Transforming Growth Factor β1 in Liver Carcinogenesis: Messenger RNA Expression and Growth Effects

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Abstract
Transforming growth factor β1 (TGF-β1) is a potent inhibitor of hepatocyte proliferation. Since loss of sensitivity to growth inhibition is thought to contribute to the development of neoplasia, we analyzed the expression of TGF-β1 mRNA during hepatocarcinogenesis in vivo and in cultured liver epithelial cells (oval cells) obtained from carcinogen-treated animals. We found that TGF-β1 mRNA increases in the liver during carcinogenesis and that, at the early stages of the process, oval cells but not hepatocytes contain the growth factor mRNA. Moreover, immortalized, nontumorigenic oval cells (LE/6 cell line) continued to produce TGF-β1 mRNA in culture. TGF-β1 message markedly decreased upon cell transformation, but message levels, although generally low, were variable in various tumor cell clones. A consistent feature of the tumorigenic cell lines was a loss of sensitivity to TGF-β1 growth inhibition. Tumor cells could bind TGF-β1 with similar capacity as normal cells and had the same type of receptors (M. 280,000, 85,000, and 65,000) capable of binding iodinated TGF-β1, suggesting that the loss of sensitivity to TGF-β1 in transformed liver epithelial cells involves postreceptor mechanisms. Further studies showed that c-myc is not a target for TGF-β1 in liver epithelial cells and that TGF-β1 no longer induces fibronectin mRNA in transformed cells. The data presented are consistent with the hypothesis that TGF-β1 secreted during liver carcinogenesis may inhibit the proliferation of normal cells while providing a selective advantage for the growth of cells that are "partially transformed" and are unresponsive to the factor.

Introduction
Transforming growth factor β is a highly conserved M. 25,000 homodimeric polypeptide synthesized by a wide variety of cell types in vivo and in vitro (1). Although originally described as a transforming growth factor, the biological effects of TGF-β on cell growth and differentiation are complex and vary according to cell type (2, 3). The best characterized of these effects include stimulation of fibroblast proliferation, inhibition of epithelial cell growth, and enhancement of extracellular matrix formation. Several distinct forms of TGF-β have been isolated and characterized, but the primary functions of the various TGF-β species are not yet known (1). However, the fact that most cells examined possess functionally active, high affinity cell surface receptors for TGF-β (4) implies a fundamental role for this protein in normal cell physiology.

Several investigators have shown that TGF-β1 is inhibitory to hepatocyte as well as nonparenchymal liver epithelial cell proliferation in culture (5–10). We have reported that the steady-state levels of TGF-β1 mRNA increase during rat liver regeneration, a highly regulated physiological growth process (8). On the basis of these results, as well as the finding that liver nonparenchymal cells expressed TGF-β1 transcripts, we have proposed that TGF-β1 may function in vivo as a negative paracrine effector of hepatocyte proliferation to restrict uncontrolled liver growth. Further evidence supporting a biologically important function for TGF-β1 in liver homeostasis in vivo was provided by Russell et al. (11), who found that i.v. injection of active TGF-β1 into partially hepatectomized rats inhibited the early proliferative stages of liver regeneration.

Loss of sensitivity to growth-inhibitory factors is thought to contribute to the unregulated growth characteristic of tumor cells (12, 13). Transfection of fibroblastic cell lines with the H-ras oncogene leads to both an increase in TGF-β1 mRNA expression and an altered responsiveness to TGF-β1 (14–16). It is likely, however, that the mechanisms of epithelial carcinogenesis differ significantly from transformation of mesenchymal cells. In fact, analysis of the effects of TGF-β on transformed cell lines of epithelial origin have revealed a somewhat confusing picture. Some cell lines, such as FMXII melanoma line, are unresponsive to TGF-β, whereas other lines, such as A549 lung carcinoma cells (4) and a human colon carcinoma cell line (17), are inhibited, depending on the culture conditions and the concentration of TGF-β used. Although there are several reports indicating that malignant liver cells are resistant to growth inhibition by TGF-β1 (7, 18), the relationship of changes in TGF-β1 expression to altered growth factor sensitivity during liver carcinogenesis and the mechanisms by which an altered growth response occurs remain unclear.

