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Impact of maternal diabetes type 1 on proliferative potential, differentiation and apoptotic activity in villous capillaries of term placenta

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ABSTRACT

Introduction: Maternal diabetes mellitus changes morphology and impairs function of placental capillaries. Here, quantitative parameters characterizing cell proliferation using detection of Ki67, differentiation reflected by nestin expression and apoptosis in placental capillary bed with active caspase 3 as a marker were compared in normal term placentas and placentas from pregnancies complicated by Type 1 maternal diabetes mellitus.

Methods: Specimens of sixteen diabetic placentas and eight control placentas were collected by systematic uniform random sampling. Immunohistochemical detections of Ki67, nestin, and active caspase 3 were performed in histological sections of five haphazardly chosen blocks per placenta. Twenty fields of view per section, i.e. one hundred fields of view per placenta, were used for analysis of proliferation as well as of apoptosis, and in approximately 70 capillary cross-sections per placenta the nestin-positive segments of their circumference were measured.

Results: The percentage of Ki67-positive cells counted in the capillary wall was significantly lower in diabetic group. The counts of Ki67-labelled nuclei per villous area unit were significantly lower in cytotrophoblast and capillary wall of terminal villi in diabetic placenta. The proportion of nestin-labeled segments of capillary circumference was significantly higher in placentas of diabetic group. No differences in the numbers of apoptotic cells were found between studied groups.

Discussion: The results show that the term placenta in Type 1 diabetes has lower potential to enlarge the surface area of structures involved in maternofetal transport, and that the villous capillary bed displays delayed differentiation. Those factors may participate in decreased ability of diabetic placenta to comply with fetal requirements in the final stage of pregnancy.

1. Introduction

Placental growth is driven by cell proliferation in three basic cellular compartments, i.e. trophoblast, vascular bed and villous stroma. The growth of placental cellular compartments is of proliferative character continuously from the 12th week to the end of gestation [1]. In the third trimester, the placental growth and remodeling concerns first of all terminal villi and their capillaries, and there are indications that angiogenesis, both the elongating and sprouting, is the driving force of the formation of new villi [2]. The development of placental villi is carried out by three well-balanced processes, i.e. cell proliferation, differentiation and apoptosis, and trophoblast, stromal cells and capillaries undergo those processes [3].

Maternal diabetes mellitus manifests itselfs also in altered placental capillary development. Enhanced total capillary volume, length and surface area [4–8], and enhanced capillary branching in both gestational and type 1 diabetes [9,10] suggest more active angiogenesis or, on the other hand, less active apoptosis.

The autoradiographic studies on cell proliferation brought

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varied results. It was shown that cellular proliferation expressed as labeling index is higher in central vs. peripheral part of cotyledons in normal term placentas and that labeled nuclei occur in cytotrophoblast as well as in cells of capillaries and stroma [11]. Other authors found higher amount of labeled nuclei in trophoblast than in stroma during the whole pregnancy and no differences between normal and diabetic term placenta [12], but also significantly higher ^{3}H thymidine incorporation into endothelial cells of diabetic villi [13]. No differences of proliferative activity but decreased apoptosis in villous stroma of diabetic placentas were found in the study based on Ki67 detection and TUNEL assay [14]. In a semi-quantitative study, the PCNA immunoreactivity of endothelial cells was found higher in diabetic placentas whereas the Ki67-immunoreactivity was similar in fetoplacental endothelium of both normal and diabetic placenta [15].

During angiogenesis, cell proliferation is followed by lumen formation and stabilization of the vascular wall. Those processes are determined by changes of cytoskeleton and junctional proteins. The roles of actin filaments, intermediate filaments and microtubules in regulation of angiogenesis were summarized by Bayless and Johnson [16]. Besides common cytoskeletal structures, nestin, a transitional intermediate filament protein takes part in the formation of new cells. It is expressed in dividing cells of various origin, becomes downregulated upon differentiation and replaced by tissue-specific intermediate filament proteins, and plays also an important role in placental angiogenesis [17–19]. Nestin mediates the vimentin disassembly during mitosis. Its expression was reported in the immature endothelium during angiogenesis in developing organs and tumors as well as in periendothelial cells (pericytes and smooth muscle cells) of developing vessels, namely in brain, pancreas and testis [20–24].

As for angiogenesis, the occurence of dynamic endothelial intercellular junctions in villous capillaries of mature placenta suggests a considerable plasticity of its capillary bed [25]. Studies on junctional adhesion molecules have shown altered expression of junctional proteins, which makes capillaries in diabetic placenta less stable and drives endothelial proliferation [26,27].

