

Serum Somatomedin-C Concentrations in a Rabbit Model of Diabetic Pregnancy

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SUMMARY

We have developed and validated a method for measuring immunoreactive somatomedin-C (Sm-C) in serum of rabbits, and have shown that during midgestation (11–26 days; gestation = 31 days) serum Sm-C concentrations are higher in normal pregnant animals than in pregnant diabetic animals. Sm-C concentrations in the serum of 28-day gestation fetuses of diabetic rabbits (3.14 ± 0.25 U/ml) were significantly higher than in the fetuses of nondiabetic rabbits (2.31 ± 0.23 U/ml; $P < 0.05$). Fetuses from litters of the most severely hyperglycemic diabetic mothers (glucose > 250 mg/dl) had higher serum Sm-C (3.66 ± 0.41 U/ml) than those of mothers who were mildly hyperglycemic (2.71 ± 0.2 U/ml). Although these differences were not statistically significant, fetuses from the former litters accounted in great part for the difference between the fetuses of diabetic and normal pregnancy. The diabetes-related increment in Sm-C does not appear to be due to insulin, since fetal insulin concentrations were not different between the normal and diabetic litters (normal, 50.0 ± 3.6 μ U/ml versus diabetic, 49.6 ± 7.6 μ U/ml). Despite their elevation in serum Sm-C, fetuses from litters of diabetic rabbits were growth retarded in weight (26.8 ± 6.9 g and 33.8 ± 6.9 g, diabetic versus normal pregnancy; $P < 0.05$) and in length (7.9 ± 0.7 cm and 8.6 ± 0.7 cm, diabetic versus normal pregnancy; $P < 0.025$). We speculate that these discrepancies between growth and Sm-C might be secondary to the toxic effects of glucose on embryonic growth and that later in gestation, the excessive energy provided to the fetus might stimulate Sm-C synthesis. *DIABETES* 33:590–595, June 1984.

Infants of diabetic mothers (IDMs) may be either overgrown or have intrauterine growth retardation (IUGR).^{1,2} IUGR seems to occur when the mother has advanced vascular complications of diabetes, while overgrowth is usually associated with fetal hyperinsulinism. It is not known, however, whether insulin stimulates fetal growth by increas-

ing substrate utilization, by stimulating cellular proliferation directly, or by increasing serum somatomedins.^{1–3}

To determine the effect of diabetes on maternal concentrations of somatomedin-C (Sm-C)/insulin-like growth factor I (IGF-I)⁴ and to assess the relationships among fetal serum Sm-C and insulin and fetal growth, we have carried out studies in maternal rabbits made diabetic with alloxan, and their fetuses.

MATERIALS AND METHODS

Animals and experimental design. The experimental design has been reported previously.⁵ Briefly, young adult, New Zealand white female rabbits were mated so that the time of mating was known within 3 h. After 48 and 72 h, some animals were given intravenous injections of alloxan (2,4,5,6-tetraoxypyrimidine, 4,5-dioxyuracil; Sigma Chemical Co., St. Louis, Missouri) in a total dose of 60–130 mg/kg. Each rabbit was bled by ear puncture at approximately 7-day intervals. Blood was chilled immediately, and serum was separated and frozen at -20°C until assay (2 yr in the case of Sm-C). Serum glucose concentrations were measured by the hexokinase–glucose-6-phosphate dehydrogenase reaction (Glucose Strate, General Diagnostics, Warner-Lambert Co., Morris Plains, New Jersey). Rabbits having serum glucose values greater than 150 mg/dl were termed “diabetic.” One diabetic rabbit had ketones in her blood (determined semiquantitatively using Acetest Reagent Tablets; Ames Co., Elkhart, Indiana) and was excluded from further study. Pregnant does were killed on the 28th day of gestation (term = 31 days) by intravenous injection of 300 mg of pentobarbital. Pups were promptly exposed in utero and were bled by intracardiac puncture. Crown-rump length and weight of each was recorded. In addition to sera from maternal and fetal rabbits,

