Raised Plasma Somatomedin Activity and Cartilage Metabolic Activity (^{35}S Sulphate Uptake in Vitro) in the Fetus of the Mildly Diabetic Pregnant Rat

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Summary. A mildly diabetic state was induced in pregnant rats following treatment with streptozotocin the day after mating. On day 21 of gestation, these rats had a lower plasma insulin (55 ± 9 versus 107 ± 23 mU/l for control rats; $p < 0.05$, mean $\pm$ SEM) and a reduced pancreatic area occupied by insulin-containing cells compared with control animals (0.40 ± 0.04 versus $1.03 \pm 0.08\%$; $p < 0.001$), but hyperglycaemia was not apparent. Fetuses from mildly diabetic animals were longer but not heavier than those from control rats. Plasma somatomedin activity measured by fetal rat cartilage bioassay was higher in fetuses from mildly diabetic rats (1.12 ± 0.07 versus 0.74 ± 0.05 U/ml for control fetuses; $p < 0.001$) as was cartilage metabolic activity in basal culture medium (^{35}S sulphate uptake) (1883 ± 141 versus 1473 ± 104 c.p.m./mg for control rats;

$p < 0.05$), but plasma insulin levels and the pancreatic area occupied by insulin-containing cells did not differ between the two groups of fetuses. Fetal plasma somatomedin activity, measured by fetal cartilage assay, showed a significant positive correlation with both body weight and length. It is concluded that by day 21 of gestation a small body overgrowth had occurred in the fetus of the mildly diabetic rat and this was associated with an increase in plasma somatomedin activity, but not with any abnormality of circulating insulin levels or volume density of B cells in the pancreatic islets.

Key words: Rat fetus, streptozotocin, insulin, pancreas, somatomedin, growth, cartilage.

Insulin has been postulated to act as a growth-promoting hormone in fetal life [1]. This process may involve an increase in the circulating levels of somatomedin since we have shown previously that treatment of the fetal rabbit in utero with a pharmacological dose of insulin resulted in enhanced circulating somatomedin activity and cartilage metabolic activity [2]. No increase in physical growth could be detected over the short period of study, but recently it has been reported that treatment of the fetal rat with similar amounts of insulin in utero resulted in an increase in body weight and length [3].

Induction of a mild, non-ketotic diabetes in the pregnant rat with streptozotocin was associated with enhanced levels of plasma or pancreatic insulin in the fetus [4, 5]. The experimental model of the mildly diabetic pregnant rat has been used in the present experiments to examine the relationship between fetal growth, insulin and somatomedin activity. The mildly diabetic state is characterised by impaired glucose tolerance, reduced plasma insulin and percentage area of pancreas occupied by B cells, but an absence of glucosuria.

Previous studies have shown that when fetal cartilage of appropriate gestational age is used as assay material,

fetal plasma somatomedin activity can be measured in samples to which post-natal cartilage is refractory [6, 7]. For this reason, we have measured fetal rat somatomedin activity by both fetal and post-natal cartilage bioassay systems. A preliminary report of this work has been presented [7].

Materials and Methods

Chemicals

Incubations were performed in Krebs's phosphate-saline buffer (pH 7.5) as modified by Beaton [8] and enriched with amino acids according to Daughaday and Reeder [9]. Medium was supplemented with penicillin (100 U/ml) and streptomycin (100 $\mu\text{g}/\text{ml}$) and sterilised by passage through a Millipore filter (0.45 μm) before use. Streptozotocin was obtained from the Upjohn Co., Kalamazoo, USA, and was administered in sodium citrate buffer (0.1 mmol/l, pH 4.5) at a concentration of 6 mg/ml. [^{35}S] sulphate was obtained from Amersham International, Amersham, Bucks., UK.

Animals

Wistar rats of approximately 200 g weight were used from the Departmental colony. The date of mating was assessed from the appearance

of the vaginal plug which was defined as day 0 of gestation. Pregnant rats were given freshly prepared streptozotocin (30 mg/kg body weight) IP the morning after mating, or received citrate buffer alone. Throughout gestation animals were observed for evidence of glucosuria using a semi-quantitative method (Clinistix, Ames, Slough, Bucks, UK). Of 21 rats treated with streptozotocin, three animals developed glucosuria (> 28 mmol/l) and were excluded from the study. The remaining animals were considered to be mildly diabetic, a classification later confirmed by blood glucose estimation and pancreatic histology. We have shown previously [10] that mildly diabetic rats exhibited a raised blood glucose profile during a glucose tolerance test (peak value > 15 mmol/l) compared with normal animals (peak value < 10 mmol/l). Glucose tolerance tests were not performed on animals in this study to avoid the possible influence of maternal stress on fetal growth or the endocrine system.

