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ADULT HUMAN OSTEOBLAST-LIKE CELLS DO NOT EXPRESS INSULIN-LIKE GROWTH FACTOR-I

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Summary: Previous studies using cultures of fetal rodents calvaria have indicated an important role for insulin-like growth factor-I (IGF-I) in the local control of bone formation. In this study, we have examined the expression of IGF-I in adult human osteoblast-like (hOB) cells. To detect very low levels and to distinguish between the two IGF-I mRNA transcripts Ea and Eb, which are formed by alternate splicing, we have used the reverse transcriptase-polymerase chain reaction (RT-PCR). Expression of both Ea and Eb IGF-I mRNA transcripts were found in human liver and in placenta. However, neither Ea nor Eb IGF-I mRNA could be detected under basal conditions or after stimulation with growth hormone in normal hOB cells and two human osteosarcoma cell lines with osteoblastic properties, SaOS-2 and MG-63. We conclude that adult hOB cells do not synthesize IGF-I. Thus, in contrast to its crucial role as a local regulator of skeletal remodeling in fetal rodent bone, IGF-I does not appear to have this autocrine function in adult human bone.

Insulin-like growth factor-I (IGF-I) is a polypeptide which mediates many of the anabolic effects of growth hormone (GH) on target tissues such as bone (for a review see (1)). In rat, the major site of synthesis is the liver, but syntesis in several other tissues has been detected. Studies using cultures of fetal rat calvaria have indicated an important role in the local control of bone formation (2, 3) and the regulation of IGF-I production in these cultures has been the subject of several investigations (see e.g. (3-6). The human IGF-I gene codes for two mRNA transcripts, Ea and Eb, formed by alternative splicing (7). Ea IGF-I mRNA representing exons 1-4 and exon 6 codes for a protein of 153 amino acids, whereas Eb IGF-I mRNA representing exons 1-5 codes for a protein of 195 amino acids (8). Both transcripts are expressed in the liver (9), but neither of the two proteins has been isolated and characterized. In contrast, the serum form of IGF-I is known to be a molecule of 70 amino acids coded for by the sequences in exons three and four. This serum IGF-I is usually found associated with a specific, high affinity IGF-binding protein (IGFBP), which is also the major IGFBP produced by human osteoblasts, the IGFBP-3 (10). The existence of this and other IGFBPs has complicated radioligand assays for measurement of IGF-I because they compete with the receptors or antibody for tracer protein. This could be part of the explanation for the variation in the reported IGF-I levels in the culture medium from human osteoblast-like (hOB) cells, ranging from nondetectable levels (11, 12) to more than 20ng/ml (13). The expression of

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IGF-I mRNA trancripts has, however, not been investigated. In the present study we have used the very sensitive reverse transcriptase - polymerase chain reaction (RT-PCR) to examine the expression of Ea and Eb IGF-I mRNA in adult human liver, placenta, and in different human osteoblast-like cell types, including normal and spontaneously transformed cells.

MATERIALS AND METHODS

Cells
The methods used in the isolation and culture of the normal hOB cells have been reported previously (14, 15). Briefly, trabecular bone was obtained from the iliac crest used as donor sites in adult patients undergoing bone graft procedures. The samples were dissected into small fragments and thoroughly rinsed with phosphate-buffered saline (PBS). Trabecular fragments were seeded as explants into tissue culture plates and cultured at 37°C in Eagle's minimal essential medium supplemented with glutamine, streptomycin, penicillin, and 10 % heat-inactivated fetal calf serum. Experiments were performed on cultures at first or second passages. The human bone cell populations obtained by these methods maintain the phenotype of mature osteoblasts through the culture period (16, 17). The human osteosarcoma cell lines, SaOS-2 (18) and MG-63 (19), were cultured under the same conditions as the primary human bone cells described above.

RNA-preparation
RNA from hOB cells was prepared using a guanidinium-thiocyanate procedure (20). Liver and placenta RNA were purchased from Clontech, Palo Alto, CA. The RNA concentration and purity were calculated by absorption at 260 and 280 nm.

cDNA synthesis
cDNA from total RNA from human osteoblast-like cells was synthesized with 1st-strand cDNA Synthesis Kit (Clontech, Palo Alto, CA) exactly according to the manufacturer's protocol. 1 µg total RNA was used in each reaction, and the primer used was the Oligo $(dT)_{18}$ primer.