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TABLE 1
Glucose, insulin, and glucose/insulin ratio in maternal rabbits (mean \pm SEM)

Days of gestation	Glucose (mg/dl)				Insulin (μ U/ml)				Glucose/Insulin			
	≤ 10	11-18	19-26	28	≤ 10	11-18	19-26	28	≤ 10	11-18	19-26	28
Normal	123.0 \pm 3.1 (3)	134.9 \pm 7.3 (9)	124.4 \pm 8.2 (18)	115.4 \pm 13.5 (14)	—	—	25.8 \pm 5.1 (18)	22.2 \pm 2.9 (14)	—	—	7.8 \pm 1.9 (16)	6.0 \pm 0.9 (14)
Diabetic	387.4 \pm 61.6 (8)	347.3 \pm 36.4 (10)	338.7 \pm 30.6 (11)	246.1 \pm 31.2 (11)	6.9 \pm 3.4 (2)	6.5 \pm 0.7 (2)	15.2 \pm 3.3 (11)	18.4 \pm 7.9 (11)	74 \pm 43.7 (2)	56.1 \pm 3.5 (2)	34.0 \pm 7.5 (11)	32.1 \pm 7.3 (11)
P-value	< 0.05	< 0.001	< 0.001	< 0.001	—	—	NS	NS	—	—	< 0.001	< 0.005

() = Number of maternal rabbits studied.

sera from four hypophysectomized young adult rabbits were also studied. These sera were generously provided by Drs. B. I. Posner and B. Patel, McGill University, Montreal, Canada, and by Dr. A. Vezinhet, INRA, Montpellier, France.⁶

A study of pulmonary maturation in diabetic pregnancy employing most of these same animals has been reported.⁵ Insufficient quantities of serum precluded study of all the rabbits from the first report, and serum samples from additional rabbits are now included. The following groups of sera from adult female rabbits were drawn at approximately weekly intervals: 17 sera from 5 nonpregnant normal does; 14 sera from 4 nonpregnant diabetic does; 39 sera from 18 pregnant does; 33 sera from 12 pregnant does who received alloxan, but did not become hyperglycemic (glucose < 150 mg/dl); and 48 sera from 17 pregnant diabetic does. Sera obtained from 28-day gestation fetuses were as follows: 26 sera from pups in 11 normal litters; 23 sera from pups in 10 litters obtained from rabbits treated with alloxan who re-

mained euglycemic; and 27 sera from pups of 11 litters of diabetic pregnancies. Serum insulin and glucose concentrations are retabulated in groups appropriate for the purpose of this study and include only animals in whom Sm-C concentrations are known.

Insulin and somatomedin-C radioimmunoassays (RIA).

Serum insulin concentrations were determined by a heterologous double antibody RIA using ¹²⁵I-porcine insulin, guinea pig anti-porcine insulin, and a purified porcine insulin standard.⁷ The RIA for somatomedin-C was performed by the nonequilibrium technique originally described by Furlanetto et al.⁸ and modified by Copeland et al.⁹ Human Sm-C of greater than 90% purity was used for iodinations.¹⁰ Somatomedin-C concentrations are reported relative to a human standard serum (Ortho 1788-5; Ortho Diagnostics, Raritan, New Jersey).⁹

Preparation of sera for Sm-C RIA. Each serum sample was mixed with an equal volume of 0.1 M glycine-glycine HCl buffer and incubated in a stoppered polystyrene tube at a final pH of 3.6 for 24 h at 37°C. After incubation, samples were neutralized with 1 N NaOH (3% of incubation volume). Except for the time of incubation, this procedure is identical to the methods that have been developed for the RIA of Sm-C in sheep sera.¹¹ Acid-treated sera were also subjected to gel chromatography in 1 M acetic acid. This was accomplished by eluting 0.3 ml of serum on a 1.6 \times 40.5-cm Sephadex G-50 column (Pharmacia, Piscataway, New Jersey) and collecting two fractions: the first had a K_{av} of 0-0.22 and contained the bulk of serum proteins and Sm-C binding proteins. The second had a K_{av} of 0.22-0.60 and contained the free Sm-C. Fractions were lyophilized and reconstituted in assay buffer.

