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Transforming Growth Factor-Beta Receptors and Mannose 6-Phosphate/Insulin-Like Growth Factor-II Receptor Expression in Human Hepatocellular Carcinoma

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Objective

The authors examined the expression of transforming growth factor-beta receptor (TGF-βr) types I and II and the mannose 6-phosphate/insulin-like growth factor-II receptor (M6-P/IGF-IIr) in human hepatocellular carcinoma (HCC).

Summary Background Data

Transforming growth factor-beta (TGF-β) is part of a superfamily of peptide-signaling molecules that play an important role in modulating cell growth. It is secreted as a latent complex and therefore, must be activated to elicit a biological response. Bioactivation of the TGF-β complex is facilitated by binding to the M6-P/IGF-IIr. Once activated, TGF-β exerts its effects by binding to specific cell membrane TGF-β receptors. The loss of responsiveness of hepatocytes to TGF-β has been implicated in hepatocarcinogenesis and could result from a loss in the expression of either the TGF-β receptors or the M6-P/IGF-IIr.

Methods

Human hepatocellular carcinomas and surrounding normal tissue were collected from operating room samples and snap-frozen in liquid nitrogen (n = 13). Tissues from two tumors were fixed in Omni-fix for sectioning and immunohistochemistry staining for the M6-P/IGF-IIr and TGF-β1. RNA was extracted from both normal and malignant liver tissue and analyzed using an RNase protection assay. SDS-PAGE of purified membrane hybridized with ^{125}I-TGF-β_1 and ^{125}I-IGF-II was used to determine the TGF-β type I (TGF-βrI) and type II (TGF-βrII) receptors and M6-P/IGF-IIr protein levels, respectively. Gels were quantitated by phosphorimager, and a paired t test was used for statistical analysis.

Results

In HCC, a 60% ($p < 0.01$) and 49% ($p < 0.02$) reduction in the mRNA levels for TβrI and TβrII, respectively, relative to the receptor levels in surrounding normal liver, was shown. A similar decrease in the receptor protein levels also was observed. The M6-P/IGF-IIr mRNA and protein

levels were reduced in 7 of 11 hepatocellular carcinomas. Immunohistochemical staining demonstrated an absence of intracellular TGF-β_1 and reduced M6-P/IGF-IIr in the hepatocellular carcinoma cells.

Conclusions

These results demonstrate that human HCCs have a significantly reduced expression of both the TGF-βrI- and TGF-βrII-signaling receptors for TGF-β. This may provide a selective growth advantage to the HCC by allowing them to escape the mito-inhibitory effects of activated TGF-β. Furthermore, in the subset of HCC in which the expression of the M6-P/IGF-IIr is downregulated, the bioactivation of TGF-β also may be impaired.

Hepatocellular carcinoma (HCC) is a highly malignant tumor with a poor prognosis and an annual incidence of between 250,000 and 1.2 million cases in high-risk areas, such as Southeast Asia, coastal areas of mainland China, and sub-Saharan Africa.[1] Worldwide, it is the seventh most common cancer with the highest incidence of adult malignancy in areas endemic for hepatitis B virus.[1] Chemotherapy with the most active agent, doxorubicin, only produces a 10% to 20% response rate, with very rare complete remissions. The only established treatment modality involves hepatic resection or transplantation.[1] The success of these modalities is limited, however, by the advanced stage of the tumor at the time of diagnosis. Therefore, the investigation of the molecular and cellular processes involved in hepatocarcinogenesis may offer both a better understanding of its etiology and provide new methods for its treatment.

Transforming growth factor-beta (TGF-β) is a ubiquitous multifunctional cytokine belonging to a superfamily of peptides, including Müllerian-inhibiting substances, bone morphogenic proteins, activins, and inhibins.[2,3] It can act both as an inhibitor and stimulator of cellular replication as well as control the synthesis of many components of the extracellular matrix.[4] However, TGF-β is a potent mito-inhibitor for most epithelial cells.[5–9] The TGF-β family is comprised of five structurally related TGF-β molecules: TGF-β_{1-5}, and in mammals only TGF-β_1, TGF-β_2, and TGF-β_3 are expressed.[2] This 25-kd homodimer is secreted from cells as a larger latent complex containing phosphomannosyl residues, and it must be released from this complex to be biologically active.[10] *In vitro,* conditions such as low or high pH, heat, and urea have been shown to activate TGF-β_1.[2,11,12] The binding of the latent complex of TGF-β_1 to the mannose G-phosphate/insulin-like growth factor IIr (M6-P/IGF-IIr) in the presence of transglutaminase also has been shown to facilitate the extracellular activation of TGF-β_1 by plasmin.[13]

