Abnormal ambient glucose levels inhibit proteoglycan core protein gene expression and reduce proteoglycan accumulation during chondrogenesis: Possible mechanism for teratogenic effects of maternal diabetes

(extracellular matrix/skeletal birth defects)

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ABSTRACT Using a tissue culture system based on a nearly pure population of avian precartilage mesenchymal cells, we have found that ambient glucose levels as little as 50% lower, or 100% higher, than normally present in embryonic sera are deleterious to cartilage development, as measured by the accumulation of highly sulfated proteoglycan and the corresponding cartilage-specific chondroitin sulfate core protein mRNA. Abnormal glucose concentrations in the ranges studied did not selectively influence cell replication, and the effects on chondrogenesis were not due to differences in overall protein synthesis or glucose utilization in the treatment groups. Core protein gene expression was more severely affected than accumulation of extracellular product, suggesting the existence of posttranscriptional compensatory mechanisms. The sensitivity to ambient glucose levels of both expression of the cartilage-specific chondroitin sulfate core protein gene and the accumulation of the corresponding extracellular matrix macromolecules during chondrogenesis suggest a molecular mechanism for the well-known adverse effect of maternal diabetes on embryonic skeletogenesis. The results further suggest that hypoglycemia resulting from stringent control of diabetes may also be deleterious to skeletal development.

Congenital malformations occur 3–4 times more often in offspring of insulin-dependent diabetic women than in the general population (1, 2). Skeletal defects, including partial or complete agenesis of sacral vertebrae in conjunction with hypoplasia of the lower limbs (caudal regression syndrome), are particularly common, occurring in ≈1% of children born to diabetic mothers (3). The cause of these defects is unknown, although recent clinical (1, 4–8) and experimental (9–13) studies have implicated abnormal maternal metabolism during early embryogenesis (rather than compromised vasculature or other aspects of diabetes) in their etiology.

While insulin itself can act as a teratogen (14–17), its ability to cross the placental barrier has been questioned (refs. 18 and 19; however, cf. ref. 20). Recent attention has therefore focused on abnormal maternal serum glucose levels as a potential cause of diabetes-related birth defects. Infants born to poorly controlled insulin-dependent diabetic women have a higher incidence of perinatal mortality and congenital abnormalities than those born to mothers who have been well-controlled at the critical periods for organogenesis and throughout pregnancy (8, 21, 22). However, stringent metabolic control of insulin-treated diabetic women during pregnancy may expose patients to frequent hypoglycemia (23), which is also of concern as a risk factor in fetal development (15, 24). Experimental studies of pregnant animals, and embryos in organ culture, indicate teratogenic consequences of both hyper- (10, 25, 26) and hypoglycemia (15, 16, 27, 28).

In the present study, we have used an in vitro system to determine whether abnormal glucose levels in the range encountered clinically can directly impair the development of skeletogenic tissue. During vertebrate limb development, precartilage mesenchymal cells differentiate in a spatiotemporal pattern, giving rise to the cartilaginous primordia of the limb skeleton (29–31). The mesenchyme located within 300 μm of the distal wing tip of the 5-day chicken embryo constitutes a nearly homogeneous population of precartilage cells, uncontaminated by cells of the myogenic lineage (30, 32). When these cells are grown in high-density culture, they differentiate progressively and uniformly into cartilage (30), elaborating a characteristic extracellular matrix rich in highly sulfated proteoglycans. These molecules, which largely consist of proteochondroitin sulfate (33), are major structural components of this tissue. They play a critical role in the integrity of the cartilaginous models of endochondral bones and in the functioning of growth plates during long-bone development (34).

We have used the extent of accumulation of highly sulfated proteoglycan, and of mRNA for the corresponding cartilage-specific proteoglycan core protein (35), as quantifiable end points to assess the effects of altered glucose concentrations on a specific developmental event. Our results provide direct evidence that both high and low glucose levels, in ranges that do not impair cell replication or protein synthesis, have a deleterious effect on cartilage development.