Polymerase chain reaction
Transcripts of Ea and Eb IGF-I mRNA were studied by amplification of the obtained cDNA using different primer pairs (see Table I for details). The G3PDH primers were included in the 1st-strand cDNA Synthesis Kit described above. In a 50 µl reaction, 200 ng transcribed total RNA was amplified in 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin, 200 mM of each dNTP, 50 pmol of each primer, and 1.25 U AmpliTaq DNA polymerase (Perkin Elmer-Cetus Corp, Emeryville, CA). The mixture was subjected to 30 cycles of amplification in a Perkin-Elmer 9600 Thermal cycler, using the following cycle parameters; Denaturation for 45s at 94°C, reannealing for 45s at 60°C, extension for 90s at 72°C. A denaturation step of 2 min at 94°C and a extension step for 7 min at 72°C were added to the initial and final cycle, respectively. Amplified products were separated by electrophoresis on a 1.5% agarose gel and visualized by ethidium bromide staining and ultraviolet transillumination. The identity of

Table I Primers used in PCR-amplification reactions

Primer	Primer sequence	Pos in seq
Ea and Eb (F)	5'-GTGGATGCTCTTCAGTTCGTGTGTG-3'	186-210bp
Ea (R)	5'-CCTGTAGTTCTTGTTTCCTGCACT CC-3'	467-442bp
Eb (R)	5'-CTGAGACTTCGTGTTCTTGTTGGTAG-3'	623-598bp

The primer sequences are given as forward primer (F) and reverse primers (R) The positions of the primers (Pos in seq) are given as their positions in base pairs (bp) in the published cDNA sequence (7).

the PCR product was confirmed by transfer onto a nylon membrane (Hybond N; Amersham Corp.) and hybridization to an IGF-I exon 3 genomic fragment (8). The results were analyzed on a PhosphorImager (Molecular Dynamics) after 12h exposure. A 123 bp ladder (GIBCO BRL Life Technologies Inc. Gaithersburg, MD) was used as a size marker.

RESULTS AND DISCUSSION

Detection of Ea and Eb IGF-I mRNA in Human Liver Using RT-PCR

As a control, the expression of Ea and Eb IGF-I mRNA was first examined in human liver. Using exon-specific oligonucleotide primers, PCR was performed on cDNA synthesized from total RNA from human liver. The patterns obtained after RT-PCR, gel electrophoresis, Southern blotting, and hybridization with a ^{32}P-labeled IGF-I probe are shown in Figure 1. Single bands with the expected sizes of 281 and 266bp were found. Negative controls showed that there was no contamination in the PCR. These results confirm that the amplified PCR products are Ea and Eb IGF-I mRNA and that human liver express both splicing variants. Although the RT-PCR is not directly quantitative, Ea appears to be the predominant form. Interestingly, Nagaoka et al (9) estimated that Ea was 10-fold more abundant in liver using the ribonuclease protection assay.

Normal Human Osteoblast-like Cells Do Not Express Ea or Eb IGF-I mRNA

PCR was then performed on cDNA synthesized from total RNA from human liver, normal hOB cells and placenta. The patterns obtained by ethidium bromide staining of agarose gels after gel electrophoresis are depicted in Figure 2. Our results demonstrate that both transcripts are found in liver and placenta, and Ea appears to be more abundant also in placenta. However, neither Ea nor Eb could be detected in normal hOB cells. The G3PDH PCR product confirms that the cDNA was intact. Identical results were obtained also after the stimulation with human growth hormone (hGH). Thus, no IGF-I expression was detected in normal hOB cells, neither under basal conditions, nor after treatment with hGH. This is in good agreement with a recent study using RIA by Kassem et al. (12), also showing that the IGF-I levels in normal hOB cells are not related to the actual IGF-I concentration but rather reflects the amount of IGFBP-3 present.