Programmed cell death by apoptosis is one of processes maintaining cellular balance of the organ. In human placenta, the attention has been paid mainly to this process in villous trophoblast, e.g. Refs. [28–31], but to our knowledge, only one paper mentions that apoptotic endothelial cells occur in placenta and represent 5% of apoptotic placental cells [3].

The aim of this paper was to compare the proliferative activity, to determine the degree of cell differentiation and to quantify apoptosis in capillary bed of normal and diabetic term placenta.

2. Material and methods

2.1. Material

Placental samples were taken with written informed consent of mothers, under procedures approved by the local ethics committee. All mothers, white European women, declared that they were nonsmokers. No patient with chronic hypertension, IUGR and abnormal results of Doppler velocimetry was included. Specimens of term placentas were collected from eight control mothers and sixteen mothers suffering from type 1 diabetes mellitus (T1DM), see Table 1 and Table 2.

Placental full-thickness tissue pieces were taken by the systematic uniform random sampling [9] and cut transversally into three blocks corresponding to the parabasal, middle and parachorial placental zone. Samples (approx. 21 per placenta) were fixed in 4% formaldehyde solution and after fixation and randomizing of tissue orientation were embedded in the paraffin wax.

One section of each block taken from T1DM placentas was stained with hematoxylin and eosin and evaluated for occurrence of villous chorangiosis. The criteria for chorangiosis were the occurrence of at least 10 capillary crossections in at least 10 villi found in at least one third of sections observed at 10× objective magnification.

2.2. Immunohistochemistry

Histological sections were cut at 7 μm and rehydrated. After antigen retrieval in 0.01 M citrate buffer in a microwave oven and inhibition of endogenous peroxidase activity by 1% hydrogen peroxide, the blocking of non-specific antigen binding sites was performed by preincubation with 5% normal goat serum in 0.1 M PBS buffer.

For detection of Ki67 antigen, the sections were incubated with rabbit polyclonal anti-human Ki67 antibody (Acris, Germany), diluted 1:75 in PBS with 1.5% normal goat serum, for 60 min at room temperature. The antigen-antibody complex was visualized with LSAB + peroxidase system (Dako, Denmark), the nuclei were counterstained with hematoxylin.

Nestin antigen was identified using a mouse monoclonal antibody (clone 10C2, Abcam, Cambridge, GB) in PBS with 1.5% normal goat serum (Dako, Denmark) with a dilution 1:200, for 45 min at room temperature. The visualization was performed with anti-mouse Envision system-HRP (Dako, Denmark) and counterstaining with hematoxylin.

As for active caspase 3-immunohistochemistry, incubations were performed with anti-active caspase-3 rabbit polyclonal antibody diluted 1:200 in 1.5% normal swine serum (Cell Signaling Technology) and detection using LSAB + peroxidase system (Dako, Denmark). The double immunohistochemical study was performed with the same anti-active caspase-3 rabbit polyclonal antibody diluted 1:200 and anti-CD34 mouse monoclonal antibody (clone QBEnd 10, Dako, Denmark) diluted 1:50 in 1.5% normal goat serum to visualize apoptotic endothelial cells. Images were obtained using Leica SPE confocal microscope using 40× oil immersion objective NA = 1.3. Nuclei were stained with DAPI.

Simultaneously performed control experiments with the omission of either primary or secondary antibody gave negative results.

2.3. Quantitative studies

For each quantitative study, five blocks per placenta were randomly chosen, and one section per block cut at 7 μm. All imaging and measurements were performed using Leica IM 500 program.

For evaluation of proliferative activity, the set of sections was processed for Ki67 immunohistochemistry. In twenty fields of view per section taken two fields apart at objective magnification 40×, the area of terminal villi was measured, and Ki67-labelled nuclei were counted separately in cytotrophoblast, villous stroma and villous capillary wall. Mean numbers of labelled nuclei per square milimeter were calculated.

In the same fields of view, the numbers of Ki67-positive nuclei and total numbers of nuclei in the capillary wall were counted, and the percentage of Ki67-labelled nuclei was calculated from altogether 8830 nuclei in control and 18 442 nuclei in diabetic group.

Capillary differentiation was assessed on sections processed for nestin immunohistochemistry. Six fields of view per section taken two fields apart at objective magnification 63× were used for evaluation. Capillary crossections lying completely in the upper right quadrant of the field of view, and which long axis was shorter then double short axis, were taken into measurement. Approximatelly in 70 capillary crossections per placenta, the total of nestin-labeled segments of capillary circumference was measured