Cellular Distribution of c-myc Transcripts during Chemical Hepatocarcinogenesis in Rats

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ABSTRACT

The expression of cellular myc (c-myc) was studied during early and late stages of chemical hepatocarcinogenesis in the rat using Northern blot analysis and in situ hybridization. Hepatocarcinogenesis was induced according to the resistant hepatocyte model of Solt and Farber. An uninitiated version of this model was also used to examine the expression of c-myc during proliferation and differentiation of oval cells. The expression of c-myc was increased throughout hepatocarcinogenesis starting with early preneoplastic foci and oval cells. Similar levels of c-myc transcripts were detected in oval cells and basophilic hepatocytes generated by the uninitiated version of the protocol as were found in preneoplastic foci and oval cells during hepatocarcinogenesis. Whereas c-myc expression remained elevated in late neoplastic nodules and carcinomas, it gradually declined in both "remodeling" nodules and uninitiated livers. Our data indicate that c-myc expression is elevated during the undifferentiated stages of hepatocyte development. Furthermore, the data support the hypothesis that a critical early step in chemical hepatocarcinogenesis involves a block in the normal differentiation program of the hepatocytes.

INTRODUCTION

Although altered expression of cellular oncogenes is well known in both human and experimental tumors, the exact role of these genes in the multistep process of neoplastic development has yet to be defined. A number of experimental models exist in which the stepwise development of neoplasia can be studied (1, 2). It is therefore possible to examine the possible involvement of oncogenes whose expressions are modulated at the end-stage of the tumor development at individual steps during the transformation process.

One of the best characterized of the experimental tumor model systems is the chemically induced hepatocarcinogenesis in the rat (1, 2). In this system different cell populations, which are putatively associated with certain stages in the neoplastic process, have been characterized by histological and histochemical methods (3). Furthermore the expression of a number of cellular oncogenes has been determined during both liver regeneration and chemically induced hepatocarcinogenesis (4-19).

Due to the strong association between an increased level of c-myc2 expression and cellular proliferation observed in both regenerating liver and in numerous other experimental systems, considerable interest has centered on the possible role that c-myc might play in the multistep process of hepatocarcinogenesis. Increased levels of c-myc transcripts have been found in both experimental animal and spontaneous human liver tumors (4-9, 11-19). Northern blot analysis of c-myc expression during the transformation process.

MATERIALS AND METHODS

The Solt-Farber method (21) was used to induce liver tumors in male Fischer rats (body weight, 150 g). Briefly, this consisted of initiation with 200 mg/kg of DEN followed 2 wk later by AAF administration for 2 wk (dose, 1 mg/day) with PH being performed 1 wk after starting AAF treatment. In another group of rats DEN administration was omitted, and the rats were treated only with AAF and PH. We refer to this as the uninitiated version of the carcinogenesis protocol. The in situ hybridization, RNA preparation, and Northern blot analysis were performed as described earlier (23). Briefly, frozen liver sections were prefixed in 4% paraformaldehyde and stored in 70% ethanol on 4°C until the day of the experiment. The permeabilization steps involved 20-min treatment in 0.2 N HCl at room temperature, 30 min in 2x standard saline citrate at 70°C, and 10 min in Pronase (100 μg/ml) at 37°C. The slides were then postfixed in 4% paraformaldehyde for 20 min. Prehybridization (2 h) and hybridization (24 h) were performed at 37°C in the presence of 50% formamide. After hybridization the slides were then treated with 30 min with RNase A (20 μg/ml) at 37°C and were washed with increasing stringency for 24 h. The slides were immersed in Kodak NTB-2 emulsion and exposed for 3 wk. The 2.2-kilobase-long EcoRV-HindIII fragment, a part of the second, and the whole third exon of the mouse myc gene were used as a probe. For the in situ experiments, the length of the RNA probe was adjusted to 250 to 300 bases by alkali hydrolysis (24). The "sense" RNA probe (complementary to probe) was used as control (data not shown).

Histological Methods. Frozen liver sections were used for staining with hematoxylin and eosin and for histochemical demonstration of GGT (25)
RESULTS

Northern Blot Analysis. In the regular Solt-Farber protocol, increased levels of the 2.3-kilobase-long c-myc transcript were found throughout the neoplastic process starting from 7 days after PH until the appearance of carcinomas (Fig. 1a). At 9 mo, RNA was isolated from a well-circumscribed nodule and the surrounding liver tissue. The steady-state levels of the c-myc transcripts were much higher in the nodule than in the surrounding tissue (Fig. 1a, Lanes 7 and 8), in agreement with data from Makino et al. (12).

