Vasculogenesis in complete and partial hydatidiform mole pregnancies studied with CD34 immunohistochemistry

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BACKGROUND: Defective chorionic villous vascularization is present in pregnancies complicated by absent or abnormal embryonic development. The aim of this study was to investigate the embryonic and/or maternal genomic influence on vasculogenesis in diploid complete hydatidiform mole (CHM) and in triploid partial hydatidiform mole (PHM) in comparison with normal development. METHODS: Mean villous stromal area and functional vascular area, vessels with a lumen and haemangiogenetic cords, peripherally or centrally located were measured and counted in chorionic villi of 12 CHM, 12 normal pregnancies (termination of pregnancy, TOP) and 15 PHM of which nine were without an embryo (PHM2E) and six were with an embryo (PHM1E), using quantitative CD34 immunohistochemistry. RESULTS: TOP showed significantly more vessels per chorionic villus, centrally and peripherally located (median, range), than CHM, PHM2E and PHM1E (4.0, 0–9 versus 0.0, 0–11, 0.0, 0–18 and 1.0, 0–21). CHM showed significantly more centrally located cords than PHM2E, PHM1E and TOP (1.5, 0–22 versus 1.0, 0–15, 0.5, 0–8 and 1.0, 0–2). CONCLUSIONS: Initiation of chorionic villous vasculogenesis is independent of the maternal genome (CHM). The development of an embryo, however, is obligatory for the modulation of normal vascularization resulting in a well developed vasculosyncytial membrane.

Key words: CD34/chorionic villous/complete hydatidiform mole/partial hydatidiform mole/vasculogenesis/triploidy

Introduction

Hydatidiform mole is a phenotype with two different entities having different histopathological and cytogenetic criteria: the complete hydatidiform mole (CHM) and the partial hydatidiform mole (PHM).

CHM arises from the fertilization of an empty oocyte (the maternal pronucleus is lacking or inactivated) by two haploid sperm (heterozygous) or by one haploid sperm followed by duplication of its chromosomes ( homozygous). Genetic studies have demonstrated that the majority of CHM are androgenetic and diploid with a predominantly 46 XX karyotype. The 46 XY karyotype is less common and 46 YY has never been found (Kajii and Ohama, 1977; Jacobs et al., 1980; Lawler et al., 1991). CHM is characterized by generalized swelling of the villi (stromal oedema with formation of cisterna), diffuse trophoblastic hyperplasia and absence of embryonal tissues (Szulman and Surti, 1978).

Although the villous stroma of CHM is avascular in the classical descriptions, a few studies have demonstrated blood vessels in villi of CHM without embryonal blood cells. In the majority of CHM, some vasculogenesis is present, mainly in the villi with little or no oedema (Pardinas et al., 1996; Qiao et al., 1997; van de Kaa et al., 1997).

It has been suggested that the absence of a maternal contribution to the nuclear genome in CHM (paternal origin) will result in the inability to form an embryo (Szulman and Surti, 1978). As in recent studies, however, some early embryonic development has been found (nucleated blood cells, amniotic tissue and yolk sac), it is presumed that the embryonic development will stop very early in these pregnancies (Fisher et al., 1997; Paradinas et al., 1997; van de Kaa et al., 1997; Weaver et al., 2000).

PHM is characterized by focal swelling of the villous tissue, focal trophoblastic hyperplasia and often the presence of an embryo or fetal tissue, with >90% of them being triploid (Szulman et al., 1981). Triploidy may either arise through fertilization of a haploid oocyte by two spermatozoa (diandric) or through the fertilization of a diploid oocyte by one sperm (digynic). PHM is the phenotype of diandric triploidy (Jacobs et al., 1982; Zaragoza et al., 2000). Digynic triploid pregnancies are not associated with PHM but show severely restricted embryonic growth with relative macrocephaly and a very small placenta.

