A Novel Orthotopic Tumor Model to Study Growth Factors and Oncogenes in Hepatocarcinogenesis

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ABSTRACT
An orthotopic xenograft tumor model of hepatocellular carcinoma was created by injection of Hep 3B cells directly into the liver parenchyma of nude mice. Tumors were localized primarily in the injected lobe of the liver, beginning from the third week after tumor cell implantation. Thereafter, tumors grew rapidly, and animals usually died from hepatocellular carcinoma within 2 months. Insulin-like growth factor II, an embryonic growth factor and mitogen, is overexpressed in these tumors at both mRNA and protein levels. Oncogenes, such as c-myc, c-fos, and c-jun, are also up-regulated in this model. c-Fetal protein can be detected shortly after implantation and correlates with tumor growth, and measurement of serum c-fetal protein serves as an early biomarker to monitor the effect of antitumor therapy. Using this model, we have shown that inhibition of insulin-like growth factor II expression by a short methylated oligonucleotide prolongs survival. This in situ tumor model thus provides a fast, reliable, and reproducible means to study the therapeutic effect of inhibitors of growth factors and oncogenes in liver cancer.

INTRODUCTION
HCC, or hepatoma, affects approximately 1 million people worldwide. The incidence of HCC is extremely high in Southern Asia and sub-Saharan Africa (1–3). In some countries, it is the single most common cause of cancer death in men. In East Asia, the annual incidence of HCC approaches 150 per 100,000. According to cancer mortality data collected in 1992, HCC accounts for 340,000 cancer deaths per year in China (4). It is a devastating disease with no effective therapy and no reliable methods of detection. HCC often metastasizes widely, and up to 90% tumors of >5 cm will have invaded the portal vein. Distant sites of metastatic disease include lung, bone, adrenals, and brain.

Current therapies for HCC include surgery, chemotherapy, radiotherapy, and percutaneous ethanol injection. These treatments are extremely morbid, invasive, or cytotoxic, and many patients cannot tolerate them, especially those who have extensive liver cirrhosis and poor hepatic function at the outset of therapy. The liver is very sensitive to radiation exposure, limiting the usefulness of radiation therapy. The tolerated doses of radiotherapy to the liver appear to be far below the threshold required for significant control of primary neoplasms. Usually, nonspecific supportive therapy is the only treatment that can be offered. As a result, the 5-year survival rates for patients have been disappointing, usually in the range of 16–25% (5).

Recently, IGF2 has become an attractive therapeutic target for human tumors. This growth factor is frequently overexpressed in human tumors, including HCC, lung cancer, breast cancer, colorectal cancer, leiomyosarcoma, and Wilms’ tumor (6–11). We and others have shown that hepatic IGF2 mRNA abundance varies with hepatitis B virus expression in a coordinated fashion in hepatitis B virus-transgenic mice that develop HCC (12, 13). Moreover, hepatic malignancies frequently develop in mice that carry the IGF2 transgene (14). β-Cell oncogenesis is markedly inhibited in IGF2 knockout transgenic mice (15). In a SV40 transgenic model, animals carrying the mutated IGF2 allele developed fewer and smaller liver tumors than those carrying the normal IGF2 allele (16). Thus, IGF2 appears to be a second “survival” signal for oncogene-induced abnormal cell growth in a variety of tumors (17). Because there are no apparent clinical symptoms associated with IGF2 deficiency in adults and because IGF2 is highly expressed in malignant tissue, targeting this growth factor may provide a novel therapy for HCC patients who cannot tolerate other therapies.

In our laboratory, we have examined the inhibition of growth factor transcription as a therapeutic modality for HCC. In searching for an effective therapy for HCC, it is necessary to have a reliable animal liver cancer model. Creating tumors by s.c. injection of tumor cells in the flank region of athymic nude mice is the most common tumor model used (18, 19). This model is easily created in nude mice and requires only minimal technical skills. The therapeutic effect can easily be observed by systemic administration or local intratumoral injection of anti-cancer drugs. However, this model may not be representative of naturally occurring HCC. Hepatic tumors, of course, normally
reside within the liver parenchyma, where drug metabolism and transformation often occur. Thus, the pharmacodynamics and pharmacokinetics of drug therapy may be greatly different for intrahepatic as compared with peripheral tissues.