The level of c-myc transcripts was also determined in animals subjected to the uninitiated version of the Solt-Farber protocol (Fig. 1b). Similar levels of c-myc transcripts were observed in both initiated and uninitiated animals for the first 2 wk after partial hepatectomy (Fig. 1a, Lanes 4 and 5, versus Fig. 1b, Lanes 3 and 4). However, the level of c-myc transcripts rapidly declined in the uninitiated liver to levels indistinguishable from normal liver 3 wk after PH.

Histology. Histological alterations during rat liver carcinogenesis have been studied extensively (1), and the morphological changes induced by the Solt-Farber protocol have been described earlier (2, 22, 23). Briefly, a prominent oval cell proliferation emerges 1 wk after PH. The oval cells first appear in the perportal areas of the liver but gradually infiltrate the whole lobule. One wk after PH numerous basophilic staining foci composed predominantly of newly formed hepatocytes are apparent. Some of these foci as well as the oval cells stain positively for GGT, while other foci are GGT negative. These basophilic foci can then develop into putatively preneoplastic nodules which then have two options. They can either remodel into apparently normal liver or progress into carcinoma.

In the uninitiated model the histological picture was similar for the first 2 wk following PH. However, only the oval cells were GGT positive, while all the foci of basophilic hepatocytes were negative. The light microscopic picture of these livers gradually returned to normal, and 10 wk after the PH the regular lobular structure was established. However, some remaining oval cells were consistently observed in the perportal areas.

In Situ Hybridization. The c-myc transcripts were exclusively localized in the oval cells 1 wk after PH (Fig. 2a) in the Solt-Farber model. These data are in agreement with results reported from Fausto’s laboratory (14), which demonstrated elevated levels of c-myc transcripts by Northern analysis in oval cells isolated by centrifugal elutriation during choline-deficient induction of hepatocarcinogenesis. A wk later the expression of c-myc was similarly increased in all the basophilic foci (Fig. 2b). The grains were relatively evenly distributed inside the foci (Fig. 2b), and no consistent differences were observed in the expression of c-myc transcripts between the GGT-positive and -negative foci (Fig. 3, a and b). The grain count did not significantly change as the nodule size increased, and the oval cells continued to show increased expression of c-myc transcripts (data not shown). Furthermore, both persistent hyperplastic nodules and primary hepatocellular carcinomas continued to display increased levels and a relatively uniform distribution of c-myc transcripts (Fig. 4).

At early timepoints in the uninitiated model, c-myc transcripts were expressed in both oval cells and basophilic hepatocytes at similar levels to those seen in the complete Solt-Farber model (Figs. 5 and 6). However, 3 wk after PH and at later timepoints, in agreement with the Northern blot analysis (Fig. 1b), we were unable to detect the c-myc transcripts in the liver.

DISCUSSION

In this paper we have demonstrated by Northern blot analysis increased levels of c-myc transcripts throughout chemical hepatocarcinogenesis in the rat induced by the Solt-Farber protocol. We have also shown increased levels of c-myc transcripts for a short period in livers of animals subjected to the uninitiated version of the same model. Furthermore, using the in situ hybridization technique we were able to demonstrate an increased expression of c-myc in oval cells and basophilic hepatocytes at early stages of hepatocarcinogenesis and later in the foci, nodules, and carcinomas. In the uninitiated version of the model an increased expression of c-myc was restricted to oval cells and basophilic hepatocytes, and this expression returned to normal level 3 wk after PH.

Increased c-myc expression has been found during hepatocarcinogenesis using 3'-methyl-4-dimethylaminobenzene (12, 13) and choline-deficient diet (14), in the tumors produced by 2-amino-3-methylimidazo[4,5-f]quinoline (26) and aflatoxin B1 (4), and in both Peraino’s (7) and Pilot’s protocols (15). However, little data are available on the cellular distribution of the c-myc expression during hepatocarcinogenesis. Yaswen et al. (14) isolated oval cells and hepatocytes by centrifugal elutriation during choline-deficient diet-induced hepatocarcinogenesis and found increased levels of c-myc mRNA only in the oval cells. However, Beer et al. using Pilot’s protocol (15) isolated GGT-positive cells during hepatocarcinogenesis by using GGT antibody affixed to Petri dishes, but could not detect an increased
Fig. 2. a, expression of the c-myc oncogene in the oval cells of a liver treated with the Solt-Farber protocol 1 wk after PH. × 750; bar, 12 μm. b, expression of c-myc oncogene in a basophilic staining focus of a liver treated with the Solt-Farber protocol 2 wk after PH. The distribution of the grains inside the focus is relatively even. × 150; bar, 60 μm.

