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Increased levels of multiplication-stimulating activity, an insulin-like growth factor, in fetal rat serum

(somatomedin/fetal growth/radioimmunoassay/somatomedin-binding protein)

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ABSTRACT **Multiplication-stimulating activity (MSA), purified from medium conditioned by the BRL-3A rat liver cell line, previously has been shown to be closely related to the human somatomedins or insulin-like growth factors. A radioimmunoassay was utilized to measure MSA levels in sera from fetal, maternal, and young rats. A serum somatomedin-binding protein was found to interfere in the radioimmunoassay by competing with antibody for binding ^{125}I-labeled MSA. Therefore, prior to radioimmunoassay, sera were filtered on Sephadex G-75 in 1 M acetic acid to dissociate and separate somatomedin activity from the binding protein. Concentrations of MSA by radioimmunoassay were 20- to 100-fold higher in fetal rat sera (1.8–4.4 μg/ml) than in maternal sera. MSA levels gradually decreased after birth, reaching maternal levels by day 25 of extrauterine life. MSA concentrations in fetal rat sera also were found to be correspondingly high by a rat liver membrane radioreceptor assay and a competitive binding protein assay using rat serum somatomedin-binding protein. The findings of higher levels of MSA in fetal than in maternal rat sera and the gradual decline in MSA serum concentrations after birth are in direct contrast to total somatomedin activities measured by bioassay. Thus, MSA may function as a growth factor in the fetal rat whereas other somatomedins may play a role in stimulating growth during extrauterine life.**

Dulak and Temin (1, 2) reported the purification of multiplication-stimulating activity (MSA) from serum-free medium conditioned by a rat liver cell line (BRL-3A). MSA has been shown to be closely related to the human somatomedins or insulin-like growth factors (3–8), the proposed mediators of growth hormone's anabolic action on skeletal tissue *in vivo* (9). Thus, MSA, somatomedin A, IGF I (insulin-like growth factor), and IGF II all compete with each other for binding to receptors on purified membranes or on cells in culture and for binding to a binding protein in serum (3–8). Additional biological properties that relate MSA to the somatomedins include: (*i*) weak insulin-like metabolic activity *in vitro* including stimulation of glucose oxidation in fat (1, 10, 11) and competition for ^{125}I-labeled insulin binding to insulin receptors (10); (*ii*) ability to stimulate $^{35}SO_4$ incorporation into cartilage (1, 8, 11); and (*iii*) mitogenic activity for cells in tissue culture (1, 4, 12).

We recently reported evidence that MSA is produced by explants of fetal rat liver in organ culture (11). Using a radioimmunoassay for MSA, we now show that high concentrations of MSA are found in fetal rat serum and are 20- to 100-fold higher than the levels in maternal sera or sera of rats 25 days old.** We propose that MSA may play a role in growth of the rat fetus.

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MATERIALS AND METHODS

Animals. The fetuses were removed from timed pregnant rats (Sprague–Dawley; from Zivic–Miller Laboratories, Allison Park, PA) under ether anesthesia and decapitated. One- to 30-day-old rats were sacrificed by decapitation. Blood was allowed to clot at 4°C and serum was obtained after centrifugation.

MSA Purification. The details of the MSA purification scheme are reported elsewhere (13). Two MSA species, MSA II-1 and III-2, have been purified to homogeneity, their molecular weights are 8700 and 7100, respectively (13).

Radioiodination of MSA. MSA II-1 and III-2 were labeled with ^{125}I by a modified chloroamine-T procedure (10). Specific activity of ^{125}I-labeled MSA (^{125}I-MSA) ranged from 50 to 190 Ci/g (1 Ci = 3.7×10^{10} becquerels).

Chromatography of Rat Serum on Sephadex G-75. Rat serum was diluted with an equal volume of 2 M acetic acid and, after 0.5-hr incubation at room temperature, was filtered on Sephadex G-75 in 1 M acetic acid (room temperature). When individual fractions were to be radioimmunoassayed, aliquots were taken to dryness in a Savant Speed-Vac Concentrator, redissolved in water, and again taken to dryness. When column fractions were pooled prior to radioimmunoassay, the pool was lyophilized in 50-ml polypropylene tubes, and the precipitate was taken up in a small volume of 1 M acetic acid, taken to dryness in the Speed-Vac Concentrator, dissolved in water, and taken to dryness again.