MATERIALS AND METHODS

Isolation and Culture of Cells. Cells were prepared by trypsin treatment of distal wing bud tips from Hamburger–Hamilton stage 25 (36) chicken embryos (Avian Services, Frenchtown, NJ) as described (30, 37). Cells were plated at 10–μl spots (2.5 × 10³ cells per ml) (38) in 24-well culture plates (Falcon no. 3047 or Costar no. 3024). After 1 hr at 38°C and 5% CO₂/95% air, 1 ml of Ham’s F-12 medium (GIBCO) containing 10% fetal bovine serum was added to each well, and the plates were returned to the incubator. Medium was completely replaced on each successive day of culture. In experiments in which the concentration of glucose was varied, media were prepared by using powdered glucose-free Ham’s F-12 medium, obtained as a special order from GIBCO, and a concentrated glucose solution. Glucose concentrations were determined by a glucose oxidase method (Glucose Analyzer; Beckman).

Quantitative Alcian Blue Staining of Spot Cultures. After designated times, cell layers were washed with Earle’s bal-
anced saline solution, fixed for 10 min with 3% acetic acid adjusted to pH 1.0 with HCl, and stained overnight with 0.5% alcin blue 8GS made up in the same solution (39, 40). Cell layers were washed twice with 3% acetic acid adjusted to pH 1.0 to remove unbound stain and then extracted overnight at 4°C with 8 M guanidinium chloride. Extracts from each well were removed to a 96-well microtiter plate and their absorbance at 600 nm was read spectrophotometrically (40) with a Bioket EIA Reader (Bio-Tek, Burlington, VT).

Assay for Incorporation of Sulfate into Proteoglycan. Cells were grown as described, but with the addition of 5–7 μCi of Na35SO4 per ml (carrier free; 1 Ci = 37 GBq; Amersham) to the medium. Medium was collected on each day and pooled for each spot culture. At the end of the 6-day culture period, each cell spot was homogenized in 1 ml of F-12 medium. Cell spot homogenates and pooled media were digested overnight at 37°C with 100 μg of proteinase K per ml. Aliquots of each digest were spotted onto Whatman 3 MM filter paper and completely dried. Filter paper strips were washed with five changes of 1% cetylpyridinium chloride (400 ml) in 0.3 M NaCl, as described (41). Strips were then dried and cut into squares containing individual samples. These, in turn, were subdivided into eighths and suspended in an Aquasol 2 gel for liquid scintillation counting.

In Vitro Radiolabeling of Cells and Determination of Relative Cell Proliferation and Protein Synthesis. Cultures were prepared as described and grown in the presence of 0.06 μCi of [3H]thymidine per ml (Amersham TRK-686) or 0.22 μCi of [3H]leucine per ml (Amersham TRK-636) in medium containing appropriate concentrations of glucose. Radioactive medium was replaced each day. After 6 days of culture, acid-precipitable radioactivity in each cell layer was determined by liquid scintillation counting.

RNA Isolation. RNA was isolated from cells according to the procedure of White and Bancroft (42) with modifications. Equivalent numbers of cells were harvested from 24-well culture plates and suspended in cold 10 mM Tris-HCl, pH 7.0/1 mM EDTA. Cells were lysed by the addition of two successive 0.1-ml aliquots of 5% Nonidet P-40 with a 5-min interval. Nuclei were removed from the lysates by centrifugation for 3 min at 13,500 × g. The cytoplasmic supernatant was extracted with 1 vol of phenol/chloroform [1:1 (vol/vol) buffered with 10 mM Tris-HCl, pH 7.0/1 mM EDTA/100 mM NaCl]. This was followed by an additional extraction of the supernatant with 1 vol of chloroform/isoamyl alcohol (24:1, vol/vol). To the final aqueous supernatant was added 0.2 vol of 37% formaldehyde (final concentration, 7.4%) and 1 M sodium phosphate (pH 6.5) was added to bring the solution to 25 mM sodium phosphate. The mixture was then incubated at 60°C for 15 min and stored at -70°C. Replicate cultures were homogenized in high salt buffer and analyzed for DNA content by the Hoeschst 33258 dye binding method (43). For analysis, volumes of cytoplasmic extract corresponding to equivalent amounts of DNA were applied with suction to GeneScreen (DuPont), which was presoaked in 25 mM sodium phosphate (pH 6.5), using a slot-blot manifold. The RNA was covalently crosslinked to the membrane by exposure to shortwave UV light for 2 min.

