Increase in matrix metalloproteinases from endothelial cells exposed to umbilical cord plasma from high birth weight newborns

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MEAN BIRTH WEIGHT HAS INCREASED in the past decades in most Western countries, and the proportion of macrosomic infants (birth weight > 4 kg) has risen (5, 28, 29). The main reasons for this apparent increase in birth weight are changed maternal lifestyles and increased prevalence of overweight and obesity during pregnancy. Increased health risk as a consequence of high weight at birth has gained growing attention (7, 34). For this apparent increase in birth weight are changed maternal intake of saturated fat during pregnancy or suckling is associated with features of the metabolic syndrome and endothelial dysfunction in the offspring (3).

The vascular endothelial cells are sensitive to metabolic and inflammatory changes such as those present in obesity (3, 33). In line with this, a consistent finding in most studies is impaired endothelial-mediated vasodilation in overweight subjects (30, 33, 38). It is therefore possible that the vascular endothelium of newborns with different birth weights may have been modulated during fetal life to exhibit different functional properties (3). It is, however, not known which endothelial functions might be modified and what the regulatory mechanisms for such changes might be. One possibility is that the fetal vascular endothelium is affected by humoral factors originating from the maternal circulation.

Matrix metalloproteinases (MMP) play an important role in the turnover of extracellular matrix components, including those of the vascular wall (24, 32). They also have been linked to the development and progression of cardiovascular diseases and features of the metabolic syndrome (15, 25, 37). These proteolytic enzymes are secreted from various cells, including the endothelium, and a change in MMP is considered as a possible marker of altered endothelial function.

In the present study, we asked whether umbilical cord plasmas from newborn with HBW and NBW could affect MMP activity derived from cultured human umbilical vein endothelial cells (HUVEC).

MATERIALS AND METHODS

Study subjects. The protocol was approved by the Regional Ethical Committee for Health Region South in Norway and conformed to the Helsinki Declaration. Women undergoing cesarean section on maternal request at the Division of Obstetrics, Rikshospitalet-Radiumhospitalet Medical Center, in the period from December 2004 to June 2005 were asked to donate umbilical cord blood remaining after standard clamping procedures. Seven women who gave birth to NBW...
babies (3.1–3.6 kg) and six women who gave birth to HBW babies (4.0–5.0 kg) gave written consent.

All women were of Caucasian origin, healthy, and did not use any medication regularly. Their current and previous pregnancies had been uncomplicated, and none of the women had metabolic syndrome, diabetes, or cardiovascular disease. All babies were delivered uncomplicated at term, i.e., between 38 and 40 complete weeks of gestation.

Collection of umbilical cord blood samples. Immediately after delivery, the umbilical cord vein was cannulated, and a 4-mL blood sample was collected in vacutainer tubes containing 0.5 mL buffered citrate (0.129 mmol/l). Thrombocytes were removed by centrifugation and subsequent filtering before the cord plasma samples were stored at −80°C until further use.

HUVEC cultures. HUVECs were isolated from freshly obtained human umbilical cords from donors different from the mothers of the included NBW and HBW infants, by collagenase digestion according to the protocol of Jaffe et al. (21). HUVECs were grown in DMEM (Sigma-Aldrich, Oslo, Norway) containing 1 g/l glucose, 10% fetal calf serum, 10 IU/ml heparin (Leo Pharma, Oslo, Norway), 10 μg/ml endothelial cell growth supplement (BD Biosciences, San Jose, CA), and penicillin (50 U/ml) plus streptomycin (50 μg/ml) in humidified air containing 5% CO2 at 37°C. The HUVECs used were passed in culture maximally three times, hence reducing the risk of changing their endothelial properties may differ according to degree of density (41). The cells were washed once in serum-free DMEM before they were resuspended in serum-free DMEM and added to test plasma in the indicated proportions.

After incubations over selected time periods, the supernatants were centrifuged (10 min, 2,500 g) and then immediately frozen at −80°C until further analyses.

Gelatinase assay. We used a gelatinase assay to measure MMP activity, as recently detailed (36). In brief, calf skin [3H]collagen (Sigma-Aldrich) was used as substrate. The acid-soluble radioactive protease products were measured in a liquid scintillation counter (Wallac Win spectral 1414, Perkin-Elmer Instruments, Oslo, Norway). NBW and HBW specimens were always included in the same assay runs. The gelatinase activity obtained from each of the supernatants of HUVECs exposed to the six HBW cord plasma samples was divided by the average gelatinase activity obtained from the supernatants of HUVECs exposed to the seven NBW cord plasma samples, hence giving the gelatinase activity ratio of HBW to NBW.

