SYNTHESIS BY CULTURED HEPATOCYTES OF SOMATOMEDIN AND ITS BINDING PROTEIN

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1. Introduction

The somatomedins (SM) are a group of hepatic polypeptide hormones of ~7500 mol. wt that are growth hormone (GH)-dependent, have insulin-like activity and stimulate the growth of a variety of cells in culture [1–6]. Their primary function appears to be the mediation of the growth-promoting effect of GH [1–3], a function strongly supported by the recent demonstration that a partially purified preparation stimulated the growth of GH-deficient Snell dwarf mice [7]. Serum SM levels are primarily increased in patients with GH-producing pituitary tumors and decreased in GH-deficient dwarfs [1–3,8–11]. The serum activity may also be regulated in part by insulin, thyroxine, cortisol, estrogen, prolactin and placental lactogen [12–20]. A unique feature of the SM is that they circulate in plasma bound to a large carrier protein [21] which influences their biologic activity and significantly prolongs their half-life [22]. In addition, the kidney plays a role in the catabolism of SM and circulating inhibitors affect SM activity [23–27]. Because of this complex pattern of regulation, previous methods for investigating hepatic SM synthesis, including liver perfusion [4,13,17] and the ability of hormones to reverse somatostatin-induced suppression of serum SM levels have given only limited information about hepatic synthesis and are unsuitable for investigating intracellular events [18]. While hepatoma cells and hepatocytes have been observed to produce SM [28,29], this is the first report of GH-sensitive, cultured normal rat liver cells that synthesize SM and the first demonstration that the liver synthesizes SM binding protein. Since these cells retain enzymatic activity and synthetic capacity for several days [30], they are potentially a good model to study the hormones that regulate the synthesis of SM and its binding protein.

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2. Materials and methods

2.1. *Materials*

Rat GH (NIH B_3, 0.9 U/mg) was provided by the Endocrine Study Section, NIH, Bethesda, MD.

2.2. *Cell culture*

Hepatocytes were obtained from male Sprague Dawley rats (250 g) 4 days after a 70% hepatectomy. Under ether anesthesia the liver was perfused with 500 ml Ca^{2+}- and Mg^{2+}-free Eagle's medium 199 containing 200 mg collagenase (Gibco), 100 U penicillin G and 1.8 mg $NaHCO_3$. The first 20 ml perfusate was discarded and the remaining recirculated for 20 min. The liver was removed, placed in 75 ml perfusion medium and shaken for 15 min at 37°C. After the Ca^{2+} concentration was adjusted to 0.35 mM, the mixture was filtered through nylon mesh. The hepatocytes were spun at 50 × *g* for 3 min at 25°C, washed with complete Eagle's medium, and suspended in the same medium at 1.5×10^6 cells/ml. Cell viability was determined by trypan blue exclusion and averaged 80%. The yield was $100–200 \times 10^6$ cells/liver. Cultures were initiated by adding 3 ml cell suspension to 5 cm Falcon petri dishes precoated with rat tail

collagen. After 4 h the cells were adherent and the medium was changed.

2.3. *Radioreceptor assay and bioassay*

The human placental membrane competitive binding assay used was a modification of the method in [10]. Details of this method are in [24,31]. SM-A (neutral somatomedin) was prepared by Dr K. Uthne from human Cohn fraction IV by acid–ethanol extraction, gel chromatography and zone electrophoresis [32–34]. The SM-A preparation used for labeling was homogeneous by electrophoresis and CM-52 cellulose chromatography and had an activity of 1250 U/mg in the radioreceptor assay. SM-A was iodinated to 80–120 μCi/μg spec. act. [35]. Radioligand and non-specific bindings averaged 23% and $< 2\%$. Normal human serum, SM-A, SM-C (basic SM) and insulin-like growth factor-I gave parallel displacement curves. Serum activity was expressed relative to a pool of normal sera for 10 healthy adults arbitrarily assigned a value of 1.0 U/ml. The mean levels ± SE were 1.06 ± 0.06 for 39 normal adults, 0.35 ± 0.03 for 9 GH-deficient subjects and 3.21 ± 0.4 U/ml for 12 acromegalic patients. The interassay coefficient of variation was 6%.

Bioactivity was measured by $^{35}SO_4$ incorporation into embryonic chicken pelvic leaflets [36]. The index of precision (λ) averaged 0.23.

