Insulin-like Growth Factor II Induced by Hypoxia May Contribute to Angiogenesis of Human Hepatocellular Carcinoma

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ABSTRACT

Insulin-like growth factor II (IGF-II) is highly expressed during hepatocarcinogenesis (P. Schirmacher et al., Cancer Res., 52: 2549—2556, 1992; B. C. Park et al., J. Hepatol., 22: 286—294, 1995). However, the mechanism of its enhanced expression is largely unknown. In this study, we show that IGF-II mRNA levels are increased within six h of exposing human hepatoma cell cultures to hypoxia, suggesting that hypoxia may be a strong stimulus for the induction of IGF-II expression in the process of hepatocarcinogenesis. This finding and the fact that hepatocellular carcinoma (HCC) is a typical hypervascular tumor (M. Mise et al., Hepatology, 23: 455—464, 1996) imply that IGF-II may play an important role in the development of neovascularization of HCC. Here we demonstrate that IGF-II substantially increases vascular endothelial growth factor (VEGF) mRNA and protein levels in a time-dependent manner in human hepatoma cells. The induction of VEGF by IGF-II was additionally increased by hypoxia. Moreover, the direct angiogenic activity of IGF-II was observed in the quantitative chick chorioallantoic membrane assay (M. Nguyen et al., Microvasc. Res., 47: 31—40, 1994). These data suggest that IGF-II may be a hypoxia-inducible angiogenic factor in HCC.

INTRODUCTION

Angiogenesis, the development of new blood vessels by sprouting from preexisting endothelium, is an important component of a wide variety of biological processes including embryonic development and organ regeneration (1). Angiogenesis also contributes to the progression of tumor growth, which depends upon neovascularization. Under physiological circumstances, the ability of a given tissue to produce a neovascular response is under strict control, i.e., the balance between factors and events favoring or interfering with neovascular response is in equilibrium (2). The persistence of a neovascular response over the time required for physiological need may switch the beneficial original program into a harmful one. Therefore, angiogenesis retains a pathological significance during the tumor growth.

HCC is generally considered to be a hypervascular tumor (3). Although hepatic arterial embolization is widely used as an effective treatment of HCC on the basis of hypervascularization of HCC, the molecular mechanism(s) involving the neovascularization has not yet been clearly defined. It has been reported recently that VEGF and bFGF may contribute to neovascularization during hepatocarcinogenesis (3, 4).

We reported previously that most of the cirrhotic and HCC tissues express IGF-II (5), which is known as a fetal growth factor (6) and stimulates the growth of HCC cells in vitro (7). IGF-II is also known to have an angiogenic activity in rat cornea assay (8). However, the specific mechanism of an enhanced expression of IGF-II in cirrhotic and HCC tissues and its relationship to an increased angiogenesis in HCC have not yet been investigated. Thus, the present work was performed to study the specific mechanism of an enhanced expression of IGF-II and its relationship to increased angiogenesis during hepatocarcinogenesis.

MATERIALS AND METHODS

Immunohistochemistry. Paraffin-embedded liver tissues from normal, cirrhotic, and HCC patients were cut at 4-μm thickness. Each section was deparaffinized and pretreated with normal mouse serum to protect against nonspecific binding. The sections were incubated with primary monoclonal mouse anti-IGF-II antibody (Upstate Biotechnology, Inc., New York, NY) at a dilution of 1:50. A secondary antibody was biotinylated rabbit anti-mouse antibody (DAKO, Carpinteria, CA). The staining of IGF-II was visualized with avidin-biotin peroxidase and 3-aminobenzidine as a chromogen (9).

Cell Culture and Hypoxic Condition. HepG2 human HCC cells (2.0 × 10⁵ cells) were plated in a 75-cm² flask and cultured in MEM (Life Technologies, Inc., Gaithersburg, MD) supplemented with 10% FBS (Life Technologies, Inc.), 100 units/ml of penicillin, and 100 μg/ml of streptomycin. After 3 days, the cells were washed with MEM and reincubated in MEM containing 1% FBS for 20 h and then incubated in the presence or absence of IGF-II (R & D Systems, Minneapolis, MN) under normoxic or hypoxic conditions. For hypoxic condition, cells were incubated at a CO₂ level of 5% with 1% O₂ balanced with N₂.

Northern Blot Analysis. Total RNAs were prepared from HepG2 cells using the Tri Reagent kit (Molecular Research Center, Cincinnati, OH). RNAs (30 μg) were electrophoresed on formaldehyde agarose gels, transferred to Zeta-Probe membranes (Bio-Rad, Hercules, CA), and probed with ³²P-labeled 0.8-kb human IGF-II cDNA fragment or 0.5-kb human VEGF cDNA fragment for standardization. Blots were stained with methylene blue (10). Quantification of bands was performed with using Image-Pro Plus (Media Cybernetics, Inc., Silver Spring, MD) and adjusted to the density of 18S rRNA.

