Transcripts of the Insulin-like Growth Factors I and II in Human Hepatoma

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ABSTRACT

Using complementary DNAs of human insulin-like growth factors as probes, expressions of the insulin-like growth factors I and II mRNA were examined in seven human hepatoma tissues and their adjacent nontumorous livers. The level of insulin-like growth factor I mRNA in hepatoma was lower than that in the nontumorous liver control. This phenomenon was probably caused by the low expression of human growth hormone receptor in hepatoma tissues. The levels of insulin-like growth factor II mRNA vary among hepatomas. Some show elevated expression; some have diminished expression compared to their nontumorous liver counterparts. In four of the seven hepatomas, expression of fetal forms of insulin-like growth factor II transcripts was observed and may represent dedifferentiation of insulin-like growth factor II expression during hepatocarcinogenesis.

INTRODUCTION

The balance between growth and terminal differentiation is well controlled in normal cells. The control is probably mediated through the growth factor requirement (1). It has been shown that many tumor-derived cell lines secrete polypeptide growth factors into their media (1) and in many cases release these lines from serum requirement. Therefore in transformed cells, the requirement for specific growth factors is lost or decreased, but nontransformed cells show an absolute requirement for growth factors to proliferate in culture. Recent work suggests that several oncogene products are similar to growth factors or their receptors. One protooncogene, c-sts, codes for the B chain of the platelet-derived growth factor (2, 3) while c-erbB codes for epidermal growth factor receptors (4). Similarly, the product of the c-fms oncogene appears to be very similar to the colony-stimulating factor 1 receptor (5). These results provide evidence to associate the alteration in the structure or expression of growth factors or their receptors to tumor formation.

The IGFs constitute a heterogeneous group with both insulin-like and growth-promoting properties. Two human IGFs have been characterized. IGF-I has been purified from human serum and sequenced (6). It is produced in response to circulating growth hormones and is active in stimulating the proliferation of a large number of cultured cells (7). Furthermore, IGF-I has been shown to induce skeletal growth in hypophysectomized rats (8). The action of IGF-II is less well defined. Some evidence in experimental animals points to a role for IGF-I in the control of the proliferation of human liver cells, colon carcinomas, and liposarcoma, have revealed elevated levels of IGF mRNA when compared to the expression in normal tissues (10-12). Similarly, there is a report of increased IGF-I content in primary human lung tumors (13). The secretion of growth factors by tumors and tumor-derived cell lines suggests that these factors may act as autocrine regulators of cell proliferation (14). To elucidate the roles of IGF growth factors in the control of the proliferation of human liver cells, this study examined the IGF transcripts in human hepatomas and their adjacent nontumorous livers by hybridizing poly(A)+ RNA from hepatomas to human IGF cDNA probes.

MATERIALS AND METHODS

Human Liver Samples. Human fetal liver, kidney, human hepatomas, and their adjacent nontumorous liver (normal liver) samples were obtained according to the regulations of Veterans General Hospital, Taipei, Taiwan, Republic of China. The surgically removed liver samples were then carefully dissected into normal and hepatoma parts and were immediately frozen and stored in liquid nitrogen until use. The dissected normal liver and hepatoma tissues were confirmed by pathological examination.

Cell Lines. Human hepatoma cell lines were maintained in DMEM supplemented with 10% FCS. Hep 3B cell line (15, 16), Hep G2 cell line (16), Huh-7 cell line (17), and cell line PLC/PRF/5 (18) were included in this study.

Treatment of a Human Hepatoma Cell Line with Human Growth Hormone. Hep3B, a human hepatoma cell line, was grown to about 70% confluence in DMEM with 10% FCS supplement. The cells were then grown in serum-free DMEM for 2 days. Afterward the media were changed to either a fresh serum-free DMEM or a serum-free DMEM supplemented with 1 μM purified human growth hormone (19). After 4 h of incubation at 37°C, the RNAs were isolated for further analysis.

Isolation of DNA and RNA from Liver Samples. High molecular weight DNA was prepared by the method of Blin and Stafford (20). The hepatoma cell lines were grown to about 70% confluence when RNA was harvested. Four h before harvest, the culture was again fed with a fresh DMEM plus 10% FCS. The total RNA was extracted by the guanidinium/cesium chloride method (21). Poly(A)+RNA was purified by fractionation using oligo(dG)•oligo(dC) cellulose chromatography as described by Aviv and Leder (22). The concentrations of DNA and RNA were determined by A260 reading and by the intensity of nucleic acid on the gel by ethidium bromide staining.

