Volume 303, number 2,3, 205–209 FEBS 11084 June 1992

Differential regulation of insulin-like growth factor binding protein (IGFBP)-2 mRNA in liver and bone cells by insulin and retinoic acid in vitro

Ch. Schmid[a], I. Schläpfer[a], M. Waldvogel[a], P.J. Meier[b], J. Schwander[c], M. Böni-Schnetzler[a], J. Zapf[a] and E.R. Froesch[a]

Division of [a]Endocrinology and Metabolism and Division of [b]Pharmacology, Department of Internal Medicine, University Hospital, CH-8091 Zürich, Switzerland and *[c]Department of Internal Medicine, University Hospital, CH-4031 Basel, Switzerland*

Received 7 April 1992

Isolated cells produce insulin-like growth factors (IGFs) and their binding proteins (IGFBPs). Two distinct cell types were studied with regard to IGFBP-2 expression: (i) rat hepatocytes, which produce IGF I at a high rate and thus regulate its plasma concentration; and (ii) rat osteoblasts, which are targets of IGF I action. IGFBP-2 expression is low in hepatocytes prepared from normal adult rats and high in calvaria cells from newborn rats. Retinoic acid stimulates IGFBP-2 production by liver cells. Insulin suppresses both basal and retinoic acid-induced IGFBP-2 mRNA expression in hepatocytes and has no such effect on osteoblasts. Retinoic acid and insulin regulate IGFBP-2 expression in a tissue-specific manner.

Insulin-like growth factor binding protein-2; Insulin; Retinoic acid; Hepatocyte; Osteoblast

1. INTRODUCTION

Insulin-like growth factors (IGFs) and their binding proteins (IGFBPs) are produced by many tissues. Their synthesis appears to be regulated by nutritional, local and endocrine factors [1]. As a result of their affinities to IGFs, IGFBPs regulate tissue distribution, half-life and actions of IGFs [2].

The liver is the main source of serum IGF I [3]. In contrast hepatocytes do not appear to produce IGFBP-3, the predominant IGFBP of serum [4,5]. It is still unclear whether the same cell type secretes IGFs and IGFBPs in parallel or whether IGFs and IGFBPs are released by distinct cell types. Thus it is of interest to study regulation of both IGF and IGFBP expression. We have previously analysed IGFBPs of conditioned media by [^{125}I]IGF II ligand blotting, a method which detects a variety of IGFBPs. Hepatocytes from adult rats released IGFBPs which are distinct from IGFBP-3; the release of these (unidentified) IGFBPs was suppressed by insulin [2,4,6]. Insulin inhibits the expression of IGFBP-1 mRNA and the release of the corresponding protein [7,8]. However, it has not yet been determined by the use of specific antibodies whether or not insulin also inhibits IGFBP-2 release by normal adult rat hepatocytes.

In contrast to liver cells osteoblasts express type-I IGF receptors and are important target cells of IGF action. In addition bone cells produce low amounts of IGFs which may act locally, i.e. as autocrine/paracrine factors. We found that calvarial osteoblasts constitutively expressed much higher IGFBP-2 mRNA levels than cultured liver cells. Triiodothyronine increases IGFBP-2 mRNA expression in osteoblasts, while retinoic acid (RA) has the opposite effect [9]. These observations prompted us to compare the regulation of IGFBP-2 mRNA levels in hepatocytes and osteoblasts and to evaluate the relative IGFBP-2 levels in the medium conditioned by these cells using immunoblot analysis.

Insulin has important effects on liver cells via its own receptors; it was also tested for effects on osteoblasts. Insulin shares with IGFs an affinity for the type-I IGF receptor but not for IGFBPs. Thus it may well affect gene expression in osteoblasts but is less likely to interfere with the secreted IGFBPs.