To investigate further the potential role of TGF-β1 in hepatocellular carcinoma development, we have used

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3 The abbreviations used are: TGF-β, transforming growth factor β; TGF-β1, transforming growth factor-β1; CDE, cholinedeficient diet containing 0.1% ethionine; FBS, fetal bovine serum; LE/6, liver epithelial cells isolated from livers of rats fed a CDE diet; E-1, tumor line derived from nude mouse tumors obtained by inoculation of cells containing the H-ras oncogene; S-T, spontaneously transformed LE/6 cells; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; poly(A)+ RNA, poly(A) RNA; poly(A)+-poly(U)-containing RNA; TEG, epidermal growth factor; cDNA, complementary DNA; p18, passage 18 (other passages are similarly designated).
an experimental animal model of chemically induced hepatocarcinogenesis which is characterized by the massive proliferation of epithelial cells with stem-like properties (19). When these cells, commonly referred to as oval cells, are placed in tissue culture and maintained under conventional conditions, they become immortalized but do not transform. Transfection of oval cells with plasmids containing the activated H-ras (E) gene or maintenance of the cells in confluent culture conditions for periods ranging from 1 to 2 months leads to cell transformation (19, 20). When inoculated into nude mice, H-ras-transfected or spontaneously transformed oval cells form differentiated hepatocellular carcinomas (19, 20). Using these two model systems, we have analyzed (a) the expression of TGF-β1 mRNA during neoplastic transformation in vivo and in vitro, (b) the effects of TGF-β1 on DNA synthesis at various stages of the transformation process, and (c) the binding of the growth factor to normal and transformed cells. Moreover, in an attempt to determine the mechanism of action of TGF-β1, we examined its effects on the expression of two genes, c-myc and fibronectin, which are associated with cell proliferation and matrix production, respectively.

Results
Expression of TGF-β1 mRNA in Whole Liver Tissue and Cells Isolated from Livers of Carcinogen-fed Rats. Since we have shown that the messenger for TGF-β1 increases during liver regeneration and have suggested that TGF-β1 functions as a growth regulator of this process, we wanted to determine whether the expression of TGF-β1 mRNA changed during liver carcinogenesis in vivo. We examined the steady-state levels of TGF-β1 mRNA by Northern blotting in poly(A)* RNA extracted from normal rat livers and the livers of rats fed the CDE diet for 4, 8, and 17 weeks (Fig. 1A). As previously reported, the abundance of the 2.5-kilobase major coding transcript for TGF-β1 is low in normal rat liver (8). After 4 weeks on the diet, a slight increase (approximately 2-fold) in the steady-state levels of TGF-β1 mRNA relative to normal liver was detected in the carcinogen-treated rats. By 8 weeks, the abundance of TGF-β1 transcripts increased approximately 10-fold above that found at 4 weeks, and it remained high at 17 weeks. Lower levels of TGF-β1 were observed in a hepatocellular carcinoma that developed at the end of 35 weeks (Fig. 1A). A smaller transcript, similar in size to that reported in other systems (21), was also present in most RNA preparations. It is not known whether this transcript codes for a protein which is homologous to TGF-β1 or whether it represents a degradation product.

To determine what cells in the liver may be the source for TGF-β1 mRNA, we purified hepatocytes and oval cells by centrifugal elutriation from CDE livers after 4 weeks of carcinogen treatment and analyzed TGF-β1 mRNA expression by Northern blot hybridization (Fig. 1B). We did not detect TGF-β1 mRNA in hepatocytes from normal or 4-week CDE livers. On the other hand, oval cells contained high levels of the major 2.5-kilobase TGF-β1 mRNA transcript. In contrast to what we observed in the whole livers, where levels of the message increased progressively during carcinogen feeding, oval cells isolated at 4, 9, and 16 weeks contained approximately the same levels of TGF-β1 message. This suggests that the increase in TGF-β1 mRNA in the whole liver may be due to an increase in the number of oval cells (22) without a change in the content of TGF-β1 per cell and/or that other liver cell types, such as sinusoidal lining cells, express TGF-β1 mRNA during the early stages of carcinogenesis.