Measurements of MMPs and cytokines. ELISA kits from Amersham Biosciences (Uppsala, Sweden) were used to measure active MMP-2 and -9 (detection limits 0.2 ng/ml) separately. To measure the total concentration of MMP-2 and -9 molecules, the sum of pro- and active MMP-2 and -9 molecules, we used p-aminophenylmercuric acetate (AMPA) to the supernatants according to the instructions of the manufacturer. AMPA converts any preformed MMP-2 or -9 molecules to their active forms. ELISA kits purchased from R&D Systems (Minneapolis, MN) were used to determine IL-1β (detection limit 1 pg/ml), IL-6 (detection limit 1 pg/ml), TNF-α (detection limit 2 pg/ml), and INF-γ (detection limit 5 pg/ml). TNF-α and INF-γ were measured with an immunoluminometric assay (Innolit; Diagnostic Products) (detection limit 3.3 nmol/l).

MMP inhibitors. To directly block the proteinase actions of MMP-2 and -9 in the HUVEC supernatants, we used two recently designed mechanism-based inhibitors of gelatinases termed inhibitor 1 and inhibitor 3 (20). Expression of mRNA for MMP-2 and -9 in the HUVECs was repressed by using specific small interfering RNA (siRNA; Santa Cruz Biotechnology, Heidelberg, Germany). The tissue inhibitors of MMP (TIMP) types 1 and 2 were determined by ELISA (Amersham Biosciences; detection limits 1.5 and 3 ng/mL, respectively).

mRNA expressions. We used Northern blot analysis to examine the mRNA expressions of MMP-2 and -9. In short, after extraction of RNA from cultured HUVECs and the subsequent transferal to a nylon filter membrane, samples were UV cross-linked before hybridization with the appropriate cDNA probes. After hybridization, the blots were washed, and radioactivity on the membrane was recorded. A quantification was performed by densitometric analysis (ImageQuant 5.2 software; Amersham Biosciences), and the obtained mRNA signal was normalized to the signal obtained by hybridization with a GAPDH probe.

Biochemical analyses. The concentrations of glucose, insulin, and lipids in the venous cord plasma samples were determined with conventional methods (Roche Diagnostics, Mannheim, Germany) at the Department for Clinical Chemistry, Rikshospitalet-Radiumhospitalet Medical Center.

Statistics. The various assays were done in triplicate for each study subject, and the corresponding median values were used for further calculations. Values are presented as means (SD). Differences between the two groups were analyzed with the Mann-Whitney U and Kruskal-Wallis tests, and a P value of <0.05 was considered significant.

RESULTS

Characteristics of the mothers and their newborns are provided in Table 1. Importantly, a statistical difference between the two groups was only detected for birth weights and birth lengths, indicating that the two groups were otherwise quite similar. We could not detect any significant differences in the cord blood concentrations of glucose, insulin, or lipids between the NBW and HBW groups (Table 2).

Gelatinase activity is enhanced in supernatants of HUVECs exposed to cord blood of HBW. Figure 1A shows that cord plasma samples from HBW infants induced a higher gelatinase activity than those from the NBW group at plasma content above 5%. Maximal values with a ratio of approximately three were obtained with 20% plasma added to the HUVEC cultures. The time course of the effects is given in Fig. 1B, which shows that this maximal gelatinase activity was evident after 48 h of incubation.

To test whether the observed increase in gelatinase activity in HUVEC supernatant exposed to HBW cord plasma was due to a higher endogenous gelatinase activity in HBW and NBW cord plasmas, gelatinase activity was measured directly in the cord plasma samples and compared with those obtained in the supernatants of HUVECs incubated with cord plasma from the same donors. Whereas some gelatinase activity could be demonstrated in both HBW- and NBW-derived plasma (with an activity ratio of ~1), the gelatinase activity found in the supernatants of HUVECs exposed to HBW samples was clearly higher (P < 0.05; Fig. 2).