Activated TGF-β molecules exert their biological effects by binding with high affinity to cell surface receptors. Cross-linking experiments with ^{125}I-labeled TGF-β_1 have identified three primary receptors: 53-kd TGF-βrI,[14] 70-kd TGF-βrII,[15] and 200–400-kd TGF-βrIII.[16] Both TGF-βrI and TGF-βrII possess intrinsic serine-threonine kinase activity[14,15] and signal through a heteromeric receptor complex.[17] Transforming growth factor-βrI requires TGF-βrII to bind ligand, whereas TGF-βrII requires the presence of TGF-βrI to signal.[17] Transforming growth factor-βrIII, also known as betaglycan, is the most abundant TGF-β binding protein expressed on the cell surface.[18] Transforming growth factor-βrIII is not needed for growth arrest[7] and does not directly participate in TGF-β signal transduction, but serves to concentrate ligand before presentation to types I and II signal transducing receptors.[16]

An elevated level of TGF-β_1 mRNA has been reported in HCC, and TGF-β_1 also is increased in the plasma of patients with HCC.[19–21] Furthermore, the loss of responsiveness of liver tumor cells to TGF-β_1 has been implicated in hepatocarcinogenesis.[22] To determine whether this loss of HCC responsiveness to TGF-β_1 could result from reduced TGF-β receptor levels, we determined the expression of TGF-βrI and TGF-βrII signaling receptors at the both mRNA and protein level and compared it with that in the surrounding normal liver. Additionally, because the M6-P/IGF-IIr functions in the bioactivation of TGF-β_1, the expression of this receptor also was determined in human HCC.

MATERIALS AND METHODS

Tissue Samples

Hepatocellular carcinoma and the corresponding surrounding normal liver from 13 patients were collected

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from freshly discarded pathology tissue at the time of surgical resection or transplantation at Duke University Medical Center. Two of these patients were excluded from this study because the RNA was found to be degraded. Of the 11 patients remaining in the study, 6 were women and 5 were men, with an average age of 56 years (range 43–84 years). These patients with HCC represented a subset with operable disease, and none had a history of hepatitis or cirrhosis. Tumor diagnosis was histologically confirmed by pathology with four well-differentiated hepatomas, six moderately differentiated hepatomas, and one primary hepatic adenocarcinoma—the differentiation degree of which was not specified. For immunohistochemical analysis, samples were fixed in Omnifix (An-Con Genetics, Inc., Melville, NY) for 48 hours and embedded in paraffin. The remaining tissues were snap-frozen in liquid nitrogen and maintained at −70 C.

Preparation of RNA

Total RNA was isolated from liver samples by the guanidium-thiocyanate acid-ethanol method (Tel-Test, Friendswood, TX).[23] RNA concentrations were determined by spectrophotometry with $OD_{260/280}$ (range 1.7–1.9). RNA integrity was determined by electrophoretic fractionation of 20 μg of total RNA on a horizontal denaturing formaldehyde-agarose (1.1%) gel with resultant visualization of ribosomal RNA bands.

Plasmids and Probe Preparation

A 172 nucleotide *SstI-BamHI* fragment from the human M6-P/IGF-IIr (bp 2958–3130) cDNA was subcloned into pBLUESCRIPTII KS (Stratagene, LaJolla, CA). A 287-nucleotide *EcoRI-BstUI* fragment from human TGF-βrI cDNA (bp 1–287) was subcloned into pGEM-7Zf(+) (Promega, Madison, WI). A 318-nucleotide *AvaI-Pst* fragment from human TGF-βrII cDNA (bp 243–561) was subcloned into pGEM-3Zf(+) (Promega, Madison, WI). A human GAPDH in pTRIPLESCRIPT (Ambion, Inc., Austin, TX) was linearized with *StyI*.