MSA Radioimmunoassay. Details of the radioimmunoassay are reported elsewhere (14). The assay utilized ^{125}I-MSA III-2 and rabbit antiserum; antigen–antibody complex was precipitated with polyethylene glycol. Specific binding was obtained by subtracting radioactivity precipitated in the presence of excess unlabeled MSA (nonspecific binding). Nonspecific binding (4–10% of input radioactivity) was approximately the same as the assay blank (radioactivity precipitated when ^{125}I-labeled MSA was incubated with carrier gamma globulin

Abbreviations: MSA, multiplication-stimulating activity; BRL, Buffalo rat liver; IGF I, insulin-like growth factor I; IGF II, insulin-like growth factor II.

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in the absence of antiserum). Results are expressed as B/B_0, in which B is specific binding and B_0 is maximal specific binding (i.e., in the absence of unlabeled MSA). B_0 ranged from 20% to 37% of input radioactivity.

Competitive Protein-Binding Assay. The competitive protein-binding assay using partially purified somatomedin-binding protein from rat serum was performed as described (7), except that the charcoal was activated by incubation for 4–7 days at 4°C in phosphate-buffered saline containing 20 mg of fatty acid-free bovine serum albumin per ml. To measure specific MSA binding in fractions from Sephadex G-75 chromatography of rat sera, aliquots were lyophilized and substituted for partially purified rat serum binding protein in the competitive binding assay. Excess unlabeled MSA (1.3 μg/ml) was added to duplicate samples to determine nonspecific binding.

Rat Liver Membrane Radioreceptor Assay. The rat liver membrane radioreceptor assay was performed as described with ^{125}I-labeled MSA II-1 and highly purified rat liver plasma membranes (4, 15).

[^{3}H]Thymidine Incorporation into DNA in Chicken Embryo Fibroblasts. The [^{3}H]thymidine incorporation assay in serum-starved chicken embryo fibroblasts was performed as described (16).

Analytical Disc Acrylamide Electrophoresis. Pooled fractions from Sephadex G-75 chromatography (1 M acetic acid) of fetal rat serum were lyophilized and analyzed in a disc acrylamide electrophoresis system containing 12.5% acrylamide at pH 2.7 in the presence of 9 M urea (17). Electrophoresis was performed in a 100 × 140 × 1.5 mm slab gel (Bio-Rad slab gel unit). The gel was cut into 1-mm slices which were extracted overnight at 4°C with 0.3 ml of borate-buffered saline containing 10 mg of bovine serum albumin per ml. Aliquots were tested in the MSA radioimmunoassay. A mixture of BRL MSA polypeptides was electrophoresed in a parallel channel of the same gel and the gel was stained for protein with Coomassie blue G-250.

RESULTS

Measurement of MSA in Fetal and Maternal Rat Sera by Radioimmunoassay. Somatomedins or insulin-like growth factors are bound to specific carrier proteins in the circulation (18–20). The somatomedins are dissociated from the binding proteins by 1 M acetic acid (21). Because the serum binding proteins bind ^{125}I-MSA (7, 19), they could interfere in the MSA radioimmunoassay by competing with MSA antibodies for binding of radioligand. In order to examine this question, fetal rat serum was chromatographed on Sephadex G-75 in 1 M acetic acid and immunoreactivity in each fraction was determined by using the MSA radioimmunoassay (Fig. 1). The elution position of the serum binding protein was located by measurement of MSA binding activity in each fraction (Fig. 1*B*). The elution position of MSA polypeptides was determined with ^{125}I-MSA II-1 and ^{125}I-MSA III-2 (Fig. 1*A*). There was a peak of apparent immunoreactivity just after the void volume, corresponding exactly to the position of the binding protein (Fig. 1*C*). A second broad peak of immunoreactivity eluted in a position corresponding to the BRL MSA polypeptides MSA II-1 (molecular weight 8700) and MSA III-2 (7100).

