Maternal homocysteine and chorionic vascularization in recurrent early pregnancy loss

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Defective chorionic villous vascularization has been suggested to be associated with embryonic death. There are no reports, however, describing chorionic vascular profiles in spontaneous miscarriage tissue. Therefore, we investigated chorionic villous vascularization by both histopathology and an image analysis system combined with CD34 immunohistochemistry in spontaneous miscarriage tissue of 19 women with recurrent early pregnancy loss (REPL). Subsequently, we studied the vascular profile parameters (median vascular area, perimeter, number of vascular elements per measured chorionic area, and the median area, perimeter and diameter per vascular element) in relation to the maternal plasma total homocysteine concentrations (an independent risk factor for REPL). The histopathological scores and the measured number of vascular elements per mm² chorionic tissue were not significantly different between women with elevated and those with normal total homocysteine concentrations. However, women with elevated total homocysteine concentrations (fasting \( >18.3 \, \mu \text{mol/l} \) and/or \( 6 \, \text{h} \) after methionine load \( >61.5 \, \mu \text{mol/l} \) ) showed (per measured chorionic area) significant smaller median vascular areas and perimeters. The single chorionic vascular elements in these women had significant smaller median areas, perimeters and diameters. Furthermore, the fasting total homocysteine was negatively correlated with the perimeter of the vascular element \( (r = -0.54; \, P < 0.05) \). In conclusion, in REPL, elevated maternal total homocysteine concentrations are associated with defective chorionic villous vascularization.

Key words: abortion/CD34/chorionic vascularization/early pregnancy loss/homocysteine

Introduction

During the past decade, the list of candidate causes for recurrent early pregnancy loss (REPL) has grown rapidly. During the early 1990s, an elevated plasma total homocysteine concentration, which has been described as a risk factor for atherosclerosis (Boushey et al., 1995; Graham et al., 1997), venous thrombosis (Den Heijer et al., 1996), neural tube defects (Steegers-Theunissen et al., 1994), placental abruption or infarction (Goddijn Wessel et al., 1996) and pre-eclampsia (Dekker et al., 1995) was also suggested to be associated with REPL (Steegers-Theunissen et al., 1992; Wouters et al., 1993). Recently, others have confirmed this observation (Quere et al., 1998; Coumans et al., 1999).

Homocysteine is a sulphur-containing intermediate in methionine metabolism, and can be catabolized in the trans-sulphuration pathway (vitamin B6-dependent) or remethylated to methionine (folate- and cobalamin-dependent). Mild hyperhomocysteinaemia can be caused by deficiencies of the relevant B-vitamins or by genetically determined reduction in the enzyme activities. Despite the high number of publications describing methionine–homocysteine metabolism in recent years, the pathogenic role of increased total homocysteine concentrations is still poorly understood. Methionine, involved in the formation of \( S \)-adenosylmethionine, is a substrate for DNA methylation. As hyperhomocysteinaemia may reflect reduced remethylation, one could postulate in relation to REPL that elevated total homocysteine is associated with an impaired DNA methylation and gene expression (Heby, 1995), possibly leading to disturbed chorionic vasculogenesis.

Recently, the development of the chorionic villous vascular system in normal human first-trimester pregnancies was investigated by quantitative CD34 immunohistochemistry (te Velde et al., 1997). This study showed a significant correlation between the menstrual age and the total number of vascular elements as well as the villous stromal area. In accordance with this was an earlier observation that embryonic death was associated with a defective chorionic villous vascularization, defined as lower vascular density (total number of blood vessels per eight villi) (Meegdes et al., 1988).

