J. Biochem. 122, 717-722 (1997)

Antisense Oligodeoxynucleotides of IGF-II Selectively Inhibit Growth of Human Hepatoma Cells Overproducing IGF-II[1]

Shwu-Bin Lin,[*,†,2] Sung-Hwa Hsieh,[*] Hui-Lun Hsu,[*] Ming-Yang Lai,[‡] Lou-Sing Kan,[§] and Lo-Chun Au[‖,2]

[*]Graduate Institute of Medical Technology, [†]Department of Laboratory Medicine, National Taiwan University Hospital, [‡]Graduate Institute of Clinical Medicine, College of Medicine, National Taiwan University, Taipei, Taiwan, [§]Institute of Chemistry, Academia Sinica, Taipei, and [‖]Department of Medical Research, Veterans General Hospital-Taipei, Graduate Institute of Medical Biotechnology, National Yang-Ming University, Taipei, Taiwan, ROC

Received for publication, April 11, 1997

Insulin-like growth factor II (IGF-II) is expressed in many developing embryonic tissues and is involved in mammalian growth and development. After birth, serum IGF-II is mainly produced by liver cells. Many reports have indicated that IGF-II is overexpressed in some hepatocellular carcinoma (HCC) tissue. These findings imply the possible importance of this growth factor in carcinogenesis. We screened four human HCC cell lines and three rat HCC cell lines and found that HuH-7 and HepG2 cells produced fivefold more intracellular IGF-II than the other cell lines. Experimental data indicate that IGF-II functions through the intracrine mode for HuH-7 cells. To study whether the overexpression of IGF-II is significant for the growth of HCC or only a consequence of HCC development, we used antisense oligodeoxynucleotides (ATON) to arrest the translation of IGF-II mRNA, and then measured the effects on cell growth. We found that the production of IGF-II was suppressed by ATON, and the decrease of IGF-II resulted in growth inhibition of HuH-7 and HepG2. ATON had no effect on the other tested cell lines, which produced lower levels of IGF-II. The growth inhibition was mainly attributed to a decrease of cell proliferative activity. The results indicate that the IGF-II-overproducing cell lines *do* depend on IGF-II for growth, and ATON of IGF-II can selectively inhibit the growth of these cells. ATON may be a potential therapeutic agent for this type of HCC *in vivo*.

Key words: antisense oligonucleotide, growth inhibition, human hepatoma cell lines, IGF-II.

Downloaded from http://jb.oxfordjournals.org/ at Peking University on October 2, 2012

Insulin-like growth factor II (IGF-II) is a 67-amino-acid polypeptide growth factor that is structurally and functionally related to insulin and insulin-like growth factor I (IGF-I). IGF-II is widely expressed in the developing embryonic tissues and is related to the growth and development of various tissues. After birth, the expression of IGF-II is progressively extinguished in almost all tissues. In normal adults, serum IGF-II at a level of hundreds of ng/ml is mainly produced by liver cells. The biological functions of IGF-II are mediated through its binding to either IGF-I or -II receptors. The function of IGF-II receptors is related to carbohydrate metabolism, whereas IGF-I receptors are geared toward the signal transduction pathway and account for the mitogenic effect of IGF-II (*1-5*). Overexpression of IGF-II or reexpression of fetal transcripts has often been found in HCC tissues including primary liver cancer and regenerating nodules (*6-13*). The role of IGF-II in oncogenesis was elucidated by a transgenic mice experiment (*14*), on the basis of which IGF-II was proposed to offer a second signal contributing to hyperproliferation in oncogene-induced tumorigenesis, since tumors were only formed when both IGF-II and SV-40 T antigen genes were introduced. In cervical cancer cell lines, IGF-II was shown to mediate EGF-induced mitogenesis (*15*). In addition to the mitogenic effect, autocrine secretion of IGF-II was also shown to be related to differentiation of skeletal myoblasts (*16*).

In this study, we found that human hepatoma cell lines HuH-7 and HepG2 overproduce IGF-II five times more than other tested HCC cell lines. To clarify the importance of IGF-II in the growth of these hepatoma cells, an antisense oligodeoxynucleotide (ATON) against IGF-II mRNA was used in order to elucidate (i) if the production of IGF-II can be suppressed through treatment with ATON; (ii) what the biological effects will be if the overexpressed IGF-II is suppressed; and (iii) if IGF-II level is an important trait for categorizing different hepatoma cells.

MATERIALS AND METHODS

Chemicals, Isotopes, and Antibodies—[^{35}S]Methionine (1,175 Ci/mmol), [γ-^{32}P]ATP (3,000 Ci/mmol), and [^{3}H]-thymidine (25 Ci/mmol) were purchased from Amersham (Amersham, UK). A rabbit reticulocyte lysate-based TNT™ *in vitro* transcription-translation system, RNase H,

[1] This work was supported by grants DOH (84-86)-H-404 from the National Health Research Institute and NSC-86-2314-B-002-147 from the National Science Council of the Republic of China.

[2] To whom correspondence should be addressed: Au, L.-C., Fax: 886-2-873-3024; Lin, S.-B., Fax: 886-2-371-1574.