2.4. *Chromatography*

Columns of Sephadex G-75 (0.9 × 70 cm) were equilibrated with either 0.1 M ammonium formate or 1 M acetic acid at 25°C. A 6 × concentrate of hepatic media (1.5 ml) was applied to each and eluted fractions tested by radioreceptor assay.

3. Results

Primary monolayer cultures of rat parenchymal liver cells were found to release ~48 U SM . 24 h^{-1} . $liver^{-1}$. Figure 1a shows the parallel dose–response curves for the standard SM serum and hepatocyte culture medium using ^{125}I-labeled SM-A as the radioligand in the radioreceptor assay. Although parallel dose–response curves were also obtained with ^{125}I-labeled SM-C (basic SM) as the radioligand, ^{125}I-labeled SM-A gave improved sensitivity (data not shown). Since all SM crossreact in this receptor assay [37], the measured value reflects total SM production. Released SM represents de novo synthesis since there is no appreciable hepatic storage of SM [2]. Cell cultures from regenerating rat livers were used because they synthesized almost twice as much SM as normal cells (data not shown) and could be cultured from inception in entirely serum-free media thereby avoiding addition of exogenous serum factors.

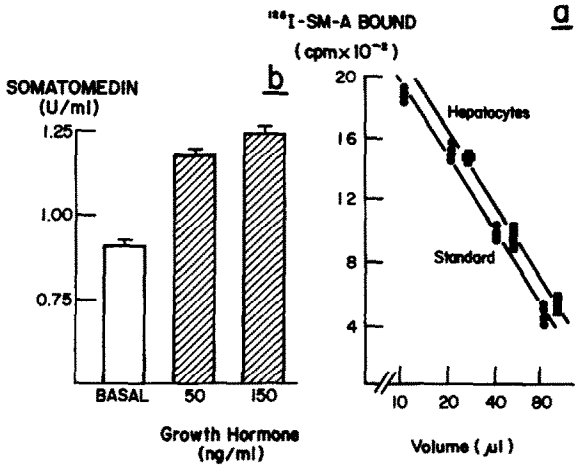

Fig.1. Somatomedin synthesis by rat hepatocytes in monolayer culture measured by radioreceptor assay. (a) The dose response curves of hepatocyte culture medium and standard normal human serum. (b) The effect of growth hormone on somatomedin synthesis. Rat growth hormone was added at 4 h and somatomedin production was measured at 24 h. The results are means (± SE) per 24 h, $n = 4$.

Hepatocytes retained their functional capacity to respond to GH stimulation with significant increases in SM production of 28% and 36% at 50 ng/ml and 150 ng/ml, respectively (fig.1b). The SM measured by radioreceptor assay was determined to be biologically active SM for the hepatocyte culture media was able to stimulate the incorporation of $^{35}SO_4$ into embryonic chick pelvic cartilage. Figure 2a shows that the log dose–response curve for hepatocyte culture medium from 5–60% was parallel to that for the standard serum. The yield calculated for this experiment was 40 U . 24 h^{-1} . $liver^{-1}$. Although this was in good agreement with the 48 U calculated by the radioreceptor assay for other experiments, the concordance could be fortuitous. Rat GH (50 ng/ml)

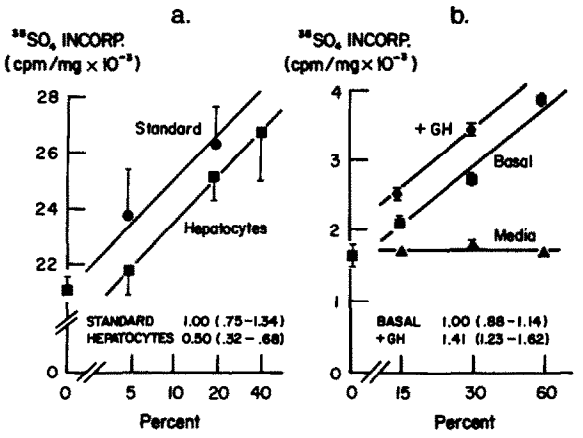

Fig.2. The synthesis of bioactive somatomedin by cultured rat hepatocytes measured by the incorporation of $^{35}SO_4$ into embryonic chicken pelvic cartilage. The viability of the cultured hepatocytes was > 95%. Each point is the mean ± SE of 5 observations. The calculated relative potencies ± 95% fiducial limits are listed at the bottom of each graph. (a) The log dose response curves of hepatocyte somatomedin compared to the standard serum. (b) The effect of 50 ng/ml of rat growth hormone on somatomedin synthesis. Growth hormone was added after 72 h and somatomedin production measured 24 h later. The lower incorporation of $^{35}SO_4$ in b was due to using 12 versus 11 day old pelvic rudiments from a different supplier and a 4 h incubation period instead of 6 h.