RESULTS

Induction of IGF-II by Hypoxia. To investigate the molecular mechanism of the enhanced expression of IGF-II in liver cirrhosis and HCC, we first conducted an immunohistochemical staining of IGF-II expression in cirrhotic and HCC tissues by using monoclonal mouse anti-IGF-II antibody. As shown in Fig. 1a, IGF-II was expressed in...
Fig. 1. IGF-II expression in human liver tissues and induction of IGF-II mRNA by hypoxia. a, IGF-II was stained with monoclonal anti-IGF-II antibody by the avidin-biotin-peroxidase method (9). Expression of IGF-II was detected evenly in the cytoplasm of proliferating hepatocytes in regenerating nodules of liver cirrhosis (LC, ×100) and hepatocellular carcinoma tissues (HCC, ×200), whereas no staining was found in normal adult liver tissue (N, ×400). b, HepG2 cells were incubated either under normoxic (N; 21% O₂ and 5% CO₂) or hypoxic (H; 1% O₂ and 5% CO₂) conditions for the indicated hours (3, 6, 12, and 24 h). Total RNAs (30 µg/lane) were analyzed by Northern blot analysis using a human IGF-II cDNA probe. Transcripts were seen at 6.0 kb for IGF-II. The filter was stained with methylene blue to assess loading differences (10). rRNA markers (18S and 28S) are indicated. The density of bands was quantified using the Image-Pro Plus (Media Cybernetics, Inc., Silver Spring, MD), normalized to the density of 18S rRNA band, and the induction fold was determined by comparison with values from normoxic controls.

Fig. 2. Time-dependent induction of VEGF mRNA by IGF-II in HepG2 cells. HepG2 cells were treated with the indicated concentrations of human recombinant IGF-II (0, 10, 50, and 100 ng/ml), and total RNAs were extracted from the cells at the indicated time points (12 and 24 h). Northern blot analysis was performed with 30 µg of total RNA/lane using a human VEGF cDNA probe. The filter was stained with methylene blue to assess loading differences. The density of bands was quantified using the Image-Pro Plus, normalized to the density of 18S rRNA band, and the induction fold was determined by comparison with values from normoxic controls.

nearly all of the cases of cirrhotic (30 of 32 cases; 93.8%) and HCC tissues (31 of 31 cases; 100%) tested, whereas it was not detected in normal adult liver tissue. IGF-II was seen in almost all of the regenerating hepatocytes of cirrhotic nodules of cirrhosis and tumor cells as well as the nontumorous surrounding tissues of HCC. Because many patients with HCC were accompanied with liver cirrhosis, which causes portal hypertension, the rapid expansion of tumor cells and portal hypertension in patients with HCC may cause oxygen and nutritional deprivation, probably leading to the generation of local hypoxic microregions in cirrhotic and HCC tissues. To study whether hypoxia induces the synthesis of IGF-II, we analyzed the expression of IGF-II in HepG2 human HCC cells grown under normoxic and hypoxic conditions. The steady-state level of 6.0 kb IGF-II mRNA from P3 promoter was significantly increased within 6 h of growth under low oxygen tensions (Fig. 1b). However, IGF-II mRNA was not induced in Chang human normal liver cells (ATCC CCL-13) under the same hypoxic condition (data not shown). These findings suggest that hypoxia may be a strong stimulus for the induction of IGF-II expression in cirrhotic and HCC tissues. In addition, this expression may be transduced by an unknown hypoxia-responsive signal pathway established in cirrhotic and HCC tissues.

Induction of VEGF Expression by IGF-II and Hypoxia. To study the function of IGF-II in the neovascularization of HCC, we examined whether IGF-II can induce the expression of VEGF gene in HepG2 cells by Northern blot analysis. Surprisingly, we found that IGF-II substantially increases VEGF mRNA in a time-dependent manner (Fig. 2). The level of VEGF mRNA in HepG2 cells was increased in response to IGF-II within 12 h and reached a maximum increase (~3.4-fold) by 24 h at the concentration range from 10 to 100 ng/ml. We then tested whether hypoxia and IGF-II can act additively or synergistically to induce VEGF gene expression in HepG2 cells. As
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Fig. 3. Induction of VEGF expression by IGF-II and hypoxia. a, induction of VEGF mRNA by cotreatment of IGF-II and hypoxia in HepG2 cells. Total RNAs from HepG2 cells cultured with or without IGF-II (50 ng/ml) under the conditions of normoxia or hypoxia for the indicated time periods (12 and 24 h) were analyzed with a human VEGF cDNA probe. Strong signal of VEGF mRNA was detected at 4.5 kb. The filter was stained with methylene blue to assess loading differences. The density of bands was quantified using the Image-Pro Plus, normalized to the density of the 18S rRNA band, and the induction fold was determined by comparison with values from normoxic controls. b, induction of VEGF protein by IGF-II, hypoxia, and IGF-II/hypoxia in HepG2 cells. HepG2 cells cultured with MEM media containing 1% FBS were exposed to hypoxia in the presence or absence of IGF-II (50 ng/ml) for the indicated periods of time. Culture media were collected and analyzed for the amount of the secreted VEGF protein by ELISA.

