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Skeletal growth of fetuses from streptozotocin diabetic rat mothers: in vivo and in vitro studies

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Summary. For largely unknown reasons severe or moderate diabetes of pregnant rats results in pronounced fetal growth retardation. Therefore, some skeletal growth parameters of fetal rats from streptozotocin diabetic mothers were studied in vivo and in vitro. Two days post conception rats were intravenously injected with 65 mg/kg body weight streptozotocin. On day 20 post conception 8 normal and 8 diabetic rat mothers received 5 μCi 3-H thymidine intraperitoneally. One day later the experiments were terminated. Fetal body weight and body length were significantly ($p < 0.05$–0.001) reduced in the hyperglycaemic rats compared to normal rats, as was the thymidine incorporation into rib cartilage ($p < 0.02$). In the cell culture colony formation from isolated chondrocytes of normal and hyperglycaemic fetuses was determined. Proinsulin, insulin (62.5–250 ng/ml), insulin-like growth factor I and II (6.25–25 ng/ml) significantly ($p < 0.05$–0.001) augmented colony formation in a dose-dependent manner, with the somatomedins being 8 times more effective than proinsulin or insulin. Isolated chondrocytes from hyperglycaemic compared to normal fetuses formed significantly ($p < 0.05$–0.001) fewer colonies in the basal state and in response to all 4 hormones. The results confirm the growth retardation of fetuses from diabetic rat mothers. A reduced responsiveness of chondrocytes from hyperglycaemic fetuses to various growth factors could be demonstrated as compared to cells from normal fetuses.

Key words: Fetal rats, skeletal growth, streptozotocin, diabetes.

The classic hyperglycaemic-hyperinsulinism hypothesis by Pedersen [1] and modified by Freinkel [2] gives an explanation for the macrosomia of infants from diabetic mothers. However, as reported recently by one group [3, 4], skeletal growth delay during early gestation as measured by ultrasound is a frequent complication of diabetic human pregnancies. This early growth delay is negatively correlated to the quality of diabetes regulation. Thus, in human pregnancy diabetes may result in growth delay during early gestation and in augmented somatic growth towards term. In fetal rats it was not possible to confirm that maternal diabetes increased fetal size. Severe diabetes of pregnant rats resulted in a pronounced fetal growth retardation, while a mild diabetic syndrome during pregnancy was associated with no or a moderate reduction in fetal size [5–7]. Why fetuses from diabetic rat mothers are smaller compared to normal rat mothers is largely unknown. Therefore, some skeletal growth parameters of fetal rats from streptozotocin diabetic mothers were studied.

Materials and methods

Wistar rats were used (Thomae, Biberach, FRG). Mating of the rats has been previously reported [9]. Briefly, one male and one female Wistar rat were caged together from 08.00 to 10.00 hours. Appearance of vaginal plug indicated the time of conception. Two days later streptozotocin (STZ) (Boehringer, Mannheim, FRG) was dissolved in citrate buffer, pH 4.5, and 65 mg STZ/kg body weight was injected into a tail vein. As previously described [9], on day 20 post conception all animals received intaperitoneally 5 μCi 3-H thymidine (specific activity 6.7 mCI/mmol, New England Nuclear, Boston, Mass., USA). One day later, after an overnight fast of 14 h, the animals were anaesthetized with Nembutal (40 mg/100 g body weight, Abbott Laboratories, Chicago, Ill., USA) and the fetuses delivered individually by Caesarian section. Body weight and nose-rump length were determined. After killing by decapitation, blood was collected from the neck vessels, centrifuged, and the serum pooled and stored at −20 °C until use. Serum glucose was determined from the pooled serum of each litter (Autoanalyzer II, Beckman, Munich, FRG).

Cartilage from ribs 3–7 of each fetus was dissected and cleaned from adherent tissue and perichondrium under a magnifier lamp. For the isolation of chondrocytes one half of cartilage from all fetuses of one litter was pooled. The remaining cartilage from each fetus was dried at room temperature for 20 h and weighed. The cartilage was

Table 1. Litter size, fetal weight, fetal length, 3-H-thymidine incorporation in vivo (cmp per mg fetal rib cartilage), maternal and fetal serum glucose of normal and streptozotocin diabetic rats