Intrahepatic HCC can be created by the intraportal injection of tumor cells (21). In this model, tumors form throughout the liver. However, the model is poorly reproducible due to the technical skills required and the relatively high perioperative mortality. In this communication, we report a reliable, reproducible, and simple model to create intrahepatic HCC in nude mice. In this model, cells from the human liver cancer cell line Hep 3B are directly injected into the left lobe of the liver. Postoperatively, all treated mice bear HCC. Tumors formed in this model secrete AFP into the circulation, which offers a reliable biomarker to monitor tumor growth and the therapeutic effects of drugs. Furthermore, growth factors and oncogenes that are frequently overexpressed in HCC are also up-regulated in these tumors. This model thus provides a convenient and reproducible tool for the screening of antitumor therapeutics for HCC.

MATERIALS AND METHODS

Cell Culture. The human HCC cell line Hep3B was purchased from American Type Culture Collection (Manassas, VA). Hep 3B cells were maintained in MEM (Life Technologies, Inc., Rockville, MD) supplemented with 10% fetal bovine serum, 200 mM glutamine, 100 mM MEM sodium pyruvate, and 5% CO2. Hep 3B cells were maintained in MEM (Life Technologies, Inc., Rockville, MD) supplemented with 10% fetal bovine serum, 200 mM glutamine, 100 mM MEM sodium pyruvate, and 5% CO2.

In Situ Tumor Model. Male BALB/c athymic (nu/nu) nude mice (age, 4–5 weeks; weight, 20–25 g) were purchased from Simonsen Laboratory (Gilroy, CA) and kept under pathogen-free conditions, fed standard food, and given free access to sterilized water. Housing and all procedures were performed according to protocols approved by the Institutional Animal Care and Use Committee at VA Palo Alto Health Care System.

The HCC model was created by direct intrahepatic injection of Hep 3B cells. Mice were anesthetized i.p. with 80 mg/kg Ketamine and 10 mg/kg Xylazine, then placed on the injection site, and light pressure was applied for 1 min to prevent bleeding. The abdomen was then closed with a 6-0 silk suture.

After tumor cell implantation, animals were kept in a warm cage, observed for 1–2 h, and subsequently returned to the animal room after full recovery from the anesthesia was documented.

Serum AFP Measurement. Blood samples (10 μl) were collected from the tail vein and suspended in 40 μl of distilled water. Samples were kept at ~80°C for AFP measurement. Ten μl of PBS-diluted blood samples were then used to measure the serum AFP level with the AFP ELISA kit (Sierra Resources International, Inc., Valrico, FL), using the protocol provided by the manufacturer.

Expression of Growth Factors and Oncogenes. Two weeks after Hep 3B cell implantation, we collected tumor tissues, peritumor tissues, and adjacent normal tissues for the measurement of growth factors and oncogenes.

Tissues (100 mg) were homogenized in 1.0 ml of solution D (4 M guanidinium thiocyanate solution containing 1% β-mercaptoethanol, 2.5 mM sodium citrate, and 0.5% sarcosyl). Total nucleic acid was extracted and purified using our previously described method (22, 23). Total nucleic acid samples were used directly for cDNA synthesis with RNA reverse transcriptase.

IGF2 expression in tumor tissues was examined by PCR amplification. Briefly, cDNA samples were amplified in a 3.0-μl reaction mixture in the presence of 50 μM deoxynucleotide triphosphate, 0.2 μM IGF2 primers, 0.25 μCi of α-dCTP (Amersham, Arlington Heights, IL), and 0.125 unit of KlenTaq1 DNA polymerase (Ab Peptides, Inc., St. Louis, MO). DNA was amplified for 32 cycles at 94°C for 15 s and 65°C for 40 s, followed by a 30-s extension at 72°C. The PCR products were separated on 5% polyacrylamide-urea and scanned for quantitation with PhosphorImager Analyzer (Molecular Dynamics, Sunnyvale, CA).

