IGF-I production by adult rat hepatocytes is stimulated by transforming growth factor- α and transforming growth factor- β_1

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Abstract

Previously, we have observed that epidermal growth factor (EGF), a potent mitogen for cultured hepatocytes, stimulates the production of IGF-I and IGF-binding proteins (IGFBPs) by cultured hepatocytes from adult rats. This study was undertaken to investigate the possibility that other growth factors of hepatic origin could specifically be involved in the regulation of IGF-I and IGFBP expression. The effects of transforming growth factor- α (TGF- α), through EGF receptors to induce a mitogenic response, and transforming growth factor- β_1 (TGF- β_1), produced by non-parenchymal liver cells and able to inhibit hepatocyte proliferation *in vivo* and in culture, have been studied in cultured adult rat hepatocytes.

Our results demonstrate that TGF- α and TGF- β_1 significantly stimulate IGF-I and IGFBP secretion by cultured hepatocytes but no change in the abundance of IGF-I and IGFBP mRNAs was observed with respect to controls. Cycloheximide is able to inhibit both basal and TGF-stimulated release of IGF-I and a similar effect was elicited by octreotide, the somatostatin analog, known to directly affect hepatic IGF-I gene expression.

Our findings show the role of the liver in the secretion of IGF-I and IGFBPs, not only under endocrine and nutritional control but also under autocrine and paracrine control.

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Introduction

Insulin-like growth factor-I (IGF-I) is a polypeptide growth factor that plays an important role in postnatal development (1). The majority of IGF-I circulates bound to specific high-affinity binding proteins (IGFBPs) which are involved in the clearance, tissue distribution and biological activity of IGF-I (2). To date, the complete primary structures of six distinct IGFBPs have been determined from cDNA clones and the proteins have been named IGFBP-1, IGFBP-2, IGFBP-3, IGFBP-4, IGFBP-5 and IGFBP-6 (2). Adult liver is the main source of circulating IGF-I since it has the highest tissue level of IGF-I mRNA (3, 4). IGF-I production by the liver is achieved very early during postnatal life as a function of the hormonal status of the rat (5, 6). In the adult rat, serum IGF-I concentrations are controlled by growth hormone, insulin and nutritional factors (7–9). Direct effects exerted on IGF-I mRNA levels by amino acid availability were observed in cultured hepatocytes (10). Previously, we have observed that epidermal growth factor (EGF), the potent mitogen for cultured hepatocytes and mainly produced in the salivary glands,

increases IGF-I production (11), which could lead the cell to undergo DNA synthesis. In cultured adult rat hepatocytes which do not express type I IGF receptors (12) IGF-I did not seem to exert any mitogenic activity, indicating that the stimulation of liver IGF-I production by EGF could not be correlated with the mechanisms leading to DNA synthesis. The aim of this study was to ascertain whether other growth factors of hepatic origin, such as transforming growth factor- α (TGF- α), synthesized by hepatocytes and acting through EGF receptors to induce a mitogenic response (13), and TGF- β_1 , produced by non-parenchymal liver cells and able to inhibit hepatocyte proliferation *in vivo* as well as in culture (14), could be specifically involved in the regulation of hepatic IGF-I production by cultured hepatocytes. Our results demonstrate that both TGF- α and TGF- β_1 greatly stimulate IGF-I and IGFBP production by rat hepatocytes cultured in serum-free medium, confirming at the same time the opposite direct effect exerted by TGF- α and TGF- β_1 on hepatocyte DNA synthesis. No change in IGF-I mRNA level was seen when TGF- α and TGF- β_1 were added to the culture medium, and no variations in IGFBP-1 and

IGFBP-4 mRNA levels were observed in all tested conditions.

Materials and methods

Materials

Chemicals employed in this study were of analytical or reagent grade and were obtained from Sigma Chemical Co., St Louis, MO, USA or Collaborative Research (Waltham, MA, USA). Rat IGF-I (15) and rat IGFBP-1 (16), -2 (17) and -4 (18) cDNA probes were kindly provided by Dr C B Bruni from the University of Naples, Italy, and Dr S Shimasaki, Department of Cell Biology, The Scripps Research Institute, La Jolla, CA, USA. The human glyceraldehyde-3-phosphate dehydrogenase (G3PDH) cDNA probe was purchased from Clontech Laboratories Inc., Palo Alto, CA, USA. The probes were labeled to high specific activity by multiprime DNA labeling systems (Amersham International plc, Amersham, Bucks, UK) using [32 P]dCTP (NEN Du Pont, Du Pont De Nemours GmbH, Bad Homburg, Germany, specific activity 3000 Ci/mmol).