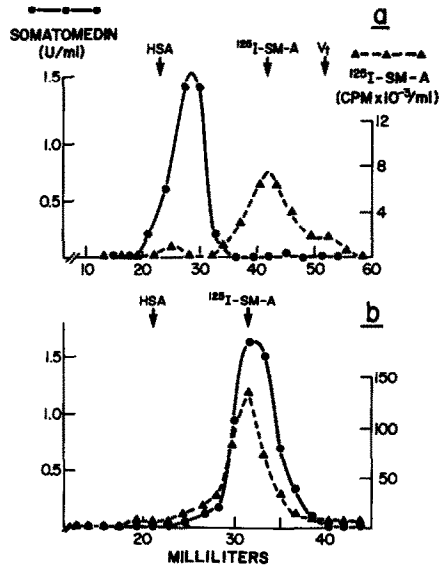

Fig.3. Chromatography of hepatic somatomedin on Sephadex G-75 (0.9 × 70 cm). (a) Chromatography at pH 7.4. (b) Chromatography in 1 M acetic acid. Recoveries were 85% and 78%, respectively. Column markers were human serum albumin (HSA), ^{125}I-labeled SM-A, and pyridine (V_t).

stimulated SM release by 41% (fig.2b) compared with 28% by radioreceptor assay.

To learn whether cultured hepatocytes synthesized free or bound SM, culture media was chromatographed on Sephadex G-75 at neutral pH to determine the approximate size of the SM complex. Under these conditions all of the SM synthesized by hepatocytes eluted just after human albumin and well ahead of free ^{125}I-labeled SM-A consistent with 4–5 × 10^4 mol. wt (fig.3a). Since free SM can be separated from its binding protein by chromatography on Sephadex under acidic conditions, hepatic SM was applied to a similar column equilibrated with 1 M acetic acid and found to coelute with free ^{125}I-labeled SM-A (fig.3b) at ~8000 mol. wt. On rechromatography at neutral pH the free hepatic SM did not aggregate and then coeluted with free ^{125}I-labeled SM-A, demonstrating that the original large molecular weight SM complex observed at neutral pH also contained the SM binding protein (data not shown).

4. Discussion

Rat hepatocytes in monolayer culture were found to synthesize SM continuously for at least 96 h and respond to maximal GH stimulation (150 ng/ml) with an increase of SM production of 36%. Measurements of SM synthesis by bioassay and radioreceptor assays were in good agreement indicating that the material released was bioactive.

The use of hepatic parenchymal cells in monolayer culture offers many advantages for the study of the synthesis of SM and its binding protein. These cells have been shown [30] to maintain for several days metabolic functions characteristic of intact liver cells, synthesis of glycogen and albumin, gluconeogenesis from 3-carbon precursors, sensitivity to insulin and glucagon and active microsomal enzymes [30]. They are a uniform population of cells that synthesize SM for at least 4 days, and respond to ng amounts of GH. They allow many variables to be controlled and thus far are the only method available for long term studies and investigating the intracellular mechanisms regulating SM synthesis. Perfused livers and suspension

cultures have limited usefulness because of decreased viability and deteriorating function was exemplified by the progressive fall in SM release near to zero after 3 h perfusion [13]. Since in perfused hypophysectomized rat livers GH only maintained control levels of SM release and merely slowed the decreasing release from normal livers, these effects of GH could be due to increased cell survival or decreased catabolism of SM rather than actual stimulation of SM synthesis. This might explain the need for pharmacologic doses of GH as opposed to ng amounts with cultured cells.

SM in plasma is transported bound to a carrier protein and very little if any SM exists in a free state [21,22,38]. It is known that the binding protein is a major determinant of the half-life of SM, for free SM is cleared by the body within minutes whereas the half-life of bound SM is 3–5 h in rats [2]. The production of this binding protein by hepatocytes establishes that the liver is its site of synthesis and that primary monolayer cultures can also be used to investigate its regulation. This protein has a special importance to endocrinology in that it is the only known binding protein for a polypeptide hormone. Whether the synthesis of the binding protein is independent [38] or correlated to [39] that of SM and how its synthesis is regulated are of major interest in understanding the biology of SM.

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