The oligonucleotide primers used for quantitating IGF2 expression were 3038: (5’ primer; TTGGAAGAACTTGCAGACT); (3’ primer; TTGGAAGAACTTGCAGACT). This pair of IGF2 primers crosses IGF2 intron 8 (intron in mouse IGF2), such that genomic DNA and mRNA give different sizes of PCR products, which can be easily separated on 5% polyacrylamide-urea gel for IGF2 quantitation.

The PCR primers used for the measurement of other growth factors and oncogenes include the following: (a) IGFIR, 626 (5’ primer; CGTCTGGGACTACATTGTGGGGA) and 627 (3’ primer; GGAGACATTGCTCTTGGTGCA); (b) c-myc, TV622 (5’ primer; GATTTCTGCTCTTCCTGAGCAGGAT) and TV623 (3’ primer; CTCCACGAGAAGTTGACCTGACTG); (c) α-F, G043 (5’ primer; TATTGTGGCTCCACACTGCAATA) and G047 (3’ primer; ACTTATCTGCAGTACATTGGTA); (d) c-Jun, 1324 (5’ primer; GAGACGGGACCTTATGGCTACAGT) and 1519 (3’ primer; GCCCGTGGCTGGACTACCTAG); (e) c-Fos, 6550 (5’ primer; AGGGAGCTGACTGATACTCCAAG) and 6551 (3’ primer; ATCCGCGAGGAGTGGTGACCTG); and (f) β-actin, 774 (5’ primer; GGGAATTCATCAAATCGGAGCAGGAGGAGGAG) and 775 (3’ primer; GGAAGCTTACCAAGTGCCGACAGA). β-Actin was used as an internal control for PCR reaction.

Immunohistochemical Staining. After deep anesthesia with 80 mg/kg Ketamine and 10 mg/kg Xylazine, tumor-bearing nude mice were transcardially perfused with 4% paraformal-
dehyde solution. The tissue slice containing tumors was dissected out and fixed in 4% paraformaldehyde solution for 4 h. The samples were embedded in paraffin blocks according to standard procedures. Twenty 5-μm tissue sections were cut and embedded in paraffin.

Expression of AFP, c-myc, and IGF2 was examined by immunohistochemical staining (24). Briefly, formalin-fixed and paraffin-embedded sections were deparaffinized and incubated with different dilutions of antisera (1:50 for AFP, 1:50 for c-myc, and 1:100 for IGF2; Santa Cruz Biotechnology). Cells were stained with AEC Detection Kits (Vector Laboratories, Inc., Burlingame, CA).

Administration of MON1 Oligonucleotide to Tumor-bearing Mice. We have previously shown (25) that MON1, a methylated oligonucleotide, specifically inhibits IGF2 expression in two liver cancer cell lines, Hep 3B and Hep G2, and prolongs the tumor survival in Hep 3B-implanted nude mice. In another batch of animals, we examined the MON1-induced chemosensitizing effect of cisplatin on tumor growth in this orthotopic tumor model.

One month after Hep 3B implantation, nude mice were divided into four groups: (a) 12 mice, control, PBS (100 μl i.v. biweekly); (b) 6 mice, IGF2 inhibitor MON1 (7 mg/kg i.v. biweekly); (c) 6 mice, cisplatin (0.5 mg/kg i.v. biweekly); and (d) 6 mice, MON1 (7 mg/kg) + cisplatin (0.5 mg/kg). Testing materials (PBS, MON1, and cisplatin) were injected into the tail vein under sterile conditions. Mice were continuously observed until tumor death was recorded for all animals.

