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Research paper

Maternal undernutrition during early pregnancy inhibits postnatal growth of the tibia in the female offspring of rats by alteration of chondrogenesis

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ABSTRACT

Epidemiological research has suggested that birth weights are correlated with adult leg lengths. However, the relationship between prenatal undernutrition (UN) and postnatal leg growth remains controversial. We investigated the effects of UN during early pregnancy on postnatal hindlimb growth and determined whether early embryonic malnutrition affects the functions of postnatal chondrocytes in rats.

Undernourished Wistar dams were fed 40% of the daily intake of rats in the control groups from gestational days 5.5–11.5, and femurs, tibias, and trunks or spinal columns were morphologically measured at birth and at 16 weeks of age in control and undernourished offspring of both sexes. We evaluated cell proliferation and differentiation of cultured chondrocytes derived from neonatal tibias of female offspring and determined chondrocyte-related gene expression levels in neonatal epiphysis and embryonic limb buds.

Tibial lengths of undernourished female, but not male, offspring were longer at birth and shorter at 16 weeks of age ($p < .05$) compared with those of control rats. In chondrocyte culture studies, stimulating effects of IGF-1 on cell proliferation ($p < .01$) were significantly decreased and levels of type II collagen were lower in female undernourished offspring ($p < .05$). These phenomena were accompanied by decreased expression levels of *Col2a1* and *Igf1r* and increased expression levels of *Fgfr3* ($p < .05$), which might be attributable to the decreased expression of specificity protein 1 ($p < .05$), a key transactivator of *Col2a1* and *Igf1r*.

In conclusion, UN stress during early pregnancy reduces postnatal tibial growth in female offspring by altering the function of chondrocytes, likely reflecting altered expression of gene transactivators.

1. Introduction

Maternal nutrition during pregnancy and lactation plays a pivotal role in the health of human offspring. Previous epidemiological studies show that maternal undernutrition (UN) during early gestation increases the risk of obesity, cardiovascular disease, and mental illness, whereas UN during mid and late gestation is correlated with reduced glucose tolerance and obstructive airway disease, respectively (Roseboom et al., 2001; Schulz, 2010). Moreover, related mechanisms have been demonstrated in line with "fetal origins hypothesis" and "*in utero* programming" concepts (Barker and Osmond, 1986; Lucas, 1998; Roseboom et al., 2011; Sayer and Cooper, 2005; Victora et al., 2008). Maternal dietary components also have strong effects on epigenetic processes during specific periods of fetal and early postnatal development.

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In particular, UN promotes changes in gene expression patterns of stem or progenitor cells following epigenetic modifications, leading to altered tissue composition and function and predisposition to adult diseases including metabolic syndrome, and cardiovascular disease (Chango and Pogribny, 2015).

In Japan, the incidence of low birth weight infants has tended to increase since the 1990s (Health at a Glance, 2013) and currently affects approximately 10% newborns. Moreover, data from the Ministry of Education, Culture, Sports, Science and Technology, show that heights of children and adolescents born after the latter half of the 1990s in Japan were more than those of the parental generation, whereas ratios of leg length to total height [(height − sitting height)/height] were lesser than those of the parental generation (Ministry of Education, Culture, Sports, Science and Technology in Japan, 2013). In addition, increases in average and sitting heights of girls aged 6–16 years and born in 1996–1998 were 41–42 cm and 20–21 cm, respectively, but those in their paternal generation were 44–45 cm and 20–21 cm, respectively (Ministry of Education, Culture, Sports, Science and Technology in Japan, 2013). These data indicate that adolescent leg growth was decelerated in the younger generation. In separate studies, leg lengths were strongly and negatively correlated with insulin resistance and coronary heart disease risk (Ferrie et al., 2006; Gunnell et al., 1998; Smith et al., 2001), potentially reflecting biological responses to nutrition during development. Similarly, coincident increases in the incidence of low birth weight infants and ratios of low leg length to total height in the present generation of children and adolescents suggest that UN during pregnancy is associated with shorter limbs of offspring. Previous studies mentioned that leg length in adulthood was correlated with birth weight, but suggested that leg growth after birth was independent of prenatal nutrition because there was no strong correlation between birth weight and leg length when they were adjusted for birth length (Ferrie et al., 2006; Gunnell et al., 1999; Wadsworth et al., 2002). However, Kusin et al. showed that infants were taller at the age of 5 years when their mothers were given high-energy supplements rather than low-energy supplements during the last trimester (Kusin et al., 1992). Therefore, prenatal nutrition may affect leg growth and height after birth through fetal programming during endochondral ossification of the femur and tibia. We hypothesized that undernutrition during early pregnancy also affects postnatal leg growth because 70% of the stature is attained by the fetus in 28 weeks of gestation (Strauss, 1997). Herein, we characterized effects of maternal UN during early pregnancy on postnatal growth of hindlimb bones and chondrocyte properties in rat offspring following gestational UN. We assessed gene expression profiles and histone modifications in neonatal tibial epiphyses and defined the related molecular fetal programming mechanisms.