level of c-myc mRNA in these early putative preneoplastic cells. These investigators did, however, find increased levels of c-myc transcripts in the late stages of hepatocarcinogenesis. According to Makino et al. (12) the increased expression of c-myc may be a causative factor in the carcinogenesis process. However, Beer and Pitot (15) have proposed that the increased expression of c-myc observed late in the hepatocarcinogenesis process is merely a consequence of cellular alterations reflecting the continuous cell proliferation which characterizes the late stages of the process. There exists, however, a considerable body of data that are inconsistent with any direct correlation between the expression of c-myc and cell proliferation. Cote et al. (16) studied c-myc expression in five different Morris hepatomas but did not find any correlation between growth rates and levels of c-myc expression. Huber and Thorgeirsson (19) found that the c-myc gene is constitutively expressed during both the rapidly dividing and nondividing phases of the Hep-G2 hepatoma cell line. Yaswen et al. (14) have demonstrated increased c-myc expression with time in oval cells, but Sirica and Cihla (28) reported decreasing DNA synthesis in the same cell population. There is a general agreement that the c-myc gene plays a role only during the G1 phase of the normal cell cycle (27, 29). The c-myc transcripts were evenly distributed inside all the foci examined in the present experiment (Fig. 4). If c-myc expression is exclusively turned on as a consequence of cellular proliferation, then one has to postulate that all the foci are precisely synchronized, which seems unlikely. Furthermore, we have demonstrated (23) that the in situ technique is capable of detecting differences between single cells in gene expression.

The c-myc gene is expressed in the liver not only as a result of cell proliferation but also during differentiation. Cote (13) raised the possibility that, in the Morris hepatoma and 3'-
dimethylaminoazobenzene carcinogenesis, the lack of differentiation and not the state of proliferation may be responsible for c-myc expression. The c-myc gene is also expressed in the liver during fetal development in both humans (8) and in rats (14). Usually it is very difficult to separate the process of differentiation from that of proliferation. However, treatment of rat liver epithelial cells with TGF-β has a strong antiproliferative effect in vitro (31). Concomitantly, under appropriate conditions, TGF-β can induce the differentiation of these cells. In this system the expression of the c-myc gene is increased when the proliferation is decreased and the epithelial cells differentiated toward hepatocytes.3

Furthermore, an active differentiation process is ongoing for the first 5 to 6 wk in both the complete and uninitiated Solt-Farber models (22), and in agreement with the data described above, we could demonstrate c-myc expression in these differentiating cell populations (i.e., oval cells, basophilic foci). Most importantly, there is a fundamental difference between the two models. The c-myc gene is expressed only temporarily in the uninitiated model and disappears with the “terminal” differentiation of the basophilic hepatocytes (Fig. 1b), as has been observed in other tissues (32). In contrast, increased levels of c-myc expression are observed throughout the neoplastic process in the complete Solt-Farber model (Fig. 1a).

A possible, and perhaps the most likely, explanation for the disruption of the regulation of the c-myc gene is that the initiation step in hepatocarcinogenesis results in a permanent block in the normal differentiation pathway for the immature hepatocytes. This hypothesis therefore predicts that the real targets for the initiation step in hepatocarcinogenesis would be the immature population of hepatocytes. Kaneko (30) has demonstrated that the c-myc gene is hypomethylated in human fetal liver and in hepatocellular carcinomas. These data support our hypothesis that the initiation step may cause disruption at the genomic level which keeps the c-myc gene in an upregulated state.
myc, HEPATOCARCINOGENESIS, AND DIFFERENTIATION

Fig. 4. Expression of c-myc oncogene in a hepatocellular carcinoma, induced by the Solt-Farber protocol 1 yr after initiation. The grains are relatively evenly distributed above the tumor cells, but there are practically no grains above the lymphocytes (arrow). × 300; bar, 30 μm.

Fig. 5. Expression of c-myc oncogene in the oval cells of a liver treated with the uninitiated version of the Solt-Farber protocol 2 wk after PH. × 750; bar, 120 μm;

Fig. 6. Expression of c-myc oncogene in a basophilic focus induced by the uninitiated version of the Solt-Farber protocol 2 wk after PH. The expression is relatively even in the focus (inset from the focus). × 75; bar, 120 μm; inset magnification, × 300.

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