Retinoic acid (RA) was used as a model compound to modulate gene expression. It has been shown that RA stimulates the expression of the phosphoenolpyruvate carboxykinase gene in liver cells [10]; the activity of the corresponding enzyme, which is important for hepatic gluconeogenesis, is down-regulated by insulin. RA is teratogenic and has important effects in embryogenesis and cell differentiation; RA modulates a variety of

Correspondence address: Ch. Schmid, Division of Endocrinology and Metabolism, Department of Internal Medicine, University Hospital, CH-8091 Zürich, Switzerland. Fax: (41) (1) 255 4447.

mRNA levels in osteoblasts [11]. Thus both hepatocytes and osteoblasts respond to RA.

2. MATERIALS AND METHODS

2.1. *Reagents*

Human monocomponent insulin was a gift from Novo-Nordisk (Gentofte, DK); it was dissolved in 0.004 N HCl to 100 μg per ml, then further diluted in albumin-containing cell culture medium (see below). Recombinant human IGF I was a gift from Ciba-Geigy, Basel; it was dissolved in 0.1 M acetic acid at 1 mg per ml. All-*trans*-retinoic acid (RA) was purchased from Sigma, dissolved at a concentration of 2×10^{-3} M in absolute ethanol in subdued light just prior to use, and kept in the dark. The aliquots of the stock solutions were diluted in cell culture medium to give a final concentration of 0.05% ethanol. The ethanol concentration in the control cultures was adjusted to 0.05%. Human serum albumin (HSA) was purchased from the Swiss Red Cross and was treated with charcoal to remove fatty acids and steroid hormones [12].

2.2. *Cell culture*

Hepatocytes were prepared from 200 g male adult Sprague–Dawley rats by perfusing the liver in situ with bacterial collagenase as described previously [13] and plated (day 0) at a density of 7×10^4 cells/cm^2 on type-I collagen-coated (Serva, Heidelberg, GFR) dishes (6 cm diameter, Falcon Primaria) in Williams E medium containing fetal calf serum (FCS, 10%), glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 μg/ml) (all from Gibco), insulin and dexamethasone (both 10^{-7} M). 3 h later cells attached, and the medium was replaced with fresh medium containing insulin and dexamethasone (10^{-7} M each) but no FCS. The following day media were replaced with serum- and hormone-free medium and incubated for 60 min at 37°C before adding test medium, i.e. Williams E medium containing 0.2 g/l HSA.

Osteoblast-like cells were prepared from newborn rat calvaria and grown in medium containing 5% FCS as described elsewhere [14]. 24 h prior to starting the test period the cells were rinsed with serum-free medium and kept in Williams E medium containing insulin and dexamethasone (both 10^{-7} M) and were subsequently treated as described for the hepatocytes.

2.3. *Extraction of total RNA and Northern analysis*

2.3.1. RNA preparation

Upon removal of the culture media for IGFBP analysis (see below) cells were quickly rinsed 3 times with ice-cold PBS, and collected in PBS by scraping them from the dishes with a rubber policeman (the material from triplicate wells was pooled) and centrifugation for 10 min at 4°C. The pellets were lysed in guanidinium isothiocyanate (BRL, Gaithersburg, USA) for preparation of total RNA according to Chirgwin [15], and concentrations were measured by measuring UV absorbance at a wave length of 260 nm. RNA was stored at −80°C until assayed.

2.3.2. Northern blots

RNA was denatured (15 min at 65°C) and equal aliquots (20 μg/lane) were size-fractionated in formaldehyde-containing 1% (w/v) agarose gels, transferred to Nylon filters (Hybond-N, Amersham) by capillary blotting, and fixed by UV-crosslinking according to standard procedures.

2.3.3. cDNA probes

The following cDNAs were used: IGFBP-2 [16], alkaline phosphatase (liver/bone/kidney type; [17]), IGF I [18] and β-tubulin [19] cDNA. Inserts were excised from the plasmids and purified by preparative gel electrophoresis. 50 ng aliquots of cDNA were labeled by random oligonucleotide priming [20] using a commercial kit (Boehringer) and deoxycytidine 5′-[α-^{32}P]triphosphate (250 μCi, specific activity 3,000 Ci/mmol, from Amersham). The radiolabeled cDNA probes had specific activities of $1–4 \times 10^9$ cpm/μg DNA.

