Morphologic alterations and immunohistochemical analysis of alpha-fetoprotein and CD34 in chorionic villi of anembryonic pregnancy

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ABSTRACT

Objective: To investigate the morphology of chorionic villi using light and electron microscopy, especially the expression of alpha-fetoprotein (AFP) in trophoblastic cells and the process of maturation and margination vasculogenesis proper using CD34 immunohistochemistry in tissues from the first trimester of pregnancy loss due to anembryonic pregnancy in comparison with embryonic pregnancy.

Methods: The study consisted of 2 groups: 9 patients with anembryonic pregnancies and 9 patients with embryonic pregnancies between 6 and 10 weeks of gestational age registered at the Department of Gynecology and Obstetrics, University Hospital of Karadeniz Technical University, Turkey, from March 2003 to December 2004. We examined the chorionic villi using light and electron microscopy. For immunohistochemical staining, we used AFP and CD34.

Results: Microscopically, pathologic changes were shown in syncytiotrophoblast cells of anembryonic pregnancies and AFP was strongly expressed by villous trophoblastic cells compared to embryonic pregnancies. We determined the CD34 positivity in both groups. In anembryonic pregnancies, vascular elements were much fewer in number compared with embryonic pregnancies (p<0.001) and were located in the formed of hemangioblastic cords.

Conclusion: Placental vasculogenesis is a basic feature in all types of pregnancy and a relationship exists between trophoblast cells and vessels in the chorionic villi with the potential to influence each other’s functions. Defective chorionic villus vascularization is associated with embryonic death. This study may support the hypothesis, as suggested by previous studies, that anembryonic pregnancy results from early embryonic death and subsequent reabsorption rather than from the nondevelopment of an embryo.


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nembryonic pregnancies are probably the earliest form of missed abortion, in which the embryo dies a considerable time before the first ultrasound examination. Embryonic remnants, such as bones or secondary yolk sac fragments, which are undetectable by sonographic or macroscopic examinations, are often found during microscopic examinations. These observations support the hypothesis that most anembryonic pregnancies result from early embryonic death and subsequent reabsorption rather than from

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the nondevelopment of an embryo. The frequency of anembryonic conceptus among clinically recognized pregnancies may be as high as 16% and these are usually miscarried between 8 and 14 weeks, but are now commonly identified by ultrasonographic imaging in early pregnancy. Despite their common occurrence, the etiology of anembryonic conceptus is poorly understood, suggesting that many are chromosomally abnormal. Alpha-fetoprotein (AFP) is the first alpha-globulin to appear in the blood of vertebrates during ontogenesis. Human AFP appears early in the embryonic period and traces have been detected as early as at 4 weeks of gestation. The serum concentration of AFP rises rapidly thereafter until it reaches its peak level at 10-13 weeks of gestation, and then decreases steadily. In maternal serum, there is a steady increase in the AFP level to a peak of approximately 32-34 weeks, which is followed by a rapid decrease towards term. The remarkable persistence of this fetal protein during evolution suggests that it must be important for growth and development and only small amounts of AFP enter the maternal circulation. The presence of increased AFP in maternal peripheral blood during pregnancy is also a useful marker of fetal development defects and multiple pregnancies. Maternal serum AFP levels are within the normal range in most anembryonic pregnancies, suggesting that they may in fact be embryonic pregnancies in which the embryo died before the first sonographic examinations. Vascularogenesis in normal first trimester of chorionic villi is characterized by the transformation of mesenchymal cells into hemangioblastic cell cords, which are the precursors of the capillary endothelium and hematopoietic stem cells. The vascular lumen is formed by the dehiscence of the intercellular clefts. Chorionic villi of normal first trimester pregnancies contain more vessels than hemangioblastic cords (maturation) and these vessels are located mainly peripherally (margination), forming a normal vasculosyncytial membrane. It has been suggested that defective chorionic villous vascularization, demonstrating inadequate vasculogenesis and abnormal development of the vasculosyncytial membrane, is seen in pregnancies complicated by embryonic death and is even more pronounced in anembryonic pregnancies. The monoclonal antibody against CD34 antigen in human endothelial cell membranes and hematopoietic progenitor cells proved to be a useful marker of villous vascular endothelial cells in normal first trimester pregnancies. Using CD34 immunohistochemistry, not only vessels with a lumen, but also hemangiogenetic cords can be visualized, in contrast with hematoxylin and eosin (H&E) staining. There have been few studies defining morphological findings on chorionic villi of anembryonic pregnancies. The aim of this study is to investigate the morphology of chorionic villi using light and electron microscopy, especially the expression of AFP of chorionic villi and the process of maturation of luminized vessels from hemangioblastic cell cords and margination of vessels forming the vasculosyncytial membrane proper using CD34 immunohistochemistry in anembryonic pregnancy.