TGF-β1 mRNA Expression in Untransformed and Transformed Liver Epithelial Cells in Vitro. We next wanted to determine whether transformation of oval cells in culture would involve a decrease or suppression of TGF-β1 mRNA expression. Such a loss in synthesis of an inhibitory factor would suggest that neoplastic transformation of liver cells may involve the interruption of a negative autocrine loop.

Untransformed, untransformed LE/6 cells in culture express large amounts of the 2.5-kilobase TGF-β1 transcript during long-term passage in culture (23). To test the effects of transformation on TGF-β1 mRNA expression, we used two different in vitro systems of liver cell transformation, both leading to the production of differentiated hepatocellular carcinomas. In the first, LE/6 cells were transfected with the E1 H-ras oncogene. The second system involved maintaining LE/6 cells in confluent conditions for periods ranging from 1 to 2 months with infrequent changes in medium. After five to six passages (approximately 8 months), the cells spontaneously trans-
formed (20). When injected into nude mice, the ras-transfected or spontaneously transformed cells produced moderately well-differentiated hepatocellular carcinomas with similar morphology to tumors which develop in the liver of rats fed the carcinogenic diet for periods of 35–52 weeks (19, 20).

Transfection of LE/6 cells with the EJ gene caused a 40% decrease in TGF-β1 message (Fig. 2, A and C). We inoculated these cells in nude mice, harvested the tumors, and established several transformed lines (EJ-T lines) in culture. TGF-β1 mRNA levels in the tumor lines were 3–4-fold lower than in untransformed cells (Fig. 2, A and C). A similar, major decrease in the levels of TGF-β1 message was detected in spontaneously transformed LE/6 cells (S) and in the tumor line (S-T line) derived from these cells (Fig. 2, B and D). In the spontaneous transformation system, the levels of TGF-β1 message were approximately 3-fold lower in the transformed cells before inoculation (Fig. 2B, S) than in either the early (p18) or late (p55) passage untransformed lines (Fig. 2B and D) and were decreased further in the tumor line (S-T line). Thus, using two different in vitro models for hepatocellular carcinoma development, we found a suppression of TGF-β1 mRNA expression as the cells become tumorigenic.

To determine whether the decreased expression of TGF-β1 message was consistently associated with liver cell transformation, we next examined the levels of TGF-β1 transcripts in a series of clonal nontumorigenic and tumorigenic lines (Fig. 3A). LE/6 cells do not normally form colonies in soft agar, but a small proportion of late passage cells become anchorage independent when grown in medium containing EGF. For these studies, we selected six nontumorigenic cloned lines which origi-
nated from p55 untransfected LE/6 cells grown in soft agar in the presence of EGF (Fig. 3A, Lanes 8–13) and seven tumorigenic clonal lines (Lanes 1–7) derived from an EJ-transformed tumor cell line (EJ-T, Lane 15). Clones of late passage LE/6 cells which were capable of anchorage-independent growth but were nontumorigenic, expressed, with one exception (Lane 12), levels of TGF-β1 mRNA similar to the uncloned line (Fig. 3A, Lane 14). On the other hand, in the tumorigenic clones, the abundance of TGF-β1 transcripts was generally low but showed a significant amount of variability.

