Production of Hepatocellular Carcinoma by Oval Cells: Cell Cycle Expression of c-myc and p53 at Different Stages of Oval Cell Transformation

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ABSTRACT

In rats maintained on a carcinogenic diet (choline deficient containing 0.1% ethionine), the levels of c-myc and p53 mRNAs increased by 4 wk after animals were placed on the diet. Cell isolation studies showed that the change in c-myc takes place in oval cells, while p53 increases predominantly in oval cells but also in hepatocytes. To determine whether this increase is a consequence of cell proliferation or is associated with transformation, we have developed an in vitro model of hepatocarcinogenesis using epithelial cells isolated from the livers of rats fed the carcinogenic diet. When maintained in vitro with in frequent subculture, this cell line (LE/6) undergoes spontaneous transformation. Inoculation s.c. of the transformed cells into nude mice yields tumors histologically identified as hepatocellular carcinoma. We have used these cell lines to compare the cell cycle expression of c-myc and p53 mRNAs in untransformed, partially transformed, and tumorigenic LE/6 cells. We find that the expression of both genes is under cell cycle control in untransformed and partially transformed cells. However, complete transformation of this cell line is associated with constitutive expression of myc but not p53 transcripts. On the basis of this work we suggest that constitutive expression of c-myc may be a late event in hepatocarcinogenesis.

INTRODUCTION

Enhanced expression of both myc and ras protooncogenes has been demonstrated in a number of different experimental models of hepatocarcinogenesis (1–8). In previous work, we found that levels of c-myc and c-Ki-ras, but not c-Ha-ras, transcripts were significantly elevated throughout the course of liver carcinogenesis in the rat, and we speculated that one of these genes might be involved in the progressive evolution of liver tumors (4). Although the bulk of the experimental evidence accumulated to date indicates that activated ras genes are not a consistent feature of hepatocarcinogenesis, Nagy et al. (8) found by in situ hybridization that c-myc transcripts are specifically elevated in oval cells, immature hepatocytes, and primary liver tumors relative to surrounding liver tissue.

Since it is known from both in vitro and in vivo studies that the expression of myc mRNAs increases as cells are stimulated to divide (15–21), it has been difficult to determine whether increased expression of this gene is indicative of molecular events specifically related to transformation or rather is a consequence of changes in gene expression in the actively proliferating oval cells. In addition to genetic alterations such as amplification, rearrangement, or mutation, c-myc can be activated by mechanisms leading to a loss of cell cycle control of its expression. Constitutive expression of c-myc associated with transformation has previously been shown in transformed cell lines derived from mouse fibroblasts (17), human colon carcinomas (22), and a rat liver tumor (23), suggesting that this might be a general feature of malignant transformation. On the other hand, in rat tumors induced by diethylnitrosamine and phenobarbital, the expression of c-myc and c-Ha-ras transcripts was variable and only increased late in hepatocarcinogenesis (5). Furthermore, in the same experimental system, when immunohistochemical techniques were used to localize myc proteins in specific cell types, increased levels of myc were detected in neoplastic as well as normal hepatocytes (7). Thus, the issue of whether altered expression of cellular oncogenes, in particular that of c-myc, are essential in initiation or progression of chemically induced rat liver tumors has yet to be established.

To determine if constitutive expression of myc is associated with liver carcinogenesis and, if so, at what stage in the carcinogenic process deregulation occurs, we have developed an in vitro system using cultured oval cells in various stages of transformation. Using this system we have examined the cell cycle expression of two genes, myc and p53, which are known to be activated early in the G1 phase of the cell cycle, in immortalized, anchorage-independent, and tumorigenic oval cells. We also describe the kinetics of expression and cellular distribution of both genes during hepatocarcinogenesis. We find that both genes are increased in the rat livers throughout carcinogen administration and that myc, but not p53, transcripts are constitutively expressed when oval cells become tumorigenic.

MATERIALS AND METHODS

Animals. Male Sprague-Dawley rats (Charles River, Wilmington, MA) weighing 130 to 150 g were fed a CDE diet prepared by Teklad Test Diets, Madison, WI. Poly A+ RNA was isolated from the livers fed a CDE diet, we found that increased expression of myc occurred preferentially in the oval cell population, not in differentiated hepatocytes (4). These studies have been extended by Nagy et al. (8) who found by in situ hybridization that c-myc transcripts are specifically elevated in oval cells, immature hepatocytes, and primary liver tumors relative to surrounding liver tissue.