Material from the region of the column corresponding to the elution position of the binding protein produced a nonparallel dose–response curve in the MSA radioimmunoassay (data not shown). Also, when the binding protein region was pooled, lyophilized, and again treated with 1 M acetic acid, no low molecular weight immunoreactivity was generated (data not shown). Thus, the apparent immunoreactivity present in the binding protein region is not due to incomplete dissociation of

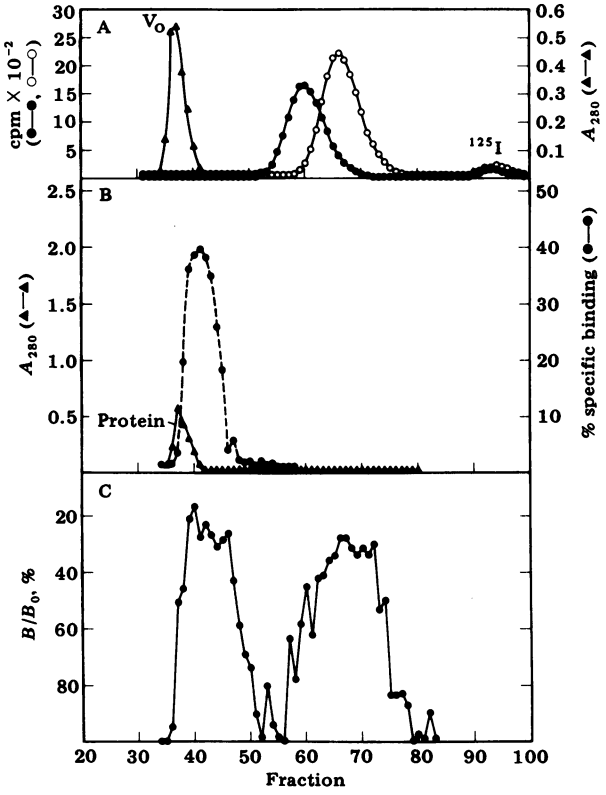

FIG. 1. Chromatography of fetal rat serum on Sephadex G-75 in 1 M acetic acid. The sample volume was 4.0 ml, and 5.0-ml fractions were collected. (*A*) Column (94 × 2.5 cm) was calibrated with bovine gamma globulin (10 mg), ^{125}I-MSA II-1 (●), ^{125}I-MSA III-2 (○), and Na^{125}I. The elution position of gamma globulin (▲) was determined by measuring A_{280}; radioactivity in individual fractions was measured to determine the elution positions of ^{125}I-MSA. (*B*) One-half milliliter of a pool of fetal rat serum (30 fetuses, 19-day gestation) was chromatographed. Protein elution was determined by measuring A_{280}. Duplicate 0.5-ml aliquots from each fraction were lyophilized and specific ^{125}I-MSA binding was measured. Specific binding is expressed as % of total cpm added to the assay tube. (*C*) Immunoreactivity in the MSA radioimmunoassay was measured on a 3.0-ml aliquot from each column fraction after chromatography of the fetal rat serum.

somatomedin activity from the binding protein. Our data do not rule out the possibility that part of the immunoreactivity found in fractions containing the binding protein is due to a heretofore unrecognized form of MSA that can not be dissociated from the binding protein by 1 M acetic acid.

Maternal rat serum and amniotic fluid were also chromatographed on Sephadex G-75 and fractions analyzed as in Fig. 1 (data not shown). The elution positions of the binding protein and peaks of immunoreactivity were the same as for the fetal sample; however, the level of immunoreactivity was much lower in the maternal and amniotic fluid samples.

We conclude that the serum binding protein interferes in the radioimmunoassay by competing with MSA antibody for ^{125}I-MSA binding. Therefore, all measurements of MSA immunoreactivity on rat sera were made after Sephadex G-75 chromatography of the sera. Fractions corresponding to the second peak of immunoreactivity (fractions 56–75, Fig. 1*C*) were pooled, lyophilized, and tested in the radioimmunoassay.

Fetal and maternal sera (2-ml samples) were chromatographed on Sephadex G-75 in 1 M acetic acid; fractions corresponding to the second peak of immunoreactivity in Fig. 1*C* were pooled, lyophilized, and tested in the MSA radioimmunoassay (Fig. 2). The dose–response curves for the fetal and maternal samples were parallel to those of the MSA II and MSA