Given the variability in TGF-β1 message expression in various tumorigenic clones, we investigated whether there would also be variations in the expression of the message between the clonal tumor cells in culture and the tumors that they produced. Total RNA was extracted from two of the EJ-T clonal derivatives (clone 3-T and clone 12-T) and from their corresponding nude mouse tumors and analyzed for TGF-β1 mRNA expression by Northern blot hybridization (Fig. 3B). Message abundance was similar for clone 3-T cells in vitro (Lane 1) and in the tumor (Lane 2), but tumors produced by clone 12-T (Lane 2) had higher levels of TGF-β1 than 12-T cells in culture (Lane 1). Furthermore, the levels of the message in both tumors could be modulated and, in the case of clone 3-T, almost abolished by treating the nude mice with 0.1% phenobarbital in their drinking water (Fig. 3B, Lane 3). Morphologically, the tumors produced by these two clones in phenobarbital-treated and untreated animals were heterogeneous, but both tumors contained large areas of well-differentiated hepatocellular carcinoma. However, in the absence of in situ hybridization data, we could not determine what cells in the tumor expressed the message and whether message expression varies with the degree of cell differentiation. Nevertheless, the experiments with phenobarbital indicated that modulation of TGF-β1 levels in the tumors had no effect on tumor growth or morphology.

Effects of TGF-β1 on DNA Synthesis in Early and Late Passage Untransformed Cells. LE/6 cells proliferate in medium containing 10% fetal bovine serum until they reach confluence, at which point they cease dividing. To determine whether LE/6 cells were growth inhibited by TGF-β1, we first examined the effects of different doses of TGF-β1 on the incorporation of [3H]thymidine in uncloned, exponentially growing early and late passage LE/6 cells. Exposure of p18 and p65 cells to different concentrations of TGF-β1 for 48 h led to a dose-dependent inhibition of DNA synthesis (Fig. 4A). At 15 ng/ml, DNA synthesis of both early and late passage cells was inhibited by approximately 54%.

Effects of TGF-β1 on DNA Synthesis in Transformed Cells. We determined whether transformation of liver cells was associated with a consistent loss of sensitivity to the growth-inhibitory effects of TGF-β1. We found that, in contrast to untransformed cells, EJ-T cells were only 8% inhibited by 1.0 ng/ml of TGF-β1 and 21% inhibited by 5.0 ng/ml, making these cells approximately 4.5-fold less sensitive than the parental untransformed cells (Fig. 4B). In two clonal lines (clone 3-T and clone 12-T) derived from EJ-T cells, 5 ng/ml TGF-β1 inhibited DNA synthesis by 15%, whereas DNA synthesis in clone 3-T was stimulated by TGF-β1 treatment at this concentration. At low concentration (1 ng/ml), TGF-β1 stimulated DNA synthesis in both clonal lines. Similar results were obtained with the spontaneously transformed cell lines (data not shown). These experiments with transfected and spontaneously transformed cells are in basic agreement with the work of Houch et al. (18) and demonstrate that tumor cells lose sensitivity to growth inhibition by TGF-β1 and, in some cases, may even be stimulated by low concentrations of the factor.

Effects of TGF-β1 on Anchorage-independent, Nontumorigenic Liver Epithelial Cells. To determine at what stage of the transformation process loss of sensitivity to growth inhibition by TGF-β1 occurred, we examined the effects of TGF-β1 on anchorage-independent but nontumorigenic clonal derivatives of the LE/6 cell line. For these studies, we selected two soft agar colonies, clone
2-6 and clone 3-1 (obtained from p50 LE/6 cells grown in the presence of EGF), and expanded them in a monolayer culture. TGF-β1 was growth inhibitory to clone 2-6 but not to clone 3-1 (Fig. 4A). Thus, at least in some clones, loss of sensitivity to TGF-β1 may occur prior to tumorigenic transformation.

**TGF-β1 Receptor Studies.** To learn whether the decrease in sensitivity of ras-transfected cells to the antiproliferative effects of TGF-β1 was associated with a loss of receptors for the growth factor, we performed binding studies of 125I-labeled TGF-β1 in the presence or absence of excess unlabeled TGF-β1. All cell lines studied exhibited similar specific binding of 125I-TGF-β1. The values for specific binding were 2.3, 3.4, and 2.6% of total 125I for LE/6, EJ-T, and clone 3-T cells, respectively. Affinity labeling studies revealed the presence of specific, multiple receptor proteins with an M, of 280,000, 85,000, and 65,000, respectively, in untransformed and transformed liver epithelial cells (Fig. 5). These receptor species are similar to those detected in membranes obtained from rat adrenals (Fig. 5, Panel A), as well as in many different cell types (24). The M, 65,000 receptor species was faintly labeled (Fig. 5), although it was clearly detectable on the original autoradiograms. The variation in intensity of the band corresponding to the M, 280,000 protein may result from unequal loading of membrane proteins on the gel. Although we could not determine whether there were small differences in receptor number or affinity among the cell lines, the results show that TGF-β1 receptors are present in both untransformed and tumorigenic liver epithelial cells.

