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Effect of epidermal growth factor on insulin-like growth factor-I (IGF-I) and IGF-binding protein synthesis by adult rat hepatocytes

A. Barreca [a], A. Voci [b], F. Minuto [c], M. de Marchis [b], E. Cecchelli [a], E. Fugassa [b], G. Giordano [a] and G. Gallo [d]

Cattedra di [a] Endocrinologia and [c] Fisiopatologia Endocrina, DiSEM, and [b] Istituto di Fisiologia Generale, University of Genova, Genua, Italy, and [d] Cattedra di Fisiologia Generale, University of Urbino, Urbino, Italy

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Summary

Growth hormone has been established as a primary regulator of IGF-I gene expression in adults, not only in liver but also in many extrahepatic tissues. We considered the possibility that IGF-I production by adult rat liver could also be stimulated by epidermal growth factor (EGF), a peptide known to be involved in liver regeneration. Chromatographic analysis performed after acid treatment of conditioned media revealed the presence of both immunoreactive (IR) IGF-I and IGF binding protein (IGFBP). Both IR IGF-I and IGFBP were present in the conditioned medium of adult rat hepatocytes in basal conditions. The stimulation of IGF-I and IGFBP secretion by EGF appears to be dose-dependent with a significant increment already evident at 5 nM. That EGF stimulates secretion is supported by the finding that IGF-I and IGFBP-1 mRNA levels are increased after EGF supplementation. We conclude that adult rat hepatocytes spontaneously produce IGF-I and IGFBP, and that EGF is able to increase their synthesis and secretion. This non-growth hormone-dependent regulation of IGF-I and IGFBP-1 production by adult rat hepatocytes in culture indicates an important autocrine/paracrine role for IGF-I, particularly during liver regeneration after extensive organ mass loss.

Introduction

Although growth hormone has been established as a primary regulator of IGF-I gene expression in adults, not only in liver but also in many extrahepatic tissues (Mathews et al., 1986; Roberts et al., 1986, 1987; Hynes et al., 1987), other growth factors, i.e. epidermal growth factor (EGF), fibroblast growth factor (FGF), and platelet-derived growth factor (PDGF) have been found to stimulate IGF-I production by several cells (Clemmons et al., 1981; Clemmons, 1984, 1985). Production of both IGF and IGF binding proteins (IGFBP) by liver has been reported by many authors (Moses et al., 1979; Schalch et al., 1979; Spencer, 1979; Binoux et al., 1982; Minuto et al., 1984; Scott et al., 1985). Recently, growth hormone (GH) and insulin have been shown to

Correspondence to: A. Barreca, Cattedra di Endocrinologia, DiSEM, University of Genova, I-16132 Genua, Italy.

stimulate accumulation of IGF-I mRNA transcripts (Scott et al., 1985; Norstedt and Moeller, 1987; Johnson et al., 1989) as well as IGF-I secretion in culture medium of adult rat hepatocytes. Three structurally distinct but homologous IGFBP have now been characterized in terms of nucleotide sequence and molecular structure (Martin and Baxter, 1986; Mottola et al., 1986; Brinkman et al., 1988; Lee et al., 1988; Wood et al., 1988; Luthman et al., 1989). Two of these are non-glycosylated forms (IGFBP-1 and IGFBP-2). IGFBP-1 levels are inversely related to insulin and GH concentration (Drop et al., 1984; Povoa et al., 1984; Suikkari et al., 1988, 1989; Hardouin et al., 1989), whereas a glycosylated form (IGFBP-3) is GH-dependent (Moses et al., 1976; Furlanetto, 1980).

Although EGF has been widely used to stimulate DNA synthesis in cultured hepatocytes, its role in normal and regenerating liver is not very clear. It has been established that hepatocytes actively sequester injected EFG by means of a large number of receptors for this growth factor (Michalopoulos, 1990), and that regenerating hepatocytes actively produce transforming growth factor-α (TGF-α) (Mead and Fausto, 1989), a polypeptide known to act through the EGF receptor. In these conditions, the number of EGF receptors and the EGF-dependent tyrosine kinase activity are reduced by down-regulation (Rubin et al., 1982; Raper et al., 1987), thus supporting a role for EGF in initiating liver regeneration (Earp and O'Keefe, 1981).