Cell culture

Hepatocytes were isolated from male Wistar rats (180–200 g, Nossan Breeding, Correzzana, Italy) as previously described (19). Trypan blue exclusion testing indicated that the viability of the cells was greater than 85%. Hepatocytes were resuspended at a concentration of 2×10^5 cells/ml in serum-free Dulbecco's modified Eagle's medium (DMEM) supplemented with essential and non-essential amino acids (basal medium), insulin (0.1 U/ml) and dexamethasone (10^{-7} mol/l). Aliquots of cell suspension were plated on 60 and 100 mm tissue culture dishes (Costar, Cambridge, MA, USA) coated with collagen (type III, Sigma). After 90 min, culture medium was replaced with basal medium containing 0.25% BSA (Fraction V, cell culture tested, Sigma). Experiments were begun on cultures that had been incubated for 24 h of adaptation to the *in vitro* environment (time 0) and exposed as indicated to vehicle alone (control) or to various concentrations of TGF- α and/or TGF- β_1 from human platelets, both endotoxin and cell culture tested from Sigma (TGF-treated). Monolayers were checked for cell viability with Trypan vital stain in normal saline before and after incubation of hepatocytes with additives.

[3 H]Thymidine incorporation studies

Hepatocytes cultured for the first 24 h in basal medium containing 0.25% BSA (control) were then exposed to TGF- α (40 ng/ml) and/or TGF- β_1 (2 ng/ml) and pulsed with [3 H]thymidine (5 μ Ci/ml, New England Nuclear, Dreieich, Germany) at various time in culture. Incubations were carried out at 37 °C for 2 h and measurement

of DNA synthesis was done according to McGowan *et al.* (20).

IGF-I RIA and IGFBP assay

After 24 h of culture in the presence of test substances or vehicle alone, the production of IGF-I and IGFBPs was measured in the media of hepatocytes. A media pool (20 ml; 2×10^5 cells/ml) was dialyzed overnight against 1 mol/l acetic acid in Spectrapore 6 membranes, lyophilized, reconstituted in 0.5 mol/l acetic acid/0.6 mol/l NaCl and gel filtered on a Sephadex G-50 column (1.6 $\times$ 90 cm) equilibrated with 0.1 mol/l acetic acid/0.15 mol/l NaCl, pH 2.75. Fractions were collected at 0.1 K_{av} intervals, lyophilized, reconstituted in PBS and analyzed for immunoreactive IGF-I (Medgenix Diagnostics SA, Fleurs, Belgium) and IGF binding activity. The IGFBP assay was performed by the method of Hinz & Liu (21) as slightly modified by us (11).

RNA isolation and Northern blot analysis

At various times in culture, IGF-I and IGFBP-1, -2 and -4 mRNA abundances were determined in adult rat hepatocytes cultured in the presence of TGF- α (40 ng/ml) and/or TGF- β_1 (2 ng/ml) or vehicle alone (control). Cells were rapidly frozen in liquid nitrogen and total RNA was prepared using the guanidinium isothiocyanate–phenol–chloroform method (22). RNA samples obtained under each study condition and derived from a pool of four 100 mm plates were quantified by measuring absorbance at 260 nm and fractionated in a 1% agarose gel. Samples were then transferred to nylon membranes and processed for Northern blot analysis as previously reported (11). To ensure even loading, the membranes were stripped and rehybridized with a human G3PDH cDNA probe. The signal intensity of the autoradiograms was quantified by a Hoefer (San Francisco, CA, USA) GS-300 densitometer controlled by an IBM-AT personal computer.

Results

The time-course of [3 H]thymidine incorporation into DNA by cultured hepatocytes isolated from the adult rat is depicted in Fig. 1. Hepatocytes were cultured in basal medium for 24 h of adaptation to the *in vitro* environment then shifted to fresh DMEM in the presence or absence of TGF- β_1 and/or TGF- α , used as positive control. As can be seen the addition of TGF- α (40 ng/ml) to the culture medium increased the incorporation of [3 H]thymidine into DNA with a maximum after 24 h decreasing thereafter, whereas the exposure of hepatocytes to TGF- β_1 (2 ng/ml) did not exhibit any stimulant effect. When TGF- β_1 was added together with TGF- α , it prevented the TGF- α -induced [3 H]thymidine incorporation into hepatocytes, antagonizing the stimulatory effect of TGF- α .

Figure 1 Time-course of [3 H]thymidine incorporation into DNA by hepatocytes isolated from the adult rat. Monolayers were cultured in basal conditions (♦) for the first 24 h then in the presence of TGF- α (40 ng/ml ▲) or TGF- β_1 (2 ng/ml ▼) or both (■) as described under Materials and methods. Hepatocytes were pulsed for 2 h with [3 H]thymidine and collected at indicated times during the next 72 h in culture to evaluate DNA synthesis (11). Results are typical of five separate experiments, each experiment using triplicate culture dishes for each condition.