However, few studies have been reported on chorionic villous vascularization in spontaneous early pregnancy loss (Meegdes et al., 1988; Rehder et al., 1989; Rockelein et al., 1991; Salafia et al., 1993), usually based on a histological score in correlation with cytogenetic findings. There are no reports in which vascular profiles of single vascular elements, which can be assumed as more exact determinants of the vascular function, have been described in spontaneous miscarriage tissue. Quantitative analyses of vascular profiles can be made by a computerized image analysis system (e.g. Vidas®plus system), which is frequently used in tumour angiogenesis research (Weidner, 1995). Therefore, to investigate the possible pathogenic role of elevated total homocysteine concentrations in REPL, we studied spontaneous miscarriage tissue of 19 women with REPL, in particular the chorionic villous vascularization, by both histopathology and the Vidas®plus image analysis system.
(including CD34 immunohistochemistry) and correlated it with the maternal variables in homocysteine metabolism.

Materials and methods

Subjects

Between September 1994 and February 1998, 131 consecutive Caucasian women suffering from REPL and without previous B-vitamin supplementation were referred to our hospital. Subgroups of these patients had contributed to previous studies (Nelen et al., 1997a,b, 1998). All women were screened for chromosomal rearrangements, severe uterine anomalies, antiphospholipid antibodies, and thyroid dysfunction by a routine investigational procedure (Wouters et al., 1993). Twenty-four women fulfilled the following inclusion criteria: (i) no possible other causes for REPL than hyperhomocysteinaemia; (ii) no factors possibly interfering with homocysteine metabolism (e.g. medication); (iii) routine histological investigation of the last miscarriage preceding the methionine-loading test; and (iv) ultrasonographical or histopathological evidence of embryonic death. After exclusion of a twin pregnancy (n = 1), insufficient number of chorion villi in sections (n = 2), and a histological suspicion of infection or chromosomal abnormalities (n = 2), 19 cases were finally eligible to participate in the study.

Early pregnancy loss was defined as the spontaneous ending of a pregnancy within 16 weeks of menstrual age, excluding ectopic and molar pregnancies. Recurrent early pregnancy loss was defined as at least two consecutive spontaneous abortions following conception from the same partner. Gestational age (in weeks) was calculated by the menstrual age at the moment of the curettage or spontaneous expulsion, as well as by the maximum measured crown–rump length (n = 17), collected from the clinical record. The Institutional Review Board of the University Hospital Nijmegen approved the study. Before participation, written informed consent was obtained from all subjects.

Methionine-loading test

Methionine–homocysteine metabolism was investigated by a standardized oral methionine-loading test (Wouters et al., 1993). At the time of measurement, none of the women was pregnant or lactating. Elevated total homocysteine was defined as a total homocysteine concentration fasting and/or after-load exceeding the 95th percentile of a group of 104 healthy parous controls (Nelen et al., 2000). For the fasting total homocysteine concentration this was above 18.3 µmol/l, and for the after-load concentration was >61.5 µmol/l. Folate was considered to be decreased if the serum concentration was below 6.8 nmol/l (5th percentile).

Blood samples for measurement of plasma total homocysteine concentrations were drawn in ethylenediamine tetra-acetate (EDTA) vacutainer tubes of 4 ml and centrifuged within 30 min at 3000 g for 10 min. The plasma was separated and stored at −20°C. Plasma total homocysteine concentrations were measured using a high-performance liquid chromatography (HPLC) technique and fluorimetric detection (Te Poel-Pothoff et al., 1995). Dry and heparinized vacutainer tubes of 10 ml were used for collecting venous blood samples to assay folate (serum and red cells), pyridoxal 5'-phosphate (an active form of vitamin B6) (whole blood) and vitamin B12 (serum) concentrations. Folate and vitamin B12 concentrations were measured simultaneously with Dualcount SPB (solid phase boil) Radioassay (Diagnostic Products Corporation, Los Angeles, CA, USA), as described previously (Mooij et al., 1991). Determination of pyridoxal 5'-phosphate was performed using a HPLC technique (Schrijver et al., 1981).