2.3.4. Hybridization procedure

Prehybridization and hybridization were carried out at 42°C in a hybridization incubator (GFL model 7601, Burgwedel, Germany): filters were prehybridized in a solution containing 50% (v/v) of deionized formamide, 5 × Denhardt's solution (0.02% Ficoll and 0.02% polyvinylpyrrolidone), 5 × SSPE (1 × SSPE = 0.18 M sodium chloride, 0.1 M monosodium phosphate, 0.01 M EDTA, pH 7.4), 0.2% SDS and 100 μg/ml of single-stranded sheared salmon sperm DNA. The hybridization buffer contained, in addition, about 2×10^7 cpm of heat-denatured [^{32}P]cDNA probe (1 ng/ml).

After hybridization for 48 h the membranes were washed in 0.1 × SSC/0.1% SDS solution, starting at room temperature and finishing with three washes at 54°C (or 45°C as indicated in legends to figures) for 20 min each, and were then exposed at −80°C to an X-Omat AR-5 film (Kodak) in cassettes equipped with intensifying screens to visualize [^{32}P]cDNA–mRNA hybrids.

2.4. *Processing of cell culture media and immunoblot*

Conditioned culture media were collected and processed for immunoblot analysis of IGFBPs. Briefly, media from 3 identically treated dishes were pooled and (9 ml) dialyzed against 0.1 M ammonium bicarbonate, lyophilized and dissolved in 200 μl H_2O for blot analysis of 20 μl samples: SDS-PAGE (15%) under non-reducing conditions, transfer to nitrocellulose, and incubation of blots with an antiserum raised in rabbits to IGFBP purified from BRL-3A cells, i.e. rat IGFBP-2 ([21]; antibody 3695, 1:500) and alkaline phosphatase staining using a second antibody (goat anti-rabbit IgG alkaline phosphatase conjugate) for detection of IGFBP-2.

3. RESULTS

Hepatocytes and osteoblasts produce and release IGFBPs into the culture medium as assessed by [^{125}I]IGF II ligand-blot analysis [2,4,6]. Analysis of

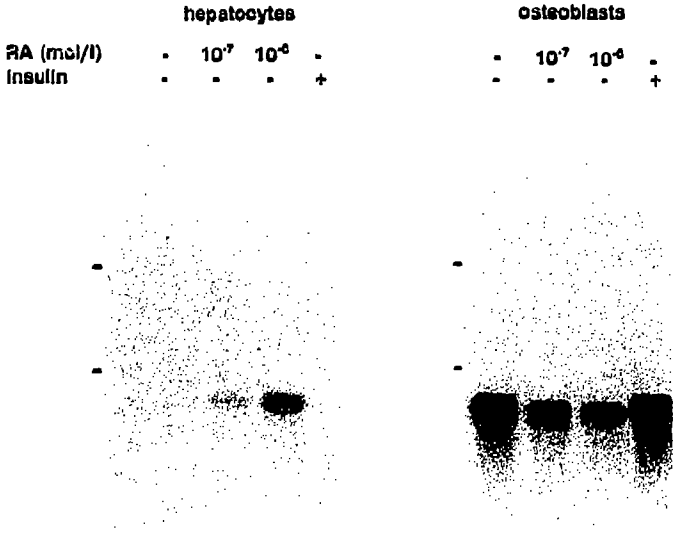

Fig. 1. IGFBP-2 mRNA in rat hepatocytes and osteoblasts exposed to retinoic acid (RA, 0.1 and 1 μM) and insulin (10 nM) for 24 h. Both cell types were handled in parallel for the last 48 h of culture and exposed to test media for the last 24 h, as described in section 2. Equal amounts of RNA (as confirmed by estimation of ribosomal RNA; positions indicated by bars) were analyzed. After hybridization to IGFBP-2 cDNA the filter was washed at 54°C and exposed to an X-ray film for detection of IGFBP-2 mRNA.