Figure 2 shows IGF-I production by adult rat hepatocytes cultured for 24 h in serum-free medium supplemented with varying doses of TGF- β_1 and/or TGF- α . No cytotoxic effects by any of the TGF concentrations employed, as judged by the Trypan blue exclusion test, were observed. As shown in the figure, IGF-I already

present in the conditioned medium of hepatocytes cultured in basal conditions was produced in significantly greater amounts when cells were cultured in the presence of TGF- β_1 . The increase in IGF-I production was positively correlated with the dose of TGF- β_1 in the range 0.5–2 ng/ml, final concentration. When

Figure 2 IGF-I and IGFBP production by rat hepatocytes cultured in the presence of TGF- α and/or TGF- β_1 . Cells were cultured for 24 h in serum-free medium containing 0.25% BSA and for an additional 24 h in the absence (control, C) or presence of increasing concentrations of TGF- α (10–40 ng/ml) and/or TGF- β_1 (0.5–2 ng/ml). Immunoreactive IGF-I (shaded bars) and IGF binding activity (open bars) accumulating in the culture media were analyzed as detailed in Materials and methods. Results are the means $\pm$ S.E.M. of triplicate samples obtained from five separate experiments. *** P = 0.001 compared with control (Bonferroni multiple t -test, Graphpad Instat software, San Diego, CA, USA).

hepatocytes were incubated for 24 h with varying doses of TGF- α (10–40 ng/ml) maximal IGF-I production occurred at a concentration of 40 ng/ml TGF- α . The effect of 40 ng/ml TGF- α was comparable to that elicited by 2 ng/ml TGF- β_1 . No further increase was observed with higher concentrations of TGF- α or TGF- β_1 (data not shown). When hepatocytes were supplemented with TGF- α (40 ng/ml) together with TGF- β_1 (2 ng/ml) the increase in IGF-I production was of the same extent as that observed in the presence of each. As Fig. 2 shows, IGF binding activity was evaluated at the same time. IGFBPs were already present in the conditioned medium of adult rat hepatocytes cultured in basal conditions and a stimulation was evident when TGF- α or TGF- β_1 was added to the culture medium at the same concentration at which maximal IGF-I production occurred. The effect of TGF- α on IGF binding activity was comparable to that elicited by TGF- β_1 .

Figure 3 shows the effect of TGF- α or TGF- β_1 on IGF-I production by rat hepatocytes cultured in the presence of cycloheximide, a protein synthesis inhibitor, or octreotide, a somatostatin analog known to directly inhibit the gene expression of IGF system components in the liver (23, 24). The doses of the drugs employed did not affect cell viability. It can be seen that the cycloheximide concentration (0.1 μ g/ml) able to inhibit protein synthesis in cultured hepatocytes by 90% (11) significantly depressed IGF-I production by hepatocytes cultured in basal medium. Moreover the presence

of cycloheximide completely abolished the increase in IGF-I production induced by the addition of TGF- α or TGF- β_1 to the culture medium. Basal secretion of IGF-I did not seem to be affected by the addition to the culture medium of octreotide (3 ng/ml) that significantly reduced IGF-I production in TGF- α - or TGF- β_1 -treated hepatocytes.

To check the possible effect on IGF-I and IGFBP genes we cultured hepatocytes up to 36 h in basal medium or in the presence of TGF- α (40 ng/ml) or TGF- β_1 (2 ng/ml), concentrations able to exhibit maximal increase of IGF-I production.

Figure 4 shows a representative Northern blot analysis of total RNA isolated from adult rat hepatocytes after 1 h of incubation with or without TGF- β_1 or TGF- α and hybridized with IGF-I and IGFBP-1, -2 and -4 probes. This figure shows the characteristic 1.6 kb IGFBP-1, 2.6 kb IGFBP-4 and 1.3 kb G3PDH messages expressed by rat hepatocytes within the time selected for the experiment. Major bands of hybridization to the rat IGF-I probe are evident at 7.0 and 1.7 kb, and in a broad but defined region between 0.8 and 1.2 kb.

IGF-I, IGFBP-1 and -4 messages were clearly expressed by rat hepatocytes cultured in basal medium while IGFBP-2 mRNA was not detected, in agreement with other authors (25). No effect was elicited by TGF- α or TGF- β_1 on IGF-I mRNA levels and on mRNAs encoding for IGFBP-1 and -4. No effect was exhibited even at the other incubation times in all conditions tested (data not shown).