The monoclonal antibody against CD34 antigen in human endothelial cell membranes and haemopoietic progenitor cells proved to be a useful marker of villous vascular
endothelial cells in normal first trimester pregnancies (te Velde et al., 1997) and complicated pregnancies in the first and second trimester (Qiao et al., 1997; Lisman et al., 1998; Lisman and Exalto, 1999).

In a recent study, using CD34 immunohistochemistry, we have demonstrated an abnormal development of the vasculosyncytiotrophoblastic membrane in pregnancies complicated by embryonic death and even more in anembryonic pregnancies. It was concluded that vasculogenesis is a basic feature in all types of pregnancy and is subsequently modulated directly or indirectly by embryonic signalling (Lisman et al., 2004).

The aim of the present study is to investigate the embryonic and/or maternal genomic influence on vasculogenesis in CHM and in PHM, with chromosomes exclusively and predominantly, respectively, of paternal origin, in comparison with normal development.

**Material and methods**

**Case selection**

Records of patients with CHM, PHM or termination of pregnancy (TOP) at the Departments of Gynaecology and Obstetrics at the Spaarne Hospital and at the Academic Medical Center, University of Amsterdam during recent years were retrieved. CHM were proved to be all diploids by DNA analysis. All cases of PHM were proved to be triploids either by DNA analysis or by karyotype. Two groups of 12 pregnancies each (CHM and TOP) and one group of 15 pregnancies (PHM) subdivided into one group of nine PHM without an embryo and one group of six PHM with an embryo or fetus, were recruited by selecting consecutive cases fulfilling the following inclusion criteria: group I, CHM (not containing embryonic signs such as a stunted embryo, yolk sac or amnion on ultrasound); group II, subdivided into group IIA, PHM without an embryo (PHM – E; not containing embryonic signs such as a stunted embryo, yolk sac or amnion on ultrasound) and group IIB, PHM with an embryo or fetus [PHM + E; crown–rump length (CRL) or DBP available with or without positive heart action]; group III, TOP (CRL available, positive heart action). The Dutch Central Molar Registration, University Medical Center St Radboud, Nijmegen revised all group I, IIA and IIB slides. As the selection was based on ultrasound criteria, we preferred the term PHM without an embryo over early embryonic demise in PHM pregnancy although the latter seems to be more appropriate from a morphogenetic point of view.

Gestational age (GA) was calculated based on the CRL measured in a viable state of the embryo (TOP and PHM + E) or based on the last menstrual period (CHM and PHM – E). In three cases of PHM + E, the embryo was in a viable condition; in the other three cases, the embryo was in the post-mortem state before dilatation and curettage (D&C).

In all cases, routinely phosphate-buffered formalin-fixed paraffin blocks were retrieved and stained with haematoxylin and eosin (H&E) and anti-CD34 antibody.

According to Dutch law, no approval of the Institutional Review Board was obligatory for the performance of these histopathological measurements. However, this study was performed with the approval of the Science Committee of the Spaarne Hospital.

**Immunohistochemistry**

Placental tissue sections (4 μm thick) were cut and mounted on 3-aminopropyl-triethoxy-silane-coated slides. Incubation with monoclonal mouse-antiCD34 antibody (BioGenex, San Ramon, CA) was performed at room temperature for 1 h, after blocking endogenous peroxidase activity. Detection of the primary antibody was performed using biotinylated rabbit anti-mouse antibody (DAKO A/S, Copenhagen, Denmark) and streptavidin–biotin–horseradish peroxidase complex (sABC/HRP, DAKO A/S, Denmark). The peroxidase reaction was visualized using diaminobenzidine/H2O2 [0.05% (w/v)/0.03% (v/v)].

**Analysis of vasculogenesis**

Slides of placenta tissue of CHM and PHM were examined at a magnification of × 40 (field diameter 4500 μm) and placenta tissue of TOP was examined at a magnification of × 100 (field diameter 1800 μm) by one trained observer, blind to the group and duration of the pregnancy. For each case, 15 randomly selected mesenchymal or immature intermediate villi (without stromal connective tissue fibres to rule out stem villi) were evaluated. According to a previously performed pilot study to obtain stable running means for villous stromal area, functional vascular area and vascular elements, 15 villi appeared to be sufficient for a stable outcome of measurements. The outcome of measurement for the different variables, studying 15 villi per pregnancy specimen, yielded an intra-observer variability of <15% (unpublished data). In 15 villi of each pregnancy, the total amount of vascular elements was counted.

