Effect of Iron Deficiency on Placental Cytokine Expression and Fetal Growth in the Pregnant Rat

Lorraine Gambling, Zehane Charania, Lisa Hannah, Christos Antipatis, Richard G. Lea, and Harry J. McArdle

The Rowett Research Institute, Bucksburn, Aberdeen AB21 9SB, United Kingdom

ABSTRACT

Iron deficiency anemia is the most common nutritional disorder in the world. Anemia is especially serious during pregnancy, with deleterious consequences for both the mother and her developing fetus. We have developed a model to investigate the mechanisms whereby fetal growth and development are affected by maternal anemia. Weaning rats were fed a control or iron-deficient diet before and throughout pregnancy and were killed at Day 21. Dams on the deficient diet had lower hematocrits, serum iron concentrations, and liver iron levels. Similar results were recorded in the fetus, except that the degree of deficiency was markedly less, indicating compensation by the placenta. No effect was observed on maternal weight or the number and viability of fetuses. The fetuses from iron-deficient dams, however, were smaller size at birth, and Godfrey et al. [5] have shown that maternal Fe status may be a risk factor for adult disease. Several studies have shown that Fe deficiency during pregnancy, both in humans and in animal models, results in long-term problems for the offspring, such as increases in blood pressure [6], diminished brain function [7–12], and compromised immune system development [13–16]. The mechanisms underlying these effects have not been clarified, but clearly, growth and development in utero may play an important part.

The placenta is the pathway for delivering the majority of nutrients to the developing fetus. Consequently, any stress that alters placental development or function is likely to have consequences for the developing fetus. Placental function is regulated, at least in part, by a wide spectrum of cytokines, which are produced both locally and distally. One cytokine that has attracted a lot of interest is tumor necrosis factor α (TNFα). Indeed, a pivotal role for this cytokine during pregnancy has been suggested [17]. Elevated levels of TNFα at the maternal-fetal interface are associated with early and midpregnancy failure in rodents and with premature labor in humans [18–20]. However, TNFα is also produced at low levels in placental and decidual immune cells during normal, healthy pregnancies and, therefore, is thought to be beneficial for pregnancy. It is reported to induce apoptosis of placental cells and, therefore, may be important in trophoblast turnover and remodeling [21]. Data also suggest that TNFα may regulate placental steroid production by the placenta and down-regulate amino acid transfer [22]. Because the suggested beneficial and detrimental roles of TNFα are concentration dependent, the regulation of TNFα expression at the maternal-fetal interface must be crucial for successful placental development and function.

The relationship between Fe status and cytokines has been the subject of many studies, most of which have concentrated on the effect of cytokines on Fe uptake or metabolism (see, for example, [23] for a recent report). Recently, however, several groups have examined the effect of Fe status on TNFα production. Scaccabarozzi et al. [24] have shown that Fe supplementation increased and that derferrioxamine (DFO), an Fe chelator, decreased the production of TNFα by monocytic cells. This same effect has been obtained in the leukemic cell line THP-1 [25] and in mice treated with DFO [26]. Similar data were also obtained in Kupffer cells, in which loading with Fe reduced the sensitivity to lipopolysaccharide [27]. To our knowledge, how this relationship operates in the placenta has not been examined, but given the association between elevated concentrations of TNFα and problems with pregnancy, this question is clearly of considerable importance. In this paper, therefore, we examine the effect of maternal Fe deficiency on TNFα and TNFα receptor levels in the placenta.

Since its identification in placenta [28], leptin has been the subject of considerable investigation. Results of several studies suggest it is important in the maintenance of preg-
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FIG. 1. Effect of maternal Fe deficiency on maternal blood and serum hematological parameters. Hematological parameters were measured in maternal blood and serum at Day 21 of gestation. A significant ($P < 0.02$) diet-dependent decrease was observed in hematocrit (A), hemoglobin (B), serum Fe (C), and transferrin saturation (D). No significant change was observed in TIBC (E). Data are presented as mean ± SEM ($n = 34$). Statistical analysis was carried out by one-way ANOVA.

Our data show a clear correlation between maternal Fe status, placental cytokine levels, and fetal development. Although we cannot demonstrate causality, the data suggest a relationship that may explain at least some of the effects of maternal Fe deficiency on her offspring.