**Effects of TGF-β1 on the Steady-State Levels of c-myc mRNA.** Because transformed cells have cell surface receptors for TGF-β1, one possible mechanism for their loss of TGF-β1 sensitivity could be an alteration in a postreceptor signaling response involving the expression of protooncogenes. Since in mouse keratinocytes (25), TGF-β1 inhibits c-myc expression, we examined the effects of TGF-β1 on the steady-state levels of c-myc mRNA in untransformed and transformed liver epithelial cells. LE/6, EJ-T, and clone 3-T cells were plated at approximately one-fourth confluence to assure that the cultures were in the logarithmic phase of growth prior to exposure to TGF-β1. Cells were incubated overnight in medium without TGF-β1 and on the following day were fed fresh medium with or without 5 ng/ml TGF-β1. After 16 or 72 h exposure to TGF-β1, RNA was harvested, and c-myc expression was analyzed on Northern blots (Fig. 6). Although the overall levels of c-myc were approximately 5-fold higher in the EJ-T cells as compared to the untransformed cell line, the expression of c-myc mRNA was not inhibited by TGF-β1 in either line. We have not examined time points earlier than 16 h after TGF-β1 exposure. However, in systems in which c-myc expression is inhibited by TGF-β1, the inhibition of c-myc expression is stable over time (25–27). Therefore, unlike mouse keratinocytes (25), human endothelial cells (26), and human cervical cells (27), the growth-inhibitory response of immortalized, untransformed liver epithelial cells to TGF-β1 does not appear to be mediated through an alteration in the steady-state levels of c-myc mRNA. Similar results have been obtained with cultured hepatocytes exposed to TGF-β1 and may indicate that the mechanisms of TGF-β1 growth inhibition differ in liver cells and keratinocytes.

**Effects of TGF-β1 on the Synthesis of Fibronectin mRNA.** In addition to its effects on cell proliferation, TGF-β1 can induce the synthesis of proteins important in the regulation of extracellular matrix formation (1). This effect is complex and involves both the increase in levels of specific mRNAs as well as alterations in the proteolytic degradation of extracellular matrix proteins (1). Moreover, it has been shown that TGF-β1 induces type I

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*J. E. Mead and N. Fausto, unpublished observations.*
procollagen mRNA synthesis in primary cultures of hepatocytes (28). On the basis of these observations, we determined whether the liver epithelial cell lines expressed fibronectin mRNA and whether the message could be induced by TGF-β1. We found that TGF-β1 increases fibronectin mRNA transcripts by approximately 15-fold in untransformed cells. However, in two tumor lines where constitutive expression of the message was high, TGF-β1 had no effect on the expression of fibronectin mRNA (Fig. 7). Thus, reduced sensitivity of liver epithelial cells to growth inhibition by TGF-β1 is accompanied by a lower inducibility of fibronectin mRNA.

Discussion

In this paper, we show that the steady-state levels of TGF-β1 mRNA increase in the early stages of liver carcinogenesis, before the development of tumors. Oval cells that proliferate as a consequence of feeding the carcinogen are an important source for the message, and when placed in culture these cells continued to produce TGF-β1 mRNA. Work in progress using immunocytochemical methods for TGF-β1 detection shows that at the early stage of carcinogenesis the message contained in these cells is translated into the peptide. Although the expression of TGF-β1 mRNA by immortalized, untransformed oval cells in culture remained approximately the same at early and late passages, the message level decreased drastically after transfection of the cells with the E1 ras oncogene or in cells transformed by a modification of the culture system. In both systems, TGF-β1 mRNA was even lower in cell lines derived from the nude mice tumors. However, a more extensive analysis of clonal tumor lines showed that the expression of TGF-β1 mRNA in these lines, although generally low, was quite variable. On the other hand, a consistent feature of all transformed lines was their loss of sensitivity to the inhibitory effect of TGF-β1. In some lines, the peptide at low concentrations even had a stimulatory effect on DNA synthesis.