Table 1. Characteristics of the mothers and their newborns

<table>
<thead>
<tr>
<th>Parameter</th>
<th>NBW (n = 7)</th>
<th>HBW (n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal age, years</td>
<td>33.1 (5.6)</td>
<td>34.2 (4.0)</td>
</tr>
<tr>
<td>Parity</td>
<td>0.9 (0.9)</td>
<td>1.2 (0.8)</td>
</tr>
<tr>
<td>Body mass index, kg/m²</td>
<td>28.2 (4.7)</td>
<td>27.4 (4.8)</td>
</tr>
<tr>
<td>Duration of pregnancy, days</td>
<td>265 (7)</td>
<td>272 (10)</td>
</tr>
<tr>
<td>Infant gender</td>
<td>3 females/4 males</td>
<td>2 females/4 males</td>
</tr>
<tr>
<td>Birth weight, g</td>
<td>3.426 (302)</td>
<td>4.247 (301)*</td>
</tr>
<tr>
<td>Birth length, cm</td>
<td>49.9 (0.9)</td>
<td>53.2 (1.7)*</td>
</tr>
</tbody>
</table>

Values are means (SD). Body mass index data were obtained before pregnancy. *P < 0.05 between the normal birth weight (NBW) and high birth weight (HBW) groups.
Increased gelatinase activity due to enhanced MMP-9. The gelatinase assay used mostly reflects the activity of the two gelatinases MMP-2 and -9 (24). We therefore used ELISA kits to measure directly the concentrations of active MMP-2 and -9 proteins. In line with data obtained with the gelatinase assay, addition of 20% cord plasma yielded an apparent maximal concentration of active MMP-9 protein in supernatants of HUVECs exposed to HBW cord plasma, whereas the concentration of active MMP-9 protein in HUVECs that had NBW cord plasma added remained virtually unchanged with increments in cord plasma supplements (Fig. 3A). Concentrations of active MMP-2 protein also increased with higher HBW and NBW cord plasma contents, but no significant difference was found between the two groups (Fig. 3B). Similar to the findings from the gelatinase assay, maximal concentrations of both the active MMP-2 and -9 proteins were obtained after 2 days of culturing HUVECs (data not shown).

To test whether the enhanced concentration of MMP-9 in the supernatants of HUVECs with added HBW plasma could be due to elevated levels of MMP-9 in the cord plasma of HBW, we measured the concentration of this molecule in the cord plasma of NBW and HBW infants. The MMP-9 concentration in NBW plasma was similar \( (P > 0.05) \) to that of HBW plasma, namely, 0.31 (0.026) and 0.30 (0.028) ng/ml, respectively.

The ELISA assays measured the prevailing concentrations of active MMP-2 and -9 proteins. To determine the total concentration of MMP-2 and -9 proteins, we added AMPA to activate preformed MMP-2 and -9 proteins. With this approach, the concentrations of total MMP-9 protein in HUVEC supernatants exposed to 20% plasma for 2 days were 6.4 (0.3) and 3.1 (0.2) ng/ml \( (P > 0.05) \) for HBW and NBW, respectively. The corresponding concentrations of total MMP-2 protein were 5.6 (0.4) and 5.8 (0.4) ng/ml \( (P > 0.05) \) for HBW and NBW, respectively. Thus cord plasma from HBW infants stimulated production and/or release particularly of MMP-9.

Reportedly, IGF-1 can stimulate MMP, and it is associated with increased infant size at birth (19, 27). The cord plasma concentrations of IGF-1 did, however, not differ \( (P > 0.05) \) between NBW and HBW, with the values being 7.8 (1.8) and 7.4 (1.5) nmol/l, respectively.

Specific inhibition of MMP-2 and -9. To further study MMPs in HUVECs exposed to HBW cord plasma, we used two inhibitors that specifically block the enzymatically active sites of the gelatinases MMP-2 and -9 (20). Addition of these inhibitors caused a marked decrease in MMP-2 and -9 activities in supernatants of HUVECs exposed to 20% HBW cord plasma (Fig. 4). The results strongly suggest that MMP-9 is the major gelatinase responsible for increased gelatinase activity in HUVECs exposed to HBW cord plasma.

### Table 2. Glucose-, insulin- and lipid concentrations in venous cord blood

<table>
<thead>
<tr>
<th>Glucose, mmol/l</th>
<th>Insulin, pmol/l</th>
<th>Cholesterol, mmol/l</th>
<th>LDL, mmol/l</th>
<th>HDL, mmol/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>NBW ( (n = 7) )</td>
<td>3.8 (1.2)</td>
<td>21.0 (8.4)</td>
<td>1.6 (0.3)</td>
<td>0.7 (0.2)</td>
</tr>
<tr>
<td>HBW ( (n = 6) )</td>
<td>4.6 (1.3)</td>
<td>32.2 (25.9)</td>
<td>1.7 (0.5)</td>
<td>0.6 (0.2)</td>
</tr>
</tbody>
</table>

Values are means (SD). LDL, low-density lipoprotein; HDL, high-density lipoprotein.
Inhibitors decreased profoundly active MMP-2 and -9 from both the NBW- and the HBW groups (Table 3).