RNA by hybridization to a cDNA specific for IGFBP-2 revealed marked differences (Fig. 1): osteoblasts prepared from calvariae of newborn rats express high basal levels of IGFBP-2; by contrast, IGFBP-2 mRNA is barely detectable in RNA from hepatocytes prepared from adult rats. IGFBP-2 mRNA levels are increased by RA in hepatocytes but decreased in osteoblasts. RA exerts these effects in a dose-dependent manner. Insulin inhibits IGFBP-2 mRNA expression in hepatocytes but does not affect this parameter in bone cells (Fig. 1). Under the same conditions (24 h test) IGF I at 10 nM had no effect on IGFBP-2 mRNA expression in both hepatocytes and osteoblasts.

IGFBP-2 mRNA levels are low in hepatocytes freshly isolated from normal adult rats. In hepatocytes, time-course experiments with 0, 2, 6 and 24 h time points, respectively, reveal that the RA-mediated increase of IGFBP-2 mRNA levels occurs rapidly and is even more pronounced after 24 h. However, the stimulation by RA is abolished when insulin is simultaneously present (Fig. 2A). In contrast, insulin does not affect RA-stimulated mRNA levels of alkaline phosphatase (Fig. 2B). IGF I mRNA, by contrast, is stimulated by insulin and barely affected by RA treatment. As expected, IGF I mRNA levels are particularly high in freshly isolated hepatocytes (Fig. 2C).

Hepatocytes prepared from adult rats and grown in primary culture produce large amounts of IGFBPs as