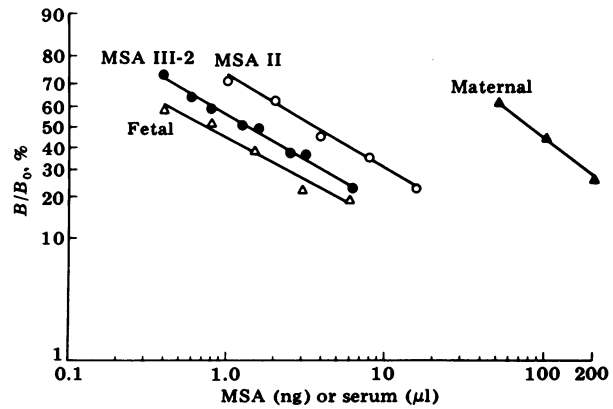

FIG. 2. Measurement of MSA levels by radioimmunoassay in fetal and maternal rat sera. Fetal rat serum (2 ml from a pool of 30 fetuses, 21-day gestation) and maternal serum (2.0 ml from a pool of 28 rats, 19-day gestation) were chromatographed on Sephadex G-75 in 1 M acetic acid. Fractions corresponding to the second peak of immunoreactivity in Fig. 1C were pooled and radioimmunoassayed. MSA III-2 and MSA II (II-1, 2, 3, and 4) were used as standards. The data are expressed as B/B_0 on a logit scale versus ng of MSA standard or μl of serum equivalent added per assay tube.

III-2 standards. Based on the dose–response of the MSA II standard, the level of MSA was 4.4 μg/ml in the fetal sample and 0.04 μg/ml in the maternal sample.

To determine interassay variability for the radioimmunoassay, a sample of rat serum was chromatographed on Sephadex G-75 and the same pool was analyzed in seven separate immunoassays. The mean (±SD) was 1.18 ± 0.15 μg/ml. Another serum pool was chromatographed eight times over Sephadex G-75 and each of the eight pools was assayed in a separate MSA radioimmunoassay. The mean (±SD) was 1.29 ± 0.27 μg/ml.

The requirement for Sephadex G-75 chromatography of sera prior to measurement in the MSA radioimmunoassay raises the possibility of losses of MSA during the chromatography and lyophilization steps. Nine micrograms of MSA II was added to a 2-ml sample of rat serum known to contain low levels of MSA by radioimmunoassay. The sample containing exogenous MSA was chromatographed on Sephadex G-75 in 1 M acetic acid, and the usual fractions were pooled and assayed. Recovery was 73%. When 1/10th the amount of MSA II (0.9 μg) was added to the same serum sample, followed by chromatography and assay, the recovery was 44%. We conclude that recovery of MSA from the Sephadex G-75 column was satisfactory and not strongly dependent on the amount of MSA in the sample. However, these recovery experiments do indicate that our radioimmunoassay measurements underestimated the actual concentrations in rat sera and suggest that the underestimation was greatest for sera containing lower amounts of MSA. When small volumes (less than 0.5 ml) of serum were chromatographed on Sephadex G-75 columns, recovery of immunoreactivity was decreased appreciably. Consequently, a 2-ml sample of serum was routinely chromatographed for radioimmunoassay. This requirement necessitated the use of pools of fetal rat serum and prevented measurements on sera from individual fetuses.

Disc Acrylamide Gel Electrophoresis of MSA Immunoreactivity in Fetal Rat Serum; Comparison with Mobility of BRL MSA Polypeptides. Fig. 1 shows that the elution volume of MSA immunoreactivity in fetal rat serum corresponds to the elution position of ^{125}I-MSA II-1 and ^{125}I-MSA III-2 after Sephadex G-75 chromatography. An analytical system with greater resolving power was chosen to examine further the identity of fetal serum MSA immunoreactivity with MSA polypeptides purified from the BRL-3A rat liver cell line. Fetal rat serum was initially chromatographed on Sephadex G-75 in 1 M acetic acid and fractions corresponding to fractions 56–75 in Fig. 1C were pooled and lyophilized. The fetal sample was electrophoresed on a disc acrylamide gel (pH 2.7, 9 M urea) (Fig. 3). A mixture of BRL MSA polypeptides was electrophoresed in parallel. The channel containing the fetal sample was sliced and eluates from the slices were tested in the MSA radioimmunoassay.

MSA immunoreactivity was detected in regions of the gel corresponding to the position of MSA II and MSA III polypeptides. In the region of the gel corresponding to BRL MSA II polypeptides, MSA II standard was used to calculate the amount of MSA present, whereas in the region of the gel corresponding to BRL MSA III polypeptides, MSA III-2 standard was used to calculate the amount of MSA present in the eluate. We conclude that the mobilities of the immunoreactive MSA in fetal rat serum correspond to the mobilities of MSA II and MSA III polypeptides derived from the BRL-3A rat liver cell line. There appears to be a greater amount of MSA II than MSA III in fetal rat serum.