Statistical Analysis. Animal survival data after IGF2 inhibitor (MON1) treatment were entered as the format of Kaplan-Meier Life Table and presented as the cumulative survival plot (26). Statistical differences in animal survival after

Fig. 1 Liver tumors formed from intrahepatic injection of Hep 3B cells. A, survival after Hep 3B cell implantation. Time of death was recorded as weeks after Hep 3B cell implantation. No tumor deaths were observed in mice that received PBS injection in place of Hep 3B implantation. B, H&E staining of liver tumor. Four weeks after Hep 3B cell implantation, the implanted lobe of nude mice was collected, fixed with 4% paraformaldehyde, and embedded in paraffin blocks for H&E staining. Note the abundance of newly formed blood vessels in tumors. C, tumor formed in the liver of nude mice. Photo was taken 4 weeks after Hep 3B cell implantation. No tumors were found at autopsy in nude mice that received intrahepatic PBS (control).
MON1 treatment were analyzed by the Mantel-Cox log-rank test (27) provided in the GB-STAT program software (Dynamic Microsystems, Inc., Silver Spring, MD). $P < 0.05$ was considered statistically significant.

RESULTS

Tumor Development after Hep 3B Cell Implantation.
After Hep 3B cell implantation, tumors formed in the livers of nude mice. Two weeks after the implantation, a small white tumor mass ($3 \times 4$ mm) was often observed on the surface of the lobe where Hep 3B cells were injected. Thereafter, these tumors grew rapidly in the injected lobe and began to spread to other lobes. Beginning 4 weeks after injection, death was observed in tumor-bearing animals. Two months after Hep 3B cell implantation, more than 90% of untreated animals died of tumor burden (Fig. 1A).

In the early stages, tumors formed in the injected lobe, and a clear boundary between tumor and normal tissues was apparent. Tumors formed from Hep 3B cell implantation demonstrated newly formed blood vessels, indicating a rich blood supply for the tumor (Fig. 1B).

By the end of the study, tumors expanded to fill the abdominal cavity, and some tumors were externally visible (Fig. 1C). At autopsy, much of the normal liver was replaced by tumor. Tumors were not limited to the injected lobe but could be seen in other lobes as well (Fig. 1C). Grossly normal liver tissue was observed to ring the large tumor mass. No metastases to other organs were observed in any of the animals tested. Thus, tumors formed from Hep 3B cell implantation were very localized in the injected lobe and expanded into other lobes at a late stage.

Expression of IGF2 in Implanted Tumors. In a subset of mice, animals bearing liver tumors 4 weeks after Hep 3B cell implantation were sacrificed, and tissue was collected for the analysis of IGF2 expression. IGF2 mRNA transcripts measured by PCR were in very low abundance in the surrounding normal liver tissue (Fig. 2A, Lanes 1–4). In tumors formed from Hep 3B cells, however, the IGF2 mRNA levels were increased (Lanes 5–8) by 30-fold in comparison with normal liver tissue.

Overexpression of IGF-II protein in the tumors was confirmed by immunohistochemical staining (Fig. 2B). Normal liver sections contained very little IGF2 staining. In tumors, however, a strong staining signal was observed in a majority of cells.

IGF-2 exerts its mitogenic effect through the IGFIR. Tumors formed from the implanted Hep 3B cells also expressed increased levels of IGFIR mRNA (Fig. 2A).

AFP as a Biomarker of Tumor Development. Serum AFP was barely detectable in normal mice and in blood samples collected at the time of Hep 3B cell implantation in study mice. Serum AFP levels were easily detected 4 weeks after the surgery (Fig. 3A). Thereafter, serum AFP levels increased dramatically and correlated directly with tumor growth in animals.

AFP mRNA was not detected in normal liver tissues by PCR (Fig. 3B), and no AFP was seen in sections by using immunohistochemical staining (Fig. 3C). In tumor tissue, AFP was detected at high levels by both mRNA and protein analysis (Fig. 3C).

Expression of Oncogenes. We measured the mRNA expression for c-myc, c-jun, and c-fos in the tumor samples (Fig. 4). All three oncogenes, including c-jun, c-fos, and

Fig. 2 Expression of IGF2 in tumors. A, IGF2 and IGFIR mRNAs in normal and tumor tissues. IGF2 and IGFIR mRNAs were amplified by PCR, and β-actin was used as PCR internal control. B, IGF2 protein in normal and tumor tissues measured by immunohistochemical staining. Formalin-fixed and paraffin-embedded sections were stained with antisera for IGF2 (Santa Cruz Biotechnology). IGF2 proteins were stained a red-brown in the section. Note the increased staining for IGF-II in the tumor specimen.
**c-myc** were markedly up-regulated in tumors taken from injected mice. Thus, this animal model may also be useful for investigating the effect of these oncogenes in the development of hepatoma.