RNasin nuclease inhibitor, and pGEM-4 plasmid for the *in vitro* translation-arrest experiment were purchased from Promega (Madison, WI). Recombinant IGF-II (rIGF-II), DNase I, *o*-phenylenediamine (OPD), neutral red, and *p*-nitrophenyl phosphate were purchased from Sigma (St. Louis, MO). A polyclonal antibody specific to human IGF-II was obtained from Austral Biological (San Ramon, CA) and a monoclonal antibody specific to both rat and human IGF-II was obtained from Amano (Troy, VA). These antibodies showed no cross-reactivity to IGF-I. An HRP-conjugated second antibody (goat anti-rabbit) was purchased from Zymed Laboratories (San Franscisco, CA).

Synthesis of Oligodeoxynucleotides—Phosphorothioate oligodeoxynucleotides (ODNs) were synthesized in the trityl-on mode by an Applied Biosystems model 391 DNA synthesizer and used in all experiments. The A, G, C, and T phosphoramidites, controlled pore glass supports and sulfur reagent were purchased from Glen Research Corporation (Sterling, VA). The ODNs were deblocked and cleaved from the solid support with concentrated ammonia water using a standard procedure. After evaporation of the ammonia, the deprotected ODNs were purified on Sep-Pak C18 cartridges (Millipore Corporation, Milford, MA) as reported previously (*17*).

In Vitro Translation-Arrest Experiment—Recombinant phigf2 plasmid containing 1.1 kb human IGF-II cDNA (*18*) was purchased from American Type Culture Collections. The IGF-II cDNA was excised by *Eco*RI and then inserted into pGEM-4 at the *Eco*RI site. A recombinant vector, pGEM4IGF, with IGF-II cDNA oriented downstream of the SP6 promoter was selected and used for screening ATONs effective against the translation of IGF-II (*19*). The *in vitro* screening protocol described in a previous report was followed (*20*). Briefly, the plasmid was added to the reticulocyte lysate system in the presence of SP6 RNA polymerase, RNase H, and the antisense ODN being tested. Translation-arrest caused by ODNs was evaluated in terms of the amount of *de novo*-synthesized [^{35}S]methionine-labeled prepro-IGF-II after gel electrophoresis separation and quantitation by Phosphor Image Quant (Molecular Dynamics, Sunnyvale, CA).

Cell Lines and Cell Culture—Four human hepatoma cell lines, HuH-7, HepG2, HA22T, and Hep3B, as well as three rat hepatoma cell lines, GN6TF, GP7TB, and GP10TP, were tested. These cells were cultivated in DMEM containing 10% fetal calf serum in an atmosphere of 5% CO_2 at 37°C. The cells were cultivated in 10-cm dishes for ELISA and in microtiter plates at a density of 5×10^3 cells/200 μl medium per well for cell proliferative activity or viable cell quantitation assays. ODNs were added to the culture media right after subculturing the cells.

Quantitation of IGF-II—The cells were cultivated to subconfluence. Conditioned media were harvested and the IGF-II was extracted with acid ethanol (*21*). The cells (2×10^7 cells) were lysed on ice in 200 μl of lysis buffer containing 20 mM Tris-HCl (pH 7.5), 2 mM β-mercaptoethanol, 2 mM EDTA, 2 mM EGTA, 10% glycerol, and 0.5 mM phenylmethanesulfonyl fluoride. After centrifugation of the samples at $3{,}000\times g$ for 10 min, total protein was quantitated with a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA). The IGF-II content was determined by an ELISA-based assay using NUNC-immuno module maxisorp F16 strip-well plates (Nunc, Kamstrup, Denmark). The wells were first coated with 100 μl antigen-containing coating buffer, which was composed of 1.6 g/liter Na_2CO_3, 3 g/liter $NaHCO_3$, 0.2 g/liter NaN_3, and cell lysate (containing 100 μg total protein), conditioned medium or rIGF-II solution (containing 2-100 ng rIGF-II). The coated plate was then subjected to blocking and successive incubations with an anti-IGF-II antibody, HRP-conjugated second antibody, and OPD solution as a standard ELISA protocol (*22*). The reactions were stopped by the addition of H_2SO_4 and the product was measured by A_{490} using an ELISA reader.

Cell Proliferative Activity Assay—Cells were grown in 96-well plates for 24 h with or without the addition of ODN. [^{3}H]Thymidine (1 μCi) was then added to the medium for a 4-h pulse labeling. After that, the cells were washed three times with PBS, trypsinized, and collected on a glass fiber filter by a Packard Filtermate 196 cell harvester (Downers Grove, IL). The [^{3}H]thymidine incorporation of the cells was counted by a Packard Direct Beta Counter MATRIX™ 96.