	Normal rats ($n=8$)	Diabetic rats ($n=8$)	p
Litter size	9.4 ± 1.0	9.1 ± 1.1	NS
Fetal weight (g)	5.61 ± 0.13	4.75 ± 0.11	<0.001
Fetal length (cm)	5.13 ± 0.05	4.85 ± 0.06	<0.05
3-H-incorp. (cpm/mg)	298 ±38	116 ±18	<0.02
Serum glucose (mmol/l)			
Maternal	5.8 ± 0.3	24.6 ± 1.5	<0.001
Fetal	3.6 ± 0.3	14.8 ± 1.1	<0.001

Mean ± SEM; NS = not significant

then dissolved in 0.4 ml concentrated formic acid at 70 °C for 3 h. Ten ml Bray solution was added and the radioactivity counted in a liquid scintillation counter (Isocap 300, Searle, Frankfurt/M., FRG) [9].

Isolation and culture of the chondrocytes has been previously reported [10]. Briefly, half of the fetal rib cartilage from one litter was minced and incubated for 30 min at 37 °C in Ham's F-10 medium supplemented with 0.25% (w/v) trypsin and 2 mg/ml collagenase CLS II Worthington (150 U/mg, Seromed-Biochrom AG, West Berlin, FRG). After centrifugation at 300 *g* for 10 min, the sediment was resuspended in 20 ml Ham's F-10 medium supplemented with 10% (v/v) fetal calf serum (FCS), 200 mmol/l L-glutamin, 0.1 ml MEM vitamins (Seromed), and centrifuged at 300 *g* for 10 min. In the trypan exclusion test [11] the viability of the single cell suspension was 82 + 5% (mean ± SD). The growth of chondrocytes in vitro was determined with a clonal proliferation assay. The culture technique is based on the observation that the use of semisolid culture medium prevented the sedimentation of cells and therefore enabled proliferating chondrocytes to form colonies. In 96 hole micro-culture plates (Greiner, Nürtingen, FRG), 400 chondrocytes were incubated in 100 µl BM-Whissler-Medium (Boehringer, Mannheim, FRG). One ml BM-Wissler-Medium was supplemented with 5% (v/v) heat inactivated fetal calf serum (FCS incubated for 60 min at 70 °C), 40 mg (w/v) gentamycin (BYK-ESSEX, Munich, FRG), 5 µl MEM vitamins, 0.1 mol/l 2-mercaptoethanol (v/v), 1.0% (v/v) methylcellulose (Fluka, Buchs, Switzerland) and different concentrations of hormones: human biosynthetic insulin and proinsulin 62-250 ng/ml, insulin-like growth-factor I and II 6.25-25 ng/ml. The cultures were incubated without medium change for 14 days at a temperature of 37 °C, an atmosphere of 5% O_2, 5% CO_2, and a 95% relative humidity. Results are expressed as percent colonies of inserted viable chondrocytes.

Biosynthetic human insulin and proinsulin were generously provided by Dr. K. Schlüter, Eli Lilly, Bad Homburg, FRG, while insulin-like growth factor I and II (IGF I, IGF II) were a generous gift of Dr. J. Zapf, University of Zürich, Switzerland.

Statistical analysis

Statistical analysis was performed with Student's paired or unpaired t-test; $p < 0.05$ was chosen to represent statistical significance.

Results

In vivo studies

Serum glucose concentrations of the diabetic rats and their fetuses were elevated compared to normal rats (Table 1). In both groups of rats fetal serum glucose concentrations were significantly reduced to about 60% compared to the corresponding maternal concentrations (normal rats, $p < 0.002$; diabetic rats, $p < 0.001$).

The mean litter size of the control and the diabetic rats did not differ from each other. As shown in Table 1, fetal body weights were lower in hyperglycaemic fetuses compared to normal fetuses. The mean weight reduction was 24%. Length was also reduced in hyperglycaemic fetuses compared to normal fetuses an average of 4.6%. Finally, the incorporation of thymidine into fetal rib cartilage was determined. Twenty-four hours after the injection of thymidine less radioactivity was found in the rib cartilages of hyperglycaemic fetuses compared to normal fetuses.

In vitro studies

Four hundred isolated chondrocytes from normal or hyperglycaemic fetuses were cultured for 14 days. The addition of insulin, proinsulin, IGF I or IGF II augmented the colony formation in a dose-dependent fashion (Figs. 1 and 2). On a molar basis IGF I and IGF II were approximately 8 times more effective in stimulating clonal growth than insulin or proinsulin. No difference in colony formation was observed between IGF I and IGF II or between insulin and proinsulin. Finally, clonal growth from chondrocytes of normal rats and hyperglycaemic rats was compared. Under basal conditions and after the addition of the 4 hormones, clonal growth was significantly less ($p < 0.05$-0.001) when cells from hyperglycaemic fetuses were cultured compared to chondrocytes from normal fetuses.