Electrophoresis and Detection of IGF DNA. Genomic DNA (7.5 μg) isolated from human fetal liver, paired human nontumorous liver, and hepatoma were digested with restriction enzyme, EcoRI. DNA fragments were then separated by electrophoresis in a 0.8% agarose gel, transferred to nitrocellulose paper, and hybridized to the nick-translated 32P-labeled IGF cDNA probes under the conditions described (23-25). The blots were washed in 15 mM NaCl/1.5 mM sodium citrate plus 0.1% sodium dodecyl sulfate at 50-55°C and exposed to Fuji X-ray film at ~70°C using a Kyokko intensifying screen. The human IGF-I cDNA used is a 727-base pair pair 1 fragment of pIGF-I (26). The human IGF-II cDNA used is a 713-base pair pair 1 fragment of pIGF-II (27).

Electrophoresis and Detection of RNA. Poly(A)+ RNA (3 μg) isolated from fetal tissues, nontumorous livers, hepatomas, and hepatoma cell lines were denatured with glyoxal and applied to a 1.2% agarose gel for electrophoresis (24, 25). The RNA was transferred to nitrocellulose paper and hybridized with nick-translated 32P-labeled DNA probes under the conditions described (24, 25). The blots were washed in 15 mM NaCl/1.5 mM sodium citrate plus 0.1% sodium dodecyl sulfate at 50-55°C and exposed to Fuji X-ray film at ~70°C using a Kyokko intensifying screen. The human IGF-I cDNA used in a 727-base pair pair 1 fragment of pIGF-I (26). The human IGF-II cDNA used is a 713-base pair pair 1 fragment of pIGF-II (27).
50–55°C and exposed to Fuji X-ray film at ~70°C using a Kyokko intensifying screen. Besides the cDNA probes of IGF-I and -II (26, 27), other probes used in this study were an 840-base pair PstI cDNA fragment of pHAF-2 which served as an AFP probe (28), an 847-base pair SacI cDNA fragment of pghr501-1 which served as a human GH receptor probe (29), and a pHFBA-1 plasmid which served as a β-actin probe (30). The human ornithine decarboxylase cDNA and human GAPDH cDNA were as described in Refs. 31 and 32.

**RESULTS**

Expression of IGF-I mRNA. The poly(A⁺) RNAs were isolated from seven hepatoma patients’ hepatoma parts and their adjacent nontumorous livers. The transcripts of IGF-I were examined by Northern blot analysis using human IGF cDNAs as probes. Multiple species of IGF-I transcripts were expressed in liver samples of hepatoma patients (Fig. 1A). The detectable sizes were 7.0, 5.2, 4.7, 2.4, and 0.9 kilobases. In some samples such as HL, the major transcript was 7.0 kilobases long, while in sample MT, a broad band about 0.9 kilobase long was predominant. While the size of transcripts may vary among patients, in pairs of samples examined, every hepatoma part showed a decrease in the level of IGF-I mRNA when compared to their nontumorous liver counterpart. The low IGF-I mRNA level was also observed in four of the human hepatoma cell lines studied, Hep G2, Huh-7, PLC/PRF/5, and Hep 3B. In human fetal liver, the level of IGF-I mRNA is also low. Its size is about 5.2 kilobases, the same size as that observed in human hepatoma cell lines. In the fetal kidney, a 7.0-kilobase transcript was detected as well. The different level in IGF-I mRNA expression shown in Fig. 1A could not be attributed to the difference in the amount of the sample loaded since a comparable level of β-actin mRNA expression was observed when a similar set of filters was probed with a human actin probe (Fig. 1B).

The expression of IGF-I is believed to be under the growth hormone regulation (7); therefore, the study included whether the low expression in hepatoma was from the impairment of the hormonal response. A hepatoma cell line, Hep 3B, was grown in a serum-free medium with or without growth hormone supplement. The levels of IGF-I, GAPDH, and ornithine decarboxylase mRNA were then assayed. Fig. 2 shows that the mRNA level of ornithine decarboxylase, a growth hormone-responsive gene (33, 34), responds to growth hormone stimulation. This suggested that a growth hormone receptor was functional in Hep 3B cell line. By contrast, transcription of a constitutive gene, GAPDH (35), was about equivalent in both conditions. Under these conditions, there is about a 4-fold enhancement of IGF-I mRNA level in the Hep 3B cell line when grown in the medium with growth hormone. The result demonstrates that Hep 3B cells have a functional growth hormone receptor and the IGF-I gene in hepatoma cells is responsive to growth hormone stimulation.