We next inhibited the expression of mRNA for MMP-2 and -9 using specific siRNA molecules. The mRNA expressions were markedly reduced after addition of these siRNAs (Table 3), indicating that the increase in MMP-9 was mainly because of increased production of this protein.

Unaltered concentrations of proinflammatory cytokines and TIMPs in HBW. To further explore how addition of cord plasma from HBW stimulated cultured HUVECs to produce active MMPs, we asked whether the plasma and HUVEC supernatant concentrations of proinflammatory cytokines and of specific TIMPs were altered. However, we were not able to demonstrate any significant changes between the HBW and NBW groups in the concentrations of a panel of possible candidate molecules (Table 4).

**DISCUSSION**

In this study, we show that addition of cord plasma from HBW newborns induced more gelatinase activity, a marker of MMP activity, originating from cultured primary HUVECs than did cord plasma of NBW newborns. In contrast, no difference in MMP activity was found in cord plasma from the two weight groups, indicating that the observed effect was dependent on endothelial cells. The increased MMP activity was mainly a result of enhanced mRNA expression of the MMP-9 subtype and augmented production of MMP-9 protein, whereas the mRNA expression and protein synthesis of the MMP-2 subtype apparently did not differ between the two weight groups. Furthermore, the upregulation of MMP-9 could be inhibited by specific blockers targeting either mRNA expression of MMP-9 or its enzymatic activity. The mechanism by which HBW plasmas induced MMP-9 in endothelial cells remains to be clarified. The difference in MMP-9 could partly be due to HUVEC-mediated change in the balance between stimulators and inhibitors of MMPs. Although MMP-2 can activate MMP-9, we did not find any enhancing effects of HBW plasmas compared with NBW plasmas on MMP-2 activity, suggesting that MMP-2 did not affect MMP-9 in our cell cultures (16). Although MMP-2 is generally considered a noninducible MMP in contrast to MMP-9, the unchanged MMP-2 between our two weight groups might indicate a certain specificity of HBW plasmas in inducing endothelial MMPs (40). Moreover, we could not detect any differences in the supernatant or cord plasma concentrations of a range of proinflammatory cytokines and TIMPs between the HBW and NBW groups.

Because all of the plasma samples of HBW and NBW infants were always compared in parallel to HUVECs from the same donor, differences in endothelial cell properties could not account for the present findings. Use of HUVEC was considered particularly relevant because the purpose of the study was to test whether endothelial cell properties may

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**Table 3. Inhibition of MMPs**

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Parameter</th>
<th>NBW (n = 7)</th>
<th>HBW (n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Inhibitor 1</strong></td>
<td>Reduction active MMP-2</td>
<td>61 (11)</td>
<td>66 (8)</td>
</tr>
<tr>
<td></td>
<td>Reduction active MMP-9</td>
<td>66 (9)</td>
<td>71 (9)</td>
</tr>
<tr>
<td><strong>Inhibitor 3</strong></td>
<td>Reduction active MMP-2</td>
<td>63 (8)</td>
<td>71 (10)</td>
</tr>
<tr>
<td></td>
<td>Reduction active MMP-9</td>
<td>63 (7)</td>
<td>69 (9)</td>
</tr>
<tr>
<td><strong>siRNA</strong></td>
<td>Reduction active MMP-2</td>
<td>72 (9)</td>
<td>75 (12)</td>
</tr>
<tr>
<td></td>
<td>Reduction active MMP-9</td>
<td>69 (10)</td>
<td>72 (8)</td>
</tr>
<tr>
<td></td>
<td>Reduction mRNA-MMP-2</td>
<td>86 (9)</td>
<td>91 (8)</td>
</tr>
<tr>
<td></td>
<td>Reduction mRNA-MMP-9</td>
<td>61 (7)</td>
<td>76 (8)</td>
</tr>
</tbody>
</table>

Values are means (SD), in percent. The reduction in active matrix metalloproteinases (MMP) is based on the change in the active MMP concentration after addition of inhibitor or small interfering RNA (siRNA) compared with no treatment. The reduction in mRNA is based on the change in the ratio of mRNA-MMP to mRNA-GAPDH after addition of siRNA compared with no treatment.