As human fibroblasts and fetal rat hepatocytes are responsive to the stimulatory effect of EGF on IGF production (Clemmons, 1984; Richman et al., 1985), we wanted to ascertain whether adult rat hepatocytes are still responsive to this stimulatory effect, and whether any of the powerful mitogenic action of EGF on liver could be mediated by IGF-I.

Materials and methods

Cell culture

Hepatocytes were isolated from male Wistar rats (180–200 g) as previously described (Fugassa et al., 1983) and 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 of Eagle's medium (DMEM), insulin (0.1 U/ml) and dexamethasone (10^{-7} M); 3 ml of cell suspension were plated on 60 mm collagenated tissue dishes and, after 90 min, DMEM containing 0.25% bovine serum albumin (BSA) was replaced for the incubation medium. After 24 h of culture, monolayers were exposed to EGF (EGF-treated) or vehicle alone (control).

Separation of cell-derived IGF-I-like immunoreactivity from IGF binding proteins

The conditioned media of hepatocytes (20 ml; 2×10^5 cells/ml) were acidified with 0.5 M acetic acid-0.15 M NaCl (pH of the mixture was < 3), and gel-filtered on a Sephadex G-50 column (1.6 $\times$ 90 cm) equilibrated with 0.1 M acetic acid-0.15 M NaCl, pH 2.75. Fractions corresponding to 0–0.20, 0.21–0.40, 0.41–0.70 and 0.71–1 K_{av} were pooled, lyophilized, reconstituted in phosphate-buffered saline and analyzed for IGF-I immunoreactivity and IGF-I specific binding activity.

IGF-I radioimmunoassay and IGF binding protein assay

IGF-I was measured by radioimmunoassay using antibodies and [^{125}I]IGF-I provided by the Nichols Institute (San Juan Capistrano, CA, USA); the standard curve was performed by recombinant Met-IGF-I. The sensitivity of the assay is 90 pg/ml and the between-assay coefficient of variation is 7.5%.

Specific binding of IGF-I was measured by overnight incubation at 4°C of 0.1 ml samples with about 20,000 cpm of [^{125}I]Thr^{59}-IGF-I (Amersham, UK), in 0.05 M Na phospate buffer, pH 7.4, containing 2.5 g/l BSA in a final volume of 0.5 ml. Protein-bound IGF-I was separated by addition of 0.5 ml of a 20 g/l charcoal suspension in assay buffer containing 20 g/l BSA, and centrifugation at $3000 \times g$ for 20 min in a refrigerated centrifuge. Specific binding was determined in each sample as the difference between the radioactivity bound both in the absence and in presence of a saturating amount of partially purified IGF-I.

[^{3}H]Thymidine and [^{3}H]leucine incorporation studies

Hepatocytes, cultured in DMEM containing 0.25% BSA (control) or test substances (EGF, IGF-I), were labeled with [^{3}H]thymidine (5 μCi/ml, New England Nuclear, Dreieich, Germany). Incubations were carried out at 37°C for 2 h, and measurement of DNA synthesis was done according to McGowan et al. (1981).

Hepatocytes cultured as above were labeled for 1 h with 2 μC/ml [^{3}H]leucine (40 Ci/mmol, New England Nuclear).

The incorporation of the labeled precursors into acid-insoluble material was evaluated as previously described (Gallo et al., 1987). Results are expressed as percent of the incorporation (dpm/μg of DNA for [^{3}H]thymidine or dpm/mg of protein for [^{3}H]leucine) recorded in the absence of test substances.

RNA isolation and Northen blot analysis

At indicated times, cells were rapidly frozen in liquid nitrogen and total RNA was prepared using the guanidinium isothiocyanate/cesium chloride method (Chirgwin et al., 1979). The integrity of each RNA sample was verified by gel electrophoresis and the quantity determined spectrophotometrically. Samples were stored at −80°C until used.