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2 To whom requests for reprints should be addressed.
3 The abbreviations used are: CDE diet, choline-deficient diet containing ethionine; poly A+, polyadenylate-containing RNA; FBS, fetal bovine serum; EGF, epidermal growth factor; SSC, standard saline citrate; SDS, sodium dodecyl sulfate; cDNA, complementary DNA; CD, choline deficient.
4 In this paper, the term cultured oval cells is used to describe a liver epithelial cell line derived from the livers of rats fed a choline-deficient diet for 6 wk.
of rats fed the CDE diet for 4, 8, and 16 wk, by the method of Chirgwin et al. (24) as previously described (4). Purified populations of oval cells and hepatocytes were obtained from the livers of rats fed the CDE diet for 4, 9, and 17 wk by centrifugal elutriation. Total RNA was then extracted from cell pellets (4).

Cell Culture. The untransformed LE/6 cells were derived from oval cells purified from the livers of rats fed the CDE diet for 6 wk as described (14). Cell lines were maintained in Dulbecco’s modified Eagle medium:Ham’s F10 (1:1) supplemented with 10% FBS, 1 μg/ml of insulin, 0.5 μg/ml of hydrocortisone, and antibiotics and passaged once a week. All experiments were performed on stocks of cells frozen at defined passages. Growth curves were performed with each cell line.

Cloned lines LE/6c2-5, LE/6c2-6, and LE/6c3-1 were established from LE/6 passage 50 cells which were stimulated to grow in soft agar by the addition of 10 ng/ml of EGF to the growth medium. Several individual colonies were picked with a Pasteur pipet, cloned once more in soft agar, and finally expanded in monolayer culture as clonal populations. All experiments were performed within 5 passages of cloning.

Transformation of LE/6 Cells. LE/6 passage 25 cells were plated at a concentration of 5 x 10⁴ cells/10-cm tissue culture dish in medium containing 10% FBS. Cultures were fed fresh medium weekly and were passaged on a bimonthly basis until they appeared morphologically altered (approximately 6 mo).⁵ Thereafter, cells were passaged as needed for specific experiments. Tumorigenicity of the cultures was assayed in nude mice by the s.c. inoculation of 1 x 10⁶ cells/animal. When tumors had reached a diameter of 2 to 3 cm, tumor tissue was carefully excised from surrounding normal mouse tissue and fixed in formalin for histological examination.

Growth Studies. Cells were plated at a density of 5 x 10⁴ cells/10-cm plate in medium containing 10% FBS and grown to confluence without the addition of fresh medium. After thorough washing with serum-free medium, confluent monolayers were incubated for 3 days (untransformed cell lines) or 4 days (soft agar clones and transformed cell lines) in medium containing 0.1% FBS. Complete growth arrest with this protocol was monitored in the cell lines by autoradiography. In quiescent cultures of each cell line, less than 1% of the cells was dividing at the time of serum stimulation. Cells were stimulated to divide by the addition of fresh medium containing 10% FBS followed by RNA isolation at the indicated time points.

Northern Blot Analysis. Twenty-μg of total RNA isolated by the method of Chirgwin et al. (24) or 5 μg of poly A⁺ RNA were separated in 1.1% agarose/formaldehyde gels, transferred to nitrocellulose filters, and hybridized with ³²P-labeled probes as previously described (4). To ensure that equivalent amounts of RNA were loaded in each lane, all gels were stained with ethidium bromide prior to transfer. In addition, after hybridization with c-myc and p53, RNA blots were washed 2 times in 0.1× SSC:0.1% SDS at 100°C for 15 min and rehybridized with a 1.35-kilobase BglII fragment of the gene for the insulin receptor. The RNA transcripts for this gene remain constant during cell proliferation.

Southern Blot Analysis. DNA was extracted from cultured cells according to standard techniques (25), digested with EcoRI, and analyzed according to the method of Southern (26). Filters were hybridized with DNA probes labeled to a high specific activity with [³²P]ATP by nick translation in hybridization buffer containing 4x SSC, 50% formamide, 0.016% bovine serum albumin, 0.016% Ficoll, 0.016% polyvinyl pyrrolidone, 25 μg/ml of yeast RNA, and 10% (w/v) dextran sulfate. The filters were then washed 4 times for 5 min in 2x SSC:0.1% SDS at room temperature and twice for 15 min at 50°C in 0.1x SSC:0.1% SDS.

DNA Probes. The DNA probes used in these studies were the following: for myc, the SalI-PstI fragment of p-v-myc corresponding to the third exon of v-myc and a 2.2-kilobase insert of pRmyc1.5 which contains 300 base pairs of the first exon of the rat myc gene and 1.9 kilobases of sequences upstream of the first exon (Ref. 27, provided by Dr. D. Steffen); and for p53, the 350-base pair XhoI-PvuII fragment from the murine p53 cDNA clone pp53-271 (Ref. 28, provided by Dr. M. Oren).