MATERIALS AND METHODS

Experimental Animals

Experiments were performed using weanling female rats of the Rowett hooded lister strain. They were group-housed in cages under constant temperature and humidity. Controlled illumination with a 12L:12D photoperiod was maintained to ensure regular estrous cycles. Forty female weanling rats were fed control diet for 2 wk before being randomized into four groups. The first group of rats ($n = 16$) remained on the control diet (50 mg kg$^{-1}$), whereas the remaining three groups ($n = 8$ each) were placed on experimental diets of reduced Fe content (37.5, 12.5, and 7.5 mg kg$^{-1}$). All diets were freely available, and body weights were recorded three times per week throughout the experiment. All groups were fed these diets for 4 wk before mating. The rats were mated with males of the same strain. Mating was confirmed by detection of a vaginal plug, and this day was denoted as Day 0. The female rats were maintained on the same diet throughout pregnancy and were killed at Day 21 of gestation. All experimental procedures were approved and conducted in accordance with the U.K. Animals (Scientific Procedures) Act of 1986.

Tissue Samples

On Day 21 of gestation, the dams were killed by stunning and cervical dislocation. The numbers of fetuses and placentas were counted, and the number of resorption sites observed in the uterus was recorded. Placentas associated with healthy fetuses were weighed and either fixed in neutral buffered formalin (10% [v/v]) overnight (4°C), followed by storage in 70% (v/v) ethanol, or frozen in liquid nitrogen before being stored at −70°C. Livers, hearts, kidneys, lungs, and brains from eight fetuses, chosen from each mother at random, were rapidly dissected, weighed, and frozen in liquid nitrogen. Livers were dissected from all dams, removed, and immediately frozen in liquid nitrogen before being stored at −70°C.
TABLE 1. Effect of maternal Fe deficiency on maternal growth and fertility. a

<table>
<thead>
<tr>
<th>Diet group</th>
<th>50 mg kg⁻¹</th>
<th>37.5 mg kg⁻¹</th>
<th>12.5 mg kg⁻¹</th>
<th>7.5 mg kg⁻¹</th>
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<tbody>
<tr>
<td>Pregnancy (%)</td>
<td>100</td>
<td>75</td>
<td>100</td>
<td>87.5</td>
</tr>
<tr>
<td>Maternal body weight (g)</td>
<td>335 ± 5</td>
<td>344 ± 10</td>
<td>330 ± 13</td>
<td>332 ± 3</td>
</tr>
<tr>
<td>Total no. of fetuses</td>
<td>13.0 ± 0.7</td>
<td>13.8 ± 1.3</td>
<td>13.4 ± 1.2</td>
<td>14.9 ± 0.7</td>
</tr>
<tr>
<td>No. of live fetuses</td>
<td>12.0 ± 0.7</td>
<td>13.0 ± 1.4</td>
<td>11.0 ± 1.6</td>
<td>14.0 ± 0.8</td>
</tr>
</tbody>
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a Values are mean ± SEM.

Hematological Measurements

Maternal and fetal hematocrit were measured by drawing blood into heparinized capillary tubes, which were then centrifuged in a high-speed hematocrit centrifuge (Universal 32R, Hettich; Scientific Laboratory Supplies, Countbridge, U.K.) and read in a microhematocrit reader. Five hundred microliters of maternal blood were collected into heparinized tubes as necessary. Standards (Spectrosol; BDH). Appropriate quality controls were included.

CT). A standard curve for Fe was prepared from commercially available rum. The Fe contents of these samples were determined by graphite-pressing the ratio of serum Fe:total Fe and TIBC.