Human Osteosarcoma Cells Do Not Express IGF-I

To extend this unexpected observation, RNA was prepared from SaOS-2 and MG-63, two human osteosarcoma cell lines with documented osteoblastic properties (18,19) and the expression of Ea and Eb IGF-I mRNA was examined by RT-PCR. Figure 3 shows that IGF-I mRNA was absent in both cell lines. Again, this was not due to a degraded RNA since the G3PDH PCR product was found in both cell lines. Our results are in agreement with a study by Mohan et al. in which no IGF-I could be detected in SaOS-2 and MG-63 when IGF-II was added to avoid interference with IGFBPs (11) and should explain the remarkably low levels of the IGF-I protein detected in the human

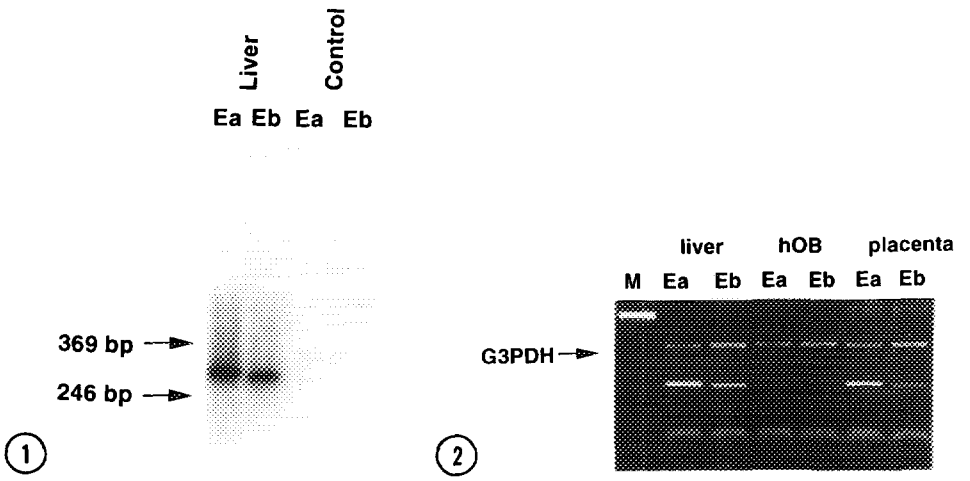

Figure 1.
Detection of Ea and Eb IGF-I mRNA in human liver using RT-PCR. PCR products obtained by amplification of human Ea and Eb IGF-I cDNA after reverse transcription of RNA from human liver or a control without cDNA were run on an agarose gel and the Southern blot was hybridized with a ^{32}P-labeled exon 3 IGF-I probe.

Figure 2.
Normal adult human osteoblast-like cells do not express Ea or Eb IGF-I mRNA. Total RNA from liver, normal human osteoblast-like (hOB) cells, and placenta were used as templates for cDNA synthesis. 10% portions of the cDNA were amplified by PCR using primers for Ea and Eb IGF-I, and then resolved on a 1.5% ethidium bromide-agarose gel. As a control of the cDNA, primers for glycerol-3-phosphate dehydrogenase (G3PDH) were included in all PCR reactions. The marker (M) was a 123 bp ladder.

osteosarcoma cell line TE-85 using radioligand assays (11, 21). Our results emphasize the importance of using valid methods to measure IGF-I, and points to an important difference between fetal rodent and adult human bone tissue.

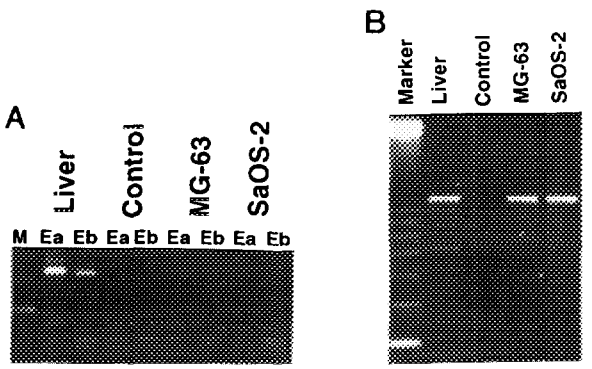

Figure 3.
Human osteosarcoma cells do not express IGF-I.
(A) Total RNA from liver, a control with no added RNA, MG-63, and SaOS-2 were used as templates for cDNA synthesis and then amplified by PCR using primers for (A) Ea and Eb, and (B) G3PDH. The marker (M) was a 123 bp ladder.

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