Statistical methods. In general, comparisons between groups of independent data were performed using the *t* test. For comparisons of serum Sm-C concentrations in the pregnant and nonpregnant adult rabbits, one-way analysis of variance was performed to determine whether systematic differences existed among the determinations obtained at different times within a group of rabbits. If there were no differences among these, the means were used in a *t* test to determine the significance of differences between groups.¹² To determine significant differences between groups of rabbit fetuses, the mean value of each litter was used in a *t* test, rather than the values of each individual fetus.¹² Other comparisons were performed using linear regression analysis to determine if there were correlations

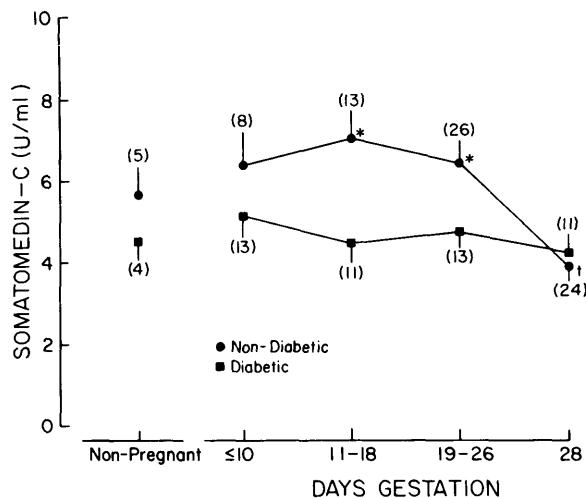


FIGURE 1. Serum Sm-C in normal and diabetic pregnant rabbits. Data are presented as means \pm SEM. The number of rabbits studied at each gestational age is in parentheses. Sera from normal nonpregnant rabbits (17 samples) were obtained from 5 rabbits at approximately weekly intervals, while those from diabetic nonpregnant animals (14 samples) were obtained from 4 animals (serum glucose = 296 ± 36.4 mg/dl, mean \pm SEM). At the times in gestation indicated, (*) indicates significant differences between normal and diabetic animals ($P < 0.05$). (†) Represents significant differences ($P < 0.005$) between this gestational age and each of the other gestational ages in the nondiabetic pregnancy rabbits.

TABLE 2

Glucose, insulin, and glucose/insulin ratio in fetal rabbits (mean \pm SEM; 28 days gestation)

	Glucose (mg/dl)	Insulin (μ U/ml)	Glucose/Insulin
Normal pregnancy	69.0 \pm 8.0 (11)	50.0 \pm 3.6 (19)	1.77 \pm 0.39 (10)
Diabetic pregnancy	218.2 \pm 36.2 (11)	49.6 \pm 7.6 (11)	5.9 \pm 1.1 (11)
P-value	< 0.001	NS	< 0.001

Normal pups were obtained from 21 litters. Ten of these were from litters in which the mother had been given alloxan but had not become glucose intolerant. These results are pooled because there were no significant differences in the glucose or Sm-C values. () = Number of litters studied.

between variables. The significance level was chosen to be $P < 0.05$. All calculations were performed using Hewlett-Packard (Corvallis, Oregon) programs for an HP-97 calculator.

RESULTS

Validations of the Sm-C RIA. When we attempted to measure Sm-C in unprocessed serum, the interference by somatomedin binding proteins made it impossible to distinguish between sera from normal and sera from hypophysectomized rabbits. After exposure to acid for 24 h, however, the mean Sm-C concentration in normal rabbits was 5.67 ± 0.7 U/ml (mean \pm SEM; $N = 5$) and in hypophysectomized rabbits was 0.79 ± 0.06 U/ml ($N = 4$). To ensure that exposure to acid was providing an accurate estimate of the total Sm-C, pools of representative acid-exposed sera (6 serum samples from each of the following 6 pools: normal and diabetic maternal rabbit sera, normal pups and pups of diabetic mothers, and normal and diabetic nonpregnant sera) were gel chromatographed in 1 M acetic acid, a method used to separate somatomedin from its binding proteins.¹³⁻¹⁴ The Sm-C measured after chromatography averaged $80.8 \pm 7.9\%$ (SEM; $N = 6$) of that measured in the acid-exposed sera. The values obtained from the pooled samples using the two methods correlated well ($r = 0.96$, $P = < 0.001$). The slight reduction in activity after chromatography probably resulted from losses during the procedure, rather than artifact caused by binding of ¹²⁵I-Sm-C by binding protein in the RIA itself. This presumption is based on the fact that, when purified Sm-C was added to acid-exposed sera, $98.7 \pm 0.3\%$ ($N = 18$) of it could subsequently be measured in the RIA.

When fresh rabbit sera, not previously exposed to acid, was eluted on acid-gel columns, the Sm-C concentrations measured in the fraction coeluting with free Sm-C were 2–3 times higher than those estimated in acid-treated sera, although the two correlated significantly (linear regression analysis; $r = 0.91$, $P < 0.001$). These fractions were found to contain small molecular weight binding proteins (of about 18–24,000 daltons) when the fractions were affinity labeled by cross-linking of ¹²⁵I-Sm-C with disuccinimidylyl suberate¹⁵ (data not shown). It appears, therefore, that after acid-gel chromatography some binding protein components migrate

with free Sm-C on Sephadex G-50 columns and retain the capacity to bind Sm-C. This artifactually influences the RIA. Furthermore, since acid-treated serum migrates in the appropriate chromatographic fraction and does not contain binding proteins (no cross-linked binding proteins are apparent in these fractions; data not shown), it appears that prolonged acid exposure of sera (24 h) renders binding proteins incapable of binding.

Serum glucose, insulin, and Sm-C in maternal rabbits. Serum glucose concentrations were markedly increased in the diabetic animals throughout gestation, but their insulin concentrations were not significantly different from those of nondiabetic animals (Table 1). The elevated glucose/insulin ratios of the diabetic animals, however, indicate that insulin secretion was impaired. The data from 30 nondiabetic pregnant rabbits include samples from 12 pregnant animals that were given alloxan, but did not become glucose intolerant. Since Sm-C concentrations in these two groups of nondiabetic pregnant rabbits were not different, their values were pooled. Although there was no significant difference between the Sm-C values in normal nonpregnant rabbits (5.67 ± 0.7 U/ml) and the nonpregnant diabetic rabbits (4.53 ± 0.48 U/ml; $P = NS$), the Sm-C in normal pregnant animals was significantly higher than in pregnant diabetic animals studied between 11 and 26 days of gestation (Figure 1).