In an attempt to find the mechanisms that may account for the loss of TGF-β1 sensitivity, we further found that untransformed oval cells contained receptors for TGF-β1 and that these receptors were not lost after cell transformation. Cross-linking studies showed that both transformed and untransformed oval cells contained membrane proteins of M, 280,000, 85,000, and 65,000 that were capable of binding iodinated TGF-β1. It is of interest to note that the high molecular weight binding protein was not found in membranes isolated from whole livers or in hepatocytes in primary cultures. Although loss of sensitivity to TGF-β1 in the tumor cells seems to involve a postreceptor mechanism, it was not caused by differential effects of the growth factor on the expression of the c-myc protooncogene.

Lin et al. (9) have previously shown that the sensitivity of normal liver epithelial cells to TGF-β1 was lost with passage in culture, whereas other work by the same group demonstrated that TGF-β1 had a variable effect (inhibition or stimulation) in tumor cell lines (29). In our work, we compared cells of p18 and p55 but found no differences in their sensitivity to growth inhibition by TGF-β1. However, sensitivity changes may have occurred at earlier passages (before p18), which were not available for study. In LE/6 cells, only major changes in growth properties involving anchorage-independent growth or tumorigenicity led to decreases in the sensitivity to TGF-β1-induced growth inhibition. Similarly, McMahon et al. (7) showed that aflatoxin B-transformed normal neonatal rat liver epithelial cells were completely insensitive to TGF-β1, whereas their untransformed counterparts were strongly inhibited by low doses of the peptide. On the other hand, Wollenberg et al. (30) found that DNA synthesis in cells obtained from liver tumors could be inhibited by TGF-β1. These different observations are difficult to explain but may result from the diverse sources of liver epithelial cells used to establish cell lines and generate tumors (7-9) as well as the use of freshly isolated cells by Wollenberg et al. (30).

Since TGF-β1 is such a potent inhibitor of DNA synthesis in epithelial cells in vitro and in particular of hepatocytes in culture, it has been proposed that a loss of sensitivity to the antiproliferative effects of TGF-β1 may be an important step in epithelial carcinogenesis (12, 13, 24). This view is supported by the work of Houck et al. (18), showing that oncogene transformation of liver epithelial cells is associated with a loss of sensitivity to growth inhibition by TGF-β1. Furthermore, Liu et al. (29) showed that liver epithelial tumor cells frequently secrete active TGF-β1 and that these cells are either insensitive to TGF-β1 or produce TGF-α, which may balance the inhibitory effects of TGF-β1. Our finding that transformed oval cells invariably lost sensitivity to TGF-β1 growth inhibition and that a similar loss was present in some, but not all, lines of anchorage-independent nontumorigenic cells indicates that release from TGF-β1 autocrine growth inhibition in oval cells may be associated with the transformation of these cells and might even occur before acquisition of tumorigenicity.

