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Expression of insulin-like growth factor-IA and factor-IB mRNA in human liver, hepatoma cells, macrophage-like cells and fibroblasts

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The human insulin-like growth factor-I (IGF-I) gene codes for two transcripts, IGF-IA and IGF-IB mRNAs, formed by alternative splicing. In this study, the expression of these IGF-I mRNA transcripts was examined using human liver, hepatoma cells, macrophage-like cells and fibroblasts. The reverse transcription-polymerase chain reaction revealed that these cells contained both IGF-IA mRNA (representing exons I, II, III and V) and IGF-IB mRNA (representing exons I, II, III and IV). Interestingly, an RNase protection assay using ^{32}P-labeled IGF-IA and IGF-IB exon-specific cRNA probes demonstrated that IGF-IA mRNA was 10-fold more abundant than IGF-IB mRNA in these cells. However, there was no difference in the stabilities of IGF-IA and IGF-IB mRNAs. These observations indicate that IGF-IA mRNA is more expressed than IGF-IB mRNA in these cells independent of their stabilities.

Insulin-like growth factor-I; Alternative splicing; mRNA; Polymerase chain reaction; RNase protection assay

1. INTRODUCTION

The human insulin-like growth factor-I (IGF-I) gene is composed of at least 5 exons located over 85 kb on chromosome 12 [1–10]. Recent studies have revealed that the IGF-I gene codes for two mRNA transcripts, IGF-IA and IGF-IB mRNAs, formed by alternative splicing [1,7–10]. IGF-IA mRNA (representing exons I, II, III and V) codes for a 17.5-kDa protein of 153 amino acids, whereas IGF-IB mRNA (representing exons I, II, III and IV) codes for a 21.8-kDa protein of 195 amino acids [1,7–10]. Although IGF-IA and IGF-IB mRNAs have been identified, neither protein has yet been isolated and characterized. In contrast, the serum form of IGF-I, referred to as somatomedin C, is known to be a 7.6-kDa molecule of 70 amino acids, which is coded for by the sequences in exons II and III of the IGF-I gene, and is probably formed from the proteolytic cleavage of larger precursor proteins directed by IGF-IA and/or IGF-IB mRNA transcripts [1,7–10]. It is becoming evident that the tissue forms of IGF-I with a molecular mass of 18–25 kDa are produced by liver, alveolar macrophages, and fibroblasts [11–13]. However, the expression of IGF-IA and IGF-IB mRNA transcripts in these cells has not been examined and the relationship of the tissue IGF-I molecules to the putative protein products of IGF-IA and IGF-IB mRNA transcripts is not clear.

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Abbreviations: IGF-I, insulin-like growth factor-I; RT-PCR, reverse transcription-polymerase chain reaction; bp, base pair

In this study, therefore, we have evaluated the expression of IGF-IA and IGF-IB mRNA transcripts in liver, hepatoma cells, macrophage-like cells and fibroblasts, and found that these cells express both IGF-IA and B mRNA transcripts, but IGF-IA mRNA is 10-fold more abundant.

2. MATERIALS AND METHODS

2.1. *Cells*

HUH-7 (human hepatoma cell line, JCRB0403) and SF-TY (human skin fibroblasts, JCRB0075) were obtained from the Japanese Cancer Research Resources Bank (JCRB, Tokyo). U937 (human macrophage-like cell line, CRL1593) was obtained from the American Type Culture Collection (Rockville, MD). Normal human liver was obtained at a post-mortem examination. In some experiments, MRC-9 (human lung fibroblasts, CCL212) obtained from JCRB and human alveolar macrophages (>90% pure) obtained by bronchoalveolar lavage [14] were also used.