Histopathology

Formalin-fixed and paraffin-embedded miscarriage tissues of 19 women were used. Standard 5 µm-thick haematoxylin and eosin-stained microscopical sections were used for pathohistological scoring. The sections were scored on a scale for multiple features (villous oedema, fibrosis, vascularization and intervillous fibrin) (Figure 1a) by two authors (W.N., J.B.). The definitive scoring list was completed jointly. In cases of disagreement, the ultimate scoring was obtained by consensus with a third investigator (A.H.). During this procedure, the investigators were blinded to the clinical data and previous histological examinations.

Immunohistochemistry

Paraffin sections (3 µm thickness) were mounted onto polylysine-coated slides and dried overnight at 37°C. Paraffin sections were dewaxed in xylene and rehydrated in a standard series of graded alcohols, and endogenous peroxidase was blocked by 1% H2O2 in methanol for 15 min followed by rinsing in phosphate-buffered saline (PBS, pH 7.4). After pre-incubation with 1% bovine serum albumin (BSA) in PBS–TWEEN 20, CD34 antigen was detected by the monoclonal mouse anti-CD34 antibody (QBEnd; Biogenex, San Ramon, CA, USA) 1:2 in PBS, overnight at 4°C. Detection of the primary antibody was performed using biotinylated horse anti-mouse (Vector Laboratories, Burlingame, CA, USA) and horseradish peroxidase avidin–biotin complex (ABC Elitekit; Vector Laboratories). Finally, peroxidase was visualized with 0.05% diaminobenzidine (DAB; Sigma, St Louis, MO, USA)/0.15% H2O2 (Sigma) in PBS for 5 min at room temperature. All incubation steps were followed by three washes in PBS of 5 min. Subsequently, the slides were slightly counterstained with Mayer’s haematoxylin, dehydrated, and finally mounted (Figure 1b).

Image analysis

Image analysis was performed using the VidasPlus system (Kontron GmbH, Eching, Germany). Microscope images were recorded using a three-chip CCD camera (DXC-325P; Sony, Tokyo, Japan) mounted on a conventional light microscope (Axiostop; Carl Zeiss, Jena, Germany) using a 20× objective (numerical aperture = 0.5). Resulting images, measuring a field of 200×212 µm, were stored on magneto-optical discs (Borsu Systema, Lelystad, The Netherlands) as true colour (24-bit RGB) images. For each case, 40 images with parts of intermediate villi were randomly recorded and evaluated by one author (W.N.) who was blinded to the clinical and histopathological data. Forty images were found to be sufficient to obtain stable running means (data not shown) for the different variables assessed as described below. Each villus was recorded only once. Image pixels were divided by the system into ‘object pixels’ (pixels resulting from CD34-stained cells) and ‘background pixels’, based on the intensities of the signals. Neighbouring object pixels were grouped together to form objects that possibly corresponded to vascular elements (Figure 1c).

Where necessary, interactive correction was performed, implying exclusion of field areas without chorionic villous tissue, closing of vessel lumina that were not completely surrounded by stained cells (Figure 1d), and erasing of falsely recognized non-vascular objects. The following vascularization variables calculated by the computerized system were used: (i) per image: % vascular area, vascular perimeter and vascular count; and (ii) per vascular element: the area, perimeter and diameter.

Statistics

The data were presented as medians (minimum–maximum) because of the skewed distributions. For statistical analyses of the different
field and vascular element parameters per woman, the median value for each parameter was chosen to be representative for that woman. Statistical comparisons between groups were performed using Mann–Whitney U-test. Correlation analyses were performed using Spearman’s rank correlation tests. In the case of a significant correlation, a stepwise univariate analysis of variance with dummy variables was performed to estimate possible confounding. A two-sided P-value <0.05 was considered to be statistically significant. Statistical analyses were performed with SPSS for Windows Release 8.0 Standard Version (1997, Chicago, USA).

Results

The major characteristics of the study population, including the variables of the homocysteine metabolism measured, are presented in Table I. The histopathological scoring and the data obtained by the Vidasplus system are described in Figure 2 and Table II. Six women with elevated total homocysteine concentrations were compared with 13 women having total homocysteine concentrations within the normal range.