Age Dependence of Serum MSA Measured by Radioimmunoassay. Sera from fetal, newborn, and young rats were chromatographed on Sephadex G-75 in 1 M acetic acid as in Fig. 1; fractions corresponding to 56–75 were pooled, lyophilized, and tested in the MSA radioimmunoassay. The MSA levels were increased in the fetus from day 19 to day 22 and then gradually declined after birth (Fig. 4). Concentrations approximating the low levels found in maternal serum were reached by day 25 of extrauterine life.

Measurement of MSA in Fetal Rat Serum by the Radioreceptor and Competitive Binding Protein Assays. In order to confirm the finding of high MSA levels in fetal rat serum determined by radioimmunoassay, two other assays were used to measure MSA concentration. MSA, IGF I, IGF II, and somatomedin A have been shown to compete for ^{125}I-MSA II-1 binding to a rat serum binding protein (5, 7). To measure activity in fetal rat serum, the rat serum binding protein was used with ^{125}I-MSA II-1 as a competitive binding assay. Fetal rat serum was chromatographed on Sephadex G-75 in 1 M acetic acid, and fractions corresponding to 56–75 in Fig. 1C were pooled and lyophilized. When this Sephadex G-75 pool was allowed to compete for ^{125}I-MSA II-1 binding to the rat serum binding protein, the dose–response curve was parallel to that of the MSA II standard. Based on the MSA II standard, the concentration of MSA in the fetal rat serum was 2.7 μg/ml.

The same Sephadex G-75 pool derived from fetal rat serum was tested in a radioreceptor assay using highly purified rat liver membranes and ^{125}I-MSA II-1. MSA, IGF I, IGF II, and somatomedin A previously have been shown to compete for ^{125}I-MSA II-1 binding in this assay (4, 5). When material in the Sephadex G-75 pool was allowed to compete for ^{125}I-MSA II-1 binding to rat liver membranes, the dose–response curve was parallel to that of the MSA II standard. Based on the MSA II standard, the concentration of MSA in fetal rat serum was calculated to be 3.5 μg/ml.

The concentration of MSA in the same Sephadex G-75 pool from fetal rat serum as measured by radioimmunoassay was 3.8 μg/ml. Thus, the level of MSA in fetal rat serum was essentially the same by three different assays.

Comparison of Somatomedin Activity in Maternal and Fetal Rat Sera by Bioassay. The finding of higher levels of MSA by radioimmunoassay in fetal rat serum than in maternal rat serum is in contrast to the report of lower levels of somatomedin activity in fetal rat serum found by the rat costal cartilage bioassay (22). Another bioassay that has been shown to measure a growth hormone-dependent activity in rat serum is the [^{3}H]thymidine incorporation assay in chicken embryo