Quantitation of Viable Cells—Cells were grown in 96-well plates for 48 or 70 h with or without the addition of ODNs. The media were aspirated and the cells were washed once with 200 μl of PBS followed by a neutral red (NR) assay (*23*) or an acid phosphatase (ACP) assay (*24*). For NR assay, 200 μl of DMEM and 50 μl of NR solution (0.15% NR in 0.1 M phosphate buffer, pH 6.0) were added to each well and the plate was then incubated in a CO_2 incubator for 1 h. After that, the solutions were withdrawn, the cells were carefully washed with PBS, and 150 μl of elution buffer (1 : 1 mix of 0.1 M NaH_2PO_4 and 95% ethanol) was added. The plate was gently rocked for 15 min and the absorbance at 540 nm of each well was measured by an ELISA reader. For ACP assay, 100 μl of sodium acetate buffer containing 0.1% Triton X-100 and 100 mM *p*-nitrophenyl phosphate was added to the washed cells. The plate was incubated at 37°C for 2 h. The reaction was stopped by the addition of 10 μl of 1 N NaOH, and the coloration was quantitated by an ELISA reader at 410 nm. The data presented are averages derived from triplicate experiments.

Assay for Cell Apoptosis—A TUNEL kit (TdT-mediated dUTP nick end labeling kit) from Boehringer Mannheim Gmbh Biochemica (Mannheim, Germany) was used to detect apoptotic cells *in situ*. The cells (2×10^4 cells in 400 μl DMEM) were cultivated in 8-well chamber slides (Nunc, Naperville, IL) for 16 h, then the ODNs were added to each well. After incubation for 24 and 48 h, the media were withdrawn and the protocol recommended by the manufacturer was followed. Briefly, the cells were successively treated by fixing with paraformaldehyde solution for 30 min at room temperature, submerging in permeabilization solution for 2 min on ice, and incubating with TUNEL reaction mixture for 60 min at 37°C in the dark. Following each incubation, the slides were rinsed with PBS. The apoptotic cells were revealed by their fluorescent nuclei under a fluorescence microscope.

RESULTS

Screening of IGF-II-Overproducing Cell Lines and Inhibition of IGF-II Production by ATONs—To screen cell lines which overexpress IGF-II, conditioned media and cell lysates of human hepatoma cell lines, HuH-7, HepG2,

Downloaded from http://jb.oxfordjournals.org/ at Peking University on October 2, 2012

HA22T, and Hep3B, and rat hepatoma cell lines, GN6TF, GP7TB, and GP10TA, were assayed for IGF-II. Cell lysates of HuH-7 and HepG2 showed approximately 5-fold higher levels of IGF-II than the other cell lines (Fig. 1). All of the conditioned media, however, had undetectable levels of IGF-II.

To gain insight into the question of whether overexpression of IGF-II is important for survival or proliferation of HuH-7 and HepG2 cells, ATON was used to suppress the production of IGF-II. The effectiveness of ODN for translation arrest of IGF-II was first screened *in vitro* using a transcription-coupled translation assay method developed in our laboratories (*19, 20*). The ability of ODN to inhibit 22-kDa prepro-IGF-II production was determined. The complementarity of the tested ODN to the IGF-II mRNA and the relative effectiveness in translation-arrest are shown in Table I. All four ATONs showed some inhibitory effect on translation of IGF-II mRNA; IG161 and IG20, which are complementary to the 5′ non-coding region and the translation initiation region of the mRNA, respectively, proved to be more effective than IG162 and IG17. Control ODNs, SIG, and CD, did not inhibit IGF-II synthesis at all. The results of the cell culture experiments presented below were obtained using IG161 as ATON, and both SIG and CD as control ODNs. When IG161 was added to the culture medium of HuH-7 cells, it was found that the decrease of intracellular IGF-II was dose-dependent (shown in Fig. 2). Intracellular IGF-II content decreased to 36% after the cells had been treated with 20 μM IG161 for 24 h, while the control ODN, SIG, showed less than 10% inhibitory effect.

Growth Inhibition by ATON Is Attributable Mainly to the Inhibition of Cell Proliferation, but Not to Cell Apoptosis—After 48 h treatment with ODNs, total numbers of viable cells were quantitated by counting with a hemocytometer after trypan blue staining. NR uptake and ACP assays were also performed. These three assays gave similar results. Total viable cells decreased as the concen-

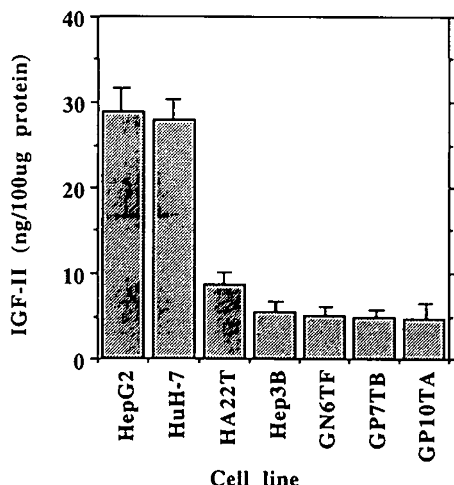

Fig. 1. **Intracellular IGF-II contents of different hepatoma cell line.** The cells were subcultured in low serum medium (containing 0.5% FCS) and grown to subconfluence. IGF-II contents in the cell lysates were measured by ELISA.