Ribonuclease Protection Assay

Hybridization of RNA probes to cellular RNA was performed by *in vitro* transcription of linearized cDNA clones in the presence of α^{32}P-CTP (DuPont NEN, Boston, MA) to produce labeled antisense probes as per manufacturer's specifications (Ambion MAXIscript Kit, Ambion Inc., Austin, TX). Gel-purified cRNA probes (250 cpm receptor/probe, 100 cpm GAPDH) were hybridized in solution with 30 μg of total RNA. After 16 hours of hybridization at 55 C, samples were digested with RNase (5 μg/mL RNase A, and 10 units/mL RNase T1), precipitated, and resuspended in gel-loading buffer. Protected fragments were resolved on a 6% polyacrylamide, 7 mol/L urea-denaturing gel. The quantity of each protected fragment was determined by phosphorimager analysis (Molecular Dynamics, Sunnyvale, CA). Volume integration of the receptor cRNA protected probe was normalized to the volume integrated for the protected band of GAPDH for that lane to obtain a ratio (receptor protected signal/GAPDH protected signal) for use in data analysis.

Membrane Isolation/Affinity Labeling

Liver membrane isolation was performed by methods previously described with modifications.[24] Affinity labeling of the TGF-β and M6-P/IGF-II receptors was performed by methods previously described.[25] Briefly, 75 μg of membrane protein was incubated with 3 ng ^{125}I-TGF-β (DuPont NEN, Boston, MA) or 3 ng ^{125}I-IGF-II (Amersham Inc., Arlington, IL) in a total volume of 50 μL incubation buffer (100 mmol/L HEPES, pH 7.8, 118 mmol/L NaCl, 1.2 mmol/L $MgSO_4$, 8.8 mmol/L dextrose, 5.0 mmol/L KCl, 1% bovine serum albumin [BSA]). Samples were incubated at 15 C for 2 hours. Cross-linking was performed by addition of 0.25 mM bis, sulfosuccinimidyl substrate (Pierce, Rockford, IL) for 15 minutes on ice. Membranes were then collected by a 10-minute centrifugation in a microfuge, resuspended in 40 μL of sample buffer, boiled for 5 minutes, loaded on a 12% EP-Tris/glycine minigel (Schleicher & Schuell, Keene, NH), and electrophoresed for 2 hours at 150 V. The gels were then dried on Whatman filter paper (Whatman, Maidstone, UK) and placed on phosphorimager screens for quantification.

Immunohistochemistry

The techniques used to immunohistochemically stain for TGF-β_1 and M6-P/IGF-IIr have been previously described.[26,27] Briefly, liver tissues were fixed in Omnifix, paraffin embedded, and 6-μm sections were placed on glass slides for immunohistochemical staining. After deparaffination, sections were exposed overnight at 4 C to antibodies TGF-β_1 CC(1–30), TGF-β_1 (78–109), and M6-P/IGF-II C1. Identical concentrations of nonimmune rabbit immunoglobulin G were used as controls. Transforming growth factor-β_1 CC(1–30) is a polyclonal antibody corresponding to the first 30 amino acids of mature TGF-β_1 and primarily stains components of the extracellular matrix.[28] Transforming growth factor-β_1 (78–109) is a rabbit polyclonal antibody we produced to

amino acid residues 78–109 of the mature TGF-β_1 molecule. With the use of Western blots and enzyme-linked immunosorbent assays, it has been shown that it recognizes TGF-β_1, has no detectable cross-reactivity with TGF-β_3, and has a <5% cross-reactivity with TGF-β_2. It primarily stains intracellular epithelial cells in a manner identical to that for the LC(78–109)[12] and LC(1–30).[29] The C1 antibody is a polyclonal antibody that binds specifically to the M6-P/IGF-IIr.[30] Tissues were washed 3 × 5 minutes with phosphate-buffered saline/bovine serum albumin (PBS/BSA) and immunoperoxidase-stained according to the rabbit immunoglobulin G Vectastain Elite ABC Kit (Vector Laboratories, Burlingame, CA) protocol. The color reaction was developed with diaminobenzidene, and the sections were counterstained with hematoxylin.