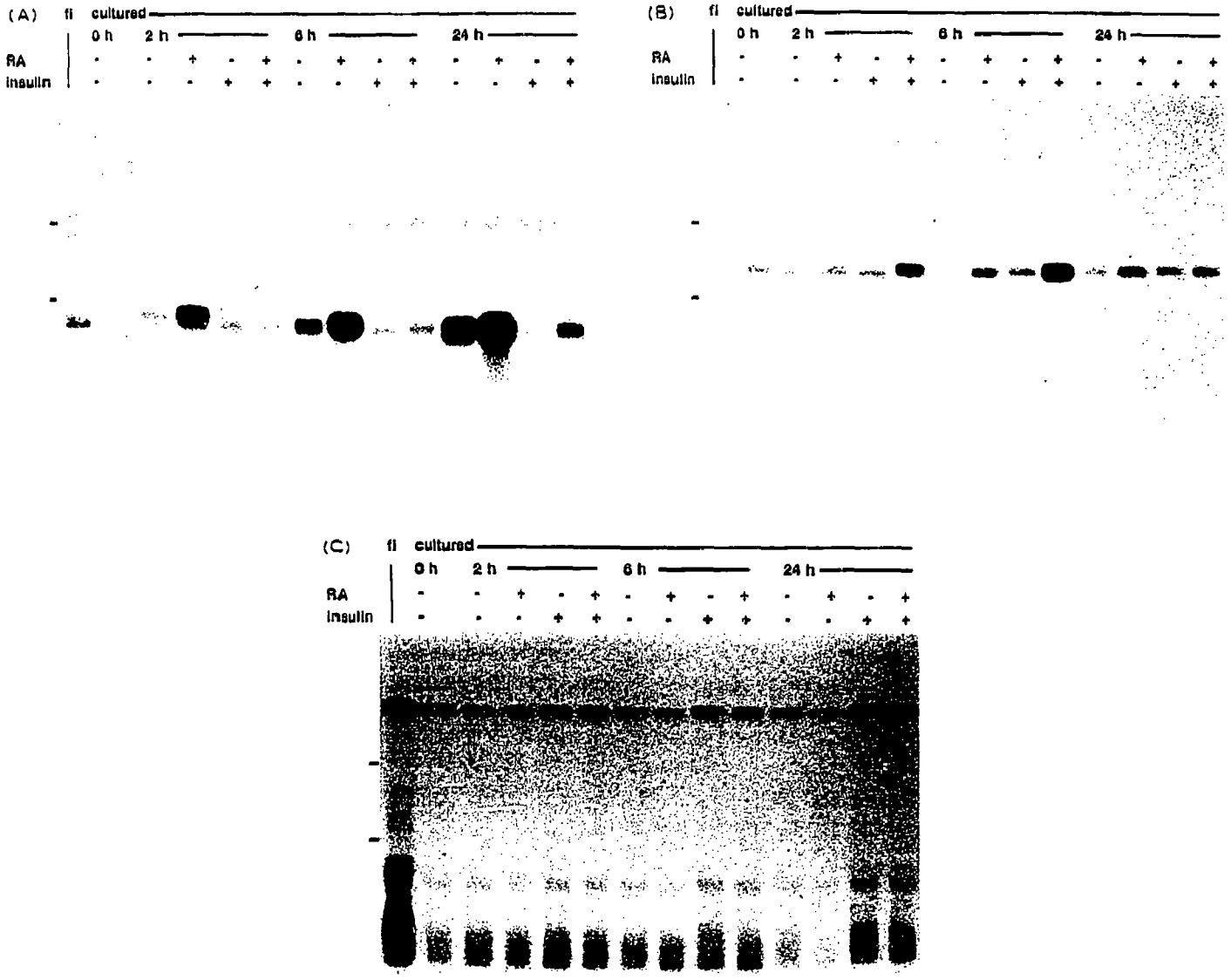

Fig. 2. IGFBP-2 (A), alkaline phosphatase (B) and IGF I (C) mRNA in freshly isolated rat hepatocytes and during a test started (0 h) 1 day after plating: cells were exposed to retinoic acid (RA) and insulin (10 nM) for 2, 6 and 24 h, respectively. Hepatocytes were prepared and exposed to test media (time point 0 h) as described; some dishes were stopped immediately (0 h), and some after 2, 6 and 24 h of incubation. After hybridization to IGFBP-2 cDNA (A), the filter was washed at 45°C, stripped free of IGFBP-2 cDNA, hybridized to alkaline phosphatase (liver/bone/kidney type) cDNA and washed at 55°C (B). The filter was also hybridized to IGF I cDNA, washed at 55°C and exposed for autoradiography (C). The experiment shown is representative of 4 independent tests.

assessed by IGF ligand-blot analysis. However, RNA isolated from these cells gives only a weak hybridization signal with IGFBP-2 cDNA (Figs. 1 and 2) indicating that the majority of IGFBPs may not correspond to IGFBP-2. Therefore, we looked for IGFBP-2 in the conditioned culture medium by means of immunoblots with a specific antiserum which had been raised against rat IGFBP-2 (Fig. 3). IGFBP-2 is identified at the 32 kDa protein position in our blot procedure; as it is the predominant IGFBP in serum of neonatal (but not adult) rats, such serum is shown for comparison in Fig. 3. Little IGFBP-2 is detected in media conditioned by hepatocytes incubated in control medium (which contains no protein other than BSA and no hormones). RA-treated hepatocytes release much more IGFBP-2 into the medium than do control cells (Fig. 3). Another prominent band at around 69 kDa is detected by the immune serum in all hepatocyte-conditioned media and also in rat sera. It appears that RA and insulin affected