Methods. The study consisted of 2 groups: the anembryonic pregnancy group (AEP) of 9 women who had anembryonic pregnancies between 6 and 10 weeks of gestational age were registered at the Department of Gynecology and Obstetrics, University Hospital of Karadeniz Technical University, Turkey, from March 2003 to December 2004. An anembryonic pregnancy was suspected when a fetal pole could not be identified with a gestational sac volume of >2.5 ml, and diagnosis was confirmed during subsequent ultrasound examinations. The embryonic pregnancy group (EP) consisted of 9 healthy women from the same population with apparently uncomplicated pregnancies that underwent termination for unwanted pregnancies during the same period of gestation. A written consent was obtained from the patients before the procedure. The Ethical Committee of the Medical Faculty of Karadeniz Technical University, Turkey approved the consent forms and protocol to use the tissue. Chorionic villi samples which were obtained by dilatation and curettage (D&C) were kept in 10% formaldehyde solution for light microscopic examination. After fixation, dehydration and embedding in paraffin wax, 5 µm serial sections were cut with a microtome (Leica RM 2150) and stained with H&E. The slides were examined using a light microscope (Olympus BX50) at 10X and 40X magnifications. In each specimen, regularity of the chorionic villus, structure of the trophoblast layers and the development of the chorionic villi were evaluated in a blind fashion and recorded by histologists and a pathologist. Electron microscopic samples were fixed in phosphate-buffered glutaraldehyde (4%) (pH 7.4) at 4°C for 24 hours and postfixed in phosphate-buffered osmium tetroxide (1%) for 4 hours, dehydrated through an ethanol series and embedded in epoxy resin. Ultra-thin sections were cut with glass knives and an ultramicrotome (Leica Reichert Ultracut S). Semi-thin sections through the chorion villus were then made and stained with toluidine blue dye. Then 600 angstrom-thin sections were made from a selected area of the tissue defined by the semi-thin section, and these sections were contrasted with...
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The ultrastructure of the chorionic villi was observed under a transmission electron microscope (Jeol 1010 C). The chorionic villi were cut into sections 4 µm thick and mounted on histogrip coated slides (Zymed, USA), dewaxed, dehydrated, and placed in citrate buffer solution. To eliminate endogenous peroxidase activity, the slides were kept in 3% hydrogen peroxide for 5 minutes and washed with phosphate buffered saline (PBS); pH; 7.4, 3 times. To unmask antigens, an antigen retrieval procedure was performed by treating the samples in a microwave oven at 600W for 4 minutes. After cooling for 20 minutes at room temperature, the sections were washed with PBS. The following primary antibodies were used: Monoclonal mouse anti CD34 (Neomakers, USA) to identify the vascular structure and anti AFP (DAKO, USA) to identify trophoblastic cells. After 30 minutes incubation with blocking solution sections were incubated with the primary antibodies at 4°C for one hour. Immunohistochemistry was performed using a horseradish-peroxidase labeled streptavidin biotin kit (Zymed, USA). The resulting signal was developed with Chromogen AEC (Zymed, USA). The sections were counterstained with Mayer’s hematoxylin solution and mounted with ultramount plus. Replacing the primary antibodies with the appropriate non-immune IgG or isotypes performed negative control staining. Photomicrographs were taken with a light microscope (Olympus BX50). Tissue sections from chorionic villi were evaluated for AFP protein localization and intensity. All samples for each individual antibody were exposed to the same protocol and were stained using the same duration of staining. The intensity of immunoreactivity was semi-quantitatively evaluated as follows: positively stained cells were grouped according to the following categories: negative = no staining, 1+ = weak but detectable, 2+ = moderate or distinct, 3+ = intense. For each tissue, an H-score value was calculated using the formula \[H\text{-score} = Pi (i+1)\]. Each slide was evaluated under a light microscope using 40X original magnification (Olympus BX50), and the percentage of cells for each intensity within these areas was determined by histologists and a pathologist who did not know to which group the slides belonged. Monoclonal mouse anti-CD34 antibody was used to describe whether the hemangioblastic cords and vessels were with or without a clear-cut lumen depicted in the process of maturation. The process of margination was illustrated by describing whether these cords and vessels were located peripherally or centrally. For statistical analysis of vascular element parameters, the median value for each parameter was used. Statistical comparisons between groups were performed using the Mann-Whitney U-test. A \(p\)-value of <0.05 was considered to be statistically significant.