b

![Graph](image)

Fig. 4. IGF-II-induced angiogenic response in the quantitative CAM assay (11). IGF-II was mixed in a collagen gel embedded between two pieces of mesh and placed on the top of the CAM. Type I collagen gel containing sucralfont alone was used as control, and the angiogenic activity of IGF-II was quantitated as the percentage of the squares in the top mesh that contained blood vessels. As shown in Fig. 4, IGF-II significantly induced angiogenesis at a concentration range from 50 to 500 ng. Among the applied doses, 300 ng of IGF-II increased angiogenesis most effectively ($P < 0.001$), which is higher than that of 100 ng of bFGF, a potent angiogenic factor (12). Similar direct angiogenic activity of IGF-II in the rat cornea was reported recently (8).

DISCUSSION

HCC is one of the most frequent malignant tumors, particularly in Asian and African populations (13). HCC is a typical hypervascular tumor. Small-sized and well-differentiated HCCs usually exhibit no remarkable development of tumor vessels, whereas advanced-stage HCC is richly supplied with blood vessels. The majority of HCC samples exhibited strong expression of VEGF and bFGF mRNA in tumorous tissues (3). VEGF mRNA expression was significantly higher in liver tumors with strong or moderate angiographic staining than in those with weak or no angiographic staining (3). Therefore, VEGF was suggested to play an important role in the development of neovascularization in liver tumors. Several other factors expressed in HCC as well as these factors may contribute to the development of HCC neovascularizations.

We reported previously that most of the cirrhotic and HCC tissues express IGF-II (5). IGF-II is a polypeptide hormone that has a role in fetal development because it is the predominant IGF in fetal life (6). As shown in Fig. 1a, IGF-II was expressed in nearly all of the cases of cirrhotic and HCC tissues tested. Although IGF-II was reported to be expressed in normal adult liver (14, 15), we could not detect IGF-II protein in normal adult liver tissues. The possible reason for this conflicting result was perhaps due to the limitation of sensitivity of the immunohistochemical staining technique, although a low level of IGF-II protein was present in normal liver tissues. A similar negative
result was already published by ourselves (5) and D’Errico et al. (16). Together with our previous study (5), IGF-II may play an important role in hepatocyte proliferation in regenerating nodules and in tumor cell proliferation in HCC tissues (17). Moreover, Kraft et al. (7) have shown that IGF-II is mitogenic for human hepatocellular carcinoma cells. However, the specific mechanism of an enhanced expression of IGF-II in cirrhotic and HCC tissues is largely unknown. In our study, Northern blot analysis revealed that hypoxia significantly induced the expression of the IGF-II gene, especially the 6.0-kb message derived from the P3 promoter (Fig. 1b). We also detected a 2.2-kb message from the P3 promoter and a 4.8-kb message from the P4 promoter. However, these two messages were not significantly increased by hypoxia, compared with that of the 6.0-kb message derived from the P3 promoter. This result indicated that hypoxia may be a strong stimulus for the induction of IGF-II expression in cirrhotic and HCC tissues.

Although it has been postulated that hepatitis B virus could play a role in the regulation of IGF-II gene expression in cirrhotic and HCC tissues (18), such hypothesis does not explain the overexpression of IGF-II in a wide range of non-hepatitis B virus-associated tumors (19) including breast tumor (20, 21), neuroectodermal tumor (22), rhabdomyosarcoma (23), and Wilms’ tumor (24). Rather, we suggest that the shortage of blood supply due to portal hypertension in liver cirrhosis and rapid proliferation of tumor cells in HCC may eventually lead to local hypoxia, which may be a strong stimulus for the synthesis of IGF-II and other angiogenic factors.

Hypoxia is known to be a very important stimulus for the new vessel formation seen in tumor angiogenesis by stimulating the expression of VEGF (25–27). In our study, the expression of VEGF gene in HepG2 cells was increased by treatment of IGF-II or hypoxia (Figs. 2 and 3). Moreover, the combination of hypoxia and IGF-II brought an additive induction of VEGF mRNA and protein. Therefore, VEGF might be induced not only directly by local hypoxia but also by IGF-II in HCC. These results suggest that IGF-II functions in the angiogenic process of HCC indirectly by increasing the secretion of VEGF, which may accelerate the progression of HCC by promoting the development of new blood vessels.

We also examined whether IGF-II has a direct angiogenic activity by using the quantitative CAM assay (11) in which IGF-II was incorporated in a collagen gel and slowly released to the CAM. As shown in Fig. 4, IGF-II elicited an angiogenic response. This result coincided with the data of Volpert et al. (8) in which IGF-II induced angiogenesis in rat cornea.

Taken together, we suggest that hypoxia may play a key role in the overexpression of IGF-II in hepatocarcinogenesis and that the secreted IGF-II functions as an angiogenic factor directly as well as indirectly through increase in the synthesis of VEGF in an autocrine manner.

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