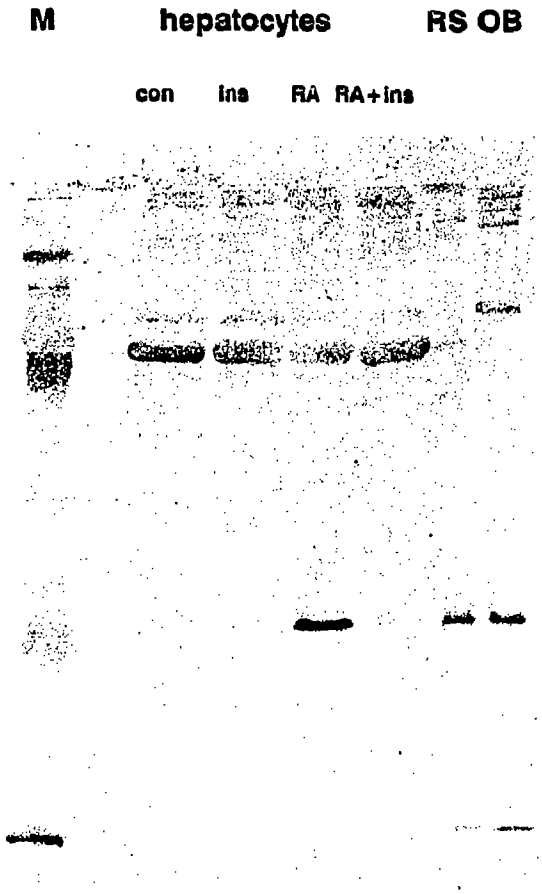

Fig. 3. Immunoblot analysis for IGFBP-2 accumulating in serum-free culture media of adult rat hepatocytes exposed to insulin (10 nM) and RA (1 μM) for 24 h (as in Figs. 1 and 2) compared to IGFBP-2 of neonatal rat serum (RS, 2 μl) and of serum-free culture medium conditioned by rat osteoblasts (OB) over 24 h. The apparent molecular mass of IGFBP-2 is 32 kDa, just above the 30 kDa marker (carbonic anhydrase). The 69 kDa marker is albumin.

the production of IGFBP-2 specifically and markedly but the intensity of the 69 kDa band only marginally. Moreover, hardly any effect of RA on IGFBPs is detectable in [^{125}I]IGF II ligand blots of the same culture media (not shown). This finding suggests that comparable amounts of other, more abundant IGFBPs are released into the medium, irrespective of RA treatment.

4. DISCUSSION

Cultured rat hepatocytes and osteoblasts produce and release IGFBP-2 into the medium, as demonstrated by hybridization of RNA to a specific cDNA probe, as well as by immunoblot analysis of IGFBP-2 in culture media. Hepatocytes prepared from adult rats and grown in primary culture release several, mainly non-glycosylated IGFBPs and very little, if any, IGFBP-3. The release of these IGFBPs is suppressed by insulin which also inhibits IGFBP-2 mRNA expression [4,6]. However, there have been no previous reports showing that insulin also decreases IGFBP-2 production of normal adult rat hepatocytes in vitro. In addition our data show that RA has a specific stimulatory effect on IGFBP-2 expression which occurs rapidly and persists for at least 1 day, and that the increased IGFBP-2 mRNA levels result in increased IGFBP-2 production. RA stimulates expression of many mRNA species. Alkaline phosphatase mRNA may serve as an example of a RA-induced mRNA which is not suppressed by insulin. Insulin stimulates IGF I mRNA expression [22] whereas RA has little effect on this parameter under the same conditions (Fig. 2C). RA only regulates IGFBP-2 and has no detectable effect on other IGFBPs indicating that this effect of RA is specific. Since the effect of RA is of considerable magnitude by immunoblot analysis but barely detectable on the [^{125}I]IGF II ligand blot it is likely that IGFBP-2 constitutes only a minor percentage of total IGFBPs released by hepatocytes, at least when they are kept without RA. These other, as yet unidentified IGFBPs have a molecular weight comparable to that of IGFBP-2 so that the ligand blot cannot be used to quantitate changes of IGFBP-2. IGFBP-2 mRNA levels correlate with the amount of IGFBP-2 in the medium: (i) osteoblasts express higher mRNA levels and produce more IGFBP-2 than hepatocytes; (ii) RA induces and insulin inhibits both IGFBP-2 mRNA and IGFBP-2 release in hepatocytes.