Figure 3 Cycloheximide and octreotide effects on IGF-I accumulating in the culture medium of hepatocytes isolated from adult rat. Cells were cultured for 24 h in basal conditions and for an additional 24 h without (control, C) or with TGF- α (40 ng/ml) or TGF- β_1 (2 ng/ml) in serum-free medium containing cycloheximide (Cex, 0.1 μ g/ml) or octreotide (Octr., 3 ng/ml). Data are the means $\pm$ S.E.M. of triplicate samples obtained from five separate experiments. *** P = 0.001, * P = 0.05 (treated vs control, Bonferroni multiple t -test, Graphpad Instat Software).

Figure 4 Representative autoradiograms of Northern blots showing IGF-I and IGFBP-1 and -4 mRNAs in adult rat hepatocytes cultured for 1 h in the absence (lane 1) or presence of 40 ng/ml TGF- α (lane 2) or 2 ng/ml TGF- β_1 (lane 3). To ensure even loading the membranes were stripped and rehybridized with a human G3PDH cDNA probe.

Discussion

The results of this study demonstrate that TGF- α , able to induce mitogenic response, and TGF- β_1 , able to inhibit hepatocyte proliferation both *in vivo* and *in vitro*, greatly stimulate IGF-I production by cultured hepatocytes from the adult rat. The increase in IGF-I production was positively correlated with the dose of TGF- α and TGF- β_1 , the maximal stimulation being elicited at 40 and 2 ng/ml respectively. By comparing the specific potency of the doses of TGF- α (40 ng/ml) and TGF- β_1 (2 ng/ml) eliciting the same increase in IGF-I production by cultured hepatocytes it appears that TGF- β_1 is 100 times more potent on a molar basis with respect to TGF- α . However, their respective but not additive capacity to stimulate hepatocyte IGF-I production may indicate a final common mechanism of action involving an increased biosynthesis of IGF-I peptide. Further studies are needed to elucidate this point.

The effect of TGF- α and TGF- β_1 in liver cells represents *de novo* synthesis since it was completely abolished by the presence of cycloheximide. Interestingly TGF- α - and TGF- β_1 -stimulated IGF-I production by cultured hepatocytes was significantly reduced by octreotide, the somatostatin analog able to inhibit specifically hepatic IGF-I expression.

The increased TGF- α - and TGF- β_1 -stimulated accumulation of IGF-I and IGFBPs in the culture medium was not apparently supported by any change in IGF-I and IGFBPs mRNA levels, which is different from what happens in EGF-treated hepatocytes (11). It could be possible that in TGF-treated hepatocytes an increase of IGF-I mRNA corresponds to a similar rate of degradation, therefore the message steady-state level seems

unchanged. On the other hand, the growth factor regulation could occur at a translational level or through a stimulated secretion of the peptide stored in the cells. The latter hypothesis seems less likely because it conflicts with the data reporting the absence of IGF-I intracellular stores (9). Since there is substantial evidence that it is the balance between IGF-I and IGFBP levels which determines the free IGF-I available for interaction in a given target tissue, we reported that an increase of IGF binding activity occurred when hepatocytes were cultured in the presence of TGF- α or TGF- β_1 demonstrating a similar synthesis and availability of IGF-I.

What is the physiological significance of TGF- α - and TGF- β_1 -enhanced IGF-I production by hepatocytes? Regarding the TGF- α -stimulated IGF-I increase, since it occurred before the peak of DNA synthesis a possible role of IGF-I in hepatocyte proliferation could be envisaged. However, direct treatment of hepatocytes with IGF-I did not stimulate DNA synthesis, in agreement with a previous report (11). Furthermore type I IGF receptors considered responsible for IGF-I-induced mitogenic action are not present in hepatocytes from adult rat liver (12). Only in the case of regenerating rat liver, when IGF-I receptors are expressed on hepatocyte cell membrane, could autocrine and paracrine interaction of the growth factor with its own receptor stimulate liver proliferation. However, data are not available supporting a clear-cut role for IGF-I in the regulation of liver growth.

TGF- β_1 is a pluripotential polypeptide acting in an extremely diverse array of biological activities. It is a potent modulator of IGF-I production in mouse bone cells where IGF-I is thought to act in the local regulation of bone remodeling (26). As far as liver is concerned, TGF- β_1 has been postulated to play a role in fibrogenesis related to liver disease (27). A role, if any, in normal adult hepatocytes has not been elucidated yet. In this study we demonstrate that TGF- β_1 increases IGF-I production but does not stimulate S phase, confirming that IGF-I itself does not necessarily lead hepatocytes to undergo DNA synthesis as occurs for other cell types (28). In such conditions TGF- β_1 , which is produced by non-parenchymal cells, could represent a physiological modulator of IGF-I production acting via a paracrine mechanism.

In conclusion, our observations, even if they do not elucidate the physiological significance of the TGF- α - and TGF- β_1 -increased IGF-I production, add further evidence to the idea that the central role of the liver in the secretion of IGF-I is not only under endocrine and nutritional control but also under autocrine and paracrine control.

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