Mast cells (MC) and histamine (HA) in the pathophysiology of diabetic placenta.

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Abstract: Human placental tissue contains relatively high amounts of histamine (HA) accumulated mostly in the vesicular structures of mast cells (MC). On the basis of neutral protease composition, human MC have been classified into two phenotypes: MCT (tryptase–positive, chymase–negative MC) and MCTC (tryptase–positive, chymase–positive MC). Degranulation of MC releases locally numerous vasoactive, angiogenic and proinflammatory mediators in addition to HA. The role of placental MC and MC-related mediators in normal course and complicated pregnancies are still under investigation. Accumulated data indicate that inflammatory-like processes and changed angiogenesis observed in diabetic placenta may be caused by an imbalance between tissue needs and availability of the MC-related mediators. This presentation, based on author’s own scientific experience and the results of others, considers placental MC number and their heterogeneity (MCT/MCTC ratio), HA concentration in placental tissue, density of the network of placental vessels, and histamine H1, H2 and H4 receptors expression, with respect to the pathophysiology of diabetic placenta. Analyzed material was limited to the class C of diabetes in pregnancy (after White), the last stage without recognized vascular changes. The results showed that in diabetes class C increased density of the villous network of vessels correlates with both significantly (p < 0.05) higher MC number and increased HA concentration. MCT/MCTC ratio was higher (p < 0.05 in diabetic pregnancy as well as the immunoreactivity for H4. In conclusion, overview of the results may lead to the suggestion that MC and HA contribute to abnormal function of the placenta in diabetes class C.

Key-Words: mast cells, histamine, human placenta, placental vessels, diabetic pregnancy, trophoblast

1 Introduction
The placenta forms connection between growing fetus and uterine wall and is an unique, temporary, highly specialized organ required for the development of the embryo and fetus [1]. It is the only organ in the female human body that serves a vital function and then becomes obsolete. Among many functions and roles of the placenta the most important include: metabolic (synthesis of glycogen, cholesterol and fatty acids), transport and exchange (gases, nutrients, waste products) within maternal-placental-fetal interface, endocrine (synthesis and secretion of the pregnancy supporting hormones), immunological (immuno-suppressive function that protects the fetal "allograft" from from T cell–mediated immune aggression as well as – to some degree – protective role against infectious agents) [2]. The placenta begins to develop upon implantation of the blastocyst into the maternal decidua and grows throughout pregnancy. After approximately 12-13 weeks (end of the first trimester of gestation) development of the maternal blood supply to the placenta is completed [3]. Thus, normal placentation, development and growth of the human placenta are crucial for physiologic course of pregnancy with preserved fetal well-being.

Human placental tissue contains a relatively high amount of histamine (HA). Granular structures within the mast cell cytosol are the main source of placental HA and many other pro-inflammatory mediators and angiogenic factors, but HA may be released from trophoblast cells as well [4]. The role of MC in placental development has been described in some papers, particularly in the context of MC-derived substances involved in angiogenesis [5,6]. Accumulation of MC at sites of a new vessel formation and their specific activation in situ causes vigorous MC-mediated angiogenesis. Degranulation of MC increases local concentration of HA, vascular endothelial growth factors (VEGFs), tumor necrosis factor (TNF), interleukin-8 (IL-8), basic fibroblast
growth factor (bFGF), and many others. Angiogenic properties of HA in the placental tissue are mainly mediated through its receptors H1, H2 and H4 [7,8].

In humans, a strict classification into mucosal and connective tissue-type MC is not possible [9]. On the basis of neutral protease composition two phenotypes have been distinguished: MCT (tryptase-positive, chymase-negative MC) and MCTC (tryptase-positive, chymase-positive MC). Changes in the placental MC heterogeneity pattern may predispose to certain placental pathologies associated with local inflammation [10].

Diabetes mellitus is one of the most common endocrine-related medical complications of pregnancy [11]. Diabetes causes a complex changes to the structure and function of the uterofetoplacental unit. Thus, the course of diabetic pregnancy can have a serious consequences for the mother and the developing fetus [12]. The placenta in diabetes has attracted much interest largely because it is supposed that placental damage may be partially responsible for the unduly high incidence of fetal complications observed in diabetic pregnancies. Clinical reports and histopathologic studies of the placenta have confirmed an increased incidence of inflammatory reactions in diabetes. It was suggested that this proinflammatory background may be correlated with changed MC number and MC heterogeneity as well as abnormal HA receptors expressions [13]. Diabetes class C (after White) is the last stage without recognized vascular changes in light microscopy.

2 Aim of the study
This work summarizes findings from both our present and recent past studies. The aims were to examine comparatively (normal pregnancy versus diabetes class C) in the tissue samples:
- MC number and heterogeneity
- HA concentration and density of the network of placental microvessels as well as
  - H1, H2 and H4 receptors expressions in respective trophoblast cultures.