On the morning of day 21 of gestation, rats were killed painlessly and the fetuses delivered by Caesarean section. Each fetus was weighed to an accuracy of 10 mg and the nose-tail tip length measured to the nearest 0.5 mm using a sliding jig of the type described by Hughes and Tanner [11]. Fetal blood was collected as a litter pool after decapitation and maternal blood taken from the vena cava. Plasma was separated by centrifugation at 4°C and stored at -20°C . Maternal and fetal pancreases were collected and fixed in Bouin's solution.

Dissection and Incubation of Cartilage

Individual costal cartilages were taken from ribs 3–7 of each fetus, cleaned of extraneous muscle and the costochondral junction area discarded. Each cartilage rib was divided into sections 1–2 mm long. The basal uptake of [^{35}S] sulphate by costal cartilage from each animal was assessed in duplicate by the addition of four cartilage sections to incubation tubes containing buffer (0.9 ml) and isotope (1 μCi , 100 μl). Stimulated uptake was obtained from duplicate incubations in which the Krebs's phosphate-saline buffer was supplemented with pooled plasma from adult male rats (10%, v/v). Incubations were performed at 37°C for 48 h before the reaction was terminated by the addition of boiling water. Cartilage was washed, weighed to an accuracy of 0.1 mg and prepared for liquid scintillation counting at constant efficiency as described by McConaghey [12]. Results are expressed as c.p.m./mg wet weight cartilage and the mean value of duplicate incubations was used in calculations.

Somatomedin and Insulin Assay

Plasma somatomedin activity was assayed by two methods. Firstly by the post-natal pig cartilage bioassay, and secondly using fetal rat cartilage from the control litters; both assay systems having been described previously [6]. The term 'somatomedin activity' describes the stimulatory plasma activity detected by cartilage bioassay and is probably a net effect resulting from the stimulatory actions of somatomedins balanced by the negative influence of plasma inhibitors. Several modifications to the fetal cartilage assay were introduced. One cartilage section from each of four animals from the same litter was incubated in medium supplemented with [^{35}S] sulphate (1 μCi , 100 μl) and test or standard rat plasma to a final volume of 1 ml. Plasma was added at concentrations of 2.5, 5 and 10%. Cartilage was incubated for 48 h at 37°C and prepared for liquid scintillation counting as described earlier. Results are expressed as relative potencies compared with a standard adult rat plasma of defined potency 1.0 U/ml. Estimates of plasma potency were acceptable if analysis of variance showed them to be valid by the criteria described previously [13]. The mean index of precision (λ) for ten assays using fetal rat cartilage was 0.27 ± 0.03 .

Plasma insulin levels were measured by radioimmunoassay [14] using [^{125}I] insulin (Amersham International, Bucks., UK), insulin binding reagent (RD12) from Wellcome, Beckenham, Kent, UK, and a rat insulin standard. Duplicate plasma aliquots (50 μl) were assayed and the limit of sensitivity was 15 $\mu\text{U/l}$. Separation of free from bound insulin was by centrifugation. Inter- and intra-assay coefficients of variation for this assay are 12 and 5%, respectively. Blood glucose was measured by a glucose oxidase technique [15].

Histology

Tissue was transferred from Bouin's reagent to 70% ethanol before embedding in paraffin wax. The tail portion of the adult pancreas attached to the spleen was embedded. All fetal pancreases from one litter were embedded together and treated as a single unit of tissue, including both head and tail regions. Sections of 6 μ thickness were stained specifically for insulin by the unlabelled antibody enzyme peroxidase/antiperoxidase method of Sternberger et al. [16]. The percentage area of pancreatic tissue occupied by insulin-containing cells was estimated by automatic image analysis using a Quantimet 720 (Cambridge Instruments, Cambridge, UK). For adult tissue, a sample of 80 fields of view covering 30.8 mm² of each section was selected. In some fetal tissue sections the total area was < 80 fields in which case the total tissue area was observed.

Statistical Methods

Statistical differences between experimental groups were calculated by Student's t-test between mean values, or by the paired t-test. Mean litter values for body weight, body length and pancreatic area of B cells were used in statistical analyses. Coefficients of regression were calculated by the least squares method. All analyses were calculated from the number of litters.

Results

The litter size, body weight, and nose-tail length of fetuses from control and mildly diabetic rats are shown in Table 1. On day 21 of gestation, nose-tail length was significantly greater in the offspring of mildly diabetic rats, but body weight did not differ from that of control animals. Mean litter number was similar in both groups. Fetuses were also examined for the frequency at which macrosomia occurred with the litters. Macrosomia was defined as a fetus with body weight exceeding $+ 2$ SD of the mean fetal weight of the control animals, an adaptation of the methods described by Kim et al. [17]. The frequency of macrosomia in control fetuses was one in 149 and in fetuses of mildly diabetic rats was six in 210. The increase in maternal weight over the period of gestation was similar in both groups and averaged 78 ± 12 g.