Results. There was no difference in staining intensity among different specimens within the same group. Semiquantitative and H-score system results are shown in Table 1. In the light microscopic findings of embryonic pregnancies, the villi were covered by 2 cell layers. The outer layer was the syncytiotrophoblast; immediately under this was a layer composed of cytotrophoblasts. All embryonic pregnancies showed tertiary villus stage development with blood vessels in the connective tissue. In the light microscopic findings of anembryonic pregnancies, the structure of chorionic villi formed by cytotrophoblasts and syncytiotrophoblasts was more evident and the lengths of syncytiotrophoblasts were greater in anembryonic pregnancies compared with embryonic pregnancies under light microscopic examination. Chorionic villus pathology of anembryonic cases showed an arrest at the secondary villus stage without any blood vessels in the connective tissue in 2 of the cases and a small number of vessels in the connective tissue were present in 5 anembryonic cases.

A positive AFP immunoreactivity was observed in cytoplasm of the cytotrophoblast of both embryonic and anembryonic pregnancies. However, this reaction was strongly positive, especially in the cytoplasm of the syncytiotrophoblast in anembryonic pregnancies (Figure 1) while it was weak or moderately positive in embryonic pregnancies (Figure 2). CD34 positivity was determined in both groups (Table 2). The vascular

Table 1 - Immunohistochemical staining intensities for alpha-fetoprotein of cytotrophoblast and syncytiotrophoblast cells groups with H-score in embryonic and anembryonic pregnancies.

<table>
<thead>
<tr>
<th>Trophoblasts</th>
<th>Anembryonic pregnancy (n=9)</th>
<th>Embryonic pregnancy (n=9)</th>
<th>(p) value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cytotrophoblasts</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td>287</td>
<td>181</td>
<td>&lt;0.0001*</td>
</tr>
<tr>
<td>H-scores min</td>
<td>268</td>
<td>175</td>
<td></td>
</tr>
<tr>
<td>Maximum</td>
<td>296</td>
<td>191</td>
<td></td>
</tr>
<tr>
<td><strong>Syncytiotrophoblasts</strong></td>
<td></td>
<td></td>
<td>&lt;0.0001*</td>
</tr>
<tr>
<td>Median</td>
<td>293</td>
<td>178</td>
<td></td>
</tr>
<tr>
<td>H-scores min</td>
<td>287</td>
<td>176</td>
<td></td>
</tr>
<tr>
<td>Maximum</td>
<td>297</td>
<td>182</td>
<td></td>
</tr>
</tbody>
</table>

*\(p<0.0001\), the Mann-Whitney U non-parametric test was used to compare the median H-score values.
Figure 1 - Anembryonic pregnancy. Immunocytochemical localization of alpha-fetoprotein (AFP) in cytotrophoblasts (white arrow) and syncytiotrophoblasts (black arrow) are strong positive (AFP; 40X).

Figure 2 - Embryonic pregnancy. Immunocytochemical localization of alpha-fetoprotein (AFP) in cytotrophoblasts (white arrow) and syncytiotrophoblasts (black arrow) are moderate positive (AFP; 40X).

Figure 3 - Anembryonic pregnancy. Immunoreactivity of CD34 in peripherally (p) and centrally (c) located vascular structure [vessel (v), hemangiogenic cords (h)] (CD34; 20X).

Figure 4 - Embryonic pregnancy. Immunoreactivity of CD34 in peripherally (p) and centrally (c) located vascular structure [vessel (v), hemangiogenic cords (h)] (CD34; 20X).

Table 2 - The number of vascular elements in chorionic villi of anembryonic and embryonic pregnancies.

<table>
<thead>
<tr>
<th>Vascular elements</th>
<th>Anembryonic pregnancy n=9 (range)</th>
<th>Embryonic pregnancy n=9 (range)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Vessels</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peripheral</td>
<td>1.11 (0.00-3.00)</td>
<td>30.77 (12.00-45.00)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Central</td>
<td>1.66 (0.00-3.00)</td>
<td>3.33 (0.00-8.00)</td>
<td>NS</td>
</tr>
<tr>
<td>Total</td>
<td>2.77 (1.00-5.00)</td>
<td>34.11 (14.00-49.00)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>Hemangioblastic cords</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peripheral</td>
<td>0.44 (0.00-1.00)</td>
<td>7.00 (4.00-9.00)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Central</td>
<td>4.55 (3.00-7.00)</td>
<td>1.11 (0.00-2.00)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Total</td>
<td>5.00 (3.00-8.00)</td>
<td>8.11 (5.00-11.00)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

NS - no significance. All values statistically significant except for central vessel value.
elements of the anembryonic pregnancies were much fewer in number compared with those of the embryonic pregnancies ($p<0.001$) and they were mostly centrally located in the formed of hemangioblastic cords (Figure 3). CD34 immunoreactivity was strongly positive in vessels of embryonic pregnancies and most of them were located peripherally (Figure 4) ($p<0.001$). The electron microscopic findings on chorionic villus of embryonic pregnancies were seen in a normal structure but in anembryonic pregnancies were observed as follows: different size of branched microvilli on the lumen side of syncytiotrophoblasts, widespread circular or oval vacuoles in the cytoplasm, multiple crescent or oval-shaped electron dense mitochondria between vacuoles, and peripherally located chromatin of the nucleus. Between the basal plate and syncytiotrophoblast layer continuities in the syncytium were seen. In cytotrophoblasts, there were a few different sizes of vacuoles, dispersed oval or bacillus-shaped mitochondria in plasma, regular-shaped granular endoplasmic reticulum (GER), smooth endoplasmic reticulum (SER) with a mildly widened cistern, euchromatic nucleus and irregular membrane at the base of the cell, and electron dense cell junctions, between cytotrophoblasts and syncytiotrophoblasts.