Discussion

The assumption that insulin may be a fetal growth factor was primarily derived from human pathology. The large babies of diabetic mothers are thought to be the result of increased insulin concentrations in the fetus, while the products of the opposite hormonal environment are small babies, such as in congenital diabetes mellitus [2, 12, 13]. In fetal rats it was difficult to confirm that maternal hyperglycaemia increased fetal weight and length.

Severe diabetes of pregnant rats, as in BB-rats [14] or as induced by 50-65 mg STZ/kg body weight in normal rats, resulted in severe growth retardation [5, 15]. Fetuses of diabetic BB-rats had, in addition to low body weights, had less ossification centres and a reduced calcium and phosphorous content of the skeleton. In one recent study fetuses of normal rats were injected twice with STZ or citrate buffer. The interfetal STZ injection resulted in stunted fetal growth without hyperglycaemia. Plasma insulin concentrations and pancreatic insulin contents were lower in STZ treated fetuses compared to buffer treated controls [16]. Thus, it appears that fetal rats need appropriate amounts of insulin for normal growth. Attempts to reproduce in fetal rats the

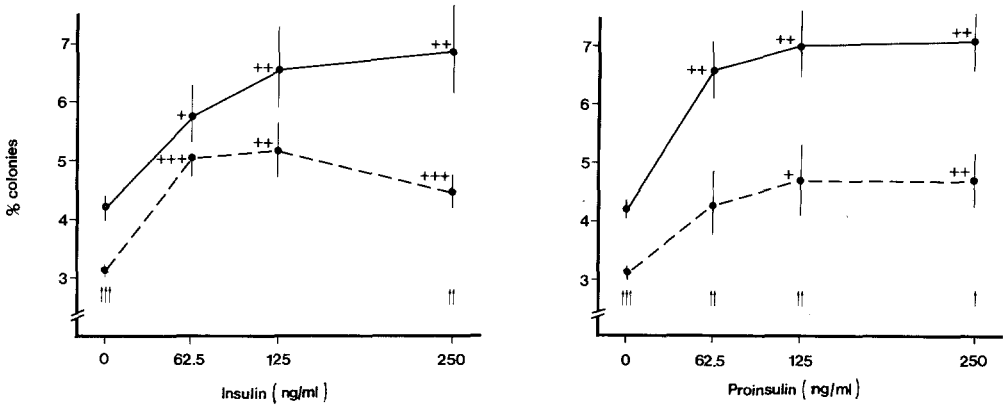

Fig. 1. The effect of insulin and proinsulin on clonal growth of isolated chondrocytes from normal ●—● ($n=8$) and hyperglycaemic ○-----○ ($n=8$) 21-day-old fetal rats. Comparison versus basal: $p+<0.05$; $++<0.01$; $+++<0.001$. Arrows ↑ indicate comparison between normal and hyperglycaemic group: $p\uparrow<0.05$; $\uparrow\uparrow<0.02$; $\uparrow\uparrow\uparrow<0.002$.