Total RNA (20 μg) was fractionated in a 1%

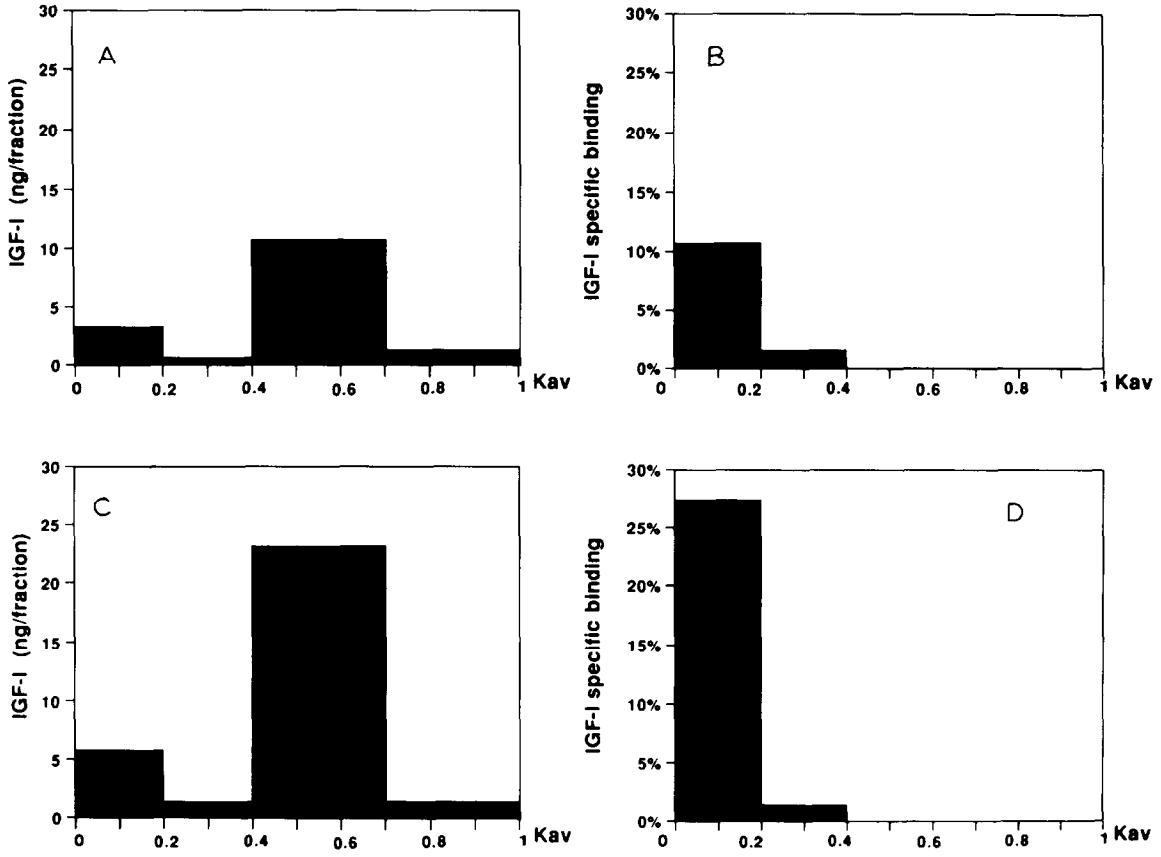

Fig. 1. Sephadex G-50 elution profiles of immunoreactive IGF-I and IGF-I binding capacity in conditioned media of adult rat hepatocytes. Cells were cultured for 24 h in serum-free medium containing 0.25% BSA and for an additional 48 h in medium without (panels *A* and *B*) or with (panels *B* and *D*) 10 nM of EGF. The conditioned media were acidified and gel-filtered on a Sephadex G-50 column equilibrated with 0.1 M acetic acid-0.15 M NaCl. Fractions corresponding to 0–0.20, 0.21–0.40, 0.41–0.70 and 0.71–1 K_{av} were pooled, lyophilized, and reconstituted in phosphate-buffered saline for IGF-I and IGF-I-specific binding capacity analysis.

agarose gel containing 2.2 M formaldehyde in 20 mM Mops, pH 7.0, 5 mM sodium acetate and 1 mM Na_2EDTA. After transfer to nylon membrane by capillary blotting, prehybridization was performed for 2 h at 42°C in a solution containing 5 × SSPE (1 × SSPE = 0.15 M NaCl, 0.01 M NaH_2PO_4, 1 mM EDTA), 50% formamide, 5 × Denhardt's solution, 0.5% sodium dodecyl sulfate (SDS) and 100 g/ml denaturated salmon sperm DNA (Sambrook et al., 1989). Hybridization was carried out for 20 h at 42°C in fresh buffer containing ^{32}P-labeled probes (rat IGF-I cDNA probe; human IGFBP-1, IGFBP-2 and IGFBP-3). Probes were labeled to high specific activity by Multiprime DNA labeling systems (Amersham, UK) using [^{32}P]dCTP (specific activity > 3000 Ci/mmol, NEN, Germany). After hybridization the filters were washed for 10 min twice in 2 × SSPE and 0.1% SDS and twice in 0.1 × SSPE and 0.1% SDS at 42°C. Filters were then exposed to Amersham Hyperfilm-MP films at −80°C using intensifying screens. Autoradiograms were scanned using a Hoefer GS-300 densitometer controlled by an IBM AT personal computer. Equivalent amounts of RNA/lane were assessed by monitoring 28 S and 18 S rRNA in ethidium bromide stained parallel gels.