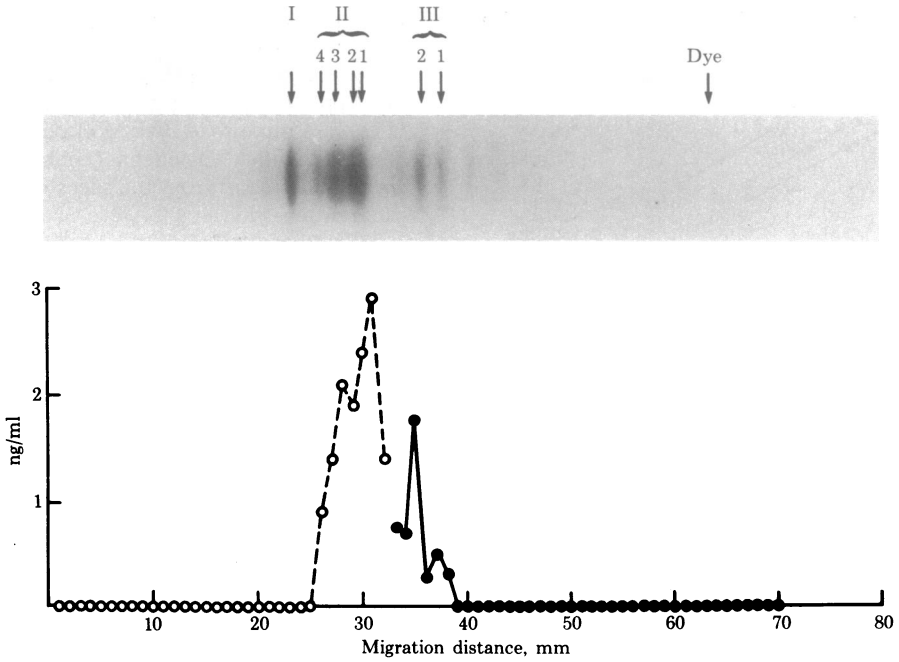

FIG. 3. Disc acrylamide electrophoresis of immunoreactive MSA from fetal rat serum. Fetal rat serum (1 ml from a pool of 280 fetuses, 19-day gestation) was chromatographed on Sephadex G-75 in 1 M acetic acid and fractions corresponding to the second peak of immunoreactivity in Fig. 1*C* were pooled and lyophilized. This pool was electrophoresed in an acid/urea/disc acrylamide system. Aliquots (5 μl) from gel slice eluates were tested in the MSA radioimmunoassay. For eluates of gel slices at 25–32 mm, the amount of MSA was calculated based on the MSA II standard (○); for gel slices at 33–39 mm, the amount of MSA was calculated based on the MSA III-2 standard (●). The amount of MSA is expressed in ng/ml of reaction mixture in the radioimmunoassay tube. Fifty micrograms of a mixture of BRL MSA polypeptides (MSA I; MSA II-1, 2, 3, and 4; MSA III-1 and 2) was electrophoresed in parallel and the gel was stained for protein. The top of the gel is at the left; the position of the methylene blue dye is at the right.

fibroblasts (16). When this assay was used to measure somatomedin activity in fetal and matched maternal samples, the level in maternal serum was slightly higher than in fetal serum. Based on the MSA II standard, the level of MSA in the fetal sample was 2.8 μg/ml (compared to 3.8 μg/ml by radioimmunoassay). Thus, in a bioassay that presumably measures other members of the somatomedin family in addition to MSA, higher activity was found in the maternal sample than in the fetal sample.

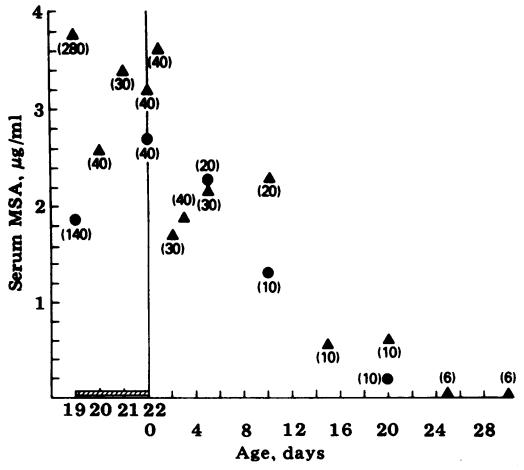

FIG. 4. Age dependence of MSA levels by radioimmunoassay in fetal and young rats. Blood was collected from fetal and maternal rats between 19 and 22 days of gestation and from young rats at ages 1, 2, 3, 5, 10, 15, 20, 25, and 30 days. Aliquots (2 ml) of serum were chromatographed on Sephadex G-75 in 1 M acetic acid, and fractions corresponding to the second peak of immunoreactivity in Fig. 1*C* were pooled. The pools were tested in a dose–response fashion (five concentrations, in duplicate) in the MSA radioimmunoassay. The number of fetuses for each pool is shown in parentheses. When two different serum pools from rats of the same age were assayed, the results are indicated by different symbols (●, ▲). The range of maternal serum MSA levels is indicated by the crosshatched bar in the lower left of the figure.

DISCUSSION

MSA has been purified from serum-free medium conditioned by a rat liver cell line (BRL-3A). MSA previously has been shown to be closely related to the human somatomedins somatomedin A, IGF I, and IGF II in various radioreceptor and competitive protein binding systems (3–8). Using BRL MSA antiserum and ^{125}I-labeled BRL MSA in a radioimmunoassay, we now find that MSA levels are 20- to 100-fold higher in fetal rat serum than in maternal serum.

Somatomedins are bound to specific carrier proteins in the circulation (18–20). If antiserum in a somatomedin radioimmunoassay is directed against a portion of the somatomedin molecule not sterically hindered by the binding protein, then the somatomedin–binding protein complex would be expected to behave identically to free somatomedin in the assay. This appears not to be the case in the radioimmunoassay for MSA because the stripped binding protein competes with antibody for ^{125}I-MSA binding. It is possible, of course, that the binding protein has been altered by treatment with 1 M acetic acid and that the native MSA binding protein complex would be recognized by MSA antibody. To avoid interference by the serum binding protein, we have chromatographed all serum samples on Sephadex G-75 in 1 M acetic acid prior to assay. The acetic acid dissociated the somatomedins from the binding protein (21), and Sephadex G-75 chromatography separated somatomedins from the larger binding protein.