TABLE I. **Effectiveness of ODN for translation arrest of IGF-II determined by *in vitro* transcription and translation assay.**

ODN	Sequence (5′→3′)[a]	Target region[a]	% inhibition
IG161	d-TCTGCCTCGCAGTTGG	−25 to −10	82±5
IG162	d-CATTGGTGTCTCTCTG	−13 to +3	70±4
IG17	d-GCATTCCCATTGGTGTC	−7 to +10	68±6
IG20	d-CATTGGTGTCTCTCTGCCTC	−17 to +3	81±4
SIG[b]	d-TGTCTCCCAGGCGGTT	no	0±5
CD[b]	d-TCCTAGGTAGTACCGTG	no	0±5

[a]The underlined sequence, CAT, is complementary to the translation initiation codon of IGF-II mRNA. The first codon of the translation initiation was numbered as 1. [b]SIG is a scrambled sequence of IG161 and CD is an arbitrarily selected ODN. Both are used as control sequences.

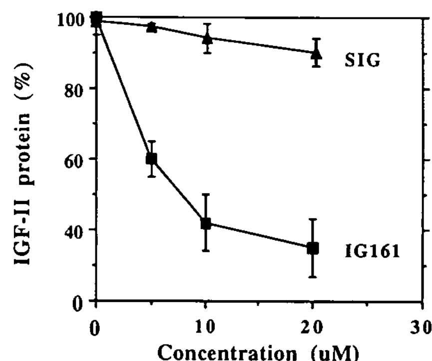

Fig. 2. **Suppression of intracellular IGF-II protein level by ODN.** HuH-7 cells were grown in 50 cm² flasks with the addition of IG161 or control ODN followed by a cultivation period of 24 h. IGF-II levels in cell lysates were determined by ELISA.

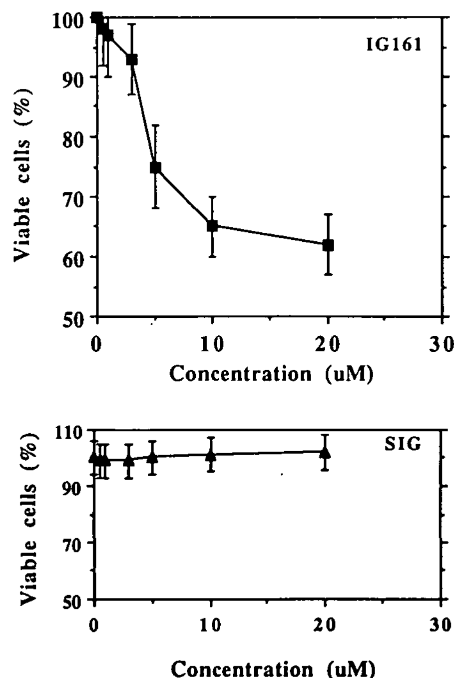

Fig. 3. **Total viable cells after ODN treatment.** HuH-7 cells were subcultured in microtiter plates at a density of 2,500 cells/well. Each well contained 200 μl of DMEM with the addition of different amounts of IG161 or SIG or without the addition of ODN (blank). After 48 h of cultivation, neutral red taken up by viable cells was measured with an ELISA reader at 540 nm. Percentage of total viable cells is presented as $[(A)_{ODN}/(A)_{blank}] \times 100$.

Downloaded from http://jb.oxfordjournals.org/ at Peking University on October 2, 2012

tration of IG161 was increased. The growth inhibition is shown in Fig. 3; a 38% decrease in total viable cells was observed in the 20 μM IG161-treated group. However, there were no effects on the control groups. The results imply a specific action of ATON and the noncytotoxic character of the phosphorothioate oligonucleotides. NR is a vital dye only taken up by viable cells and has been proven to be a reliable method for quantitating total number of viable cells (*23, 25*). Acid phosphatase is a cellular enzyme whose level has been shown to correlate well with cell number (unpublished data, *24*). We found that both colorimetric methods reflect well the amount of viable cells obtained by the trypan blue dye exclusion cell counting method which, however, is laborious and sometimes presents quite scattered data. In addition, the NR and ACP methods are rapid (taking 2-3 h) compared to the MTT method (taking 24 h) we have used previously (*26*).

In order to elucidate if the decrease of total viable cells was attributable to the retardation of proliferation or/and an increase in the incidence of cell apoptosis, the following experiments were conducted. The populations of apoptotic cells after different treatments were determined by TUNEL assay (see Fig. 4). Fragmented DNA in the nuclei of apoptotic cells was end-labeled with fluorescein-dUTP. The IG161-treated group (Fig. 4a), SIG-treated group (Fig. 4b), and blank group (Fig. 4e) showed similar results. On the other hand the positive controls, *i.e.* H_2O_2-induced apoptotic cells (Fig. 4c) and the cells treated with DNase I (Fig. 4d), showed strong fluorescence in the nuclei. The activities of cell proliferation were monitored in terms of [^{3}H]thymidine incorporation. It was found that [^{3}H]thymidine incorporation decreased in a dose-dependent manner after the treatment of HuH-7 cells with IG161 for 24 h (Fig. 5a). Control ODN caused about a 20% decrease in incorporation as compared to the blank group (no ODN added). However, the decrease showed no dose dependence. Dilution of [^{3}H]thymidine by the thymidine generated from degraded ODN could be the reason for this decrease (*27*). The real percentage of suppression therefore, should be calculated using the incorporation of the control group as the basal level. It was found that there was a 40% decrease in DNA synthesis, namely cell proliferation, at 5 μM IG161 (Fig. 5b). The dilution effect of [^{3}H]thymidine was more evident when chimeric phosphorothioate ODN (CS-ODN), which contained three phosphorothioate linkages at both