Insulin suppresses basal [6] and RA-induced (Fig. 2) IGFBP-2 mRNA levels in hepatocytes. Since basal IGFBP-2 mRNA levels are low and increase slowly in insulin-deprived (de-differentiating) hepatocytes, the inhibitory effect of insulin is more pronounced in RA-exposed hepatocyte cultures (Fig. 2). RA permits easier detection of the IGFBP-2 suppression by insulin at both the mRNA and protein level. The regulation of IGFBP-2 by insulin is specific for hepatocytes. In osteoblasts insulin, as well as IGF I, failed to affect IGFBP-2

mRNA levels. At the same time IGF I, but not insulin, stimulated the accumulation of a 32 kDa IGFBP in osteoblast culture medium, recently identified as IGFBP-2 (C.S. and J.Z., in preparation); this difference between insulin and IGF may indicate that increased IGFBP-2 accumulation in media of IGF-treated osteoblasts is due to stabilization of released IGFBP rather than to an effect on IGFBP-2 synthesis.

We have previously reported that increased hepatic IGFBP-2 expression in vivo is usually observed in conditions characterized by low insulin secretion such as streptozotocin-induced diabetes in rats and in extrapancreatic tumour hypoglycemia, and during IGF I treatment in humans [4,23,24]. These findings are compatible with our present observations on cultured hepatocytes.

IGF and IGFBP production are modulated by hormones in a specific manner at the level of their target tissues. Insulin is an important negative regulator of IGFBP-2 production in liver but not in bone cells. RA and, less effectively, triodothyronine (not shown) positively regulate IGFBP-2 mRNA expression in hepatocytes. In analogy, triiodothyronine is also less potent than RA in inducing the expression of the phosphoenolpyruvate carboxykinase gene in an adult rat hepatocyte cell line [10]. The latter enzyme is rate-limiting for hepatic gluconeogenesis and is also down-regulated by insulin. Thus, the regulation of PEPCK and IGFBP-2 mRNA expression in liver cells share common features. However, in contrast to the well-known function of PEPCK the physiological role of IGFBP-2 is not understood.

RA is the most potent naturally occurring retinoid. Difficulties in measuring tissue concentrations contributed to the reluctance to accept RA as an endogeneous humoral agent. By using improved methodology, its tissue concentrations can now be estimated to range from 0.02–0.6 μM [25].

RA plays a role in growth, differentiation and malignancy. Thus RA induces differentiation of embryonic stem cells such as the murine F9 teratocarcinoma cell line; in this particular cell line RA also induces bone/liver/kidney-type alkaline phosphatase [26]. RA also stimulates differentiation and alkaline phosphatase expression in undifferentiated bone cell lines [27,28] but not in our osteoblast system (not shown). Our observations may provide a model to further study the cell-specific control of IGFBP gene expression in vitro and suggest autocrine/paracrine functions of IGFBPs.

Acknowledgements: We would like to thank Matthew Rechler for providing us with immune serum specific for rat IGFBP-2, Gideon Rodan for alkaline phosphatase cDNA, Ani Ohannessian for expert technical assistance and Martha Salman for excellent secretarial help. This work was supported by Grant 31-9095.87 (3.046-0.87) from the Swiss National Science Foundation.