RESULTS

c-myc Expression during Hepatocarcinogenesis. Northern blots of poly A⁺ RNA isolated from the livers of rats fed the CDE for 4, 8, 17, and 35 wk were hybridized with a probe specific for the third exon of the myc gene. We found that, after 4 wk on the CDE diet, the abundance of the major coding 2.2-kilobase myc transcript was elevated approximately 8-fold above normal and remained high throughout the course of carcinogen feeding (Fig. 1A). A tumor nodule which developed after 35 wk of carcinogen treatment also contained high levels of myc RNA. We have previously reported that the expression of c-myc RNA increases in the livers of rats fed the CDE diet (4). In those studies we used the 4.5-kilobase BamHI-XhoI fragment of λ Momyc1 that contained three exons of the myc gene. This probe detected multiple, poorly resolved transcripts which made a precise determination of the kinetics of the increase in myc expression difficult.

The increase in myc expression is not due to the age of rats, since we have previously shown that the livers of rats maintained on a CD diet without ethionine for the same period of time show no increase in myc (4). The CD diet alone leads to minimal oval cell proliferation and is only carcinogenic after very long periods of time.

p53 Expression during Hepatocarcinogenesis. To determine whether other cell cycle-regulated genes are altered during liver carcinogenesis, we also examined the levels of p53 RNA in the carcinogen-treated rat livers. p53 is a nuclear protein that has...
been shown both to be involved in the control of the growth of some cells and to cooperate with the Ha-ras oncogene in the transformation of fibroblasts in culture (29). In addition, p53 is elevated in the early phase of rat liver regeneration, approximately 6 to 10 h after myc and prior to the onset of DNA synthesis (20). As we found in previous work, the levels of the major 1.8-kilobase p53 RNA were low in normal liver (20). Like myc, p53 transcripts were elevated by 4 wk on the CDE diet and remained high at 16 wk (Fig. 1B). In contrast to the pattern of myc expression, the expression of p53 transcripts decreased slightly in the tumor nodule harvested at 35 wk.

Localization of c-myc and p53 in Isolated Liver Cells. Since the initial increase in c-myc and p53 RNAs in carcinogen-treated livers coincided with the point at which marked oval cell proliferation occurs, we examined the cellular distribution of both gene transcripts in purified oval cells and hepatocytes. Hepatocytes were isolated from normal rat livers or from the livers of rats fed the CDE diet for 4 wk. After 4 wk, we were unable to obtain pure populations of hepatocytes from carcinogen-exposed rat livers. Oval cells can, however, be isolated free of other contaminating cell types for a period of up to 16 wk. The 2.2-kilobase transcript was barely detectable in hepatocytes isolated from normal liver or from carcinogen-fed animals (Fig. 2A). The levels of myc transcripts, however, were elevated approximately 3-fold in the oval cell population after 4 wk of carcinogen treatment and continued to increase with time on the diet, corresponding to the increase observed in whole livers. p53 mRNA was elevated approximately 2-fold in oval cells relative to hepatocytes from CDE livers at 4 wk but showed no further increase at 9 or 16 wk (Fig. 2B). This is in contrast to the striking increase in p53 expression that we observed in whole livers. The significance of this observation is not presently understood but may imply that other nonparenchymal cell types are expressing high levels of p53 at late stages of hepatocarcinogenesis.

Cell Cycle Expression of Protooncogenes in Early and Late Passage Nontumorigenic LE/6 Cells. We have previously reported the establishment of immortalized, nontumorigenic cell lines of oval cells purified from the livers of rats fed the CDE diet for 6 wk and demonstrated that, with passage in culture, these cells acquire some transformation-associated properties. To determine if c-myc and p53 transcripts are cell cycle regulated in immortalized, nontumorigenic epithelial cells, we examined the cell cycle expression of myc and p53 mRNAs in both early (tenth) and late (53rd) passage nontumorigenic oval cells (LE/6 cell line). To avoid cell selection, we used uncloned LE/6 cells for our initial studies. Subsequent experiments were performed with clonal lines (see below). Northern blot analysis with the myc-specific probe of RNAs from both quiescent and serum-stimulated cultures from early or late passage cells showed that levels of c-myc transcripts were low in quiescent cultures and increased approximately 5- to 8-fold early in G1 in response to serum growth factors (Fig. 3A). By the peak of DNA synthesis (48 h for early passage cultures and 12 h for late passage cells), the levels of myc had almost returned to that found in quiescent cultures. The kinetics of this increase is in basic agreement with that reported for nontumorigenic BALB/c 3T3 fibroblasts (17). As a control for the amount of RNA loaded on the gel, we examined the expression of mRNA transcripts for the insulin receptor. In other experiments we had determined that levels of insulin receptor mRNA did not change during cell proliferation. Filters were washed to remove
the *myc* probe as described in “Materials and Methods” and rehybridized with the insulin receptor cDNA probe. We found that the levels of insulin receptor transcripts were equivalent in both quiescent and serum-stimulated cultures (data not shown).