The evidence that immunoreactive MSA in fetal rat serum is identical to MSA polypeptides purified from medium conditioned by the BRL-3A cell line is as follows. (*i*) The MSA radioimmunoassay appears to be specific for MSA polypeptides. IGF I, IGF II, and somatomedin A crossreact weakly in the MSA radioimmunoassay (14). IGF II, which crossreacts most

potently, is only 10% as potent as MSA II. The low reactivity of these somatomedins, all of human origin, may be due at least in part to the species difference. Clearly, the more relevant test of specificity involves the use of somatomedins purified from the rat. Preparations of a rat somatomedin partially purified from the sera of animals bearing the growth hormone- and prolactin-secreting MStT/W15 tumor (23, 24) have shown less than 0.1% the potency of MSA II in the MSA radioimmunoassay (14). Other growth factors such as epidermal growth factor, nerve growth factor, and fibroblast growth factor also were not reactive to the MSA radioimmunoassay (14).

(*ii*) Fetal rat serum immunoreactive MSA coelutes with BRL MSA polypeptides on Sephadex G-75 chromatography and comigrates with BRL MSA polypeptides on disc acrylamide electrophoresis in an acid urea system. (*iii*) The levels of MSA in fetal rat serum measured by the rat liver membrane radioreceptor assay and a competitive binding protein assay are the same as the concentration measured by the radioimmunoassay. In the case of the human somatomedins and MSA, there has been extensive crossreactivity in various radioreceptor and competitive binding protein assays, but the potency of each polypeptide relative to the others has varied widely depending upon the assay used (3–8, 25). It is likely that the radioimmunoassay, rat liver membrane radioreceptor assay, and competitive protein binding assay all depend on different domains of the MSA molecule for reactivity. For example, reduced and carboxymethylated MSA is unreactive in the rat liver membrane radioreceptor assay (3) but fully reactive in the MSA radioimmunoassay (14). If a non-MSA polypeptide in fetal rat serum were being measured in the MSA radioimmunoassay, it would be unlikely that the radioreceptor and competitive binding assays would yield identical levels based on the MSA II standard.

The finding of high levels of MSA serum in fetal rat serum compared to maternal serum and the gradual decline in serum MSA after birth are in sharp contrast to results from bioassays (22) and the radioimmunoassay for somatomedin A (26). Somatomedin activity measured by $^{35}SO_4$ incorporation into costal cartilage has been reported to be lower in fetal rat serum than in maternal rat serum and to increase gradually in the young rat (22). Using the [^{3}H]thymidine incorporation assay in chicken embryo fibroblasts, we also find that the level of somatomedin activity is higher in maternal than in fetal serum. Similarly, the radioimmunoassay for somatomedin A reveals low levels of immunoreactivity in fetal rat serum compared to maternal serum, and the levels gradually increase after 15 days of extrauterine life (26). We conclude that MSA is the major somatomedin or IGF in fetal rat blood but in adult rats it is a minor component and other members of the somatomedin family account for most of the somatomedin biological activity. Using a competitive binding protein assay, Draznin *et al.* (27) recently found higher levels of IGF in fetal rat serum than in maternal serum. Levels gradually decreased to low levels by day 21 and then increased slowly. We suggest that this competitive binding assay measured primarily MSA in the fetal samples and other somatomedins in samples after the nadir at day 21.

A fetal rat liver organ culture system also has been shown to produce a polypeptide similar or identical to BRL MSA polypeptides (11). The fetal liver polypeptide showed dose–response curves parallel to those of BRL MSA standards in the MSA radioimmunoassay, rat liver membrane radioreceptor assay, competitive protein binding assay, and chicken embryo fibroblast bioassay. Activity in these assays comigrated with BRL MSA polypeptides in an acid/urea/disc acrylamide electrophoresis system. The fetal liver MSA represented as much as 0.2–0.8% of the total protein produced by the liver explant in culture.

The hormonal control of fetal growth is poorly understood (28). Our finding of high levels of MSA in fetal rat serum together with the observation that MSA is produced by fetal rat liver in organ culture raises the possibility that MSA may play a role in fetal growth in the rat.

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