Results

Distribution of immunoreactive IGF-I and IGF binding activity in conditioned media

Chromatographic analysis performed after acid treatment of conditioned media revealed the presence of two peaks of IGF-I-like immunoreactivity (Fig. 1). The first peak was located close to the void volume (K_{av} 0–0.20), corresponding to a higher molecular weight than that of IGF peptides. The second peak corresponded to the elution volume (K_{av} 0.41–0.70) of IGF-I. The first peak also showed specific IGF binding activity, which suggests that the immunoreactivity found in the high molecular weight region is due at least in part to the interference in the radioimmunoassay (RIA) by the free binding sites.

Effect of EGF on IGF-I and IGFBP production by adult rat hepatocytes

The increase in hepatocyte-derived IGF-I, after the addition of 10 nM of EGF, began after 24 h and was still evident at 48 h (Fig. 2).

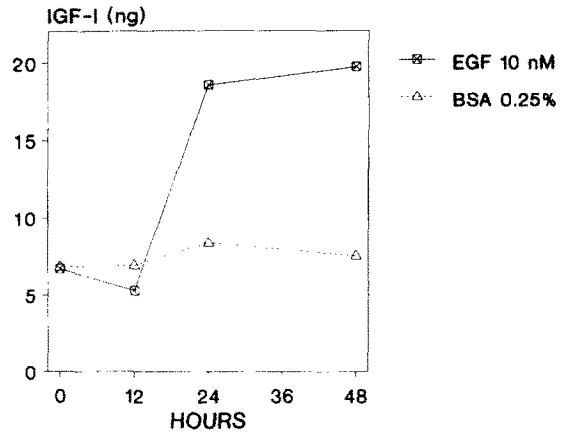

Fig. 2. Effect of EGF on IGF-I accumulated in the culture medium as a function of time in culture. Adult rat hepatocytes were cultured for 24 h in serum-free medium containing 0.25% BSA and for an additional 48 h in medium with or without (control) 10 nM of EGF.

Both IR IGF-I and IGFBP were present in the conditioned medium of adult rat hepatocytes in basal conditions; the addition of cycloheximide (0.1 μg/ml) was able to reduce the basal and EGF-stimulated IGF-I and IGFBP.

The stimulation of IGF-I and IGFBP secretion by EGF appears to be dose-dependent with a significant increment already evident at 5 nM (Fig. 3).

Effect of EGF on IGF-I and IGFBP RNA expression in adult rat hepatocytes

Since EGF was shown to clearly affect the production of IGF-I and IGFBP peptides, the relative RNA levels from EGF-treated and control hepatocytes were analyzed by Northern blot analysis. Fig. 4 shows the levels of IGF-I RNA in hepatocytes cultured with EGF. 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. The EGF-increased IGF-I production was supported by the finding that the IGF-I RNA level was 87% higher 3 h after EGF supplementation. The EGF-stimulated expression of IGF-I gene was no more evident after 10 h. Similarly, the level of RNA encoding for IGFBP-1 was increased 1–2 h after