**Discussion.** A diagnosis of an anembryonic pregnancy can be performed accurately by transvaginal ultrasonography. Numerous authors have attempted to propose a classification for early pregnancy complications. The increasing use of ultrasonography and cytogenetic techniques has modified our understanding of the pathophysiologic mechanisms of miscarriages in human pregnancy. However, the terminology used to describe some forms of early pregnancy complications is still confusing. As a large serum glycoprotein belonging to the class of onco-developmental proteins, the expression of AFP can be detected very early in pregnancy and its concentration in maternal circulation correlates positively with the vascular adaptation during pregnancy. Measurement of AFP levels in maternal serum has been widely used to predict early pregnancy complication, chromosomal abnormalities, and various fetal malformations. In patients with threatened miscarriage and, in particular, in cases of anembryonic pregnancies serum AFP levels are often within the normal range or $>95$th percentile. The cytochemical findings indicate that at least 2 anatomical pathways associated with the placenta are available for AFP entry into the maternal circulation; the AFP leaving fetal vessels can pass through the villous core and across the fibrinoid deposits at sites of discontinuity of the syncytiotrophoblast to enter the maternal circulation without passing through the cytoplasm of the syncytiotrophoblast. Jauniaux et al showed a failure of most placent albolic mechanisms, such as metabolic function, transport function and endocrine activity in anembryonic pregnancy. Johnson et al demonstrated that the trophoblast and the corpus luteum function are impaired more markedly in anembryonic pregnancies than in pregnancies that miscarrieth after the demonstration of fetal heart activity. Pathologic changes in the syncytiotrophoblast of anembryonic pregnancies using light and electron microscopy were shown in the present study. Ultrastructural changes was observed using electron microscopy in the syncytiotrophoblast suggest that the abnormal accumulation of AFP in cytotrophoblasts and syncytiotrophoblasts may be due to trophoblastic injury. The absence of discontinuation in the syncytiotrophoblast suggests trophoblastic accumulation of AFP. This accumulation may explain why maternal serum AFP levels were determined within normal ranges although fetal AFP levels were high in anembryonic pregnancies. The results of this research support the suggestion of Gitlin that placental transfer of AFP is by diffusion. Brownbill et al showed that AFP can cross the placental villus, but also proposed that there are microscopic mechanism, which explains why enhanced AFP transfer occurs in the presence of gross abnormalities of the placenta. All these data suggest that AFP may have a sequential and pluripotential functions throughout the proliferation, differentiation and immunology phenomena necessary for the development of the fetal-placental unit. However, little is known about whether AFP acts to enhance or inhibit the link between the mother and the fetus. It has been suggested that defective chorionic villous vascularization is associated with embryonic death. There have been few reports, however, describing chorionic vascular profiles in anembryonic pregnancy tissue. Therefore, in this study chorionic villus vascularization was investigated using histopathology combined with CD34 immunohistochemistry in anembryonic pregnancies. Recently, the development of the chorionic villous vascular system in normal human first–trimester pregnancies was investigated using quantitative CD34 immunohistochemistry. Meegdes et al compared chronic villous vascularization in a control group of women who underwent legal abortions with a group of spontaneous miscarriages and determined vascularized villi in 89% of the term group, 26% of the group with embryonic death, and only 9% of the group with anembryonic pregnancies. Chorionic villi of normal first trimester pregnancies contain more vessels than hemangioblastic cords (maturation).
and these vessels are located mainly peripherally (margination), forming a normal vasculosyncytial membrane. The results of Demir et al. indicated that the first signs of vasculogenesis determined by CD34 immunohistochemistry are observed at days 21-22 post-conception of pregnancy. According to Lisman et al., chorionic villous vascularization can be best explained by the theory that the origin of angiogenetic cords is independent of the embryonic development but that vasculogenesis is dependent on embryonic signals. The normal process of maturation and margination results in peripherally located luminized vessels. We observed a decreased number of total vascular elements (cords and vessels) in the anembryonic group compared with the embryonic group. In the anembryonic group, there were hardly any peripheral vessels, and this clearly illustrates a normal development of the vasculosyncytial membrane, but in the embryonic group the presence of luminated vessels indicates fetal placental unit maturation and margination. Although te Velde et al. concluded from their results that vessels with a lumen were only seen in pregnancies with an embryo; we also found few luminated vessels in anembryonic pregnancies. This fact can be explained that in the anembryonic group the embryonic structures were present before the ultrasound examination, but it was arrested in early pregnancy and embryonic tissues were subsequently re-absorbed. Nelen et al. found that in the case of early pregnancy failure the normal process of maturation and margination is disturbed. Our results support the findings of Lisman et al. It has been suggested by Stabile et al. that the so-called anembryonic pregnancies are pregnancies in which the embryo has been lost rather than pregnancies in which it never developed. An impaired fetal maternal diffusion is a plausible explanation for disturbed fetal development or even fetal death, especially if the idea is accepted that fetal-maternal diffusion begins early in pregnancy. However, if functional fetal-maternal circulation is not initiated before 12 weeks of pregnancy, then, the role of a defective chorionic vascularization in embryonic death remains to be elucidated. Lisman et al. showed that in recurrence early pregnancy loss is associated with defective chorionic villous vascularization.

In conclusion, the present study supported the view that there is a relationship between trophoblastic cells and vessels in the chorionic villi with the potential to influence each other’s functions. Defective chorionic villous vascularization resulting in early embryonic death may constitute the etiology of anembryonic pregnancies.

References