Statistical Analysis

Comparison of mean values was performed by the paired t test using the StatSoft Statistical program (StatSoft Inc, Tulsa, OK).

RESULTS

TGF-βrI, TGF-βrII, and M6-P/IGF-IIr mRNA Expression

Transforming growth factor-βrI, TGF-βrII, and M6-P/IGF-IIr were all present in both tumor and surrounding normal liver. The average mRNA level of TGF-βrI in the tumor samples showed a 60% decrease in expression as compared with that in the surrounding normal liver, with 10 of 11 samples displaying this decrease ($p < 0.01$; Fig. 1A). RNA samples also were hybridized to a TGF-βrII antisense probe to determine if a concomitant decrease was observed with TGF-βrII. The average mRNA level of the TGF-βrII in tumors demonstrated a 49% decrease in expression compared with that in the surrounding normal liver, with 8 of 11 of the tumors displaying this decrease ($p < 0.02$; Fig. 1A).

There also was a 58% decrease in M6-P/IGF-IIr mRNA expression in 7 of 11 tumors as compared with that in the surrounding normal liver. In the remaining four HCCs, the expression was twice that of surrounding normal (Fig. 2). There was no correlation between the elevation in the M6-P/IGF-IIr level and TGF-βrI or TGF-βrII expression. Three male patients and four female patients had decreases in M6-P/IGF-IIr mRNA expression. The expression of GAPDH in the tumors and surrounding normal was not significantly different.

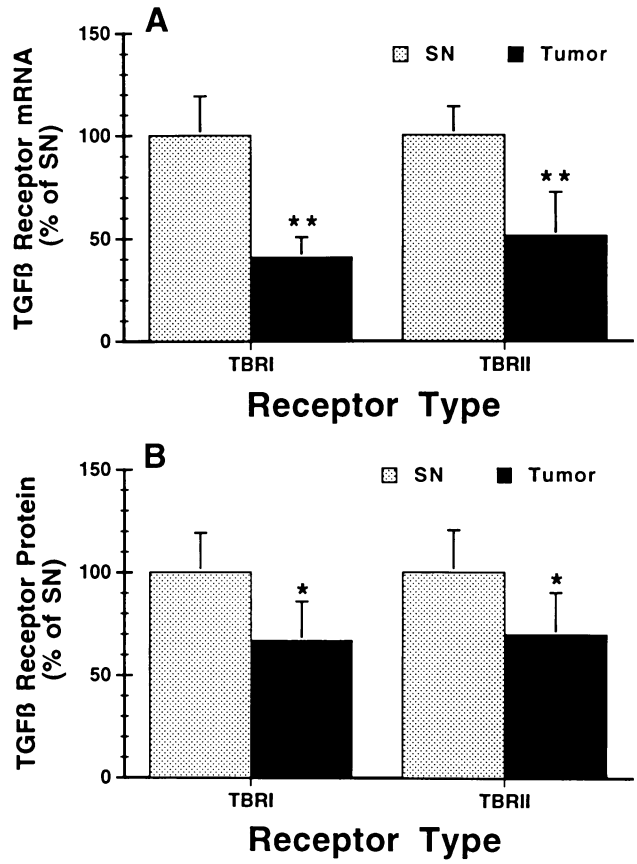

Figure 1. Transforming growth factor-βrI, TGF-βrII mRNA (A) and protein (B) expression in human HCC. The average mRNA and protein levels for these receptors are shown for HCC relative to that in surrounding normal liver. Receptor mRNA level was first normalized to that of GAPDH. Error bars represent percent standard error of the means for 11 samples; the significance levels are *($p < 0.02$) and **($p < 0.01$).

TGF-βrI, TGF-βrII, and M6-P/IGF-IIr Membrane Protein Levels

Cross-linking of ^{125}I-TGF-β_1 to plasma membranes (n = 5: 3 women, 2 men; patients chosen randomly) from HCC and surrounding normal liver demonstrated the presence of labeled proteins with migration of bands corresponding to TGF-βrI and TGF-βrII, based on their molecular weights. Quantification of relative intensities by phosphorimager analysis showed a 33% decrease in TGF-βrI expression in HCC ($p < 0.02$) compared with that in the surrounding normal liver. Similarly, a decrease of 30% was observed in tumor expression of TGF-βrII ($p < 0.02$; Fig. 1B).