The level of GH receptor mRNA was then assayed to see whether the low expression of IGF-I mRNA correlates with the level of the growth hormone receptor mRNA. By using human GH receptor cDNA as a probe, the steady state level of GH receptor RNA was found to be consistently lower in the hepatoma part as compared to the nontumorous liver counterpart (Fig. 3). Low expression was also found in fetal liver and kidney as well as in hepatoma cell lines examined (Fig. 3). Fig. 3 also shows that in some samples, such as the tumor part in sample MT, cell lines Hep G2 and Hep 3B, the level of GH receptor is very low. By using a high dose of mRNA, Hep 3B was shown to have hybridizable GH receptor mRNA detected (Fig. 3C). Apparently, the functional assay of the effect of growth hormone is more sensitive than measuring the steady state level of the GH receptor transcript. Nevertheless, the result of this study shows that there is good correlation of the mRNA level of IGF-I and GH receptor.

Expression of IGF-II mRNA. The level of IGF-II mRNA expression in the hepatomas was variable (Fig. 4), showing elevated expression (samples HL and MT), equal expression (sample CC), or diminished expression (samples BL, YF, DC, and JS) in comparison with nontumorous liver counterparts. The expression level also showed variations among hepatoma cell lines. Multiple transcripts of IGF-II have been reported in both humans and rats (12, 36). Data from this study showed that generally, in adult nontumorous liver, a 5.1-kilobase transcript was expressed while in fetal tissues such as livers or kidneys, additional transcripts (5.6 and/or 4.5 kilobases long) appear at the top.
were observed. When the size of the IGF-II transcript was examined, transcripts 5.6 and 4.5-kilobases long were detected in hepatoma parts of samples HL, MT, YF, and DC and hepatoma cell lines Hep G2 and Huh-7. Thus, there was a high incidence of expression of fetal form IGF-II mRNA in hepatomas.

DNA Restriction Patterns of IGF-I and IGF-II Genes. Alteration in transcription pattern may result from changes in gene structure and/or control of expression. To examine whether any gross change in gene structure of IGF-I and IGF-II occurred, Southern blot analysis was performed on DNA isolated from several of the tumors and their adjacent nontumorous livers. Figs. 5 and 6 show EcoRI restriction pattern of IGF-I and IGF-II, respectively. No gross gene rearrangement or amplification of IGF-I or IGF-II gene could be detected by this analysis.

Expression of α-Fetoprotein mRNA. AFP is a major serum protein synthesized during the fetal stage (37–39). Reappearance of AFP in adult serum often signals pathological conditions, particularly hepatoma and teratocarcinomas (37–39). To examine whether the expression of the fetal form of IGF-II is in any way correlated with the expression of AFP, the AFP mRNA was analyzed by the Northern blot method. Results are shown in Fig. 7. Data from Figs. 4 and 7 show that a high level of AFP expression in hepatoma is sometimes associated with the expression of fetal form of IGF-II as observed in samples HL, MT, and DC. However, expression of AFP and the fetal form of IGF-II did not completely correlate in all tumors. For example, sample YF expressed fetal form of IGF-II mRNA but no AFP mRNA was detected; in contrast, sample BL expressed AFP while its IGF-II mRNA was an adult form.

**DISCUSSION**

The liver is one of the main organs for production of IGF-I growth factor (36). In adults, the growth hormone is the major hormone regulator for the production of IGF-I (8). Besides being under hormonal regulation, the IGF-I level is also under...
developmental regulation in that IGF-I levels are low at birth and increase with age (40–42). By using human IGF-I cDNA as a probe, this study examined the expressions of IGF-I mRNA in human liver. The data demonstrated that IGF-I mRNA levels were low in the human fetal liver and increased in the adult liver. The result is consistent with others’ observation of the plasma IGF-I level (40–42). Furthermore, in both the hepatoma tissues and the hepatoma cell lines studied, the expression of IGF-I mRNA was consistently low when compared to that in nontumorous livers. Whether the regulation was exerted at the level of IGF-I transcription or posttranscription is not clear from this study. The level of the GH receptor mRNA level suggests that the low IGF-I mRNA expression in hepatoma parts probably resulted from the low level of the GH receptor. However, the GH receptor in human hepatoma is still functional as demonstrated by the response of IGF-I gene to human growth hormone stimulation in the Hep 3B hepatoma cell line studied. Therefore, the level of the GH receptor may be the limiting factor in determining the level of IGF-I transcript in human hepatoma. The data also suggest that one of the possible mechanisms involved in both hormonal and developmental regulation of IGF-I expression in human liver is at the level of the GH receptor.