Both groups showed no significant differences with regard
Table I. Distribution of the demographic and homocysteine metabolism data in the two study groups

<table>
<thead>
<tr>
<th></th>
<th>Elevated tHcy (n = 6)</th>
<th>Normal tHcy (n = 13)</th>
<th>P</th>
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</thead>
<tbody>
<tr>
<td><strong>Demographic data</strong></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Age (years)</td>
<td>33.1 (29.0–35.5)</td>
<td>33.1 (24.2–24.4)</td>
<td>NS*</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>23.1 (19.6–29.1)</td>
<td>24.2 (19.8–39.1)</td>
<td>NS</td>
</tr>
<tr>
<td>Blood pressure (mmHg)</td>
<td></td>
<td></td>
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<tr>
<td>Diastolic</td>
<td>68 (60–80)</td>
<td>70 (60–80)</td>
<td>NS</td>
</tr>
<tr>
<td>Systolic</td>
<td>113 (90–120)</td>
<td>113 (100–130)</td>
<td>NS</td>
</tr>
<tr>
<td><strong>Index pregnancy</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Menstrual age (weeks)</td>
<td>10.5 (10.0–15.0)</td>
<td>10.5 (9.0–13.0)</td>
<td>NS</td>
</tr>
<tr>
<td>CRL age (weeks)b</td>
<td>7.9 (6.4–10.1)</td>
<td>7.7 (5.9–12.0)</td>
<td>NS</td>
</tr>
<tr>
<td>Retention time (weeks)c</td>
<td>2.9 (2.1–6.7)</td>
<td>2.3 (0.8–4.6)</td>
<td>NS</td>
</tr>
<tr>
<td><strong>Miscarriages</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. in obstetric history</td>
<td>4 (2–5)</td>
<td>3 (2–4)</td>
<td>NS</td>
</tr>
<tr>
<td>Primary abortersd</td>
<td>4 (67%)</td>
<td>1 (8%)</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>≥3 miscarriages</td>
<td>4 (67%)</td>
<td>7 (54%)</td>
<td>NS</td>
</tr>
<tr>
<td>Current smoking</td>
<td>3 (50%)</td>
<td>6 (46%)</td>
<td>NS</td>
</tr>
<tr>
<td>Alcohol consumption</td>
<td>4 (67%)</td>
<td>8 (62%)</td>
<td>NS</td>
</tr>
<tr>
<td><strong>Homocysteine metabolism</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>∆Time (months)e</td>
<td>3 (1–11)</td>
<td>2 (1–18)</td>
<td>NS</td>
</tr>
<tr>
<td>tHcy (µmol/l)f</td>
<td>20.2 (12.7–33.1)</td>
<td>13.2 (7.8–17.7)</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>After-load</td>
<td>65.3 (44.3–76.2)</td>
<td>34.5 (27.1–50.9)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Folate (nmol/l)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serum</td>
<td>9.7 (4.4–20.0)</td>
<td>12.0 (6.3–20.0)</td>
<td>NS</td>
</tr>
<tr>
<td>Red cells</td>
<td>425 (310–770)</td>
<td>530 (280–880)</td>
<td>NS</td>
</tr>
<tr>
<td>PLP (nmol/l)g</td>
<td>46 (27–72)</td>
<td>41 (30–53)</td>
<td>NS</td>
</tr>
<tr>
<td>Cobalamin (pmol/l)h</td>
<td>145 (65–300)</td>
<td>280 (150–490)</td>
<td>&lt; 0.05</td>
</tr>
</tbody>
</table>

Values given as median (minimum–maximum) or as absolute number (percentage).

*Not significant.

*Gestational age calculated from the maximum measured crown–rump length (CRL) (n = 17).

*Menstrual age minus the gestational age calculated from the CRL.

*DWomen suffering from spontaneous abortions only and without pregnancies beyond 16 weeks menstrual age.

*Time interval between ending of last pregnancy and oral methionine loading test.