Hybridization Analysis. Hybridization was performed for 12–18 hr at 42°C in a solution containing 50% formamide, 0.2% polyvinylpyrrolidone (Mr, 40,000), 0.2% bovine serum albumin, 0.2% Ficoll (Mr, 400,000), 0.05 M Tris-HCl (pH 7.5), 1.0 M NaCl, 0.1% sodium pyrophosphate, 1% SDS, 10% dextran sulfate (Mr, 500,000), denatured salmon sperm DNA (200 μg/ml), and labeled probe. The cloned chicken cartilage proteoglycan core protein cDNA plasmid ST-1 (35) was labeled with 32PdCTP (3000 Ci/mmol; Amersham) using a multiprime DNA labeling system (Amersham). Prehybridization was carried out overnight in the same buffer without probe. The filters were washed twice in 2X SSC (1X SSC = 0.15 M NaCl/0.015 M sodium citrate) at room temperature for 5 min followed by two washes in 2X SSC, 1% SDS at 65°C for 45 min, and one 10-min wash in 0.1X SSC at room temperature. The membranes were subsequently exposed to Kodak X-Omat AR film for various lengths of time at -70°C with a Dupont Cronex Lightning Plus intensifying screen. The relative amounts of hybridizable RNA sequences were quantified by scanning the autoradiograms with a Hoefer GS-300 scanning densitometer interfaced to a computer programed to provide peak integration.

RESULTS

Proteoglycan Accumulation in Chondrogenic Cultures. Cultures were grown in medium with glucose concentrations of 40–50 mg/dl ("low glucose"), 80–90 mg/dl ("moderately low glucose"), 150–170 mg/dl ("normal glucose"), 260–380 mg/dl ("high glucose"), and 400–550 mg/dl ("very high glucose"). The "normal" range of values was comparable to that found in the blood of 5-day chicken embryos, the source

![Fig. 1. Proteoglycan accumulation in 6-day cultures of mesenchymal cells grown in the presence of different glucose concentrations. Control cultures were prepared with medium containing glucose concentrations shown under the hatched bars. Experimental cultures were grown in the presence of medium with glucose concentrations shown under the open bars. At the end of 6 days, cell layers were stained with alcin blue and the extracted stain was measured. Experimental cultures and controls prepared with the same cells were compared by using a matched pair two-tailed t test.](image-url)
Table 1. Incorporation of $^{35}$SO$_4^-$ into glycosaminoglycans of cell layer and medium of chondrogenic cultures

<table>
<thead>
<tr>
<th>Glucose concentration (mg/dl)</th>
<th>Fraction</th>
<th>$^{35}$SO$_4^-$ incorporation, mean cpm x 10$^{-2}$</th>
<th>% of normal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low (40–50)</td>
<td>C</td>
<td>6,495</td>
<td>82.6</td>
</tr>
<tr>
<td>Normal (150–170)</td>
<td>C</td>
<td>7,865</td>
<td>100</td>
</tr>
<tr>
<td>High (320–380)</td>
<td>C</td>
<td>6,119</td>
<td>77.8</td>
</tr>
<tr>
<td>Low</td>
<td>M</td>
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<td>82.9</td>
</tr>
<tr>
<td>Normal</td>
<td>M</td>
<td>8,460</td>
<td>100</td>
</tr>
<tr>
<td>High</td>
<td>M</td>
<td>5,943</td>
<td>70.2</td>
</tr>
<tr>
<td>Low + asc</td>
<td>C</td>
<td>13,539</td>
<td>81.4</td>
</tr>
<tr>
<td>Normal + asc</td>
<td>C</td>
<td>16,630</td>
<td>100</td>
</tr>
<tr>
<td>High + asc</td>
<td>M</td>
<td>13,794</td>
<td>83.0</td>
</tr>
<tr>
<td>Low + asc</td>
<td>M</td>
<td>5,679</td>
<td>87.1</td>
</tr>
<tr>
<td>Normal + asc</td>
<td>M</td>
<td>6,519</td>
<td>100</td>
</tr>
<tr>
<td>High + asc</td>
<td>M</td>
<td>5,430</td>
<td>83.3</td>
</tr>
</tbody>
</table>