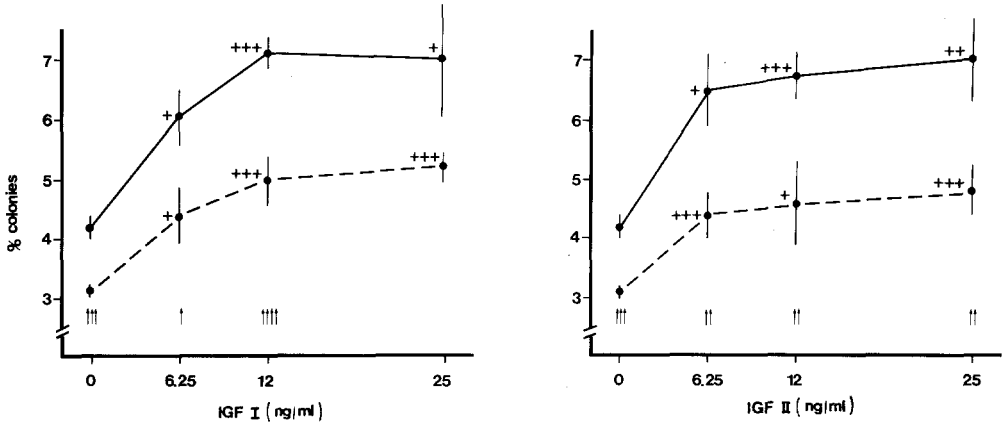

Fig. 2. The effect of insulin-like growth factor I (IGF I) and II (IGF II) on clonal growth of isolated chondrocytes from normal ●—● ($n=8$) and hyperglycaemic ○-----○ ($n=8$) 21-day-old fetal rats. Arrows ↑ indicate comparison between normal and hyperglycaemic group: $p\uparrow<0.05$; $\uparrow\uparrow<0.02$; $\uparrow\uparrow\uparrow<0.002$; $\uparrow\uparrow\uparrow\uparrow<0.001$

overgrowth seen in human infants of diabetic mothers have been less successful. Fetal body weights were determined on days 18, 20 and 22 of gestation in normal, manifest diabetic and insulin-treated diabetic rats. Fetal body weights of the manifest diabetic rats were significantly lower on all 3 days of observation. Insulin treatment nearly normalized maternal blood glucose as well as fetal body weights. However, clearly no overweight fetuses could be observed [6].

Neonatal macrosomia in maternal diabetes was induced by the injection of 30 mg STZ/kg body weight to pregnant rats. However, in these mildly diabetic animals gestation was prolonged by 12 to 40 h compared to normal rats, which may explain the increased neonatal size of mildly diabetic rats [17, 18]. When the high dose of 2 U insulin per fetus was injected on the last day of pregnancy, fetal weight and length were significantly augmented [19, 20]. Thus, it appears that, in contrast to human fetuses, rat fetuses grow at an optimal rate; further, only pharmacological doses of insulin can increase fetal size any further [21]. The results of the present study confirmed earlier reports showing that rat fetuses from severely diabetic mothers had reduced body weights and lengths as compared to control fetuses of the same gestational age [5–7]. Reduction in weight amounted 24% and 4.6% in length. A nearly identical result can be calculated from a very recent report, where fetuses from STZ-induced diabetic Wistar and Sprague-Dawley rats were studied [7]. The lower in vivo incorporation of 3-H thymidine into rib cartilage of hyperglycaemic as compared to normal fetal rats corresponded to a previous experiment, where we could show that a continuous infusion of high amounts of glucose during the last two days of pregnancy decreased the incorporation of the label into rib cartilage of small hyperglycaemic fetuses [9]. In vivo studies cannot distinguish between serum and cellular factors that may decrease cartilage growth. Therefore, growth of isolated chondrocytes was studied in vitro. Our assay of colony formation measured clonal growth of chondrocytes [10].

Besides other growth factors such as epidermal growth factor [12], insulin and the somatomedins may be involved in fetal growth [23]. In the present study IGF I, IGF II, insulin and proinsulin in a dose-dependent manner enhanced colony formation of isolated chondrocytes from normal and hyperglycaemic fetuses. In response to all four growth factors, cells from normal rats grew better than cells from hyperglycaemic animals. The results further show that the two somatomedins were about 8 times more effective than proinsulin or insulin. Compared to serum levels of normal fetal rats, IGF I and IGF II enhanced colony formation when added in physiological [24], and proinsulin and insulin in pharmacological concentrations [25–27]. A comparable result was obtained when uptake of 3-H thymidine into pieces of rib cartilage from normal fetal rats was determined [22]. In our study IGF I and IGF II were equipotent to stimulate colony formation, though

it has been claimed that IGF II is the somatomedin for the fetus [24, 28]. The result of the present study suggests that in the rat the stimulatory action of IGF II on fetal growth decreased shortly before term, when equipotency with IGF I, the predominant adult type somatomedin, was achieved.

The reduced clonal growth of isolated chondrocytes from hyperglycaemic rat fetuses in response to growth factors may explain, at least in part, skeletal growth retardation of these animals. However, it has been reported that serum of adult STZ-induced diabetic rats contained somatomedin inhibitors [29]. Very recently these low-molecular-weight inhibitors were added to mouse embryos in culture, which resulted in growth retardation of the embryos [30].

In conclusion, the results of the present study may explain, at least in part, the skeletal growth retardation of fetuses from streptozotocin diabetic rats. A reduced responsiveness of chondrocytes from these fetuses to some defined growth factors was observed. As discussed, serum somatomedin inhibitors may be equally important for the growth retardation of hyperglycaemic rat fetuses.

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