*Total plasma homocysteine concentrations.

*Whole-blood pyridoxal 5′-phosphate, an active form of vitamin B6.

*Serum vitamin B12.

tHcy = plasma total homocysteine.

to age, body mass index, diastolic and systolic blood pressure, total number of pregnancies, gestational age and the retention time of the index pregnancy, smoking habits and alcohol consumption. Women with elevated total homocysteine concentrations had significant lower median serum cobalamin concentrations (145 versus 280 pmol/l).

Although women with normal total homocysteine concentrations seemed to have a better developed chorionic vascular system and less extensive fibrosis and intervillous fibrin depositions as scored by the two examiners (Figure 2), these differences failed to reach statistical significance.

In patients with elevated total homocysteine concentrations, between 88 and 410 vascular elements per woman were obtained compared with 162 to 531 vascular elements in normohomocysteinaemic women.

The total number of vascular elements measured per mm² chorionic tissue, calculated by the Vidasplus image analysis system, was lower in women with elevated total homocysteine concentrations, but this difference failed to reach statistical significance (Table II). However, the median percentage vascular area and the median total vascular perimeter were significantly smaller in women with elevated total homocysteine concentrations. Comparing both total homocysteine groups...
Table II. Distribution of the vascular parameters calculated by the Vidas Plus image analysis system in women with elevated and normal total homocysteine (tHcy) concentrations

<table>
<thead>
<tr>
<th></th>
<th>Elevated tHcy (n = 6)</th>
<th>Normal tHcy (n = 13)</th>
<th>P</th>
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</thead>
<tbody>
<tr>
<td><strong>Per field</strong></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Vascular area (%)a</td>
<td>1.5 (0.6–3.0)</td>
<td>3.7 (1.3–7.3)</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>Perimeter (µm/mm²)b</td>
<td>8690 (3926–19 336)</td>
<td>15 501 (7 324–40 855)</td>
<td>0.05</td>
</tr>
<tr>
<td>Vascular count (n/mm²)c</td>
<td>138 (89–413)</td>
<td>246 (126–564)</td>
<td>NS</td>
</tr>
<tr>
<td><strong>Per vascular element</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Area (µm²)</td>
<td>43 (26–63)</td>
<td>58 (49–92)</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>Perimeter (µm)</td>
<td>37 (29–40)</td>
<td>45 (37–62)</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Diameter (µm)d</td>
<td>4.8 (3.6–5.9)</td>
<td>5.4 (4.7–6.5)</td>
<td>&lt; 0.05</td>
</tr>
</tbody>
</table>

Values given as median (minimum–maximum).
Comparison of both groups by Mann–Whitney U-tests.
aCD34 positively stained area/total chorionic area × 100.
bTotal perimeter of vascular elements/total chorionic area × 106.
cTotal number of vascular elements/mm² chorionic area.
dLargest diameter measured in the smallest direction.

Figure 3. Distribution of the chorionic vascular elements on the basis of their diameter in women with recurrent early pregnancy loss and elevated (□; n = 6) or normal (■; n = 13) total homocysteine concentrations. Values are given as median and 5th–95th percentiles; *, P < 0.05.

for the parameters calculated per vascular element, significant smaller median areas, perimeters and diameters were observed in women with elevated total homocysteine concentrations. Comparing women with decreased (n = 3) and normal (n = 16) serum folate concentrations, no significant differences were found in the histopathological scores or in the vascular parameters calculated by the image analysis system.

To evaluate differences in the vascular profiles between the two groups, a subdivision of the vascular elements was made on the basis of their diameter. As illustrated in Figure 3, women with recurrent early pregnancy loss and elevated total homocysteine concentrations were observed to have proportionally more vascular elements with a small diameter.