When initially placed in culture, oval cells express both

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3 N. Fausto, N. Kessman, and J. E. Mead, unpublished observations.
4 P. Grappulo, J. E. Mead, and N. Fausto, submitted for publication.
TGF-β1 and insulin-like growth factor II mRNAs, an indication that this is an immature, fetal-like population of cells (23). Most of the TGF-β1 produced by both untransformed and transformed oval cells is in a latent form that requires activation. Major experimental limitation in studying the effects of TGF-β1 on tumor growth is that sensitivity studies must be performed in vitro. Several investigators have shown that the effects of TGF-β1 differ depending on the microenvironment of the cell (31). Proof that liver tumors in vivo do not respond to TGF-β1 must await the development of new techniques to study the problem. Moreover, since tumors are heterogeneous tissues composed of a variety of cell types, in situ hybridization studies combined with immunocytochemical techniques are needed to determine what cell might be synthesizing the growth factor in vivo. Given these limitations, we can only speculate about the role that TGF-β1 plays in liver neoplasia. The data that we present are consistent with the hypothesis that the primary effect of TGF-β1 secreted by liver cells during carcinogenesis is to inhibit proliferation of surrounding nonaltered cells (perhaps hepatocytes) without restricting the growth of cells that produce the factor but no longer respond to it. This would provide a selective growth advantage to unresponsive "partially transformed" or transformed cells (29). Likewise, in this and other systems, one can only speculate about the mechanisms that regulate TGF-β1 action in normal and neoplastic cells. In the case of oval cells, loss of sensitivity to TGF-β1 does not involve loss of receptors or major changes in receptor type. An attractive hypothesis, proposed to explain similar findings in other cell types, is that loss of sensitivity to TGF-β1 is a consequence of the failure of partially or fully transformed cells to activate growth factors secreted in latent form as part of a protein complex. Finally, it is of interest that oval cells in culture are capable of producing fibronectin and could be a major source for extracellular matrix components during hepatocarcinogenesis. However, although TGF-β1 increased fibronectin mRNA in untransformed cells, the effect was no longer present in tumors cells, suggesting that liver tumor cells become insensitive to the multiple activities of TGF-β1.

Materials and Methods

Production of Hepatocellular Carcinomas in Rats. Male Sprague-Dawley rats (Charles River, Wilmington, MA) weighing 130–150 g were fed a choline-deficient diet containing 0.1% ethionine prepared by Teklad Test Diets, Madison, WI. Poly(A)⁺ RNA was isolated from the livers of rats fed the CDE diet for 4, 8, and 16 weeks by the method of Chirgwin et al. (32) as previously described (33). Purified populations of oval cells and hepatocytes were obtained from the livers of rats fed the CDE diet for 4, 9, and 17 weeks by centrifugal elutriation (34). Total RNA was then extracted from cell pellets.

Establishment of Untransformed and Malignant Liver Epithelial Cell Lines. An untransformed liver epithelial cell line, designated LE/6, was derived from oval cells purified from the livers of rats fed the CDE diet for 6 weeks as described (19). Cells were maintained in Dulbecco's modified minimal essential medium: Ham's F10 (1:1) supplemented with 10% FBS, 1 μg/ml insulin, 0.5 μg/ml hydrocortisone, and antibiotics and passaged once a week. We have previously reported that these cells were not tumorigenic in nude mice, even at relatively high passage number (19). LE/6 cells at p25 were transformed by transfection with the EJ gene, a mutated form of the H-ras protooncogene (19). After inoculation of the transfected cells into nude mice, moderately to well-differentiated hepatocellular carcinomas developed within a few weeks. A cell line designated EJ-T was established from these tumors by trypsin digestion of minced tumor tissue. Clonal populations of EJ-T were obtained by serial dilution, and several clones were selected for further study. A second method of transformation involved the maintenance of oval cells in culture under confluence for approximately 1–2 months as originally described by Yoshimura et al. (35). LE/6 cells grown under these conditions also produced differentiated hepatocellular carcinomas when injected s.c. into nude mice (20). The cell lines obtained from these tumors were named S-T ("spontaneously transformed").

Growth Effects of TGF-β1 on Liver Epithelial Cells. All of the studies were performed with TGF-β1. Early (p18) and late (p55) passage LE/6 cells as well as the ras-transformed lines were plated at a concentration of 1 x 10⁵ cells/35-mm Petri dish in complete medium and allowed to recover for 24 h. At this concentration, the cultures were approximately 80% confluent. One day after plating, cells were washed with Ca²⁺ and Mg²⁺-free Hanks' basic salt solution (GIBCO), fed fresh medium containing varying concentrations of TGF-β1, and incubated for 48 h. At the end of the incubation period, 5 μCi [³H]thymidine were added for 2 h to each well, and DNA synthesis was measured by trichloroacetic acid precipitation of nucleic acids (8). In Fig. 4, all points represent the average of duplicate determinations (cpm/mg protein) and are expressed as percentage of DNA synthesis in cells not exposed to TGF-β1. Protein determinations were performed with the Bio-Rad protein assay according to the manufacturer's instructions. TGF-β1 purified from human platelets was purchased from R & D Systems (Minneapolis, MN). [³H]Thymidine was purchased from New England Nuclear (Boston, MA).