The process of maturation was depicted by counting cords, defined as clusters of CD34-positive haemangioblastic cells without lumen formation, as well as vessels, defined as clusters of CD34-positive cells with a clear cut lumen. The process of margination was illustrated by describing whether these cords and vessels were

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**Figure 1.** Chorionic villous vascularity of normal first trimester pregnancy (a) and CHM (b). Luminalized vessels (V) and haemangioblastic cords (C), located centrally (c) and peripherally (p).
located peripherally or centrally. Peripherally was defined as situated in contact with the trophoblastic surface of the villus, contributing to the vasculosyncytial membrane as such. Centrally was defined as without any connection to the trophoblast (Figure 1a).

Morphometrical analysis
Morphometrical measurements were performed using the QPRODIT interactive video-overlay system (Leica, Cambridge, UK). The system comprises an IBM-compatible microcomputer with a video overlay board, a computer mouse and a charge-coupled device colour camera mounted on a standard light microscope. After a rough examination for fields with a sufficient number of chorionic villi, 15 villi were randomly selected by the computer using an on-screen grid. Only villi which could be visualized completely, which means including the syncytiotrophoblast, were measured. Contours of the stroma of the previously mentioned 15 villi and all the included vessels were traced manually on the computer monitor with a mouse-controlled cursor using an on-screen magnification of \( \times 100 \).

The following features were calculated: area of the villous stroma without the trophoblastic layer; functional vascular area; the percentage of villous stroma occupied by vessels; all the vessels and cords; and the amount of central and peripheral cords and vessels. The vascular density of vascularized villi was calculated separately after excluding avascular villi and was defined as the mean number of vessels per vascularized villus.

Statistical analysis
Differences between groups in patient characteristics, number of central and peripheral cords and vessels, the area of villous stroma including vessels and the vascular area were tested for significance, using analysis of variance or median tests as appropriate. Significant differences were studied further by post hoc analyses. Data were analysed using SPSS 11.5.1 (SPSS Inc., Chicago, IL).

Results

Patient’s characteristics
A total of 39 pregnancies divided into four groups were studied. Group I consisted of 12 CHMs, group IIA consisted of nine PHM – E, group IIB consisted of six PHM + E and group III consisted of 12 normal pregnancies (TOP). Patient characteristics are presented in Table I.

The mean GA of PHM – E and PHM + E was higher (93.8 and 103 days, respectively) than that of CHM and TOP (75.1 and 59.7 days, respectively); these differences did not reach statistical significance.

Morphometric measurements
The median (range) number of various vascular elements in 15 chorionic villi for the different groups is described in Table II.

CHM showed statistically significantly more cords, mainly centrally located, in comparison with PHM – E, PHM + E

Table I. Mean (SEM) patients characteristics at the time of termination of pregnancy

<table>
<thead>
<tr>
<th></th>
<th>CHM (n = 12)</th>
<th>PHM without embryo (n = 9)</th>
<th>PHM with embryo/fetus (n = 6)</th>
<th>TOP (n = 12)</th>
<th>P-valuea</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>29.9 (1.4)</td>
<td>30.1 (2.1)</td>
<td>33.0 (3.2)</td>
<td>29.8 (2.3)</td>
<td>NS</td>
</tr>
<tr>
<td>Gravidity</td>
<td>2.5 (0.54)</td>
<td>2.2 (0.28)</td>
<td>3.5 (1.03)</td>
<td>2.3 (0.59)</td>
<td>NS</td>
</tr>
<tr>
<td>Parity</td>
<td>1.1 (0.50)</td>
<td>0.89 (0.31)</td>
<td>0.83 (0.31)</td>
<td>0.5 (0.23)</td>
<td>NS</td>
</tr>
<tr>
<td>Gestational age (days)</td>
<td>75.1 (4.8)</td>
<td>93.8 (5.7)</td>
<td>103 (14.7)</td>
<td>59.7 (3.3)</td>
<td>NS</td>
</tr>
</tbody>
</table>

aANOVA.