The total homocysteine (fasting, as well as 6 h after methionine loading) and serum folate concentrations of women in the total study population were correlated with the calculated vascular data, which resulted in a negative correlation (r = –0.54; P = 0.02) between the fasting total homocysteine concentration and median perimeter per vascular element (Figure 4). After log transformation in a univariate analysis of variance model, this correlation could be described as follows: perimeter = intercept + 0.093(total homocysteine). This correlation was not influenced by the maternal age, menstrual age, crown–rump length, retention time, serum folate concentration, smoking, grade of oedema, grade of fibrosis and the total number of vascular elements per mm² chorionic tissue. All other correlations did not reach statistical significance.

Discussion

Impaired chorionic villous vascularization has been suggested as a cause of embryonic death (Meegdes et al., 1988). To our knowledge, the present study is the first to use a vascular image technique to investigate single vascular profiles in miscarriage tissue. A smaller median percentage vascular area and median total perimeter was found in women with elevated total homocysteine concentrations. In agreement with this, significantly smaller median areas, perimeters and diameters per chorionic vascular element were observed, as well as a
negative correlation between the median vascular perimeter and the fasting total homocysteine concentration. This suggests that elevated maternal total homocysteine concentrations may be a cause of defective chorionic villous vascularization.

Chorionic vasculogenesis comprises aggregates of haemangioblastic cells, which are subsequently dilated to form capillaries (Demir et al., 1989). In normal first-trimester pregnancies, this process of maturation has been characterized by an increase in the total number of vascular elements, but with a stable number of the haemangioblastic aggregates (te Velde et al., 1997). As in our study the total number of vascular elements per mm² chorionic tissue between both total homocysteine groups was not significantly different, a maturational rather than a constructional defect may be expected to be associated with elevated total homocysteine concentrations.

According to the postulated mechanism of reduced DNA methylation and gene expression, one may expect to find an impaired chorionic villous vascularization in cases of decreased serum folate concentrations, but this was not the case. One possible explanation may be the relatively small group size \((n = 3)\) of folate-deficient individuals, although alternative pathogenetic mechanisms (e.g. direct embryotoxic effects) (Obwegeser et al., 1999) should be considered. Moreover, in this study only the chorionic vascularization was investigated, but others have suggested that decidual angiogenic disturbances are also related to early pregnancy loss (Vailhe et al., 1999).

The perimeter of a blood vessel may be accepted as representing the most functional variable of all calculated vascular parameters, because it best reflects the final fetal–mammary diffusion capacity. An impaired fetal–mammary diffusion is a plausible explanation for a disturbed fetal development or even fetal death, especially if one accepts the idea that fetal–mammary diffusion begins early in pregnancy (Valentin et al., 1996; Craven and Ward, 1999). However, if a functional fetal–mammary circulation is not initiated before 12 weeks of pregnancy—as has been suggested by some authors (Hustin and Schaaps, 1987; Rodesch et al., 1992)—then the role of a defective chorionic vascularization in embryonic death remains to be elucidated. During these first weeks of pregnancy, embryonic nutrition is provided by the transfer of nutrients from the extra-embryonic coelomic cavity via the yolk sac to the embryo (Exalto, 1995). In normal first-trimester pregnancies, low extra-embryonic coelomic total homocysteine concentrations and high folate and methionine concentrations have been reported (Campbell et al., 1993; Steegers-Theunissen et al., 1997). Furthermore, these extra-embryonic coelomic concentrations were positively correlated with maternal serum concentrations. Thus, we expect also higher total homocysteine and/or lower folate extra-embryonic coelomic concentrations in the pregnancies of women with elevated plasma total homocysteine concentrations.

We realize that the investigation of pathological changes in chorionic tissue after embryonic death is limited because of the uncertainty of whether vascular changes are caused by defective development, or occurred post mortem. However, as we also found no effect of the retention time on the vascular parameters in the regression analysis—which is in agreement with observations by others (Meegdes et al., 1988)—we consider that the data on vascular parameters do reflect embryonic development.

In conclusion, in recurrent early pregnancy loss, elevated maternal total homocysteine concentrations are associated with defective chorionic villous vascularization.

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References


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