Northern Blot Analysis. Twenty μg of total RNA or 5 μg of poly(A)⁺ RNA were separated in 1.1% agarose/formaldehyde gels, transferred to nitrocellulose filters, and hybridized exactly as previously described (19) using a 1.2-kilobase BglI fragment of TGF-β1 cDNA (kindly provided by G. Bell), the Sall-PstI fragment of pv-myc corresponding to the third exon of v-myc (36), or a 500-base pair fragment of fibronectin cDNA (kindly provided by Dr. M. Rokkind). That equivalent amounts of RNA were loaded in each lane was determined by ethidium bromide staining of the agarose/formaldehyde gels prior to transfer as well as by hybridization with a cDNA probe for oval protein. Hybridization of this cDNA probe is strong in these cell lines and is not modified by cell transformation. Northern blots were routinely performed in duplicate.

TGF-β1 Binding Assay. Cells from each line were plated at a concentration of 1 x 10⁴ cells/cm² in 24-well Falcon tissue culture dishes and grown to 90% confluence. The cultures were incubated at 37 °C for 1.5 h with
0.2 μM 125I-labeled TGF-β1 (R & D Systems; approximately 26,000 cpm/Well) in binding buffer (50 mM HEPES, pH 7.5-7.8, 0.5 mM NaN3, 1.3 mM MgSO4, 1.2 mM CaCl2, 2 mM MgSO4-5 mM bovine serum albumin) in the absence or presence of excess (8 μM) unlabeled TGF-β1. Cells were then washed extensively with binding buffer and lysed in 50 mM HEPES, pH 7.5-1 mM EDTA-1% Triton X-100-1 KIU/ml aprotinin-1 μg/ml leupeptin-2.5 mg/dl phenylmethylsulfonyl fluoride. Cell-associated radioactivity was determined in a gamma counter. Each data point represents the mean of duplicate determinations, with an intraassay variation averaging 5%. Non-specific binding ranged from 2.1 to 2.9% of total 125I.

Affinity Labeling of TGF-β1 Receptors. Membranes were prepared by homogenizing approximately 1 ml of frozen packed cells in 2 ml 50 mM HEPES, pH 7.4-0.25 M sucrose-5 mM EDTA-1 KIU/ml aprotinin-1 mg/ml leupeptin-2.5 mg/dl phenylmethylsulfonyl fluoride. After centrifugation of the homogenate at 3,000 X g for 15 min, the supernatant was adjusted to 0.1 mM NaCl, 0.2 mM MgCl2, and membranes were collected by centrifugation at 30,000 X g for 40 min. The resulting membrane pellet was homogenized in a glass-teflon homogenizer in 2.5 ml of 50 mM HEPES, pH 7.5, centrifuged (40,000 X g, 40 min) and suspended in 0.5 ml of 50 mM HEPES, pH 7.5. Affinity labeling of membranes was accomplished by incubating membranes (300 μg protein) with 125I-TGF-β1 (final concentration, 4.5 ng/ml) in the absence or presence of excess unlabeled TGF-β1 (175 ng/ml). Total incubation volume was 0.2 ml, and incubation was carried out for 2 h at 25°C. At the end of this time, cross-linking was done by incubating the membranes in disuccinimidyl suberate dissolved in dimethyl sulfoxide (final concentration, 0.25 mM) for 20 min at 4°C. Membranes were collected by centrifugation, extracted with 1% Triton X-100, dissolved in gel electrophoresis buffer, and fractionated in polyacrylamide gels according to the method of Laemmli (37). The gels were dried and exposed to Kodak XAR-5 film.

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References