Fetal somatomedin activity, measured by the post-natal pig cartilage assay was significantly lower than normal adult levels but did not differ between fetuses from control and mildly diabetic rats (Table 2). Maternal plasma somatomedin activity measured by this assay was significantly lower in diabetic rats than in control animals (0.50 ± 0.04 versus 0.85 ± 0.15 U/ml, $p < 0.05$).

When measured by the fetal rat cartilage assay the somatomedin activity in fetal plasma did not differ from adult values but was significantly increased in plasma from fetuses of mildly diabetic animals compared with control fetuses (Table 2). Somatomedin measured by the fetal cartilage assay showed a positive correlation with nose-tail tip length (Fig. 1), and body weight ($r = 0.33$, $p < 0.05$). When fetuses of diabetic rats were considered alone, a significant positive correlation was maintained between somatomedin activity and nose-tail length ($r = 0.65$, $p < 0.01$), but this did not reach statistical significance for control fetuses considered alone.

Costal cartilage removed from each fetus shortly after death was incubated in plasma-free medium containing [35 S] sulphate to give an indication of the previous metabolic activity of the tissue in vivo. This basal isotope uptake was significantly raised in cartilage from fetuses of mildly diabetic animals compared to control fetuses (Table 2). Cartilage was also incubated in the presence of plasma to determine the extent to which the tissue could be further stimulated in vitro. Stimulated isotope uptake was significantly higher than basal values for cartilage but did not differ between groups.

Plasma insulin levels were significantly reduced in animals treated with streptozotocin compared to control

Table 1. Litter size, body weight and nose-tail tip length of fetuses of 21 days gestational age from control and mildly diabetic rats

	Fetal rats	
	Control (<i>n</i> = 15)	Mildly diabetic (<i>n</i> = 19)
Litter size	9.9 $\pm$ 0.6	11.1 $\pm$ 0.6
Body weight (g)	4.60 $\pm$ 0.06	4.69 $\pm$ 0.06
Nose-tail tip length (mm)	58.9 $\pm$ 0.4	60.7 $\pm$ 0.5 ^a

Results expressed as mean $\pm$ SEM (*n* = number of litters); ^a *p* < 0.01

Table 2. Plasma somatomedin activity and the incorporation of [35 S] sulphate into fetal rat costal cartilage in medium alone (basal) and medium supplemented with 10% normal rat plasma (stimulated) for fetuses from control and mildly diabetic rats

	Fetal rats	
	Control (<i>n</i> = 15)	Mildly diabetic (<i>n</i> = 19)
Somatomedin activity (U/ml)		
Pig cartilage assay	0.49 $\pm$ 0.05 ^c	0.56 $\pm$ 0.04 ^c
Fetal rat cartilage assay	0.74 $\pm$ 0.05	1.12 $\pm$ 0.07 ^{b,d}
Uptake of [35 S] sulphate by cartilage (c.p.m./mg)		
Basal	1473 $\pm$ 104	1883 $\pm$ 141 ^a
Stimulated	11333 $\pm$ 980	12445 $\pm$ 901

Results are expressed as mean $\pm$ SEM (*n* = number of litters).

^a *p* < 0.05; ^b *p* < 0.001; ^c *p* < 0.001 compared with adult standard plasma (1.0 U/ml); ^d *n* = 18

Table 3. Plasma insulin, the area of the pancreas occupied by insulin-containing cells and non-fasted glucose levels in control and mildly diabetic rats and their fetuses

	Maternal		Fetal	
	Control (<i>n</i> = 15)	Mildly diabetic (<i>n</i> = 19)	Control (<i>n</i> = 15)	Mildly diabetic (<i>n</i> = 19)
Insulin (mU/l)	107 $\pm$ 23	55 $\pm$ 9 ^a	101 $\pm$ 7	97 $\pm$ 7
Area of pancreas occupied by insulin containing cells (%)	1.03 $\pm$ 0.08	0.40 $\pm$ 0.04 ^b	0.89 $\pm$ 0.10	0.78 $\pm$ 0.06
Glucose (mmol/l)	5.52 $\pm$ 0.23	4.77 $\pm$ 0.29	4.03 $\pm$ 0.15	3.58 $\pm$ 0.33

Results expressed as mean $\pm$ SEM. (*n* = number of litters); ^a *p* < 0.05; ^b *p* < 0.001

values, although the plasma insulin levels in the fetuses of these animals did not differ from those in fetuses of control rats (Table 3). Similarly, the area of pancreas occupied by insulin-containing cells was reduced in the streptozotocin-treated rats, but not in their fetuses. Non-fasted blood glucose levels were similar between groups in both mothers and fetuses. Plasma insulin levels did not correlate with either body size or plasma somatomedin activity in the fetuses.