2.2. *Evaluation of IGF-IA and B mRNA transcripts by reverse transcription-polymerase chain reaction (RT-PCR)*

Total cellular RNA was isolated by the acid guanidinium-thiocyanate-phenol-chloroform extraction method [15]. First strand cDNA was synthesized from total cellular RNA by M-MLV reverse transcriptase (BRL, Gaithersburg, MD) using oligo-dT as a primer. Aliquots of the resulting reaction mixtures were used as templates for amplification by the polymerase chain reaction using *Thermus aquaticus* DNA polymerase, a thermal cycler (Perkin-Elmer Cetus Instruments, Norwalk, CT) and exon-specific oligonucleotide extension primers based on the nucleotide sequences of IGF-IA and IGF-IB cDNAs [7,8,16]. Primers for IGF-IA; exon I primer (20-mer) corresponding to the sequences of IGF-IA cDNA 72–91 [8] and exon V primer (20-mer) corresponding to the sequences of IGF-IA cDNA 639–658 [8]; primers for IGF-IB, exon I primers, the same primer used for IGF-IA cDNA, but corresponding to the sequences of IGF-IB cDNA 90–109 [7] and exon IV primer (20-mer) corresponding to the sequences of IGF-IB cDNA 921–940 [7]. The reaction was carried out in a 50 μl volume under recommended conditions for 30 cycles, each

consisting of denaturation (94°C, s), annealing (54°C, 15 s) and extension (72°C, 30 s). The amplified cDNAs were evaluated by agarose gel electrophoresis. The nucleotide sequences of the amplified IGF-IA and IGF-IB cDNAs were confirmed using the dideoxy chain termination method [17], with T7 DNA polymerase (Sequenase, U.S. Biochemical Corp., Cleveland, OH) after subcloning the amplified cDNAs into the riboprobe vector pBluescript SK/+ (Stratagene, La Jolla, CA). The riboprobe vectors containing amplified IGF-IA or IGF-IB cDNAs were also used for the synthesis of labeled antisense cRNA probes and unlabeled sense cRNAs as described below.

2.3. *Quantification of IGF-IA and IGF-IB mRNA transcripts by a solution hybridization/RNase protection assay*

Cellular IGF-IA and IGF-IB mRNA levels were quantified using IGF-IA and IGF-IB exon-specific cRNA probes by a solution hybridization/RNase protection assay [18,19]. The ^{32}P-labeled IGF-IA-specific antisense cRNA probe (587 bases) and IGF-IB-specific antisense cRNA probe (851 bases) were synthesized using [^{32}P]UTP (800 Ci/mmol; Amersham, Arlington Heights, IL) and the IGF-IA or IGF-IB cDNA-containing riboprobe vector. These ^{32}P-labeled antisense cRNA probes were hybridized with 10 μg total cellular RNA, and subjected to RNase treatment, purification, electrophoresis, and autoradiography. The construct standard curves, the ^{32}P-labeled IGF-IA and IGF-IB antisense cRNA probes were hybridized with various quantities of synthetic unlabeled IGF-IA and IGF-IB sense cRNAs, respectively, and the autoradiographic signals were quantified by densitometry.

2.4. *Evaluation of the stabilities of IGF-IA and IGF-IB mRNA transcripts*

Cells were incubated in the absence or presence of 10 μg/ml actinomycin D (Sigma, St. Louis, MO) at 37°C for up to 24 h in RPMI1640 (Nissui Seiyaku, Tokyo) supplemented with 2 mM glutamine, 50 U/ml penicillin and 50 μg/ml streptomycin. After the incubation total cellular RNA was isolated, and IGF-IA and IGF-IB mRNA levels were quantified by a solution hybridization/RNase protection assay.

3. RESULTS AND DISCUSSION

First, the expression of IGF-IA and IGF-IB mRNA transcripts in human liver, hepatoma cells, macrophage-like cells and fibroblasts was examined by RT-PCR. Using IGF-I exon-specific oligonucleotide primers, the sizes of amplified cDNAs would be expected to be 587 bp and 851 bp for IGF-IA and IGF-IB mRNA, respectively (Fig. 1A). RT-PCR of RNAs from liver, hepatoma cells, macrophage-like cells and