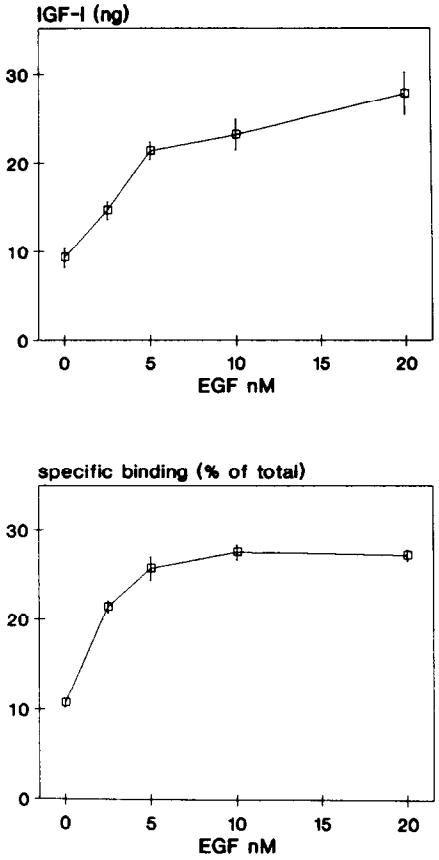

Fig. 3. Effect of EGF on IGF-I (upper panel) and IGFBP (lower panel) accumulated in the culture medium as a function of peptide concentration. Adult rat hepatocytes were cultured for 24 h in serum-free medium containing 0.25% BSA and for an additional 48 h in medium with different concentrations of EGF.

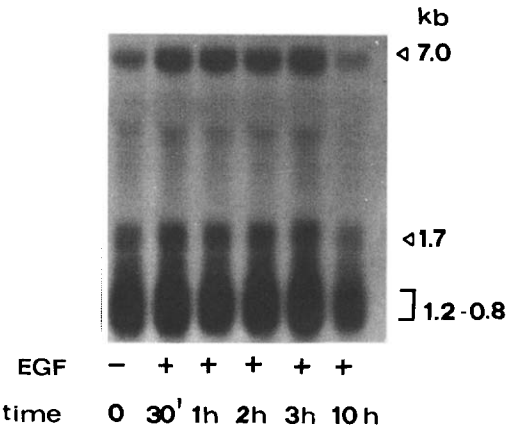

Fig. 4. Autoradiogram of Northern blot of total RNA hybridized with IGF-I cDNA probe. Total RNA samples were prepared from hepatocytes treated with EGF for different time periods.

EGF supplementation and declined to the control level within 4 h (Fig. 5). In contrast, we were unable to show any effect of EGF on the amount of IGFBP-2 RNA. In our conditions, RNA encoding for IGFBP-3 was undetectable both in control and in EGF-treated hepatocytes (data not shown).

Comparison between the effects of EGF and IGF-I on isolated hepatocytes

We then tested whether adult rat hepatocytes undergo DNA synthesis following exposure to IGF-I. As shown in Fig. 6 (upper panel), exposure of hepatocytes to synthetic IGF-I did not increase [^{3}H]thymidine incorporation at any tested concentration. EGF produced a dose-dependent increase in the dose range of 2–20 nM. Moreover, the effect of EGF was not counteracted by 100 nM of Sm 1.2, a specific anti-IGF monoclonal antibody (data not shown). In our system insulin (up to 0.5 μM) also did not appear to be stimulatory by itself, but it did have a powerful synergistic effect on the EGF stimulation of [^{3}H]thymidine incorporation (data not shown).

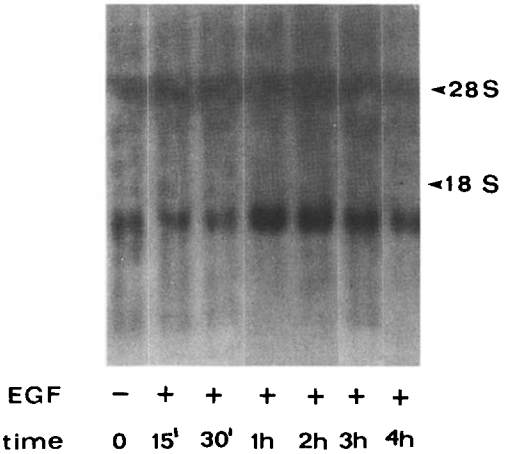

Fig. 5. Autoradiogram of Northern blot of total RNA hybridized with IGFBP-1 cDNA probe. Total RNA samples were prepared from hepatocytes treated with EGF for different time periods.