Affinity labeling with ^{125}I-IGF-II resulted in a high molecular weight band that corresponded to M6-P/IGF-IIr. Quantification showed that of those tumors with decreased M6-P/IGF-IIr mRNA, there was a similar decrease in receptor protein expression with mean decrease

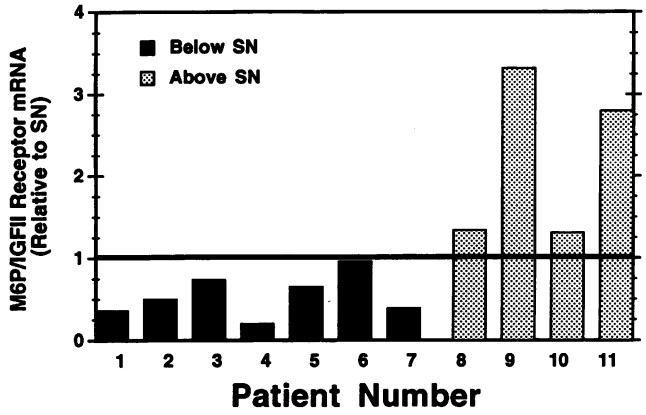

Figure 2. Mannose 6-phosphate/IGF-II receptor mRNA expression in human HCC. The mRNA level for this receptor is shown for HCC relative to that in the surrounding normal liver of 11 patients. Receptor mRNA level was first normalized to that of GAPDH. The M6-P/IGF-II receptor expression in seven HCC was below whereas in four HCC the expression was above that in the surrounding normal liver. Receptor protein level was measured in five of these HCC, and in all tumors, it directly correlated with the mRNA expression.

of 27% compared with that in the surrounding normal liver ($p < 0.03$).

Immunohistochemistry

Representative immunohistochemical staining of human HCC for the M6-P/IGF-IIr and TGF-β_1 are shown in Figure 3. Normal liver demonstrated moderate homogenous staining for the M6-P/IGF-IIr (Fig. 3A). Staining in the hepatocytes with the TGF-β_1 (78–109) antibody colocalized with that for the M6-P/IGF-IIr (Fig. 3B). In contrast, HCC had significantly lower levels of both the M6-P/IGF-IIr and TGF-β_1 than was seen in the surrounding normal liver. The intracellular distribution of the M6-P/IGF-IIr in the tumor cells also was nonhomogeneous, with the receptor predominately occurring on the cell surface membrane with intracellular aggregates at the Golgi apparatus. The tumor and normal liver stromal elements showed a similar intense staining for both the M6-P/IGF-IIr and TGF-β_1.

The TGF-β_1 CC(1–30) antibody, which primarily stains extracellular matrix, demonstrated strong staining in tumor stromal components, with lower levels of staining in HCC cells (Fig. 3C). Because total RNA isolated from tumor tissue represents both parenchymal and nonparenchymal cells, and considering the fibrotic nature of the HCC used in this study, the TGF-β receptors and M6-P/IGF-IIr in the tumor cells may be lower than the values reported, based on whole tissue RNA and protein extraction.

DISCUSSION

In this study, a significant reduction in the protein and mRNA expression of TGF-βrI, TGF-βrII, and M6-P/IGF-IIr was demonstrated in human HCC as compared with that in the surrounding normal liver. It has been postulated that during the process of carcinogenesis, epithelial cells, whose proliferation normally is inhibited by TGF-β, may escape from an autocrine or paracrine growth control by TGF-β and become autonomous.[4] The loss of responsiveness by tumor cells to negative growth control can be achieved by various mechanisms, including changes in ligand concentration, altered activation of latent forms of the ligand, modulation of number and affinity of receptors, and alteration in postreceptor pathways.[24] The aim of this study was to examine the steady-state levels of mRNA and protein expression of TGF-βrI, TGF-βrII, and M6-P/IGF-IIr in human HCC. This would give insight into the possible changes in both the response to and activation of TGF-β in human HCC.