We did not observe any significant change in the levels of *myc* RNA in proliferating late passage LE/6 cells as compared to early passage cells. Despite the fact that late passage cultures are partially transformed, that is, they grow to high saturation density and become anchorage independent in response to EGF (14), the cell cycle regulation of *myc* mRNA expression was not altered. Similarly, in both early and late passage cultures, p53 RNA levels were low in quiescent cultures and increased steadily (with some variability in early passage LE/6 cells) as cells traversed the G1 phase of the cell cycle (Fig. 3B). After one round of cell division (32 h for early passage cells and 20 h for late passage cells), cultures were again growth arrested and p53 levels were low.

**Regulation of *myc* and p53 Expression in EGF-induced Soft Agar Clones of Late Passage LE/6 Cells.** After the 50th passage, a small proportion of late passage LE/6 cells can form colonies in soft agar when EGF is added to the growth medium (14). Although none of the cell lines derived from six individual colonies produced tumors in nude mice, the ability of these cells to grow in an anchorage-independent manner indicated that the lines were partially transformed. We, therefore, examined the expression of *myc* and p53 mRNA in quiescent and serum-stimulated cultures of three individual clones, designated here as LE/6c2-5, LE/6c2-6, and LE/6c3-1 (Fig. 4). To facilitate analysis of several lines, we isolated RNA only from quiescent and proliferating cultures. We found that, like the parent late passage line, c-myc and p53 expression remained cell cycle regulated in all cloned lines, with low levels at quiescence and increased levels in proliferating cultures. In addition, the overall levels of *myc* mRNA in the three clones were very similar to those of proliferating cultures of early and late passage LE/6 cells. mRNA levels for the insulin receptor remained unchanged (data not shown), indicating that equivalent amounts of RNA were loaded in each lane.

Cyto genetic analysis of the LE/6c3-1 line showed an abnormal karyotype with 87 to 92 chromosomes per individual cell (a total of 30 cells was examined; data not shown). Thus, even though the LE/6 cells are aneuploid and are capable of anchorage-independent growth, the expression of c-myc and p53 remained tightly cell cycle regulated.

**myc and p53 Expression in Tumorigenic LE/6 Cells.** We have previously reported that LE/6 cells transfected with the Ha-ras oncogene produce well-differentiated hepatocellular carcinomas after s.c. inoculation into nude mice (14). However, since mutations in the Ha-ras protooncogene are not found in the majority of rat liver tumors (30), we developed another *in vitro* model of hepatocellular carcinoma that did not involve transfection of a known oncogene. During the course of our studies, we noticed that LE/6 cells which were passaged infrequently—no more than once or twice a month—underwent striking morphological changes. By the third bimonthly passage (approximately 6 mo), small islands of large epithelial cells with pale cytoplasm, surrounded by small densely staining epithelial cells, became apparent. Both the large and the small epithelial cells stained for glucose-6-phosphatase, indicating their probable hepatocytic lineage (31). Injection of cells (designated LE/6-T) passaged in this manner into 6 nude mice yielded rapidly growing tumors with a latency of less than 1 wk. The mechanism of transformation of these cells is not known but probably involves selection of cells which lack sensitivity to growth-inhibitory factors synthesized in dense cultures.

Despite the fact that LE/6-T cells were rapidly tumorigenic, we could successfully achieve growth arrest of this line by maintaining confluent cultures in reduced serum for 4 days. To determine if deregulation of *myc* RNA expression is associated with tumorigenicity, we examined the regulation of *myc* and p53 in cultures of the tumorigenic LE/6-T cell line. We found that *myc* RNA was high in quiescent cultures and did not increase when cultures were stimulated to divide (Fig. 5A). Levels of the insulin receptor mRNA were constant (data not shown), whereas expression of p53 RNA in the LE/6-T cells remained tightly cell cycle regulated (Fig. 5B), indicating that the loss of some aspects of normal growth control exhibited by tumorigenic cells does not lead to the deregulation of expression of all genes involved in cell proliferation.