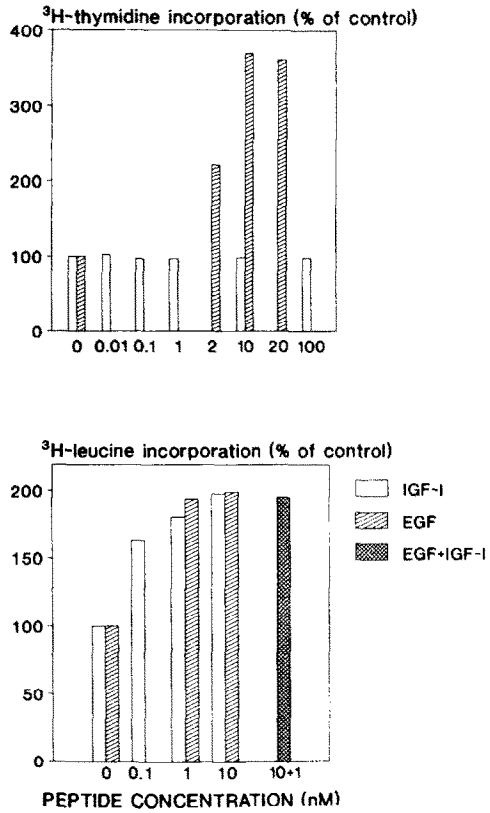

Fig. 6. Comparison between the EGF and IGF-I effects on isolated hepatocytes. Hepatocytes were cultured in the absence (control) or presence of different concentrations of test substances (EGF, IGF-I); incubations, measurement of DNA synthesis (upper panel) and incorporation of the labeled leucine (lower panel) were carried out as described in Materials and methods. Results are expressed as percent of the incorporation recorded in the absence of test substances.

In contrast, a stimulatory effect of IGF-I was evident on [^{3}H]leucine incorporation in a dose range comparable to that of EGF (Fig. 6, lower panel). The effect of maximal stimulatory concentrations of IGF-I and EGF was not additive.

Discussion

The results show that in basal conditions hepatocytes are able to produce IGF-I, and that EGF has a dose-dependent stimulatory effect on IGF-I production. That EGF increases IGF-I production is supported by the finding that the IGF-I RNA level was increased by EGF supplementation (Fig. 4).

While the expression of both IGFBP-1 and 2 has been demonstrated in liver using RNA hybridization techniques (Brinkman et al., 1988; Murphy et al., 1991), no data are yet available on the occurrence of mRNA encoding for IGFBP-3 in rat hepatocytes. In humans, the finding that serum levels of IGFBP-3 are very low in patients with liver cirrhosis (Blum et al., 1989) suggests that the binding component of the 150 kDa complex originates primarily in the liver. However, in the conditions we used, we were unable to demonstrate that adult rat hepatocytes express IGFBP-3. As it has been demonstrated that IGFBP-3 is produced by fibroblasts (Adams et al., 1984; Barreca et al., 1988; Martin and Baxter, 1988), it is possible that its production is due to the non-epithelial components of liver. The finding that IGFBP-1 expression in hepatocytes appears to be modulated by EGF (Fig. 5) indicates that this growth factor has a direct effect only on the synthesis of this IGFBP, although an EGF-stimulated secretion of intracellularly stored IGFBP-2 cannot be ruled out. In view of the fact that IGFBPs have been mostly reported to have a modulatory effect on IGF autocrine/paracrine actions, it seems noteworthy that the EGF stimulation of IGF-I production by hepatocytes is accompanied by a similar direct effect on IGFBP-1.

As for the role of IGF-I on the adult rat liver, it is interesting to note that, unlike EGF, which greatly enhances [^{3}H]thymidine incorporation by hepatocytes, IGF-I does not seem to exert any mitogenic activity on these cells (Fig. 6). This result is in agreement with the finding that, unlike fetal hepatocytes, adult hepatocytes have no type I receptors for IGF peptides (Caro et al., 1988), except during regenerating conditions. This and the finding that insulin alone, at high concentration, has no mitogenic effect suggest that the proliferative effect of IGF peptides and of insulin on hepatocytes is mainly exerted through type I IGF receptors. It seems likely that, when type I IGF receptors are expressed in the cell membrane, an autocrine/paracrine interaction of the peptide with its own receptor can take place, thus increasing the proliferation rate of regenerating liver cells. As liver is the main producer of IGF-I (Schwander et al., 1983), then the lack of IGF-I mitogenic effect on postnatal hepatocytes pro-

vides support for the hypothesis of an endocrine action of hepatic IGF-I on statural growth. On the other hand, a metabolic effect of IGF-I on liver has been described (Widmer et al., 1983; Parkes et al., 1986). This is in keeping with our data on the stimulatory action of IGF-I on amino acid uptake by hepatocytes. As type II IGF receptors and the insulin receptors are present in liver cell membranes (Krett et al., 1987; Scott and Baxter, 1987; Hartshorn et al., 1989), the stimulation of protein synthesis probably occurs by the interaction of IGF-I with these two receptors.