C, cell layer; M, medium; asc, sodium ascorbate (50 μg/ml) was added to medium. Each cpm value represents the mean for two separate spot cultures. Values of the replicates did not vary by $>$5%.

...of the precartilage mesenchymal cells. Preliminary to the studies reported here, we determined that accumulation by these cultures of highly sulfated proteoglycan, which is specifically stainable with alcian blue at pH 1.0 (39), was essentially linear over 6 days (data not shown) and that at the end of this period the cultures consisted uniformly of cells with the cartilage phenotype (30, 44). The comparisons reported below were made among 6-day cultures, unless stated otherwise.

Accumulation of highly sulfated proteoglycan was measured in cultures grown under each abnormal glucose regimen and in their matched controls. All of the abnormal treatment groups exhibited a reduction of at least 15% in alcian blue staining matrix (Fig. 1). Although the reductions were significant and of comparable magnitude for all groups, the variability in the results was lowest for the more extreme treatments ($P < 0.001$).

The observed effect on proteoglycan accumulation may have represented the impact of the abnormal glucose regimens on the overall biosynthesis or sulfation of the glycosaminoglycan component of the proteoglycan molecules, the secretion of the proteoglycans, or their assembly into a stainable matrix. To address these possibilities, we biosynthetically labeled glycosaminoglycans in cultures grown in low, normal, and high (320–400 mg/dl) glucose medium using $^{35}$SO$_4^-$, and we separately assayed all radioactivity incorporated into cetylpyridinium chloride precipitable glycosaminoglycans of the cell layers (41) at 6 days of incubation and of the pooled media collected over the entire 6-day period. Two representative experiments are shown in Table 1. The effect of each treatment regimen on the relative amounts of sulfated glycosaminoglycan in the cell layers corresponded to the alcian blue staining results in Fig. 1. The amounts of sulfated glycosaminoglycan exported into the media of these cultures were essentially proportional to those in the cell layers, indicating that the abnormal glucose levels were not acting by inhibiting retention of matrix by the cells. Moreover, the differences in treatment groups persisted when secretion and assembly of matrix was enhanced by the addition of ascorbate to the medium (Table 1).

DNA Synthesis, Protein Synthesis, and Glucose Utilization Under Different Glucose Regimens. To determine whether the abnormal glucose regimens were impairing basic cellular functions such as replication or protein synthesis, we measured acid-insoluble incorporation of $^3$H[thymidine and $^3$H]leucine in cultures grown in the presence of different glucose concentrations. There were no significant differences among the treatment groups in incorporation of these precursors (Table 2). We also measured glucose utilization as a function of ambient glucose concentration by analyzing spent medium from cultures for the loss of the sugar due to cellular uptake. Between days 5 and 6 of culture, when glucose utilization was maximal for all treatment groups, the amount of glucose extracted from the medium by a standard culture differed from normal only in the low glucose medium (Table 2).

Effect of Abnormal Glucose Levels on Proteoglycan Core Protein Gene Expression. The foregoing experiments suggested a specific impact of ambient glucose concentrations on proteoglycan synthesis or chondrogenic differentiation, rather than on general cellular functions. The mRNA for cartilage-type proteoglycan core protein is a highly specific index of cartilage differentiation, since it is undetectable in the newly isolated population of precartilage cells used in our cultures and becomes abundant during the course of chondrogenesis (45).