**Effect of the Novel IGF2 Inhibitor, MON1, on Survival Rate of Mice Implanted with Hep 3B Cells.** We have previously shown that MON1, a methylated oligonucleotide, inhibits IGF2 expression and prolongs survival in animals implanted with HCC (25). In a separate study conducted in this mouse model, we treated tumor-bearing nude mice with 0.0, 0.7, and 7.0 mg/kg MON1. At the end of the study, we found that there were no survivors in the control group 8 weeks after Hep 3B tumor cell implantation. The low-dose MON1 showed a 16.7% survival rate, and the high-dose MON1 showed a 66.7% survival rate at 8 weeks, which is significantly different from control by χ² analysis (25).

In another batch of animals, we examined the MON1-induced chemosensitizing effect of cisplatin on tumor growth in this model. One month after Hep 3B implantation, nude mice were divided into four groups, receiving PBS, IGF2 inhibitor MON1 (7 mg/kg), cisplatin (0.5 mg/kg), or MON1 (7 mg/kg) + cisplatin (0.5 mg/kg). As reported previously (25), tumors grew quickly in untreated nude mice, leading to their rapid demise. A low dose of cisplatin slightly inhibited tumor growth (data not shown) and prolonged life. However, the combination of MON1 with low-dose cisplatin significantly increased tumor survival when compared with the untreated animals (Fig. 5; P < 0.05).

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**Fig. 3** AFP as a biomarker of liver tumor growth. A, serum levels of AFP as measured by ELISA kit at baseline, 4, 6, and 8 weeks after implantation. B, measurement of AFP mRNA in tumors. AFP is highly expressed in tumors but not detectable in normal liver tissues. C, immunohistochemical staining of AFP in tumors. Strong immunostaining was observed in tumors versus absence of staining in normal tissue.
Liver Tumor and IGF-II

Thus, this is a simple, reproducible method of creating a model for the study of HCC. The tumors produced a measurable serum tumor marker, AFP. There was a rapid demise in over 90% of experimental animals, and hepatic tumor could be produced in 100% of animals by a simple intrahepatic injection of a tumor cell suspension. There was no histocompatibility mismatch, and the tumors produced a measurable serum tumor marker, AFP. Thus, this is a simple, reproducible method of creating a model for the study of HCC.

Fig. 4 Expression of oncogenes in implanted tumors. The mRNA abundance of c-myc, c-fos, c-jun, and bcl-2 was quantitated in tumors by RT-PCR. β-Actin was used as an internal control in PCR amplification.

DISCUSSION

With our orthotopic xenotransplant model using a common, readily available cell line, we have shown that an intrahepatic tumor could be produced in 100% of animals by a simple intrahepatic injection of a tumor cell suspension. There was a rapid demise in over 90% of experimental animals, and the tumors produced a measurable serum tumor marker, AFP. Thus, this is a simple, reproducible method of creating a model for the study of HCC.

Results from this animal model thus suggest that inhibition of IGF-II may provide a novel gene-based therapy for hepatoma.

The most common tumor model used today is a s.c. injection of tumor cells in the paravertebral/flank region of an immunocompromised mouse [e.g., nude, severe combined immunodeficient (SCID), and so forth]. The benefits of this model are that it is simple to perform, has low intra-procedure mortality, and allows easy visualization and monitoring of tumor growth. However, it has low fidelity to the actual environment in which the tumor cells proliferate. A capsule is often formed around the tumor, and metastases rarely occur (19). The biochemical milieu and blood supply are notably different in the skin than the source organ, especially in the case of liver tumors, where a portal system and drug-detoxifying enzymes exist. In addition, response to chemotherapeutic agents has been shown to vary depending on whether the tumor is ectopic or orthotopic (20). Thus, an orthotopic model of tumor implantation is preferable.