Relationships between the production of TGF-β and carcinogenesis have been well established. Increased production of TGF-β mRNA has been reported in gastric,[31] thyroid,[32] brain,[33] pancreatic,[34] and prostate[35] cancers, as well as in disease progression in breast cancer.[36] Furthermore, elevated levels of TGF-β at both the mRNA and protein levels have been reported in HCC,[19,20] and in the plasma of patients with hepatocellular carcinoma.[21] However, in order for TGF-β to elicit a biologic effect, the binding of active TGF-β to the high affinity signaling TGF-βrI and TGF-βrII receptors is required.[17] Several tumor cell lines, such as retinoblastoma, pheochromocytoma, neuroblastoma, and breast carcinoma cells, lack detectable expression of either TGF-βrI or TGF-βrII, and show no growth inhibition by TGF-β.[37] We found a significant decrease in both TGF-βrI and TGF-βrII in human HCC at both the mRNA and protein level. These results are similar to those observed in N-nitrosdiethylamine-initiated/phenobarbital-promoted rat liver tumors.[38] The downregulation of these receptors suggests that human HCC also may obtain a growth advantage by escaping the mito-inhibitory effects of TGF-β.

Cells that are unresponsive to TGF-β also may be aberrant in their ability to activate TGF-β from the latent complex.[39] Although some tumor cells are able to generate an active form of TGF-β in culture supernatant,[40] the extracellular activation of TGF-β by plasmin appears to be dependent on binding of latent TGF-β complex to the M6-P/IGF-IIr.[13] The level of the M6-P/IGF-IIr was decreased in 7 of 11 tumors, suggesting that in these tumors, the ability to activate TGF-β also may be diminished. Similar results in patients with hepatitis B and cirrhosis associated HCC also have been reported in a