The cDNA clone ST-1 contains an extended open reading frame encoding 379 amino acid residues of the large major proteoglycan core protein of chicken cartilage (35). We used this cDNA to assay the cytoplasmic steady-state levels of cartilage-specific proteoglycan core protein mRNA under conditions of low (40–60 mg/dl), normal (140–170 mg/dl), and high (320–390 mg/dl) ambient glucose concentrations. A representative slot blot (Fig. 2) shows a marked reduction from normal levels of steady-state levels of core protein mRNA in the low and high glucose treatment groups. Densitometric scans of this and blots from five other experiments show a mean reduction from normal levels of core protein mRNA levels of 46% for the low glucose treatment groups and 36% for the high glucose treatment groups (Table 3).

DISCUSSION

Although the diabetic condition has a variety of effects on the biosynthesis of macromolecules by established tissues (46–

Table 2. Dependence of DNA synthesis, protein synthesis, and glucose utilization on ambient glucose levels

<table>
<thead>
<tr>
<th>Glucose concentration (mg/dl)</th>
<th>$^3$H[thymidine incorporation, cpm ± SEM</th>
<th>$^3$H[leucine incorporation, cpm ± SEM</th>
<th>Glucose utilization, mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low (40–50)</td>
<td>68,765 ± 4,713</td>
<td>14,546 ± 277</td>
<td>0.29 ± 0.03</td>
</tr>
<tr>
<td>Moderately low (80–90)</td>
<td>73,937 ± 4,272</td>
<td>ND</td>
<td>0.45 ± 0.07</td>
</tr>
<tr>
<td>Normal (150–170)</td>
<td>61,718 ± 9,298</td>
<td>14,464 ± 1451</td>
<td>0.48 ± 0.06</td>
</tr>
<tr>
<td>High (320–380)</td>
<td>77,532 ± 20,124</td>
<td>15,273 ± 1585</td>
<td>0.56 ± 0.13</td>
</tr>
</tbody>
</table>

ND, not determined.

* Incorporation was determined after 6 days of growth in the presence of label. There were no significant differences between any treatment group and normal.

Stock medium and medium withdrawn on day 6 were assayed for glucose content. Glucose utilization is expressed as mg of glucose consumed by a standard culture (2.5 x 10$^6$ cells on day 0). Mean ± SEM of four or more cultures is shown.

Significantly different from normal ($P < 0.001$).
Glucose concentration

Low
Normal
High

Fig. 2. Slot blot hybridization of cytoplasmic RNA isolated from equivalent numbers of precartilage mesenchymal cells grown for 6 days in the presence of low (57 mg/dl), normal (170 mg/dl), and high (387 mg/dl) glucose concentrations. The blot was probed with 32P-labeled ST-1 cDNA (35).

48), its effects on the biochemistry of developing tissues have not been systematically studied. Our experiments show that both high and low glucose levels, comparable to those encountered clinically, can directly impair the accumulation of mRNA for chondroitin sulfate core protein and of the corresponding extracellular proteoglycan during chondrogenesis in vitro. Although the mean decline in proteoglycan accumulation under both high and low glucose regimens was on the order of 15–20%, in some experiments with each treatment group the decline was as high as 50%. This corresponds to a potentially severe impact on the formation of the cartilaginous primordia of the developing skeleton.

The critical importance of sulfated proteoglycans in development can be seen in studies of the genetic anomalies nanomelia in chickens (49) and cartilage matrix deficiency in mice (50, 51). In these mutants, hyaline cartilages contain severely reduced amounts of proteoglycan but are normal with regard to chondrocyte morphology and collagen content. Affected embryos in both species exhibit shortened and malformed limbs as a result of functional deficiencies in the cartilaginous models and growth plates. Whereas an inhibition of proteoglycan synthesis alone would therefore be sufficient for impairment of skeletal development, the effect of abnormal glucose levels may extend to other cartilage-specific gene products or to other classes of macromolecules.