To this end, orthotopic placement of intact tissue specimens has been performed, as has portal vein injection of cells (21). The former requires intact human tumor tissue, along with technically demanding surgery. Portal vein injection also requires technical skill and bypasses the usual single point tumorigenic process, instead starting out from a metastatic state.

In contrast, the model described above requires only a modest amount of technical skill and utilizes a commonly available cell line. We have shown an excellent “take” rate with a single intrahepatic injection, and we have seen intrahepatic metastases. The tumors are in their native milieu, and thus response to therapeutic agents should more closely mimic HCC tumors in humans.

We chose Hep 3B cells based on several features of this cell line. First, it is readily available (American Type Culture Collection HB 8064). Second, it bears the genome of hepatitis B and produces HBsAG. Third, it produces AFP, like most HCCs in humans (28), offering the use of a tumor marker. The AFP promoter is highly active in fetal liver but is developmentally silent after birth. AFP synthesis is frequently up-regulated in HCC. As a result, significant amounts of serum AFP are usually detectable in primary liver cancer patients who are diagnosed with poorly differentiated and highly malignant tumors (28). As opposed to some other HCC cell lines, Hep 3B cells maintain this feature of HCC and synthesize considerable amounts of AFP both in vitro and in vivo. We were able to demonstrate that serum AFP levels increased concomitantly with tumor size in xenotransplanted mice (data not shown). Thus, AFP offers a minimally invasive method of monitoring tumor progression.

In previous studies, we and others have shown that Hep 3B cells produce IGF2 at increased levels (29). In addition, primary liver cancers also produce IGF2 (30). We confirmed this finding of Schneid et al. (29) in Hep 3B cells and showed a 30-fold increase in mRNA expression compared with normal liver tissue. We also showed a large increase in immunohistochemical staining for the IGF-II product in tumor tissues compared with the surrounding normal liver tissue. In addition, IGFIR mRNA was also up-regulated in tumor tissues compared with normal liver, allowing for increased ligand-receptor binding.

Human HCC is generally a hypervascular tumor (31). In HCC, IGF2 functions as an angiogenic factor by up-regulating vascular endothelial growth factor production (32). IGF2 also...
directly stimulates the angiogenic activity in rat cornea and in human umbilical vein endothelial cells (33, 34), probably via the mechanism of activation of p38 mitogen-activated protein kinase and p125 focal adhesion kinase phosphorylation (34). Increased vascularity is also seen on H&E staining of Hep 3B-induced HCC (Fig. 1B). This offers a plausible mechanism for the mitogenic/tumorigenic activity of IGF2 in Hep 3B cells, namely, growth stimulation and neovascularization. Taken together, the high levels of IGF2 expression in HCC may facilitate tumor progression through its angiogenic activity.

This finding of increased IGF2 in tumor cells offers a target at which to aim novel therapeutic interventions. We have developed a unique oligonucleotide that inhibits IGF2 production in vitro. Upon administration of this fragment at high doses, we observed an increase in survival rate over PBS-treated controls, confirming our previous results. Thus, this model has proven useful in the screening of potential new chemotherapeutic agents. These Hep 3B-derived tumors not only showed an increased expression of AFP, IGF2, and IGFlR but also showed a notable increase in the known oncogenes c-myc and c-jun. Again, this finding in our model offers further opportunities to study common oncogenes in an orthotopic setting.

In summary, this orthotopic xenograft of a common tumor cell suspension offers many benefits over previous models. There is no need for intact tumor tissue or incision into the highly vascular liver; there are no intrasplenic or portal vein injections, thereby lowering intraoperative mortality; there is 100% engraftment at or near the site of injection; there is a high incidence of intrahepatic metastases, which mimics HCC in humans; unlike s.c. tumor models, the tumor growth is in the same environmental milieu as a naturally occurring tumor, an important factor in evaluating drug delivery; the HCC secretes and produces multiple different products that can be assayed to follow tumor progression; the cell lends itself to the creation of a luciferase-constitutive strain that can be assayed externally and noninvasively using the methods of Contag (35, 36); and it offers another model in which oncogenes and angiogenesis can be easily studied.

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