**Histological Analysis of Tumors Produced by Injection of Spontaneously Transformed Cultured Oval Cells into Nude Mice.** Microscopic examination of multiple blocks from five of the tumors revealed hepatic tumors with a variable degree of cellular differentiation. Areas of each tumor contained large cells with eosinophilic cytoplasm arranged in slender cords or trabeculae. The nuclei were most often single with a single prominent nucleolus and a heavy distinct nuclear membrane (Fig. 6A). In general, the neoplastic cells in these regions were fairly uniform in size and shape. These areas, clearly distinguishable as moderate to well-differentiated hepatocellular carcinoma, closely resembled the tumors produced in the livers of rats fed the CDE diet. Tumor giant cells, often with a single large bizarre nucleus, similar to those described in hepatocellular...
DEREGULATION OF c-myc IN HEPATOCARCINOGENESIS

Total RNA was prepared from confluent (C), proliferating (P), or mid-S-phase (S) cultures of tumorigenic LE/6 cells. Northern blot analysis was performed with myc (4) and p53 (8)-specific probes.

Cultured Oval Cells. To determine whether the loss of cell cycle control of c-myc is lost, we have analyzed the cell cycle expression of c-myc and p53 mRNA in in vitro model of cellular transformation. Campisi et al. (17) were the first to suggest that loss of cell cycle control of c-myc expression was associated with tumorigenesis. These authors compared the cell cycle regulation of several cellular oncogenes in untransformed and transformed mouse 3T3 fibroblasts and found that cell cycle control of c-myc, but not c-Ki-ras, was lost in chemically transformed fibroblasts. Huber and Thorgerisson have demonstrated that c-myc mRNA expression is deregulated in Hep G2 cells, a fully transformed, continuous cell line derived from a human liver carcinoma (23). But, since this is an established cell line, it was not possible to determine at what point in the transformation process c-myc expression became constitutive.

In order to further explore the role of c-myc overexpression in hepatocarcinogenesis and to determine at what stage of the carcinogenic process cell cycle control of c-myc is lost, we have developed an in vitro model of oval cell transformation which attempts to mimic multistep carcinogenesis in vivo. Of particular importance in evaluating the relevance of this experimental model to the biology of hepatocarcinogenesis is the fact that transformed oval cells produce moderate to well-differentiated hepatocellular carcinomas in nude mice which are similar in morphology to in vivo rat tumors. Using these cell lines, we have analyzed the cell cycle expression of c-myc and p53 mRNA at different stages of transformation. We show that even when oval cell lines are capable of EGF-induced anchorage-independent growth and could grow to high saturation densities, the expression of c-myc and p53 mRNAs remains under cell cycle control. However, at the point when the cells produce tumors in animals, we find that the expression of c-myc, but not p53, is constitutive; that is, the levels of c-myc mRNA are the same in growth-arrested and in proliferating cells. Furthermore, loss of cell cycle control of the expression of c-myc is not accompanied by amplification or gross rearrangement of the myc gene.
Fig. 6. Hematoxylin-eosin staining of tumors produced by the s.c. inoculation of spontaneously transformed cultured oval cells into nude mice. A, area of tumor showing well- to moderately differentiated hepatocellular carcinoma. × 125. B, focus of tumor containing numerous giant cells with bizarre nuclei. × 125. C, osteoid formation deep in the tumor mass. × 125.
Deregulation of c-myc in hepatocarcinogenesis

If the changes in the cell cycle expression of c-myc that we observed in vitro reflect the process of tumorigenic transformation in vivo, it would appear that deregulation of myc mRNA expression is a late step in hepatocarcinogenesis, occurring only when hepatic cells are fully transformed. However, we have no direct proof that c-myc expression becomes constitutive in vivo. Nevertheless, our results, together with those of Sirica and Cihla (34) who showed that oval cell proliferation decreases with time on the carcinogenic diet, indicate that the high levels of c-myc found in the premalignant stages of carcinogenesis are not solely due to increased cell proliferation but may reflect the state of oval cell differentiation (8).

In the present study we have focused on an analysis of the cell cycle regulation of c-myc expression at different steps in the carcinogenic process. Although we do not know how the transcription of myc is regulated in this system, Huber and Thorgerisson (24) showed that constitutive expression of c-myc in Hep G2 cells was not a consequence of increased stability of the message or amplification or rearrangement of the gene. On the basis of these studies, we cannot definitively prove a causal role for the c-myc gene in hepatocarcinogenesis. However, the finding that c-myc is constitutively expressed in liver tumor cell lines from rats and humans as well as in at least one experimental animal model of hepatocarcinogenesis suggests that the deregulation of myc expression may be an important step in the development of hepatocellular carcinomas.

References

DEREGULATION OF c-myc IN HEPATOCARCINOGENESIS

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