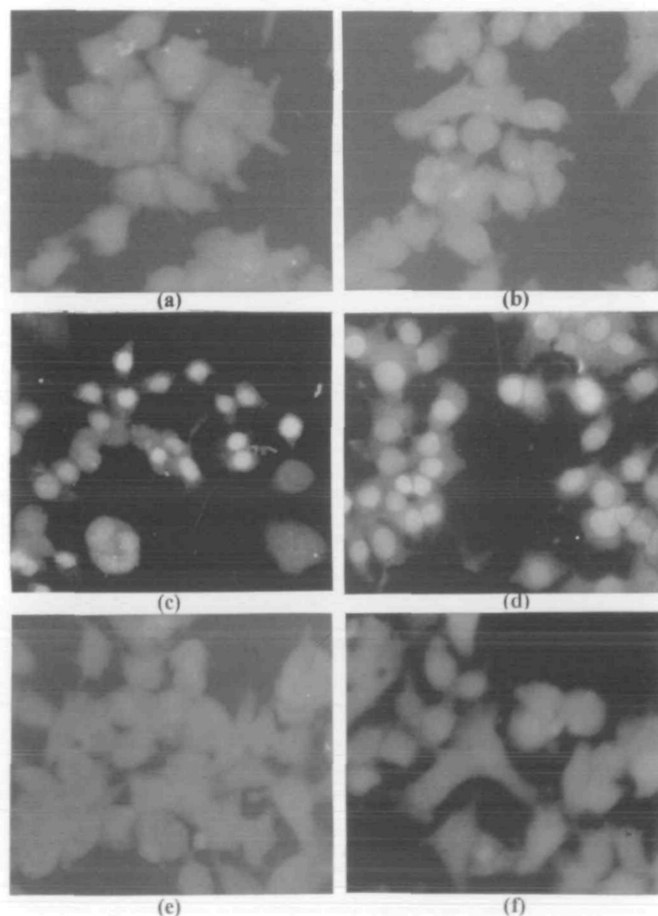

Fig. 4. **Cell apoptosis detected by TUNEL assay.** Ten thousand cells in 400 μl of DMEM were cultivated in 8-well chamber slides for 16 h, then the ODNs were added. After incubation for 48 h, the media were withdrawn and the protocol recommended by the vendor was followed. Cell shape was revealed by Evan's blue counterstaining and the nuclei of apoptotic cells appeared as fluorescent. Cells were treated with IG161 (a), SIG (b), H_2O_2 (incubating the cells in DMEM containing 4 mM H_2O_2 for 90 min, then in media without H_2O_2 for another 90 min) (c), and with DNase-I after fixation (d), without adding ODN (e) and without adding fluorescein-dUTP (f).

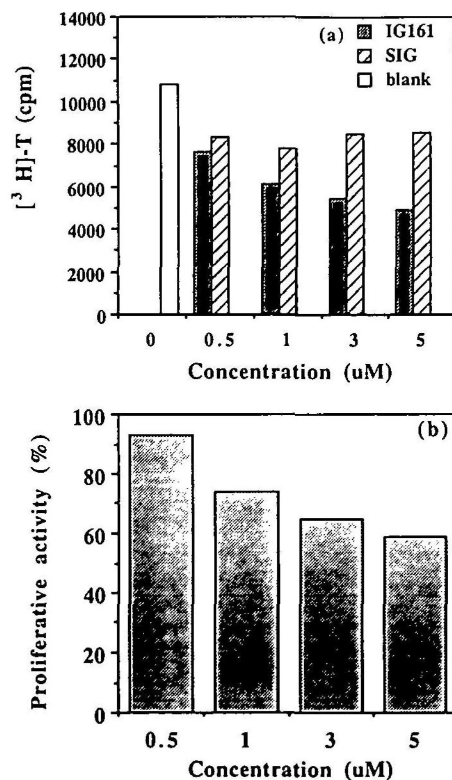

Fig. 5. **Suppression of cell proliferative activity by ODN.** HuH-7 cells cultivated in 96-well plates were treated with IG161 or SIG. After 24 h of cultivation, the cells were pulsed with [^{3}H]-thymidine for 4 h before harvest. (a) Counts per minute (cpm) of IG161-treated group, SIG-treated (control), and blank group (no ODN added). The data presented are averages derived from triplicate experiments. The standard deviation of each data point is less than 10%. (b) Percentages of cell proliferative activity, calculated from $[(cpm)_{IG161}/(cpm)_{control}] \times 100$.

Downloaded from http://jb.oxfordjournals.org/ at Peking University on October 2, 2012

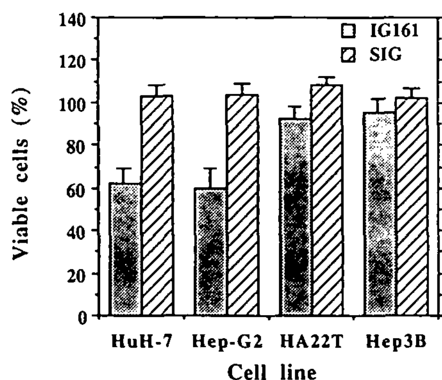

Fig. 6. **Effect of ODN on total viable cells of different human hepatoma cell lines.** The details of the experiment are described in the legend of Fig. 3. The concentration of each ODN was 20 μM.

the 3′-end and 5′-end and phosphodiester linkages in the middle of the sequence, was used. CS-ODN is a more accessible substrate for nuclease. When the cells were incubated with CS-ODN, [^{3}H]thymidine incorporation was deeply suppressed.

Action Mode of IGF-II in HuH-7 Cells—Since cell lysates of HuH-7 (and HepG2) showed high intracellular IGF-II (see Fig. 1), whereas IGF-II was undetectable in the conditioned medium, we investigated if IGF-II in HuH-7 functions through an intracrine mode. HuH-7 cells were grown in a low-serum (0.5% serum) medium containing 20 μM ATON IG161 with or without added IGF-II (50-1,000 ng/ml). It appeared that exogenous IGF-II could not relieve the growth inhibition due to ATON, which inhibited endogenous IGF-II production.

Antibody IGF-IRα(1H7) (Santa Cruz Biotech., CA) is a monoclonal antibody recognizing IGF-I receptor α subunit. It is known to inhibit binding of both IGF-I or IGF-II to the receptor and sebsequently inhibits IGF-I- or IGF-II-stimulated DNA synthesis. However, addition of this antibody to the culture medium did not cause any growth inhibition of HuH-7 cells. Thus, IGF-II seems to act in an intracrine mode in HuH-7 cells.

Selective Inhibition of the Growth of IGF-II-Overproducing Cell Lines—To answer the question of whether other HCC cell lines react similarly to treatment with ATON, the human HCC cell lines HepG2, HA22T, and Hep3B were tested. As shown in Fig. 1, HepG2 cells overproduce IGF-II, while HA22T and Hep3B do not. The results (Fig. 6) indicate that only the IGF-II-overproducing cell lines (*i.e.*, HuH-7 and HepG2) could be growth-suppressed by ATONs; the effect on HA22T or Hep3B was trivial (<10%). Additionally, ATONs of rat IGF-II were tested for the ability to inhibit the growth of rat hepatoma cell lines GN6TF, GP7TB, and GP10TA. No suppression of cell growth was found (data not shown). We therefore concluded that IGF-II *does* play an important role in cell proliferation only for those HCC cell lines overexpressing IGF-II.

DISCUSSION

In this report, we have shown that suppression of overexpressed IGF-II in hepatoma cell lines can inhibit the cell growth. The relative IGF-II protein levels of human HCC cell lines were found to be consistent with the results of Northern blot analysis reported by Su *et al.* (*6*). Their report indicated that there is more IGF-II mRNA in HepG2 and HuH-7 cells than in Hep3B cells, but no quantitative data were presented. Our results imply the intracrine nature of the overexpressed IGF-II in HuH-7 cells. These *in vitro* results coincide with the *in vivo* findings that IGF-II expression, in terms of both mRNA and protein, is increased in HCC tissue while the serum IGF-II level remains normal in the majority of HCC patients (*13*). The intracrine mode of IGF-II action has been found in breast cancer cell lines too (*28*). It was suggested that expression of different types of IGF-binding proteins (IGFBPs) may determine the disposition (intracrine/secretion) of IGF-II. For IGF-II-required cells, the intracrine mode is probably more economic than the autocrine mode. The intracrine mode also has been documented in other cases, including basic fibroblast growth factor (b-FGF) in cardiac myocytes and hepatoma cell line (*29*), growth hormone in human anterior pituitary gland (*30*), and epidermal growth factor in choriocarcinoma and placenta (*31*).

HuH-7 and HepG2 cells are able to grow in a serum-free medium (*32, 33*). Do these cells also produce other growth factors to sustain their own growth? We assayed IGF-I, transformation growth factor α (TGF-α), and basic fibroblast growth factor (b-FGF), but no detectable amounts of these factors could be found in either cell lysates or conditioned media. All of the aforementioned data suggest that the overexpression of IGF-II is important for proliferation of HuH-7 and HepG2 cells. In contrast to IGF-II, α-fetoprotein is a common oncoprotein found in hepatoma. We were able to suppress α-fetoprotein production by using antisense ODN in a previous study (*26*). However, no effect on cell growth was found. These findings indicate that these two oncoproteins, IGF-II and α-fetoproteins, play different roles in HCC development. As proposed by Christofori *et al.* (*14*), IGF-II provides second signals in oncogene-induced tumorigenesis. When both oncogene and IGF-II are present, malignant hyperproliferation of cells resulted. If the second signal is absent or inadequate, the cells undergo either apoptosis or benign proliferation. We conclude that growth inhibition of HuH-7 cells by IG161 can be attributed mainly to the retardation of cell proliferation.

We have demonstrated that suppression of overexpressed IGF-II causes growth retardation of hepatoma cells. Our findings highlight the importance of ATON against IGF-II as a potential agent for therapy of this type of HCC *in vivo*. Abnormal expression of IGFs has also been found in other cancer cells, such as colorectal, lung, and breast cancer cells (*34-37*). It would be worthwhile to examine the effects of ATON against IGF-II in these cells too. In addition, the cell lines in combination with the ATONs used in this study may provide a useful system for examining specific mechanisms of growth control of HCC by IGF-II.

We thank Ms. Ying-Chin Chen for synthesizing oligonucleotides.

REFERENCES

1. Humbel, R.E. (1990) Insulin-like growth factors I and II. *Eur. J. Biochem.* **190**, 445-462

Downloaded from http://jb.oxfordjournals.org/ at Peking University on October 2, 2012

2. Rotwein, P. (1990) Structure, evolution, expression, and regulation of insulin-like growth factors I and II. *Growth Factors* **5**, 13-18
3. Zvibel, I., Brill, S., and Reid, L. (1995) Insulin-like growth factor II: regulation of gene expression in rat and human hepatomas. *J. Cell. Physiol.* **162**, 36-43
4. Hartmann, H., Meyer-Alber A., and Braulke, T. (1992) Metabolic actions of insulin-like growth factor II in cultured adult rat hepatocytes are not mediated through the insulin-like growth factor II receptor. *Diabetologia* **35**, 216-223
5. Shapiro, E., Polonsky, K., Rubenstein, A., Kew, M., and Tager, H. (1990) Tumor hypoglycemia: Relationship to high molecular insulin-like growth factor-II. *J. Clin. Invest.* **85**, 1672-1679
6. Su, T.-S., Liu, W.-Y., Han, S.-H., Jansen, M., Yang-fan, T., P'eng, F.-K., and Chou, C.-K. (1989) Transcripts of the insulin-like growth factors I and II in human hepatoma. *Cancer Res.* **49**, 1773-1777
7. Seo, J.H. and Park, B.C. (1995) Expression of insulin-like growth factor II in chronic hepatitis B, liver cirrhosis, and hepatocellular carcinoma. *Jpn. J. Cancer Chemother.* **22** (Suppl. 3), 292-307
8. Park, B.C., Huh, M.H., and Seo, J.H. (1995) Differential expression of transforming growth factor alpha and insulin-like growth factor II in chronic active hepatitis B, cirrhosis and hepatocellular carcinoma. *J. Hepatol.* **22**, 286-294
9. Deng, T., Chen, N.L., and Jia, K.M. (1994) A study of the relationship between expression of IGF-II, IGF-IIR, HBxAg and the DNA ploidy, cell cycle of hepatocytes in hepatocarcinoma. *J. Int. Med. Res.* **33**, 743-746
10. Su, Q., Liu, Y., Zhang, J.F., Zang, S.X., Li, D.F., and Yang, J.J. (1994) Expression of insulin-like growth factor II in hepatitis B cirrhosis and hepatocellular carcinoma. *Hepatology* **19**, 788-799
11. Schirmacher, P., Held, W., Yang, D., Chisari, F., Rustum, Y., and Rogler, C.E. (1992) Reactivation of insulin-like growth factor II during hepatocarcinogenesis in transgenic mice suggests a role in malignant growth. *Cancer Res.* **52**, 2549-2556
12. Fiorentino, M., Grigioni, W.F., Baccarini, P., D'errico, A., Demitri, M.S., Pisi, E., and Mancini, A.M. (1994) Different in situ expression of insulin-like growth factor type II in hepatocellular carcinoma: an *in situ* hybridization and immuno-histochemical study. *Diagn. Mol. Pathol.* **3**, 59-65
13. Cariani, E., Seurin, D., Lasserre, C., Franco, D., Binoux, M., and Brechot, C. (1990) Expression of insulin-like growth factor II (IGF-II) in human primary liver cancer: mRNA and protein analysis. *J. Hepatol.* **11**, 226-231
14. Christofori, G., Naik, P., and Hanahan, D. (1994) A second signal supplied by insulin-like growth factor II in oncogene-induced tumorigenesis. *Nature* **369**, 414-418
15. Steller, M.A., Delgado, C.H., and Zou, Z. (1995) Insulin-like growth factor II mediates epidermal growth factor-induced mitogenesis in cervical cancer cells. *Proc. Natl. Acad. Sci. USA* **92**, 11970-11974
16. Florini, J.R., Magri, K.A., Ewton, D.Z., James, P.L., Grindstaff, K., and Androtwein, P.S. (1991) "Spontaneous" differentiation of skeletal myoblasts is dependent upon autocrine secretion of insulin-like growth factor-II. *J. Biol. Chem.* **266**, 15917-15923
17. Lin, S.-B., Chang, G.-W., Teh, G.-W., Lin, K.-I., and Au, L.-C. (1993) A simple and rapid method for the purification of oligodeoxyonucleoside methylphosphonates. *BioTechniques* **14**, 795-798
18. Bell, G.I., Merryweather, J.P., Sanchez-pescado, R., Stempien, M.M., Priestley, L., Scott, J., and Rall, L.B. (1984) Sequence of a cDNA clone encoding human insulin-like growth factor II. *Nature* **310**, 775-777
19. Huang, S.-S. (1995) Influence of insulin-like growth factor II antisense oligonucleotides on growth of hepatoma cell lines. Thesis, National Taiwan University, Taipei, Taiwan
20. Chen, T.-Z., Lin, S.-B., Wu, J.-C., Choo, K.-B., and Au, L.-C. (1996) A method for screening antisense oligodeoxyribonucleotides effective for mRNA translation-arrest. *J. Biochem.* **119**, 252-255
21. Bang, P., Eriksson, U., Sara, V., Wivall, I.L., and Hall, K. (1991) Comparison of acid ethanol extraction and acid gel filtration prior to IGF-I and IGF-II radioimmunoassays: improvement of determinations in acid ethanol extracts by the use of truncated IGF-I as radioligand. *Acta Endocrinol.* **124**, 620-629
22. Coligan, J., Kruisbeek, A., Shevach, E., and Strober, W. (1995) *Current Protocols in Immunology*, Vol. 1, chap 2, John Wiley and Sons, New York
23. Hill, E. and Ellis, M.N. (1992) Antiviral drug susceptibility testing in *Clinical Virology Manual*, 2nd ed. (Spectre and Lancz, eds.) pp. 277-284, Elsevier Science Publishing, New York
24. Connolly, D.T., Knight, M.B., Harkas, N.K., Witter, A.J., and Feder, J. (1985) Determination of the number of endothelial cells in culture using an acid phosphatase assay. *Anal. Biochem.* **152**, 136-140
25. Zhang, S.Z., Lipsky, M.M., Trump, B.F., and Hsu, I.C. (1990) Neutral red (NR) assay for cell viability and xenobiotic-induced cytotoxicity in primary cultures of human and rat hepatocytes. *Cell Biol. Toxicol.* **6**, 219-234
26. Lin, S.-B., Huang, S.-S., Choo, K.-B., Chen, P.-J., and Au, L.-C. (1995) Inhibition of α-fetoprotein production in a hepatoma cell line by antisense oligonucleotide analogues. *J. Biochem.* **117**, 1100-1104
27. Kreig, A., Yi, A., Matson, S., Waldschmidt, T., Bishop, G., Teasdale, R., Koretzky, G., and Klinman, D. (1995) CpG motifs in bacterial DNA trigger direct B-cell activation. *Nature* **374**, 546-549
28. Dubois, V., Couissi, D., Schonne, E., Schneider, Y.J., Remacle, C., and Trouet, A. (1993) Estrogen and insulin modulation of intracellular insulin-like growth factor binding proteins in human breast cancer cells: possible involvement in lysosomal hydrolases oversecretion. *Biochem. Biophys. Res. Commun.* **192**, 295-301
29. Pasumarthi, K.B., Kardami, E., and Cattini, P.A. (1996) High and low molecular weight fibroblast growth factor-2 increase proliferation of neonatal rat cardiac myocytes but have differential effects on binucleation and nuclear morphology. Evidence for both paracrine and intracrine actions of fibroblast growth factor-2. *Circulation Res.* **78**, 126-136
30. Mertani, H.C., Pechoux, C., Garcia-Caballero, T., Waters, M.J., and Morel, G. (1995) Cellular localization of the growth hormone receptor/binding protein in the human anterior pituitary gland. *J. Clin. Endocr. Metabol.* **80**, 3361-3367
31. Cao, H., Lei, Z.M., Bian, L., and Rao, C.V. (1995) Functional nuclear epidermal growth factor receptors in human choriocarcinoma JEG-3 cells and normal human placenta. *Endocrinology* **136**, 3163-3172
32. Nakabayashi, H., Taketa, K., Miyano, K., Yamane, T., and Sato, J. (1982) Growth of human hepatoma cell lines with differentiated functions in a chemically defined medium. *Cancer Res.* **42**, 3858-3863
33. Liu, J., Chen, B.-S., Tsai, T.-F., Wu, Y.-J., Pang, V., Hsieh, A., Hsieh, J., and Chang, T.-H. (1991) Long-term and large-scale cultivation of human hepatoma Hep G2 cells in hollow fiber bioreactor. *Cytotechnology* **5**, 129-139
34. Schneid, H., Seurin, D., Noguiez, P., and Le Bouc, Y. (1992) Abnormalities of insulin-like growth factor (IGF-I and IGF-II) genes in human tumor tissue. *Growth Regulation* **2**, 45-54
35. Lahm, H., Suardet, L., Laurent, P., Fischer, J., Ceyhan, A., Givel, J.C., and Odartchenko, N. (1992) Growth regulation and co-stimulation of human colorectal cancer cell lines by insulin-like growth factor I, II and transforming growth factor alpha. *Br. J. Cancer* **65**, 341-346
36. Minuto, F., Delmonto, P., Barreca, A., Alama, A., Cariola, G., and Giordano, G. (1988) Evidence for autocrine mitogenic stimulation by somatomedin-C/insulin-like growth factor I on an established human lung cancer cell line. *Cancer Res.* **48**, 3716-3719
37. Huff, K.K., Kaufman, D., Gabbay, K.H., Spencer, E.M., Lippman, M.E., and Dickson, R.B. (1986) Secretion of an insulin-like growth factor-II related protein by human breast cancer cells. *Cancer Res.* **46**, 4613-4619

Downloaded from http://jb.oxfordjournals.org/ at Peking University on October 2, 2012