The effect of abnormal glucose levels on gene expression of proteoglycan core protein could have a variety of causes, which need not be the same for the low and high glucose treatment groups. Some obvious possibilities can be excluded: we do not think that low glucose utilization in the low glucose treatment group (Table 1) is responsible for the decline in proteoglycan and its mRNA, since the moderately low glucose treatment group, which did not have reduced glucose utilization, also exhibited a significant decline in proteoglycan accumulation (Fig. 1). Moreover, in the 1 experiment of 16 in which a set of cultures that received low glucose actually utilized more of the sugar than its paired controls, proteoglycan accumulation was also reduced.

We also think it unlikely that hyperosmolarity played a significant role in the results we obtained with the high glucose cultures. When cultures with normal glucose concentrations were made osmotically equivalent to the high glucose treatment groups by addition of the nonmetabolizable sugar l-glucose, there was no corresponding decline in proteoglycan accumulation (unpublished data).

It is conceivable that cellular interaction, or some other step important in initiating chondrogenesis, is sensitive to the ambient glucose concentration. Indeed, we have recently determined that low glucose levels blunt the increase in intracellular cyclic AMP that normally occurs during chondrogenic differentiation (52). This early event in the chondrogenic pathway accompanies cell-cell interaction (53) and may influence gene expression by its role in regulating the phosphorylation of a developmentally regulated chromatin protein in the precartilage cell nucleus (54).

Whatever mechanisms are responsible for the glucose sensitivity of chondrogenesis, it is clear that the effect of abnormal glucose concentration on the steady-state levels of proteoglycan core protein mRNA is even more marked than the effect on the accumulation of extracellular proteoglycan. This suggests that the precartilage cell may have compensatory mechanisms at the level of mRNA utilization or glycosaminoglycan chain elongation, for example, that can partly correct the glucose-mediated deficit in gene expression.

The developmental events that occur in these mesenchymal cultures correspond to those that take place between the 4th and 7th week of human gestation when the embryo is most susceptible to teratogenic insult (4). The high incidence of major malformations in the offspring of diabetic women (6, 18, 55) almost certainly results from deleterious events that occur during this critical period of organogenesis.

Experiments using pregnant animals (11, 13) or whole embryo cultures (12) at stages comparable to that studied here have implicated elevated serum glucose as a causative factor in the development of malformations. Because of the multifactorial impact of high glucose on the embryo, it has been difficult to discern the cellular basis of what has been termed "fuel-mediated teratogenesis" (18). Assuming the results of the present study can be generalized to other species, they suggest that one cause of the skeletal abnormalities seen in these animal studies, as well as in the diabetic pregnancy, is a direct adverse impact of elevated glucose levels on the biosynthesis of sulfated proteoglycans in developing cartilage.

Low maternal glucose levels, which can result from stringent metabolic control of pregnant diabetic women (23), have also been suggested, on the basis of studies with pregnant animals and embryo cultures (24, 27), to adversely affect development. The results reported here show that low glucose levels, like high levels, directly impair the biosynthesis of sulfated proteoglycans in developing cartilage and are thus potentially teratogenic.

The specific mechanisms of the teratogenic effects of high and low glucose levels on developing avian cartilage require further study. It will also be important to learn whether the results reported here apply more generally to chondrogenesis in mammalian species, including humans. While the clinical benefits of the control of diabetes during pregnancy are well recognized, an understanding of the cellular impact of the diabetic milieu could lead to improved care by identifying specific risk factors to the embryo. Furthermore, the suggested deleterious effect of hypoglycemia on chondrogenesis may constitute a hazard of stringent control of diabetes during early pregnancy.

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Table 3. Effect of ambient glucose concentration on relative cytoplasmic accumulation of cartilage proteoglycan core protein mRNA

<table>
<thead>
<tr>
<th>Glucose concentration (mg/dl)</th>
<th>% of normal</th>
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<tbody>
<tr>
<td>Low (40–60)</td>
<td>54 ± 11 (n = 6; P &lt; 0.02)</td>
</tr>
<tr>
<td>High (320–390)</td>
<td>64 ± 9 (n = 6; P &lt; 0.01)</td>
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