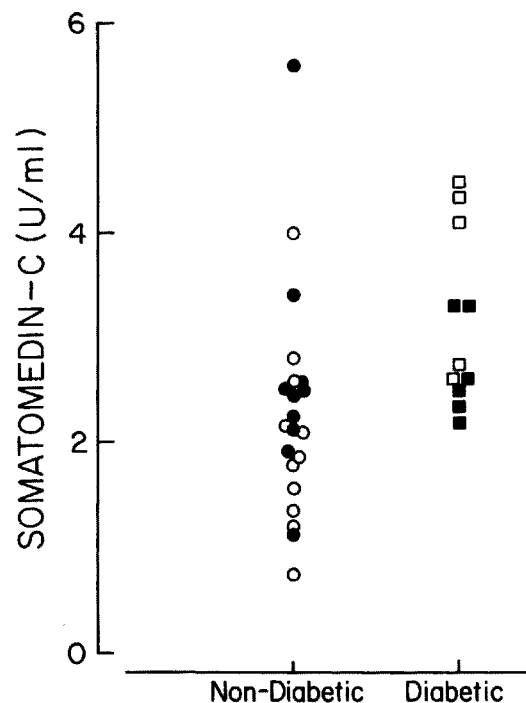


FIGURE 2. Serum Sm-C in 28-day gestation fetuses of normal and diabetic pregnancy litters. The nondiabetic pregnancy (normal) group represents 21 litters: the open circles denote litters of untreated normal pregnancy and the closed circles litters of alloxan-treated, but euglycemic, pregnancies. The diabetic pregnancy group consists of 11 litters: the open squares denote pregnancies in which the maternal serum glucose was > 250 mg/dl at 28-day gestation and the darkened squares pregnancies with maternal glucose 150–250 mg/dl. The difference between the fetuses of normal (3.14 ± 0.25 U/ml; $N = 5$) and diabetic (2.31 ± 0.23 U/ml; $N = 6$) pregnancy litters was significant ($P < 0.05$).

TABLE 3
Litter number, weight, and crown-rump length in 28-day gestation fetuses (mean \pm SEM)

	Litter size (N)	Weight (g)	Crown-rump length (cm)
Normal pregnancy	6.85 \pm 0.7 (21)	33.8 \pm 6.9 (19)	8.6 \pm 0.7 (19)
Diabetic pregnancy	7.45 \pm 0.6 (11)	26.8 \pm 6.9 (11)	7.9 \pm 0.7 (11)
P-value	NS	< 0.005	< 0.025

() = Number of litters studied.

These differences in the two groups might reflect a normal rise at midgestation, since the Sm-C in the groups were not different at term.

Serum glucose, insulin, and Sm-C in fetal rabbits. Glucose concentrations in fetuses from diabetic pregnancy litters were markedly elevated, but mean insulin concentrations were not significantly increased compared with fetuses from litters of nondiabetic mothers (Table 2). Mean Sm-C concentrations in the sera of 28-day gestation fetuses of diabetic pregnancy litters (3.14 \pm 0.25 U/ml) were significantly higher than those of normal pregnancies (2.31 \pm 0.23 U/ml; $P < 0.05$) (Figure 2). Since there was overlap between the mean Sm-C levels of fetuses from normal litters and those of diabetic pregnancies, we searched for differences among the litters of diabetic pregnancies that could account for the elevation of Sm-C. When the fetal Sm-C concentrations were grouped according to maternal serum glucose, the fetuses of the more severely hyperglycemic diabetic mothers (glucose $>$ 250 mg/dl) were higher (3.66 \pm 0.41 U/ml; $N = 5$) than those of less severely hyperglycemic (glucose 150–250 mg/dl) mothers (2.71 \pm 0.20 U/ml; $N = 6$), but the difference was not significant ($0.05 < P < 0.10$). Despite the higher Sm-C concentrations in diabetic litter fetuses, these animals weighed significantly less and had lower crown-rump lengths (see Table 3).

DISCUSSION

In this study, we have validated a method for measuring immunoreactive Sm-C in rabbit serum. The technique used for sample preparation is nearly the same as that applied to sheep serum¹¹ and consists of incubation of serum at pH 3.6. This eliminates the interference by binding proteins in the RIA, and permits distinction between normal and hypophysectomized rabbit sera. It appears to provide a better means of quantitation than acid-gel chromatography in 1 M acetic acid, since the latter does not remove all binding proteins from the Sm-C-rich fraction.

We have observed that, during midgestation, the serum Sm-C in normal rabbit does is higher than the concentration in pregrant rabbits with alloxan-induced diabetes. The reason for this difference is not obvious from our study and the number of animals studied is too small to be certain whether the difference reflects a pregnancy-related rise in normal animals, a diabetes-related depression, or both. Although serum insulin concentrations of diabetic animals were inappropriately low for the degree of hyperglycemia,

they were not significantly lower than those obtained in normal animals. In light of studies that show that serum bioactive somatomedin and immunoreactive Sm-C concentrations are reduced by suboptimal nutritional status, as well as in diabetes,^{16–18} it seems possible that the metabolic consequences of altered carbohydrate metabolism could account for the lower Sm-C in diabetic pregnant animals. One mechanism that might be important in this regard is reduction of growth hormone binding. Chemically induced diabetes causes reduction in plasma Sm-C and in liver growth hormone binding in rats, both of which are restored by insulin therapy.¹⁹

A pregnancy-related rise of Sm-C in normal animals seems probable, since elevations of serum Sm-C concentrations are known to occur during pregnancy in other mammals.³ In man, we²⁰ and others^{21–23} have reported that serum somatomedin concentrations rise progressively during the latter half of gestation. We have hypothesized that placental lactogen, a growth hormone-like peptide, is responsible for this gestational rise in somatomedin, because there is a positive correlation between serum Sm-C and human placental lactogen.^{3,20} Support for this observation comes from the observation that ovine placental lactogen raises serum Sm-C in hypophysectomized rats.²⁴ In mice, on the other hand, serum Sm-C rises early in gestation and reaches its maximal value at about 7 days (gestation is 19 days).²⁵ In this species, the serum Sm-C elevation is temporally related to an elevation of pituitary prolactin, but not to elevations of either of the murine placental lactogens.²⁶ In rabbits, placental lactogen has not been identified with certainty, and the gestational profiles of serum growth hormone and prolactin have not been characterized. Therefore, additional studies are needed to assess the relationship between serum Sm-C and growth hormone-like peptides in rabbit pregnancy.

Our fetuses of alloxan-diabetic rabbits share characteristics with those reported by others^{27–31} and, in some respects, with fetuses of other pregnant diabetic subprimates.³² In our study, as in others, fetuses of diabetic pregnancies were hyperglycemic,^{27–31} but either were not hyperinsulinemic or did not have increased pancreatic insulin,^{27,28} and were not overgrown.^{28,29} Indeed, when maternal hyperglycemia was severe, fetal growth was retarded.²⁷ Similarly, using a diabetic rat model, Kervran et al.³² did not observe an increase in weight of fetuses of mildly hyperglycemic diabetic rats (serum glucose, 100–200 mg/dl). These investigators also noted growth retardation in fetuses of severely hypoglycemic pregnancies (glucose $>$ 300 mg/dl). Unlike our rabbits, however, their rats had increased plasma insulin concentrations late in gestation. They also observed impaired insulin responses to glucose, a finding consistent with our observation that serum insulin concentrations do not correlate with the degree of hyperglycemia in the fetus.

The cause of the elevated Sm-C in rabbit fetuses of hyperglycemic mothers is not obvious, but does not appear to depend on the presence of hyperinsulinism. Under certain conditions, insulin seems to have the capacity to increase somatomedin in the fetus, since Hill and Milner found elevated bioassayable somatomedin in fetal rabbits 48 h after the administration of insulin.³³ Similarly, Spencer et al. showed that bioactive somatomedin is raised in fetal pigs

implanted with insulin-filled osmotic pumps.³⁴ In another study, Hill et al. showed that somatomedin bioactivity is elevated in term fetuses of mildly diabetic pregnant rats.³⁵ However, no elevation of plasma insulin was observed, suggesting that factors other than insulin are capable of elevating somatomedin in the fetus.

In general, it appears that the rabbit is not a good model for investigating growth mechanisms in human infants of diabetic mothers, since none of the rabbit fetuses of diabetic pregnancies were hyperinsulinemic or overgrown. On the other hand, some infants of diabetic mothers who are overgrown have no evidence of hyperinsulinism,³⁶ suggesting that in some situations the parallels between our rabbit model and man may be valid.

The occurrence of elevated Sm-C concentrations in fetuses with moderate growth retardation was not expected, since somatomedin has been shown to be a potent mitogen for cultured cells⁴ and to promote growth *in vivo*.³⁷⁻³⁸ Since high concentrations of glucose appear to be toxic to embryos,^{1,39} it is possible that the growth retardation in fetuses of hyperglycemic mothers results from early exposure to high ambient glucose concentrations. Later in gestation, the excessive energy provided to the fetus might stimulate the synthesis of somatomedin, and even partly compensate for the earlier deleterious effects of glucose excess.

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