2. Methods

2.1. Animals and study design

All experimental procedures and protocols were reviewed and approved by the animal ethics committee of Shiga University of Medical Science (Shiga, Japan). Nine-week-old male and 8-week-old female Wistar rats with body weights (BW) of 250–280 and 160–190 g, respectively, were purchased from CLEA Japan, Inc. (Tokyo, Japan) and were housed at room temperature (20 °C–25 °C) under 40%–60% relative humidity with a 12-h light/12-h dark cycle (lights on at 08:00) and free access to food and tap water. In experiments with neonatal and adult offspring, female rats were housed with one male overnight, and in experiments with embryos, female rats were housed with one male in a cage for 2 h (23:00–01:00). Embryonic day (E) 0.0 was defined as the point when a vaginal plug was observed. Pregnant rats were randomly assigned to control or UN groups for experiments with embryos, neonates, and adult rats. During the Dutch famine from December 1944 to April 1945, daily rations fell to no >800 kcal, representing 40% of rations (>2000 calories) after June 1945 (Roseboom et al., 2001). To emulate these conditions, rats in the UN group were fed 40% of the daily food intake of those in the control group, which were fed a standard AIN-93G diet (Supplementary Table S1) *ad libitum* from gestational days 5.5–11.5 as blastocyst implantation occurs around day 5.5 and hindlimb bud eruption does not occur yet at day 11.5, to examine the effects of prenatal programming of the mesenchyme on prenatal limb development (Supplementary Fig. S1). After delivery, pups were removed from their biological mothers and were nurtured by untreated foster mothers (cross-fostered) to eliminate the influence of maternal UN on nurturing of the offspring. Pups were randomly culled to eight per litter (four males and four females) on postnatal day (P) 4, and dams were fed CE-2 (CLEA Japan, Inc., Tokyo, Japan; Supplementary Table S1) during lactation. Offspring were weaned on P28 and were then fed CE-2. BWs of all offspring except for one female (death at the age of 5 weeks) were measured weekly for 16 weeks. The morphology was examined at P0.5 (males: $n = 30$, control and $n = 27$, UN; females: $n = 29$, control and $n = 32$, UN groups) and at 16 weeks of age (males: $n = 23$, control and $n = 22$, UN; females: $n = 21$ and control; $n = 22$, UN groups). Gene expression levels in the hindlimbs were examined at E13.5 and in P0.5 females, and plasma levels of IGF-1 were measured in P3 female neonates. Different litters from the same dams were used for chondrocyte culture and gene expression analysis of neonates.

2.2. Morphology

2.2.1. Neonates

Morphological measurements were performed in randomly chosen P0.5 neonates from five dams from both control and UN groups. Samples with damage such as epiphysiolysis during dissection were excluded from measurements. Body lengths of all neonates were measured using a digital vernier caliper, and proximal (males: $n = 29$, control and $n = 22$, UN; females: $n = 24$, control and $n = 25$, UN groups) and distal (males: $n = 30$, control and $n = 27$, UN; females: $n = 28$, control and $n = 32$, UN groups) epiphyseal and diaphyseal (males: $n = 29$, control and $n = 25$, UN; females: $n = 26$, control and $n = 27$, UN groups) lengths, maximum proximal (males: $n = 29$, control and $n = 25$, UN; females: $n = 28$, control and $n = 31$, UN groups) and distal femoral epiphyseal (males: $n = 30$, control and $n = 27$, UN; females: $n = 28$, control and $n = 27$, UN groups) widths, and total lengths (males: $n = 29$, control and $n = 22$, UN; females: $n = 24$, control and $n = 25$, UN groups) were measured using a dissecting microscope and ImageJ software (http://imagej.nih.gov/ij/; Supplementary Fig. S2A/B). Average diaphyseal width (AWD, males: $n = 29$, control and $n = 25$, UN; females: $n = 28$, control and $n = 31$, UN groups) was calculated using the following equation (Supplementary Fig. S2C):

$$AWD = \frac{projected\ area\ of\ the\ diaphysis}{length\ of\ the\ diaphysis}$$

The same parts were measured in the tibia (males: $n = 28$, control and $n = 27$, UN; females: $n = 29$, control and $n = 31$, UN groups), and the tibia were later fixed with 4% paraformaldehyde, embedded in paraffin, and cut into serial sections (5 μm) along the long axis of the limb. Tissue sections were stained with Alcian blue, and ratios of the height of resting and proliferative zones, prehypertrophic zones, or hypertrophic zones to total heights of epiphyseal cartilage were measured using a digital slide scanner

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