Table II. The median (range) number of vascular elements (in 15 chorionic villi) of CHM, PHM without embryo, PHM with embryo or fetus and normal pregnancies (TOP)

<table>
<thead>
<tr>
<th></th>
<th>CHM (n = 12)</th>
<th>PHM without embryo (n = 9)</th>
<th>PHM with embryo/fetus (n = 6)</th>
<th>TOP (n = 12)</th>
<th>P-valuea</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vessels</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peripheral</td>
<td>0 (0–10)b,c,d</td>
<td>0 (0–8)c,f</td>
<td>0 (0–19)</td>
<td>2.5 (0–8)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Central</td>
<td>0 (0–5)b,c,d</td>
<td>0 (0–18)f</td>
<td>0 (0–7)</td>
<td>2.5 (0–4)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Total</td>
<td>0 (0–11)b,c,d</td>
<td>0 (0–18)f</td>
<td>1.0 (0–21)</td>
<td>4.0 (0–9)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Cords</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peripheral</td>
<td>0 (0–14)e</td>
<td>0 (0–4)e</td>
<td>0 (0–16)</td>
<td>1.0 (0–7)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Central</td>
<td>1.5 (0–22)b,c,d</td>
<td>1.0 (0–15)f</td>
<td>0.5 (0–8)</td>
<td>1.0 (0–2)</td>
<td>0.001</td>
</tr>
<tr>
<td>Total</td>
<td>2.0 (0–25)b</td>
<td>1.0 (0–18)</td>
<td>2.0 (0–18)</td>
<td>2.0 (0–8)</td>
<td>NS</td>
</tr>
<tr>
<td>Vessels + cords</td>
<td>2.0 (0–26)c</td>
<td>2.0 (0–19)c</td>
<td>5.5 (0–22)</td>
<td>4.7 (2–18)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

aMedian test
bCHM versus PHM without embryo; P < 0.05.
cCHM versus PHM with embryo; P < 0.05.
dCHM versus TOP; P < 0.05.
ePHM without embryo versus PHM with embryo; P < 0.05.
fPHM without embryo versus TOP; P < 0.05.
gPHM with embryo versus TOP; P < 0.05.
and TOP. This difference, although less prominent, was also seen in PHM in which more centrally located cords were present in comparison with PHM + E and TOP. Between PHM + E and TOP, no statistically significant difference was seen with respect to cords.

In TOP, significantly more vessels with a lumen, centrally and peripherally located, were present in comparison with CHM, PHM − E and PHM + E (Figure 2). Also PHM + E showed significantly more vessels than CHM and PHM − E, but less vessels compared with TOP (not statistically significant).

**Chorionic villi characteristics**

The villous stromal area and functional vascular area (vessels with a lumen) are presented for CHM, PHM − E, PHM + E and TOP in Table III. The villous stromal area in CHM, PHM − E and PHM + E is significantly larger than in TOP. No statistically significant difference in villous stromal area was found between PHM − E and PHM + E.

The functional vascular area in CHM is significantly smaller than in TOP, PHM − E and PHM + E. Only the difference between CHM and TOP reached statistical significance.

In CHM, 0.02% of the villous area of the chorionic villi consisted of functional vascular area (vessels with a lumen), compared with 0.15% in PHM − E, 0.20% in PHM + E and 0.70% in TOP.

The prevalence of functional vascularized villi (vessels with a lumen) in the CHM group was 16%, in the PHM − E group 37%, in the PHM + E group 54% and in TOP 93%. The vascular density of these functional vascularized villi for the different groups was 2.6 for the CHM group, 3.1 for the PHM + E group and 5.6 for the TOP group.

**Discussion**

In the CHM and PHM − E groups, we observed a low number of total vascular elements (cords and vessels) compared with the PHM + E and TOP groups. A low number of luminized vessels was seen in the CHM group and in both groups of PHM as compared with the control group (TOP). As compared with all other groups, the largest number of central cords was seen in the CHM group. There was also a statistically significant smaller functional vascular area in CHM in comparison with TOP. These differences illustrate that a normal initiation of vasculogenesis is basically present in CHM pregnancies, despite the absence of an embryo or maternal-derived chromosomes. In the group of CHM, PHM − E and PHM + E, we observed a decreased number of peripheral vessels compared with the TOP control group, illustrating a decreased maturation with abnormal development of the vasculosyncytial membrane.

The prevalence of functional vascularized villi (vessels with a lumen) in the CHM group was 16%. This is in agreement with the results of van de Kaa et al. (1997), who found well formed capillaries with wide-open lumina in 17% of the cases of CHM.

**Table III.** Villous stromal area and functional vascular area (vessels with a lumen per 15 chorionic villi) in CHM, PHM without embryo, PHM with embryo or fetus and normal pregnancies (TOP), presented as mean (±SEM)

<table>
<thead>
<tr>
<th></th>
<th>CHM (n = 12)</th>
<th>PHM without embryo (n = 9)</th>
<th>PHM with embryo/fetus (n = 6)</th>
<th>TOP (n = 12)</th>
<th>P-valuea</th>
</tr>
</thead>
<tbody>
<tr>
<td>Villous stromal area (μm²)</td>
<td>379 000 (25)c,d</td>
<td>230 000 (31)f</td>
<td>162 000 (38)f</td>
<td>51 000 (2.6)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Functional vascular area (μm²)</td>
<td>81 (15)d</td>
<td>340 (72)</td>
<td>350 (34)</td>
<td>355 (25)</td>
<td>0.02</td>
</tr>
</tbody>
</table>

*aANOVA.
+bCHM versus PHM without embryo; P < 0.05.
+cCHM versus PHM with embryo; P < 0.05.
+dCHM versus TOP; P < 0.05.
+ePHM without embryo versus PHM with embryo; P < 0.05.
+fPHM without embryo versus TOP; P < 0.05.
+gPHM with embryo versus TOP; P < 0.05.
Because we found even in CHM (androgenetic in origin) the presence of centrally located cords (initiation of vasculogenesis) and signs of maturation from cords to vessels, we conclude that in the absence of the maternal genome, initiation of vasculogenesis does take place. Initiation of vasculogenesis as a sign of early embryonic development is independent of maternal imprinting. This is in agreement with the results of Zaragoza et al. (1997), who identified in four cases of CHM (confirmed androgenetic) extra-embryonic components including endothelial cells, concluding that the maternal genome is not required for initiation of vasculogenesis. Maternal genes are necessary for the development of the embryo itself and vasculogenesis is modulated directly or indirectly by embryonic signalling.

The phenomenon that the expression of genes in the human genome depends on their location on the maternal or paternal chromosome is known as genomic imprinting. In PHM, the maternal genome is present, but remains in the minority. Vasculogenesis is seen in PHM + E and in PHM + E, although maturation and margination resulting in a vasculosyncytial membrane is only seen in PHM + E. The presence of an embryo is therefore obligatory for the development of this membrane.

Retention time as seen in the group of PHM + E could be a point of discussion, because it can be argued that vascular changes occurred post-mortem. In previous studies, no effect of retention time was found on the outcome of vascular parameters (Meegdes et al., 1988; Nelen et al., 2000). Trophoblast hypoplasia with deficient vascularization appears to be the result of disturbed initiation of embryonic placentation, rather than of post-mortem changes, as found in placental studies on late second or third trimester stillborn fetuses (Genest, 1992; Hustin et al., 1996).