Immunohistochemical Analyses

Fixed placentas from the control and 7.5 mg kg⁻¹ diet groups were processed into wax blocks. Sections (thickness, 5 μm) were cut for immunohistochemistry. All epitopes were exposed by microwaving at high power in 10 mM sodium-citrate buffer (pH 6.0). Following a further wash with PBS or Tris-buffered saline (TBS) buffer, nonspecific endogenous peroxidase activity was blocked by treatment with 5% (v/v) hydrogen peroxide (Sigma) in distilled water for 5 min at room temperature. All tissue sections were exposed to a nonimmune block with normal horse or rabbit serum (150 μl in 10 ml of buffer; Vector Laboratories, Ltd., Bretton, Peterborough, U.K.) and incubated with the appropriate primary antibodies as follows: Tissue sections were incubated with goat polyclonal anti-rat TNFα (final concentration, 4 μg/ml in TBS and 1% (w/v) BSA at 4°C overnight; R&D Systems, Ltd., Abingdon, Oxfordshire, U.K.) followed by biotinylated goat anti-rabbit antibody (50 μl in 10 ml of TBS for 30 min at room temperature; Vector Laboratories). The type 1 TNFα receptor (TNFR1) was detected with mouse polyclonal rabbit anti-human TNFR1 (p55; final concentration, 3.3 μg/ml in PBS and 1% BSA at 4°C overnight; Santa Cruz Biotechnology, Ltd., Santa Cruz, CA) followed by biotinylated goat anti-rabbit antibody as described above. Leptin was immunolocalized with an in-house-produced polyclonal rabbit anti-human leptin antibody (42 μg/ml in PBS and 1% BSA at 4°C overnight) raised against recombinant human leptin (Peprotech EC, Ltd., London, U.K.) followed by incubation with biotinylated goat anti-rabbit antibody. Leptin receptor was examined using polyclonal goat anti-human leptin receptor antibody (8 μg/ml; Insight Biotech, Middlesex, U.K.), which is reactive against all splice variants. Subsequent treatment was as described above. Negative controls were performed by replacing the primary antibody with mouse immunoglobulin (Vector Laboratories) at the same concentration as the primary antibody. Sections were labeled with the avidin-biotin-peroxidase detection system (Vector Laboratories). Thereafter, sections were counterstained with hematoxylin, dehydrated, cleared in xylene (20 min), and mounted in Pertex (Cellpath Plc, Newton Powys, U.K.).

Statistical Analyses

For each dam, the litters were averaged and the data recorded as a single point (rather than treating each fetus as a single point). This is the statistically more accurate option, although it may obscure trends that might be identified with high numbers of animals. Relative organ weights were calculated as the percentage of total body weight (g 100⁻¹). All results are presented as mean ± SEM. A minimum of five measurements for each diet group was used for all analyses. Linear regression tests were used to determine statistical significance between continuous variables, whereas ANOVA was used to determine statistical significance between diet groups.

For immunohistochemical analyses, slides were scored for staining intensity on a four-point scale (0, no staining; 1, weak staining; 2, medium staining; 3, intense staining throughout region). The placenta was divided into discrete regions: trophoblast giant cells, spongiotrophoblast, labyrinth, and cytotrophoblasts. The thin, adherent rim of decidua on the maternal side of each placenta was also assessed. The data were collected from one placenta from each dam (minimum of seven in each group), and each section was scored independently by three observers. The data were analyzed by the Mann-Whitney test. Significance was assumed at P ≤ 0.05, and all analyses were carried out using Excel 6.0 (Microsoft, Seattle, WA).

RESULTS

Reduced maternal dietary Fe content had no effect on fertility and growth of the dams or on the viability and number of fetuses (Table 1). A diet-induced decrease in maternal hematocrit and hemoglobin concentration, how-
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FIG. 4. Effect of maternal Fe deficiency on fetal Fe status. Maternal Fe deficiency leads to a significant ($P < 0.0001$) diet-dependent decrease in fetal hematocrit levels (A). Fetal liver Fe levels are significantly ($P < 0.0001$) decreased in maternal Fe deficiency (B). Samples were taken at Day 21 of gestation and were baked and treated as described in Materials and Methods. The Fe content was then measured by atomic absorption spectroscopy. Data are presented as mean $\pm$ SEM ($n = 37$). Statistical analysis was carried out by one-way ANOVA.

FIG. 5. Effect of maternal Fe deficiency on fetal development. Fetal weight (A), placental:fetal ratio (B), and fetal liver relative organ weight (ROW) (C) were all significantly ($P < 0.03$) related to Fe levels in the maternal liver. Each point represents the mean value for each litter. Statistical analysis was carried out by linear regression.

However, was observed (Fig. 1). Maternal serum Fe and transferrin saturation were markedly decreased, but TIBC did not increase significantly. The Fe content of the liver is generally considered to be the most accurate measure of Fe status. In the pregnant dams, liver Fe levels were reduced as a result of decreasing Fe content of the diet (Fig. 2).

Overall, changes in maternal Fe status were reflected by alterations in placental and fetal parameters. The placental non-heme Fe level decreased (Fig. 3), as did the fetal hematocrit and fetal liver Fe (Fig. 4). Importantly, the proportional drops in Fe content in the placenta (50%) and the fetal liver (46%) were much less than that seen in the mother (69%; $P < 0.001$).