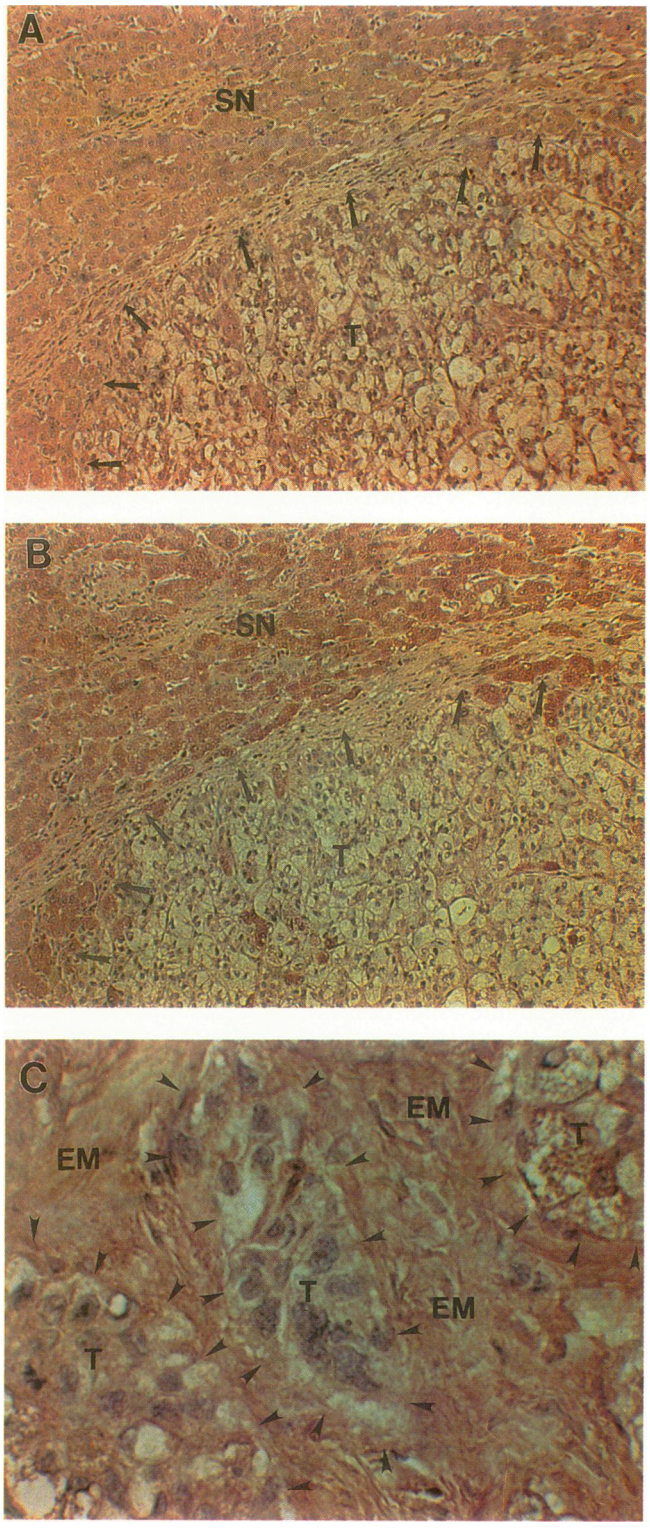

Figure 3. Immunohistochemical staining of human hepatocellular carcinoma with the M6-P/IGF-IIr C1 antibody[30] (A), the TGF-β_1 78–109 antibody[28] (B), and the TGF-β_1 CC (1–30) antibody[29] (C). Sections are representative samples showing tumor (T), surrounding normal (SN), and extracellular matrix (EM). Arrows (A and B) represent tumor/normal tissue interface. Arrowheads (C) represent extracellular matrix/tumor interface. Tissue sections exposed to nonimmune immunoglobulin G were negative for staining (data not shown). Tissue sections were counterstained with hematoxylin. Original magnification of the photomicrographs in A, B, and C are ×100, ×100, and ×400, respectively.

preliminary study by Nagy and Thorgeirsson.[41] This is important because their patient population represents a pathogenesis different from that presented in this report. The M6-P/IGF-IIr has been shown to be imprinted in a subpopulation of humans.[42] Thus, only a single allele would need to be altered in those humans in which the M6-P/IGF-IIr is imprinted to modify the expression or function of this receptor. Whether the M6-P/IGF-IIr is preferentially imprinted in those patients with reduced gene expression remains to be determined.

Insulin-like growth factor-II, a polypeptide structurally homologous to insulin, has been implicated as an autocrine growth factor in liver carcinogenesis. The cell growth effects of both IGF-I and IGF-II are mediated through the IGF-Ir,[43] whereas binding of IGF-II to the M6-P/IGF-IIr leads to its cellular internalization and degradation in the lysosomes.[44] Therefore, a decrease in M6-P/IGF-IIr in human HCC may further offer a selective growth advantage to HCC cells by a mechanism independent of TGF-β, mainly through a reduced ability to degrade IGF-II.

The association of human HCC and cirrhosis, with its concomitant fibrosis, is well established. Liver cirrhosis is a diffuse process of hepatic fibrosis and regenerative nodule formation of unknown pathogenesis.[45] Elevated levels of TGF-β have been reported in fibrotic conditions[45–48] with enhanced deposition of extracellular matrix components. However, in this study, none of the patients had cirrhosis by chart examination or pathology. Nevertheless, immunohistochemical observations demonstrated extensive stromal development in the tumors that stained strongly positive for both TGF-β_1 and the M6-P/IGF-IIr. This suggests an increased concentration of activated TGF-β_1 in the tumor stroma. Furthermore, the decreased level of TGF-β1 observed in the malignant epithelial cells of HCC, compared with those in the surrounding normal liver, may result from the decreased expression of both the M6-P/IGF-IIr and TGF-β receptors. Thus, the reduced levels of TGF-βrI, TGF-βrII, and M6-P/IGF-IIr in the tumor epithelial cells may enable the human HCC cells to escape the mito-inhibitory effect of TGF-β_1 produced by the tumor stromal elements.

We have observed a significant downregulation of TGF-βrI, TGF-βrII, and M6-P/IGF-IIr at both the protein and mRNA levels in hepatocellular carcinoma rela-

tive to the levels of these receptors in the surrounding normal liver. The reduced expression of these receptors suggests a possible molecular basis for the selective growth of human HCC, and may offer insight into newer therapeutic modalities in the future.

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