3 Materials and methods
This study was conducted in compliance with international and local laws of human experimentation and the project was approved by the local ethics committee. All subjects provided written ethical consent to the collection and use of their tissues, according to the standards published by British Medical Research Council as “Human tissue and use of biological samples: operational and ethical guidance 2001 (includes Addendum to MRC 2001 guidance following the Human Tissue Act 2004)”.

Placental samples obtained within 15 min after single pregnancies complicated by diabetes White class C (group I; N = 12; mean gestational age 253 ±8 days) were compared with the samples of placental tissue excised from twelve placentas obtained after gestationally matched preterm deliveries (group II [controls]; mean gestational age 255 ±4 days). All newborns were delivered by cesarean sections: elective in group I (in fetal interest) and in group II – due to mother’s high myopia or breech presentation of the baby in nullipara. The courses of diabetic pregnancies were normal, additional drugs were not required, except insulin. Satisfactory blood glucose levels were achieved during diabetic pregnancies as was indicated by the measurements of glycosylated fraction of hemoglobin (HbA1C). The maximal admissible level of HbA1C for our diabetic pregnant patients was 7.5% (normal range for healthy population/reference values: 5.0-7.5%). The mean (± SD) fetal/placental weights for group I and II were: 3487 ±320g/568 ±53g, and 3319 ±327g/548 ±67g, respectively.

3.1 Placental sample collection
Five specimens of placental tissue (approx. 2.0 x 2.0 x 1.0 cm)) were excised in a standardized manner: two from the region contiguous to fetal surface of the placenta (the first – from place of umbilical cord insertion, the next from peripheral region), and three from the region contiguous to maternal surface (the first one – from the central part, the next two – from peripheral regions of the placental maternal surface). Each sample was divided into 3 parts that were at once frozen in carbon dioxide, fixed in formalin and paraffin embedded, or served for the preparation of trophoblast culture in vitro.

3.2 Measurement of HA
HA concentrations were estimated fluorimetrically in placental cuts collected from the maternal surface. The method description is given elsewhere [14]. Briefly, determination of HA in this method uses pre-column derivatization with o-phthalaldehyde and reversed-phase high-performance liquid chromatography in perchloric acid extracts, followed by fluorescence detection. Excitation was set at 360 nm and emission was read at 455 nm of wavelengths. The detection limit was 250 pg per sample.

3.2 MC number (MCN) and heterogeneity
MCN in placental 5µm paraffin cuts was estimated after staining with toluidine blue or alcian blue. MC were counted in calibrated micrographs, using light microscopy with an image analysis system (Quantimet 500C+, image analysis system provided by Leica, UK). The total MCN represents the summation of MCNs from all analyzed areas of each placenta (300 images).

In order to compare heterogeneity, MC protease content was examined using a double-enzyme immunohistochemical staining technique on paraffin sections. Mouse monoclonal antibodies to human MC chymase and trypase were diluted 1:2000 – 1:3000 in tris-buffered saline (TBS, pH 7.6) and applied to tissue sections. The resultant cell suspension was filtered and fractionated by centrifugation through a 50-70% Percoll gradient. The single area analysis procedure, measurement of the total vascular area (V/EVTI), was accepted as the diameter of single lumen. V/EVTI was calculated from all analyzed areas of each placenta (300 images).

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### 3.3 Density of the microvessel network

Using light microscopy with computer morphometry for quantitative analysis (Quantimet 500C+, Leica) the vascular/extravascular tissue index (V/EVTI) was estimated in calibrated areas of the placental sections as described previously [15]. According to the picture analysis procedure, measurement of the total vascular area was performed. The total lumen area of all types of identified vessels was summed up in both groups. In order to eliminate technical error caused by unaxial section of vessel the lowest value of Ferret’s diameter was accepted as the diameter of single lumen. V/EVTI represents ratio, which is most closely correlated with intensity of vascularization. Each preparation underwent three area analyses; the single area measured with the picture analyser amounted to 8721320 µm². The total number of preparations amounted to 300 for each group.

### 3.4 Trophoblast cultures and analyses of H1, H2 and H4 expression

The cytotrophoblast cells were isolated using our modification of the method given previously by Kliman et al. [16]. This method has been described in detail elsewhere [7,17]. Briefly, placental samples were rinsed with saline to remove blood cells. Minced villous tissue was digested with 0.125% trypsin and 0.2 mg/mL deoxyribonuclease I for 30 min at 37 degrees C. Following enzymatic digestion, the resultant cell suspension was filtered and fractionated by centrifugation through a 5-70% Percoll gradient (1200 rpm for 20 min). The cell layers that accumulated between 40-50% (density 1.048-1.060 g/mL) were collected, washed and resuspended (5 x 10^6 cells/ml) in Ham’s F12/Dulbeco’s modified Eagle’s medium (1:1) with 15% fetal bovine serum. This procedure yields a highly purified preparation (approx. 95% pure) of cytotrophoblast cells with >90% viability. Established trophoblast cultures were kept in normoxia (20% O₂) at 37°F with 5% CO₂.