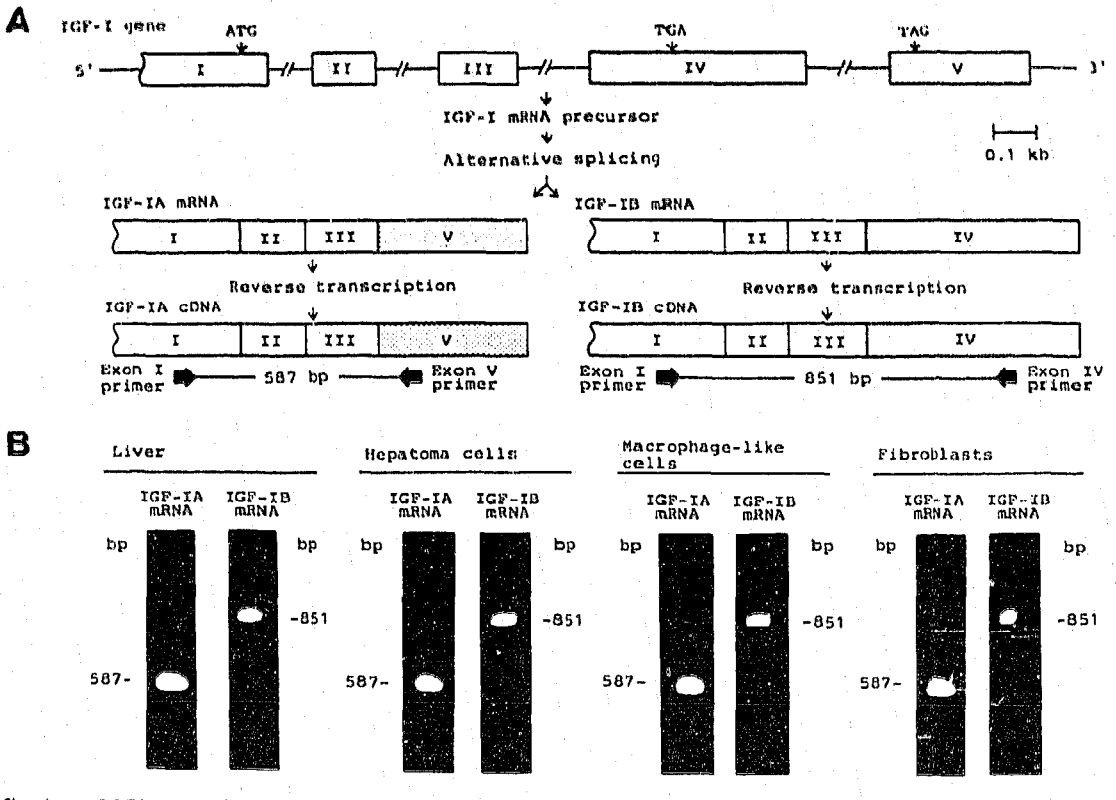

Fig. 1. Identification of IGF-IA and IGF-IB mRNA transcripts by RT-PCR. (A) Schematic representation of the human IGF-I gene and the strategy for identifying IGF-IA and IGF-IB mRNA transcripts by RT-PCR. The human IGF-I gene has at least 5 coding exons, but the complete structure of the 5′ portion of exon I (and/or the existence of additional 5′ exons) is unknown. The putative start codon (ATG) is in exon I. There are two in-phase stop codons (TGA in exon IV, TAG in exon V). IGF-I mRNA precursor is processed into IGF-IA mRNA (representing exons I, II, III and V) and IGF-IB mRNA (representing exons I, II, III and IV) through alternative splicing. After IGF-IA and IGF-IB cDNAs were synthesized by reverse transcription, cDNAs were amplified by PCR using IGF-I exon-specific primers (exon I, exon IV and exon V primers). The expected sizes of amplified cDNAs were 587 bp and 851 bp for IGF-IA and IGF-IB mRNAs, respectively. (B) Agarose gel electrophoresis analysis of amplified cDNA. After cDNAs from liver, HUH-7 hepatoma cells, U937 macrophage-like cells and SF-TY fibroblasts were amplified, DNAs were analyzed on 1% agarose gel. Left lanes: cDNAs amplified with exon I and exon V primers (corresponding to IGF-IA mRNA); right lanes: cDNAs amplified with exon I and exon IV primers (corresponding to IGF-IB mRNA). φX174/*Hae*III digest fragments were used as markers.

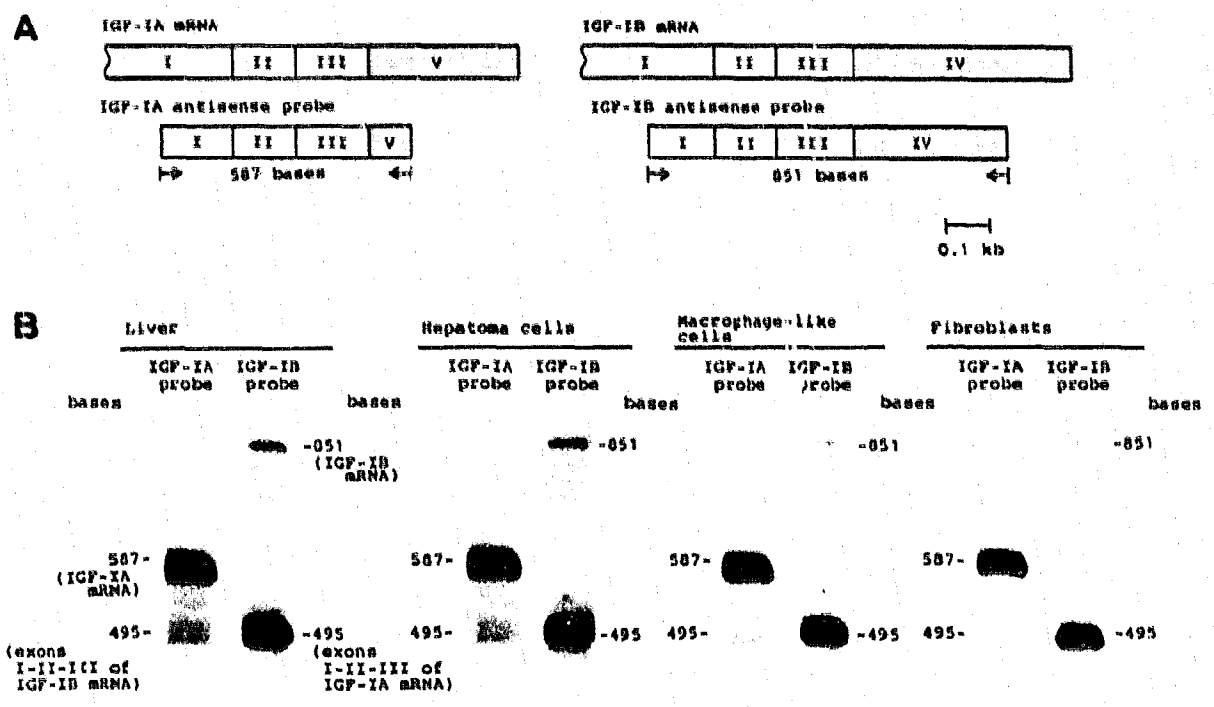

Fig. 2. Quantification of IGF-IA and IGF-IB mRNA transcripts by a solution hybridization/RNase protection assay. (A) IGF-IA and IGF-IB mRNA transcripts, and IGF-I exon-specific cRNA probes. The IGF-IA-specific antisense cRNA probe consisted of 587 bases spanning exons I, II, III and V. The IGF-IB-specific antisense cRNA probe consisted of 851 bases spanning exons I, II, III and IV. The sequences of both cRNA probes spanning exons I, II and III (495 bases) are the same and common to both IGF-IA and IGF-IB mRNAs. (B) Evaluation of IGF-IA and IGF-IB mRNA transcripts by an RNase protection assay. Total cellular RNAs (10 μg) from liver, HUH-7 hepatoma cells, U937 macrophage-like cells and SF-TY fibroblasts were hybridized with ^{32}P-labeleld IGF-IA-specific cRNA probe (left lanes) and IGF-IB-specific cRNA probe (right lanes). ^{32}P-Labeled φX174/*Hae*III digest fragments were used as markers.

fibroblasts yielded both 587 bp and 851 bp amplified cDNAs (Fig. 1B). The nucleotide sequences of the amplified cDNAs were examined, and proved to be identical to those of IGF-IA and IGF-IB cDNAs reported [7–9] (data not shown). These observations indicate that these cells express both IGF-IA and B mRNA transcripts.

Next, the amounts of IGF-IA and IGF-IB mRNA were quantified using a solution hybridization/RNase protection assay. Hybridization of IGF-IA-specific probe with cellular RNA would be expected to result in the appearance of protected bands of 587 bases (corresponding to IGF-IA mRNA) and 495 bases (corresponding to exons I, II, III of IGF-IB mRNA), and hybridization of IGF-IB-specific probe with cellular RNA would be expected to result in the appearance of protected bands of 851 bases (corresponding to IGF-IB mRNA) and 495 bases (corresponding to exons I, II, III of IGF-IA mRNA) (Fig. 2a). Figure 2B shows the results of the hybridization of these two probes with total cellular RNA from various cells. IGF-IA and IGF-IB mRNAs were present in these cells, and IGF-IA mRNA was the predominant species. The quantification of the levels of IGF-IA and IGF-IB mRNAs by densitometry demonstrated that IGF-IA mRNA was 10-fold more abundant than IFG-IB mRNA in each case, although the amounts of both mRNAs were different among these cells (Table I). IGF-IA mRNA was also more abundant than IGF-IB mRNA in human alveolar macrophages and another cell line of fibroblasts, MRC-9 (Table I). Thus, IGF-IA mRNA was the predominant species in all cells examined.

It is possible that the low abundance of IGF-IB mRNA relative to IGF-IA mRNA is due to the instability of IGF-IB mRNA. To confirm this possibility the stabilities of IGF-IA and IGF-IB mRNA were examined (Fig. 3). In the absence of actinomycin D, a transcription inhibitor, IGF-IA and IGF-IB mRNA levels were

Table I

Quantification of IGF-IA and IGF-IB mRNA transcripts

	IGF-IA mRNA	IGF-IB mRNA
Liver	0.306 ± 0.075	0.027 ± 0.006
HUH-7 hepatoma cells	0.183 ± 0.057	0.018 ± 0.005
U937 macrophage-like cells	0.150 ± 0.035	0.011 ± 0.003
Alveolar macrophages	0.203 ± 0.056	0.020 ± 0.008
SF-TY fibroblasts	0.051 ± 0.026	0.004 ± 0.002
MRC-9 fibroblasts	0.061 ± 0.015	0.006 ± 0.001

The autoradiographic signals were quantified by densitometry. The amounts of IGF-IA and IGF-IB mRNAs expressed as pg/μg total cellular RNA were calculated using standard curves constructed with various quantities of synthetic unlabeled IGF-IA and IGF-IB sense cRNAs. Values represent the mean ± SE of 3 experiments.

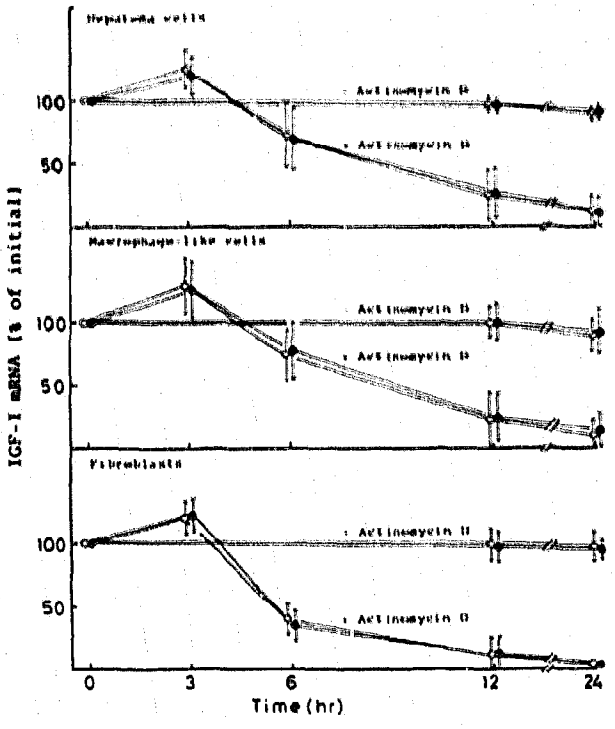

Fig. 3. Stabilities of IGF-IA and IGF-IB mRNA transcripts. HUH-7 hepatoma cells, U937 macrophage-like cells and SF-TY fibroblasts were incubated in the absence (− Actinomycin D) or presence (+ Actinomycin D) of 10 μg/ml actinomycin D for up to 24 h. After the incubation, total cellular RNA was isolated and the amounts of IGF-IA (○) and IGF-IB (●) mRNAs were quantified by a solution hybridization/RNase protection assay. IGF-I mRNA levels are expressed as a percentage of mRNA levels in cells at zero time. Values represent the mean ± SE of 3 experiments.

not changed over 24 h. On the other hand, both IGF-I mRNA levels decreased in a time dependent manner in the presence of actinomycin D. Although the half-lives of both IGF-I mRNAs of hepatoma cells and macrophage-like cells were longer than those of fibroblasts, there was no difference in the stabilities of both IGF-IA and IGF-IB mRNAs in each case. Moreover, the half-lives of IGF-IA and IGF-IB mRNAs were not different in alveolar macrophages and MRC-9 fibroblasts (data not shown). These observations indicate that IGF-IA mRNA is more expressed than IGF-IB mRNA in these cells independent of their stabilities. Furthermore, the 10-fold excess of IGF-IA mRNA in liver, macrophages and fibroblasts suggests that the major IGF-I molecules produced by these cells [11–13] are the protein products of the IGF-IA mRNA transcript, although the final proof will require more direct evidence such as the sequence analysis of the purified IGF-I proteins.

A. Consensus sequence	G U $\frac{G}{A}$ A G U - - -
B. IGF-I mRNA precursor	
Exons I-II	G U A A A U - - -
Exons II-III	G U A A G U - - -
Exons III-IV	G U A A G C - - -
Exons IV-V	U U A A G C - - -

Fig. 4. The 5′-splice junction sequences of the human IGF-I mRNA precursor. The consensus sequence of intron at the 5′ splice site (A) [20] and the sequences of introns at the 5′ splice sites of the human IGF-I mRNA precursor (B) [9] are shown. The variation from the 5′-consensus GU dinucleotide sequence is underlined.

The mechanism governing the predominant expression of IGF-IA mRNA is not clear. However, it is known that the consensus sequences which are present at the exon/intron boundaries of mRNA precursors play an important role in splicing [20–24]. Mutations in the splice junction sequences are reported to interfere with mRNA precursor splicing, e.g. mutations in the GU dinucleotide at the 5′ splice site reduce the production of correctly spliced mRNA, and instead promote the production of aberrantly spliced mRNA by the excision of the exon present upstream of the mutation through alternative splicing [24,25]. Mutations in the polypyrimidine tract at the 3′ splice site markedly reduce the efficiency of the splicing [21,26]. Interestingly, the 5′ GU dinucleotide is replaced by UU in the intron between exon IV and exon V of the IGF-I mRNA precursor (Fig. 4). This variation from the 5′-consensus sequence may reduce the splicing of exon IV to exon V and promote the 'aberrant' joining of exon III to exon V, resulting in the predominant production of IGF-IA mRNA. However, detailed mechanisms controlling the splicing of the IGF-I mRNA precursor remain to be elucidated in the future.

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