REFERENCES

[1] Humbel, R.E. (1990) Eur. J. Biochem. 190, 445–462.
[2] Zapf, J., Schmid, Ch., Binz, K., Guler, H.P. and Froesch, E.R. (1990) in: Growth Factors: From Genes to Clinical Applications (Sara, V.R. et al. eds.) pp. 227–240, Raven Press, New York.
[3] Schwander, J.C., Hauri, C., Zapf, J. and Froesch, E.R. (1983) Endocrinology 113, 297–305.
[4] Schmid, Ch., Zapf, J., Meier, P.J., Boeni-Schnetzler, M., Ernst, M. and Froesch, E.R. (1989) in: Insulin-Like Growth Factor Binding Proteins (Drop, S.L.S. and Hintz, R.L., eds.) pp. 267–272, Elsevier, Amsterdam.
[5] Scott, C.D. and Baxter, R.C. (1991) Biochemistry 275, 441–446.
[6] Böni-Schnetzler, M., Schmid, Ch., Mary, J.-L., Zimmerli, B., Meier, P.J., Zapf, J., Schwander, J. and Froesch, E.R. (1990) Mol. Endocrinol. 4, 1320–1326.
[7] Orlowski, C.C., Ooi, G.T., Brown, D.R., Yang, Y.W.-H., Tseng, L.Y.-H. and Rechler, M.M. (1991) Mol. Endocrinol. 5, 1180–1187.
[8] Powell, D.R., Suwanichkul, A., Cubbage, M.L., DePaolis, L.A., Snuggs, M.B. and Lee, P.D.K. (1991) J. Biol. Chem. 266, 18868–18876.
[9] Schmid, C., Schläpfer, I., Böni-Schnetzler, M., Zapf, J. and Froesch, E.R. (1990) in: Osteoporosis 1990 (Christiansen, C. and Overgaard, K., eds.) pp. 359–361, Aalborg ApS, Denmark.
[10] Pan, C.-J., Hoeppner, W. and Chou, J.Y. (1990) Biochemistry 29, 10883–10888.
[11] Zhou, H., Hammonds, R.G., Findlay, D.M., Fuller, P.J., Martin, T.J. and Ng, K.W. (1991) J. Bone Min. Res. 6, 767–777.
[12] Chen, R.F. (1967) J. Biol. Chem. 242, 173–181.
[13] Boelsterli, U.A., Bonis, P., Brouillard, J.-F. and Donatsch, P. (1988) Toxicol. Appl. Pharmacol. 96, 212–221.
[14] Schmid, C., Steiner, T. and Froesch, E.R. (1983) Calcif. Tissue Int. 35, 578–585.
[15] Chirgwin, J.M., Przybala, A.E., MacDonald, R.J. and Rutter, W.J. (1979) Biochemistry 18, 5294–5299.
[16] Margot, J.B., Binkert, C., Mary, J.-L., Landwehr, J., Heinrich, G. and Schwander, J. (1989) Mol. Endocrinol. 3, 1053–1060.
[17] Thiede, M.A., Yoon, K., Golub, E.E., Noda, M. and Rodan, G.A. (1988) Proc. Natl. Acad. Sci. USA 85, 319–323.
[18] Shimatsu, A. and Rotwein, P. (1987) J. Biol. Chem. 262, 7894–7900.
[19] Bond, J.F., Robinson, G.S. and Farmer, S.R. (1984) Mol. Cell Biol. 4, 1313–1319.
[20] Feinberg, A.P. and Vogelstein, B. (1983) Anal. Biochem. 132, 6–13.
[21] Yang, Y.W.-H., Brown, A.L., Orlowski, C.C., Graham, D.E., Tseng, L.Y.-H., Romanus, J.A. and Rechler, M.M. (1990) Mol. Endocrinol. 4, 29–38.
[22] Böni-Schnetzler, M., Schmid, Ch., Meier, P.J. and Froesch, E.R. (1991) Am. J. Physiol. 260, E846–E851.
[23] Böni-Schnetzler, M., Binz, K., Mary, J.-L., Schmid, C., Schwander, J. and Froesch, E.R. (1989) FEBS Lett. 251, 253–256.
[24] Zapf, J., Schmid, Ch., Guler, H.P., Waldvogel, M., Hauri, C., Futo, E., Hossenlopp, P., Binoux, M. and Froesch, E.R. (1990) J. Clin. Invest. 86, 952–961.
[25] Napoli, J.L., Posch, K.P., Fiorella, P.D. and Boerman, M.H.E.M. (1991) Biomed. Pharmacother. 45, 131–143.
[26] Gianni, M., Studer, M., Caprani, G., Terao, M. and Garattini, E. (1991) Biochem. J. 274, 673–678.
[27] Ng, K.W., Gummer, P.R., Michelangeli, V.P., Bateman, J.F., Mascara, T., Cole, W.G. and Martin, T.J. (1988) J. Bone Min. Res. 3, 53–61.
[28] Heath, J.K., Rodan, S.B., Yoon, K. and Rodan, G.A. (1989) Endocrinology 124, 3060–3068.