The sizes of IGF-I transcripts detected were 7.0, 5.2, 4.7 and 0.7 kilobases and some minors at 2.4-kilobase range. The relative abundance of each species appeared to differ among individuals and is the same in the hepatoma and its nontumorous part in any given individual. The length variation may not be caused by mRNA degradation since the integrity of the RNA preparation has been demonstrated by the intensity of ethidium bromide-stained rRNA and by the integrity of AFP RNA when the hybridized IGF-I cDNA probe was stripped from the RNA filter and reprobed with the AFP probe. The size of the IGF-I mRNA in adult human liver has been reported to be 7.7, 5.3, and 0.9 kilobases by Bell et al. (43) and 6.3, 3.7, 1.7, and 1.1 kilobases by Rotwein (44). The discrepancy in the different studies may be partly a factor of size estimation and/or an alteration in IGF-I mRNA stability and processing in different mechanisms. The human IGF-I gene has been demonstrated to be a single copy span more than 45 kilobases long, mapped on chromosome 12 (45–47). The alternative RNA processing of the primary IGF-I transcript has been shown to be responsible for at least two sizes of IGF-I mRNA (45). The possibility that there is a gross gene rearrangement or amplification of IGF-I gene in samples studied is low since Southern blot analysis showed a similar restriction pattern of the IGF-I gene in different samples.

In this study, the levels of hybridizable IGF-II mRNA in human livers were studied. Some variations in IGF-II mRNA level were observed among seven nontumorous liver samples examined. Whether differences are normal variations among individuals or are related to the disease state of the patients is not clear. Moreover, some samples, i.e., HL and MT, had enhanced IGF-II mRNA expression in the hepatoma portion as compared to that in their nontumorous portion. Whether the elevated expression is involved in any of the hepatoma-associated hypoglycemia remains to be studied (48). When the mRNA levels in fetal livers were compared with those in nontumorous adult livers, the fetal liver had a higher IGF-II mRNA expression. The difference may not be as great as has been reported (11) and is very much dependent upon which nontumorous liver was used for comparison.

The sizes of human IGF-II transcript have been reported to differ between adult and fetal livers and between tissues (11, 43). The data from this study show that in the nontumorous liver IGF-II mRNA is about 5.1 kilobases in size, while in fetal liver the major one is 5.6 kilobases, and in fetal kidney the transcripts are 5.6 and 4.5 kilobases long. Thus, the tissue-specific and differentiation-specific expressions of IGF-II mRNA are also being demonstrated. Interestingly, in about one-half of the hepatoma tissues or cell lines studied, the sizes of IGF-II mRNA were 5.6 and 4.5 kilobases, sizes that are expressed in fetal tissues. Recently, De Pagter-Holthuizen et al. (49) characterized human IGF-II gene and have identified two IGF-II promoters which are active in a development-specific manner. The expression of the fetal form of IGF-II may reflect cellular dedifferentiation in hepatoma. The fetal form of IGF-II transcript was not only detected in the hepatoma portion but was also detected in nontumorous liver of patients HL and MT. It is of interest to note that although AFP was detected in patients HL’s and MT’s hepatomas, no AFP mRNA was observed in their nontumorous livers. These data also make it impossible that the minor fetal forms of IGF-II transcripts observed in nontumorous livers could represent cross-contamination from surrounding liver or infiltration of the tumor into the normal liver.

In this study all the hepatoma tissues had IGF-I mRNA lower than that in their nontumorous liver counterparts. This agrees with the report that the plasma IGF-I level is low in human hepatoma patients (50). The data would then suggest that the uncontrolled growth in liver cancer may not mediate through the mitogenic effect of IGF-I. Similarly, IGF-II is also a mitogenic polypeptide. In rats, IGF-II concentrations in fetal serum are 20–100-fold higher than those in maternal serum (51) and are believed to play an important role in fetal development. In this respect, two hepatic tumors were shown to have an increased level of IGF-II mRNA. However, a recent study of Wilms’ tumors and adrenal pheochromocytomas has shown that the levels of IGF-II mRNA and protein are not necessarily correlated (52). Therefore, whether the increase in IGF-II mRNA in some liver tumors was reflected in its protein level, the significance of the switch to the fetal form of IGF-II expression, and the effect of fetal IGF-II in promoting tumor growth will remain to be studied.

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