After 5 days, the cultures within both groups were terminated, formalin fixed and paraffin embedded. Quantitative immunohistochemistry based on morphometric software (Quantimet 500C+) was applied for HA receptors identification in paraffin 5 µm sections of the cytotrophoblast. Human anti-H1, anti-H2 and anti-H4 monoclonal antibodies were used (dilutions 1:500, 1:500, and 1:100, respectively). Detection of primary antibodies was performed using HRP-DAB Staining Kit (R&D Systems, USA). Single analysed image area was 138692 µm². The total number of images amounted to 300 for each group.

### 3.5 Statistical analysis

All morphometric procedures were conducted twice by independent observers in a blinded fashion, and the average results recorded. Comparisons of mean HA concentration were made using Student’s t-test. For statistical analysis of V/EVTI, MCN and HA receptors expression Mann-Whitney’s U test was applied. The results are reported as mean percentage values ±SD or ±SEM of compared control values (taken as 100% for group II). The differences between groups I and II were deemed statistically significant if p < 0.05.

### 4. Results

The mean concentration of HA in diabetic placentas (group I) was significantly higher compared to controls (group II) (Tab. 1). Increased HA content was accompanied by markedly increased MCN in diabetes class C (group I), and MCN reached 146.4% of control numbers (the mean value obtained for controls: 422.7; SD ±37.2 was taken as 100%; Tab. 1). The mean V/EVTI values in the placental cuts from group I and group II were different and this finding was statistically significant (p < 0.05). Increased density of the vascular network of placental microvessels was observed in diabetes class C. (Tab. 1).

Immuneactive cells corresponding to MC were present in in great numbers particularly in regions located close to the fetal surface of the placenta (MCT predominantly) and in the clusters of connective tissue (mostly MCTC type).
**Table 1.**

Mean histamine (HA) concentration, total Mast Cell Number (MCN) and Vascular/Extravascular Tissular Index (V/EVTI) in placental sections: diabetes versus normal pregnancy.

<table>
<thead>
<tr>
<th>GROUP¹</th>
<th>Mean HA content (ng/g of wet weight ±SD)</th>
<th>Total MCN²</th>
<th>V/EVTI (mean value ±SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. DIABETES CLASS C (after White)</td>
<td>372.4* ± 35.3</td>
<td>7426* (618.8; SD ±40.6)</td>
<td>0.35* ±0.028</td>
</tr>
<tr>
<td>I. CONTROLS</td>
<td>250.1 ± 22.3</td>
<td>5072 (422.7; SD ±37.2)</td>
<td>0.25 ±0.026</td>
</tr>
</tbody>
</table>

* p < 0.05; HA

¹ Number of specimens in group I and II were equal and amounted to 300.
² In parentheses – the mean number of mast cells identified in one placenta.

**Figure 1.**

Average percentages and mean numbers of placental mast cells subtypes: diabetes versus normal pregnancy.
All identified MC were tryptase positive, whereas only 15.4% in group I, and 22.1% in group II were chymase-positive (Fig. 1). Thus, the percentage of MCT in diabetic placentas is significantly increased (p < 0.05) compared to normal pregnancy. In contrast, the percentage of MCTC in diabetes White class C is reduced (Fig. 1).

Analyses of the mean expression of H1, H2 and H4 receptors showed positive correlation between increased immunoreactivity for H4 receptor and increased V/EVTI (p < 0.05) in group I (Fig. 2). There were not statistically significant differences in H1 and H2 expressions between the studied groups (Fig. 2).

### 5. Discussion

The results showed that in diabetes class C increased density of the villous network of vessels correlates with both significantly (p <0.05) higher MC number and increased HA concentration. Increased expression of H4 receptors may be associated with increased angiogenesis and (as a result) increased density of placental villous microvessel network in diabetes class C [8,18]. Considering that HA and other MC mediators are angiogenic, and neovascularization is augmented in diabetic placentae, it is apparently in contradiction to intrauterine fetal anoxia, a well documented clinical observation in diabetes [19]. Moreover, hypoxia can intensify MC degranulation and thereby increases local concentration of the angiogenic factors [20]. Changed vasomotor activity of placental vessels may influence perfusion and oxygenation of the intrauterine environment [21,22]. It is very likely but still under investigation, that permeability of vascular wall to oxygen is deteriorated in diabetes. Using electron microscopy small vascular changes of not yet known importance have been distinguished even in diabetes class C [23].

Clinical data and histopathologic studies of the placenta have confirmed a higher incidence of
In conclusion, overview of the results may lead to the suggestion that MC and HA contribute to abnormal function of the placenta in diabetes class C. Further placental research is needed to disclose in more detailed way the nature of these multifactorial dependencies.

References:


