of Fig. 3, A and B, and we have found that the increase in the expression of p53 in most tumor RNAs is accompanied by a proportional increase in H3 expression.

In conclusion, these findings suggest that, although the p53 mRNA level is in general higher in neoplastic compared to normal or adenomatous tissue, a true overexpression is not detected.

DISCUSSION

It was initially shown (in two cases of colon carcinomas) that the expression of four growth-regulated protooncogenes (c-myc,
expression of p53 mRNA in VA13-2RA cells as it relates to the expression of p53 protein in these cell lines must be posttranscriptionally regulated (37). Thus, the functional significance of the overexpression of p53 mRNA in VA13-2RA cells as it relates to transformation and immortalization of these cells is unclear. It is however, noteworthy to mention that the clear-cut overexpression of p53 RNA in VA13-2RA is not associated with a detectable rearrangement or gene amplification of p53 locus (data not shown).

In the large majority of colon adenocarcinomas we have analyzed, the expression of p53 is increased in comparison to normal or adenomatous mucosa. This increased expression is likely to reflect the fact that a higher number of cycling cells is found in most colon tumorous tissue than in the normal or adenomatous mucosa. This is strongly supported by the proportion increased expression of histone H3. The comparison of c-myc, ornithine decarboxylase, 2A9 (35), and p53 (our current study) RNA expression relative to the expression of histone H3 in adjacent normal and neoplastic tissue alerts one to the fact that the growth fraction of cells in a given population must be taken into account when evaluating the "overexpression" of a given oncogene, especially when the gene under study is expressed in a growth-regulated fashion.

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ADDENDUM

While this work was in progress, Reed et al. (Proc. Natl. Acad. Sci. USA, 83: 3982, 1986) reported similar kinetics of p53 mRNA expression in PHA-stimulated human peripheral blood lymphocytes.

REFERENCES


11. Milner, J., and Milner, S. SV40-53k antigen: a possible role of 53k in normal human or adenomatous mucosa. This increased expression is likely to reflect the fact that a higher number of cycling cells is found in most colon tumorous tissue than in the normal or adenomatous mucosa. This is strongly supported by the proportion increased expression of histone H3. The comparison of c-myc, ornithine decarboxylase, 2A9 (35), and p53 (our current study) RNA expression relative to the expression of histone H3 in adjacent normal and neoplastic tissue alerts one to the fact that the growth fraction of cells in a given population must be taken into account when evaluating the "overexpression" of a given oncogene, especially when the gene under study is expressed in a growth-regulated fashion.
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