Data regarding fetal development can be presented in two ways: related to maternal dietary intake, or related to maternal liver Fe levels. The latter measurement is clearly a better indicator of status, taking into account individual variations in absorption, for example. Therefore, it has been used wherever appropriate as the independent variable.

Placental weight was not significantly related to maternal Fe status (data not shown), but a clear decrease was observed in fetal weight ($P = 0.01$) (Fig. 5A). Between the two extremes of dietary Fe, 50 mg kg$^{-1}$ (control) and 7.5 mg kg$^{-1}$ (15% of control), a 10% decrease in weight was observed. This decrease led to significant changes in the placental:fetal ratio ($P = 0.02$) (Fig. 5B). In addition to changes in total fetal weight, the fetal liver weights, expressed as a fraction of fetal size, were decreased by 30% ($P = 0.03$) (Fig. 5C), indicating disproportionate fetal growth. No other tissues showed a significant alteration in relative size (data not shown).

Placentas from Fe-deficient dams did not show gross morphological changes. In both control and Fe-deficient placentas, TNF$\alpha$ was localized to placental trophoblast lineages (i.e., trophoblast giant cells, spongiotrophoblast, and labyrinth) (Fig. 6, A and B). In the placentas from Fe-deficient dams, TNF$\alpha$ was significantly increased in the trophoblast-giant-cell region of the placenta ($P < 0.05$) (Figs. 6B and 7A). No significant change in the labyrinth, spon-
FIG. 6. Immunolocalization of TNFα, TNFαR1, and leptin in late-gestation placentas (Day 21) from control and Fe-deficient rats. In control placentas, TNFα (A), TNFαR1 (C), and leptin (E) were detected in all trophoblast lineages. Relative to controls, placentas from Fe-deficient rats exhibited increased TNFα (B), TNFαR1 (D), and leptin (F). The inset shows the immunoglobulin G-negative control. Significance was achieved in specific trophoblast lineages: TNFα (TGC), TNFαR1 (TGC, lab, cytotrophoblasts: data not shown), and leptin (lab). Lab, Labyrinthine placenta; Sp, spongiotrophoblast; TGC, trophoblast giant cells. Bars = 100 μm.

giotrophoblast, cytotrophoblasts, or adherent maternal decidua was observed.

The distribution of TNFαR1 in control placentas was similar to that of TNFα, with staining in most regions of the placenta. However, Fe deficiency induced a significant increase in levels in all regions except the spongiotrophoblast (P = 0.054). No changes were found in the maternal decidua (Figs. 6, C and D, and 7B).

In the control placentas, leptin was immunolocalized to all trophoblast lineages at a uniform intensity (Fig. 6E). In contrast, placentas from Fe-deficient mothers were characterized by significantly increased levels in the labyrinth (P = 0.04) and marginally increased levels in the trophoblast giant cells (P = 0.054) and spongiotrophoblast (P = 0.072) (Figs. 6, E and F, and 7C). Leptin-receptor levels produced little staining in control placentas, and Fe deficiency showed no significant changes in any region of the placenta (data not shown).

DISCUSSION

This study has examined the effects of different degrees of maternal Fe deficiency during pregnancy on Fe metabolism, placental cytokine expression, and fetal growth and development in a rat model. Our data indicate that maternal Fe deficiency causes deficiency in the fetus, but to a lesser extent than in the mother. These data are in keeping with previous studies showing that available dietary Fe is di-
rected toward the formation of new structures and the maintenance of body weight rather than toward Fe stores [39]. In particular, during pregnancy, the fetus has priority for dietary Fe over maternal stores [40], with more than 70% of dietary Fe being delivered to the fetus toward the end of gestation [41]. We have previously identified some of the mechanisms involved in the placental response to Fe deficiency. Transferrin receptor and DMT1, the protein mediating \textit{trans}-membrane transport of Fe, show increased expression at both protein and mRNA levels, whereas IREG1 (involved in Fe efflux from placenta) expression does not change and the activity of placental copper oxidase increases [42].