Although a delayed liver regeneration in hypophysectomized rats can be restored by GH administration (Uthne and Uthne, 1979), it is interesting that a non-GH-dependent regulation of IGF-I production by EGF can occur in adult liver and that this is not restricted to fetal liver. The non-GH-dependent regulation supplies a likely explanation for normal growth in the fetus, when pituitary hormones are absent, while in the adult it indicates an important autocrine/paracrine role for IGF-I, particularly during liver regeneration after extensive organ mass loss.

In conclusion, we have shown that a non-GH-dependent regulation of IGF-I and IGFBP-1 production by adult rat hepatocytes in culture can occur. The possibility of a local stimulatory effect on liver IGF-I and IGFBP biosynthesis during liver regeneration is supported by the finding that, in this condition, the TGF-α liver content is increased (Mead and Fausto, 1989). As this factor acts through the EGF receptor, it seems likely that an increased liver TGF-α level can induce an increased IGF-I synthesis, which may in turn be involved in hepatic regeneration.

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References

Adams, S.O., Kapadia, M., Mills, B. and Daughday, W.A. (1984) Endocrinology 115, 520–526.

Barreca, A., Del Monte, P., Vargiu, I., Cariola, G. and Minuto, F. (1988) J. Endocrinol. Invest. 11 (Suppl. 2), 109.

Binoux, M., Lassarre, C. and Hardouin, N. (1982) Acta Endocrinol. 99, 422–430.

Brinkman, A., Groffen, C., Kortleve, D.J., Geurts van Kessel, A. and Drop, S.L.S. (1988) EMBO J. 7, 2417–2423.

Blum, W.F., Ranke, M.B., Jenne, E., Kietzmann, K. and Bierich, J.R. (1989) in Hormonal Regulation of Growth, Vol. 58 (Frish, H. and Thorner, M.O., eds.), pp. 153–170, Raven Press, New York.

Caro, J.F., Poulos, J., Ittoop, O., Pories, W.J., Flickinger, E.G. and Sinha, M. (1988) J. Clin. Invest. 81, 976–981.

Chirgwin, J.M., Przybyla, A.E., MacDonald, R.J. and Rutter, W.J. (1979) Biochemistry 18, 5294–5299.

Clemmons, D.R. (1984) J. Clin. Endocrinol. Metab. 58, 850–856.

Clemmons, D.R. (1985) Circ. Res. 56, 418–426.

Clemmons, D.R., Underwood, L.E. and Van Wyk, J.J. (1981) J. Clin. Invest. 67, 10–19.

Drop, S.L.S., Kortleve, D.J., Guyda, H.J. and Posner, B.I. (1984) J. Clin. Endocrinol. Metab. 59, 908–915.

Earp, H.S. and O'Keefe, E.J. (1981) J. Clin. Invest. 67, 1580–1583.

Fugassa, E., Gallo, G., Voci, A. and Cordone, A. (1983) In Vitro 19, 299–306.

Furlanetto, R.W. (1980) J. Clin. Endocrinol. Metab. 51, 12–19.

Gallo, G., Voci, A., Schwarze, P.E. and Fugassa, E. (1987) J. Endocrinol. 113, 173–177.

Hardouin, S., Gourmelen, M., Noguiez, P., Seurin, D., Roghani, M., Bouc, J.L., Povoa, G., Merimee, T.J., Hossenlopp, P. and Binoux, M. (1989) J. Clin. Endocrinol. Metab. 69, 1291–1301.

Hynes, M.A., Van Wyk, J.J., Brooks, P.J., D'Ercole, A.J., Jansen, M. and Lund, P.K. (1987) Mol. Endocrinol. 1, 233–242.

Hartshorn, M.A., Scott, C.D. and Baxter, R.C. (1989) J. Endocrinol. 121, 221–227.

Johnson, T.R., Blossey, B.K., Denko, C.W. and Ilan, J. (1989) Mol. Endocrinol. 3, 580–587.

Krett, N.L., Heaton, J.H. and Gelehrter, T.D. (1987) Endocrinology 120, 483–490.

Lee, Y.L., Hintz, R.L., James, P.M., Lee,. P.D.K., Shively, J.E. and Powell, D.R. (1988) Mol. Endocrinol. 2, 404–411.