In a recent publication, in which the development of the vasculosyncytial membrane in first trimester pregnancies complicated by embryonic death and anembryonic pregnancies was studied (Lisman et al., 2004), we could not find any significant influence with regard to the number of vessels with a lumen, compared to normal first trimester pregnancies. On the contrary, villous vascularization appeared unaffected by prolonged post-mortem (pregnancies complicated by embryonic death) intrauterine retention, whereas the number of cords decreased during a prolonged retention time. Therefore, in our opinion, retention time in the three PHM + E which were in a post-mortem state before D&C did not affect vascularization.

In the group of TOP, the chorionic villi of one pregnancy showed almost no vessels peripherally as well as centrally, but did have cords both peripherally and centrally located. Although all patients underwent ultrasound to confirm embryonic viability in this group before D&C, no cytogenetic investigation was performed afterwards to rule out chromosomal abnormalities or other causes of complicated pregnancies in which abnormal development of villous vasculature can be seen (Roberts et al., 2000). DNA analysis or karyotyping was performed in the group of CHM and PHM; CHM were all diploids and PHM were all triploids.

The GA in the group of PHM – E and PHM + E is higher than in TOP. PHM – E and PHM + E were all diagnosed as PHM by the pathologist in combination with DNA analysis. Revision took place by the Dutch Central Molar Registration, University Medical Center St Radboud, Nijmegen. Molar transformation becomes more pronounced as pregnancy advances. If the study population consisted of triploids of <84 days gestation, no noticeable macroscopic features of molar change would have been seen and consequently the diagnosis would not have been PHM (Jauniaux et al., 1996).

In normal first trimester pregnancies, an increase in GA leads to an increase in the total number of vascular elements, but the amount of cords remains stable (te Velde et al., 1997). This was confirmed in the group of TOP in this study. Jackson et al. (1992) published a quantitative description of growth and maturation of chorionic villi collected at 10–41 weeks GA. They concluded that an increase of GA leads to an increase of volume and surface area of chorionic villi and chorionic villous maturation, which involves increase of capillary volume and decrease of villous diameter leading to a well developed vasculosyncytial membrane. There was no difference in vascular density in vascularized villi for the PHM + E group and the TOP group, although the prevalence of functional vascularized villi in PHM + E was 54% and in TOP 93%. In PHM + E and PHM + E, we observed mainly cords and less vessels with a lumen in relation to TOP. Obviously, in spite of a more advanced duration of pregnancy in PHM – E and PHM + E, a normal development of the vasculosyncytial membrane is only seen in TOP. Although we acknowledge the difference in GA between the various pregnancy groups, it does not seem to have influenced the results or the conclusion as drawn from this particular study.

Conclusions from the present study

We found that the villous stromal area of CHM, PHM – E and PHM + E is significantly larger than of TOP. The functional vascular area of CHM was smaller than in PHM – E, PHM + E and TOP. There was no difference found between the functional vascular area of PHM – E, PHM + E and TOP. However, the functional vascular area needs to be related to the villous stromal area. The percentage of stromal area occupied by functional vascular area in PHM – E was 0.15%, in PHM + E 0.20% and in TOP 0.70%. Although the difference between these groups is not statistically significant, it does clearly indicate a defective maturation of cords to vessels in PHM + E and even more pronounced in PHM – E and CHM. The percentage of functional vascular area as part of the stromal area is comparable with the results we found in a recent published study on chorionic villous vasculogenesis in first trimester pregnancies complicated by embryonic death and anembryonic pregnancies (Lisman et al., 2004).

In conclusion, defective chorionic villous vascularization is seen in CHM and PHM – E, and to a lesser extent in PHM + E. Chorionic villi of normal first trimester pregnancies contain more vessels than cords (maturation) and these vessels are mainly located peripherally (margination), forming a normal vasculosyncytial membrane. However, initiation of vasculogenesis is clearly present in CHM,
proving that initiation of vasculogenesis does take place in the absence of the maternal genome. Nevertheless, the developing embryo modulates the process of maturation and margination resulting in the development of a normal vasculosyncytial membrane.

References


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