The treatment in the present study has no effect on the viability or number of fetuses. This is in contrast to other reports, in which the number of offspring was significantly reduced. Toyoo [43] showed that fetal growth was dependent on the severity of maternal anemia at the beginning of pregnancy, but their treatments were so severe as to lead to a fall in maternal weight, fertility, and fetal viability. Using a marginally less severe maternal dietary restriction, Crowe et al. [6] demonstrated fetal growth restriction as a result of Fe deficiency during pregnancy at Day 20 of rat gestation. However, those authors expressed concern regarding the severity of the maternal dietary regime used, citing it as an explanation for the inconsistent result of a decrease in placental:fetal ratio. A milder dietary restriction used by Sherman and Moran [44] to investigate the effects of Fe deficiency on pregnancy showed no significant effect on fetal number, fetal weight, or placental weight. Our protocol matches more closely the one used in that study, since we also found no change in viability or fertility.

We have demonstrated that maternal Fe deficiency does induce a significant decrease in fetal weight, and that this is associated with a small rise in placental weight, giving a marked increase in the placental:fetal ratio. We can advance some hypotheses regarding possible mechanisms causing these changes. Almost certainly, direct effects of decreased Fe on fetal growth and development are involved. However, in this study, we have also shown, to our knowledge for the first time, that placental cytokine levels are altered during Fe deficiency. The changes are specific to different parts of the placenta, which may give some indication regarding the consequences of the alterations in levels.

Increases in TNF\(\alpha\) are located mostly in the region occupied by the trophoblast giant cells. The function of these cells has been studied extensively. They produce a variety of hormones and endocrine-signaling agents, including prolactin-like proteins [45] and growth hormones [46]. How expression is regulated is not well understood. In other tissues, a clear interaction occurs between TNF\(\alpha\) and, for example, prolactin, which increases TNF\(\alpha\) production and secretion in astrocytes [47], whereas TNF\(\alpha\) can alter steroid regulatory proteins (hence reducing progesterone production) and hormone receptors in corpora lutea [48]. Furthermore, Monoz et al. [49] have shown increased TNF\(\alpha\) production in Fe deficiency anemia by lipopolysaccharide-stimulated mononuclear cells [49]. How these results translate to the placenta are not clear. Studies show that TNF\(\alpha\) induces apoptosis in purified cultures of human syncytiotrophoblast and cytotrophoblast cells [21, 50], and that the pathway is mediated by TNF\(\alpha\)R1 [21]. Furthermore, Rasmussen et al. [51] have also demonstrated, in a complex series of transgenic mouse cell experiments, that TNF\(\alpha\) receptor expression is possibly more important in determining cell fate and function than simple expression of TNF\(\alpha\) [51]. Many pathways, therefore, are possible whereby our observations can be related to altered trophoblast and placental function. At present, we are testing the hypothesis that altered expression of TNF\(\alpha\)R1 is the primary response to altered Fe status.

Leptin was first identified as a placental hormone in 1997 [28], but its function is still unclear. The presence of a placenta-specific upstream enhancer on the gene suggests that it is regulated differently from that of adipose origin [52, 53]. Studies in humans have shown a correlation between cord blood and placental leptin, but not between maternal leptin and birth weight [30, 54] (reviewed in [32]). This suggests that placental leptin may promote fetal growth. Initially, the data in this study appear to contradict the hypothesis. However, growth and development are clearly dependent not on a single cytokine but on the presence of an appropriate profile of factors so that, for example, the increased leptin may be acting to counter some of the worst effects of the increased TNF\(\alpha\) levels. Some
evidence supports this idea [55]. Leptin secretion by adipocytes is regulated by TNF-α, acting through TNF-βR1 [56]. Whether this mechanism operates in placenta has not been determined, however, and the interactions between these cytokines are currently under investigation.

In summary, Fe deficiency during pregnancy exerts a considerable variety of effects, culminating in decreased fetal growth and increased placental:fetal ratio. Fetal growth and development are dependent on placental growth during early pregnancy and on differentiation and function during later pregnancy. These functions are regulated through the activity of local immune and endocrine factors. We cannot, at this stage, be certain, but it seems likely that changing patterns of cytokine production will contribute toward the inhibition of fetal growth. Whether this is the only contributory factor remains to be determined. We consider it to be unlikely, and we believe that other important changes can result in disproportionate fetal growth and postnatal problems in growth and development. This model will clearly help in unraveling the relationship between Fe status and fetal growth, which has significant clinical relevance in both the developed and the developing world. Several reports suggest that inappropriate prenatal growth patterns can be associated with problems later in life [5, 57]. At present, we cannot confirm that the outcome of pregnancy or later development will be compromised by Fe deficiency, but the data suggest that this is likely. We would argue, therefore, that this model has good potential for advancing our understanding of the mechanisms relating in utero nutrition to postnatal development.

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