Luthman, H., Soderling-Barros, J., Persson, B., Engberg, C., Stern, I., Lake, M., Franzen, A., Israelsson, M., Raden, B., Lindgren, B., Hjelmqvist, L., Enerback, S., Carlsson, P., Bjursell, G., Povoa, G., Hall, K. and Jornvall, H. (1989) Eur. J. Biochem. 225, 273–278.

Martin, J.L. and Baxter, R.C. (1986) J. Biol. Chem. 261, 8754–8760.

Martin, J.L. and Baxter, R.C. (1988) Endocrinology 123, 1907–1915.

Mathews, L.S., Norstedt, G. and Palmiter, R.D. (1986) Proc. Natl. Acad. Sci. USA 83, 9343–9347.

McGowan, J.A., Sprein, A.J. and Bucher, N.L.R. (1981) J. Cell. Physiol. 108, 353–363.

Mead, J.E. and Fausto, N. (1989) Proc. Natl. Acad. Sci. USA 86, 1558–1562.

Michalopoulos, G.K. (1990) FASEB J. 4, 176–187.

Minuto, F., Barreca, A., Del Monte, P., Buffa, C., Bevevino, R., Montale, F. and Giordano, G. (1984) Horm. Metab. Res. 12, 619–623.

Moses, A.C., Nissley, S.P., Cohen, K.L. and Rechler, M.M. (1976) Nature 263, 137–140.

Moses, A.C., Nissley, S.P., Passamani, J., White, R.M. and Rechler, M.M. (1979) Endocrinology 104, 536–546.

Mottola, C., MacDonald, R.G., Brackett, J.L., Mole, J.E., Anderson, J.K. and Czech, M.P. (1986) J. Biol. Chem. 261, 11180–11188.

Murphy, L.J., Seneviratne, C., Moreira, P. and Reid, R.E. (1991) Endocrinology 128, 689–696.

Norstedt, G. and Moeller, C. (1987) J. Endocrinol. 115, 135–139.

Parkes, J.L., Cardell, R.R. and Grieninger, G. (1986) Biochem. Biophys. Res. Commun. 134, 427–435.

Povoa, G., Roovete, A. and Hall, K. (1984) Acta Endocrinol. 107, 560–570.

Raper, S.E., Burwen, S.J., Barker, M.E. and Jones, A.L. (1987) Gastroenterology 92, 1243–1250.

Richman, R.A., Benedict, M.R., Florini, J.R. and Toly, B.A. (1985) Endocrinology 116, 180–188.

Roberts, Jr., C.T., Brown, A.L., Graham, D.E., Seeling, S., Berry, S., Gabbay, K.H. and Rechler, M.M. (1986) J. Biol. Chem. 261, 10025–10028.

Roberts, Jr., C.T., Lasky, S.R., Lowe, Jr., W.L., Seaman, W.T. and Leroith, D. (1987) Mol. Endocrinol. 1, 243–248.

Rubin, R.A., O'Keefe, E.J. and Earp, H.S. (1982) Proc. Natl. Acad. Sci. USA 79, 776–780.

Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd edn., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Schalch, D.S., Heinrich, U.E., Draznin, B., Johnson, C.J. and Miller, L.L. (1979) Endocrinology 104, 1143–1151.

Schwander, J.C., Hauri, C., Zapf, J. and Froesch, E.R. (1983) Endocrinology 113, 297–305.

Scott, C.D. and Baxter, R.C. (1987) Endocrinology 120, 1–9.

Scott, C.D., Martin, J.L. and Baxter, R.C. (1985) Endocrinology 116, 1094–1101.

Spencer, E.M. (1979) FEBS Lett. 99, 157–161.

Suikkari, A.M., Koivisto, V.A., Rutanen, E.M., Yki-Jarvinen, H., Karonen, S.L. and Seppala, M. (1988) J. Clin. Endocrinol. Metab. 66, 266–272.

Suikkari, A.M., Koivisto, V.A., Koistinen, R., Seppala, M. and Yki-Jarvinen, H. (1989) J. Clin. Endocrinol. Metab. 68, 135–140.

Uthne, K. and Uthne, T. (1979) Acta Endocrinol. 71, 255–261.

Widmer, U., Schmid, Ch., Zapf, J. and Froesh, E.R. (1985) Acta Endocrinol. 108, 237–244.

Wood, W.I., Cachianes, G., Henzel, W.J., Winslow, G.A., Spencer, S.A., Hellmiss, R., Martin, J.L. and Baxter, R.C. (1988) Mol. Endocrinol. 2, 1176–1185.