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**Fig. 3.** Preferential increase in active matrix metalloproteinases (MMP)-9 protein concentration in supernatants of HUVECs exposed to HBW-derived cord plasma for 2 days. Active MMP-9 protein concentration rose in supernatants of HUVECs that had added HBW cord plasma (A), whereas both NBW and HBW cord plasmas increased active MMP-2 protein concentration (B). Values are means (SD); n = 7 NBW and 6 HBW samples. *P < 0.05.
be affected by differences in circulating factors already present in fetal life.

A wide range of substances, such as cytokines and connective tissue elements, are involved in the turnover and maintenance of the extracellular matrix, and their relative importance might differ between various tissues and between various pathological conditions; hence, a complete survey of all potential molecules affecting the extracellular matrix was clearly beyond the scope of the present study (24). In contrast, we chose to focus solely on MMPs, since their ability to degrade components of the extracellular matrix is considered particularly relevant for the initiation and development of atherosclerosis and coronary artery disease. Consequently, circulating levels of MMPs are increasingly used as markers of progression and response to therapy of these diseases (15, 25, 37). Importantly, the possible role of MMP in the fetal circulation has hitherto received almost no attention.

Among the MMP subtypes, the gelatinase MMP-9 seems to be among the chief compounds responsible for vascular and myocardial remodeling in patients with cardiac disease (2, 35). A role for MMP-9 in placental function and labor has been suggested (14, 39); however, whether placenta-derived MMP-9 can affect fetal endothelial function is unknown.

Although we did not have any information about the nutritional intake or level of physical activity among the mothers during their pregnancies, the two study groups were quite comparable regarding demographic variables. Importantly, we only included healthy mothers who delivered at term and who had uncomplicated pregnancies. The average prepregnancy body mass index in both groups was in the overweight range, i.e., between 25 and 30 kg/m². However, because body mass index did not differ between the two experimental groups, factors related to differences in prepregnancy maternal body weight probably did not affect our results. Only cord samples obtained after planned caesarean sections were included to exclude possible contributions of various forms of stress imposed by vaginal or emergency deliveries.

The two study groups were solely selected on the basis of birth weight. Newborns of a given birth weight do not constitute a homogenous group as they may differ in body composition and other anthropometric properties (11, 31). Nonetheless, even with this relatively crude grouping of the newborns, significant differences in cord plasma properties were observed. The proportion of infants weighing >4 kg at birth has increased substantially. Intriguingly, whereas a large difference in MMP activity was noted between the NBW and HBW groups in our study, the difference in mean birth weight between these two groups was only 0.8 kg. Therefore, our findings may indicate that even small differences in birth weight can influence the development of obesity and/or cardiovascular disease in child- and adulthood.

Because only venous cord plasmas were used, the present findings could be due to differences in factors released from the placentas of HBW and NBW infants, particularly because the weight of the placenta is dependent on infant weight (8). A second source of factors is the fetal body itself, including liver, adipose tissue, and endocrine organs.

A third source of factors would be those transferred directly (transplacentally) from the mother. It is well established that mothers of HBW infants differ from those of NBW newborns in terms of body composition and nutritional status (8). There is also increased prevalence of glucose intolerance among mothers of HBW infants, which is associated with increased transfer of glucose to the fetus (7). The cord plasma concentrations of glucose and insulin in the NBW and HBW groups were not statistically different and far below those required for modulation of MMP release from HUVECs, rendering the possibility unlikely that glucose and/or insulin could have influenced our results (17).

A limitation of the present study is the lack of maternal weight information during pregnancy. Parental obesity is a strong risk factor for offspring obesity, which has both genetic and environmental components (13). Furthermore, a recent study showed that maternal weight gain during pregnancy in Icelandic women was associated with HBW newborns (26). Moreover, our group (22) recently showed that overweight and obesity track from early childhood into adulthood, further underscoring the association between overweight during early and later life.

There is accumulating evidence that several of the major diseases of later life, including metabolic syndrome, overweight, cardiovascular disease, and cancer, are associated with HBW. These diseases may be consequences of early life environment, whereby a stimulus at a critical, sensitive period of early life has adverse effects on tissue structure, physiology, and metabolism (4, 18).

In conclusion, our data show that cord plasmas from infants of HBW differ from those of NBW in the quantity or quality of one or more factors stimulating endothelial MMP activity. Such differences may reflect altered endothelial functions in fetuses destined to be HBW infants.

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**GRANTS**

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