Discussion

The dose of streptozotocin used induced a mild diabetic state in the female pregnant rat, in which blood glucose levels were not raised on day 21 of gestation, despite a reduction in the area of the pancreas occupied by insulin-containing cells and a lowered plasma insulin level compared with control animals. Such rats have an impaired response to a glucose tolerance test [10]. The glucose intolerance induced in this study is less severe than that reported by Van Assche et al. [18] who found glucosuria and raised blood glucose levels when using the same dose of streptozotocin. Plasma somatomedin activity in the mildly diabetic rats was reduced as has been reported

Fig. 1. Somatomedin activity, measured using fetal rat cartilage, in litter pools of fetal rat plasma from animals of 21 days gestational age (mildly diabetic mothers, ●; controls, ○) compared with nose-tail tip length (*r* = 0.55, *p* < 0.001). Symbols indicate the mean value for nose-tail length for each litter

to occur in the severely diabetic non-pregnant rat [19]. Fetuses from mildly diabetic rats showed a small but statistically significant increase in nose-tail tip length, but were not heavier than control rats. The similarity in body weight of fetuses from diabetic and control rats agrees with previous studies using the same dose of streptozotocin [4, 22]. However, by the day of birth the newborns of mildly diabetic rats were reported to be heavier than those of control animals [20], although this was not confirmed by other authors [4, 5].

The modest increase in body length in fetuses from diabetic rats was accompanied by a raised cartilage uptake of [^{35}S] sulphate in basal culture medium. Since tissue was removed from the animal immediately after death this reflects a greater metabolic activity *in vivo*. However, the capacity for stimulation by plasma *in vitro* did not differ suggesting that the nature of the tissue response was unaltered. Somatomedin, postulated to promote cartilage growth *in vivo* and detected by us as somatomedin activity, was significantly raised in fetuses of mildly diabetic rats when measured by the fetal rat cartilage bioassay. We have shown previously that the fetal cartilage assay will detect somatomedin activity in fetal rat plasma which cannot be detected using the post-natal pig cartilage assay [6] and this was confirmed in this study. We do not yet know whether the fetal assay detects a specific fetal form of somatomedin or whether fetal tissue has an enhanced sensitivity to one of the somatomedins present in the adult such as multiplication stimulating activity, which is many times higher in fetal rat serum than in the adult [21].

Somatomedin activity detected by the fetal cartilage assay showed a significant positive correlation with body length and weight for animals of the same gestational age, supporting a role for somatomedins as growth-promoting hormones in fetal life. The raised plasma somatomedin activity found in the fetuses from mildly diabetic rats, and the enhanced cartilage metabolic activity, suggest that somatomedin may have been involved in the observed increase in longitudinal skeletal growth.

No difference was found in the pancreatic area occupied by insulin-containing cells, or in blood glucose levels, between fetuses from the two groups of animals which agrees with previous reports of pancreatic histology at this stage of gestation [22]. However, the pancreatic content of insulin was reported to be increased in the fetus of the mildly diabetic rat [4, 5] although a corresponding increase in plasma insulin levels was seen only in one of these studies [5]. Although we have found previously that plasma insulin levels were raised in fetuses of mildly diabetic animals [10] no such increase was detectable here. No explanation for the discrepancy can be offered other than normal experimental variation since the procedures, source of animals and reagents were identical in both cases.

It is therefore difficult to associate the fetal overgrowth and enhanced somatomedin activity in the fetus of the mildly diabetic rat with an abnormality of insulin

secretion. The lack of increased body weight in these animals compared to controls could indicate that fetal insulin is more closely related to growth in fetal weight, while somatomedin activity is associated primarily with an increase in body length derived from skeletal growth. It is possible that the maternal diabetes was more manifest at an earlier stage of gestation where fetal hyperglycaemia and hyperinsulinaemia may have occurred; these no longer being apparent by day 21. This hypothesis is supported by the reported doubling of percentage area of pancreas occupied by endocrine tissue at a fetal age corresponding to day 19 of gestation in our study, in the sub-diabetic animal [22]. The anabolic response still detectable in the fetus on day 21 could well reflect events from earlier in gestation since somatomedins are held within the circulation by large molecular weight binding proteins which greatly extend their half-life [23].

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