Maternal diabetes affects cell proliferation in developing rat placenta

T.M.T. Zorn\(^1\), M. Zúñiga\(^3\), E. Madrid\(^3\), R. Tostes\(^1\), Z. Fortes\(^1\), F. Giachini\(^1\) and S. San Martín\(^2,3\)

\(^1\)Institute of Biomedical Sciences, University of Sao Paulo, Brazil, \(^2\)CREAS, Regional Centre of the Study of Healthy Foods, Valparaiso-Chile and \(^3\)Biomedical Research Centre, School of Medicine, Universidad de Valparaiso, Chile

Summary. Placentation starts with the formation of a spheroidal trophoblastic shell surrounding the embryo, thus facilitating both implantation into the uterine stroma and contact with maternal blood. Although it is known that diabetes increases the placental size and weight, the mechanisms responsible for this alteration are still poorly understood. In mammals, cellular proliferation occurs in parallel to placental development and it is possible that diabetes induces abnormal uncontrolled cell proliferation in the placenta similar to that seen in other organs (e.g. retina). To test this hypothesis, the objective of this work was to determine cell proliferation in different regions of the placenta during its development in a diabetic rat model.

Accordingly, diabetes was induced on day 2 of pregnancy in Wistar rats by a single injection of alloxan (40 mg/kg i.v.). Placentas were collected on days 14, 17, and 20 postcoitum. Immunoperoxidase was used to identify Ki67 nuclear antigen in placental sections. The number of proliferating cells was determined in the total placental area as well as in the labyrinth, spongiotrophoblast and giant trophoblast cell regions.

During the course of pregnancy, the number of Ki67 positive cells decreased in both control and diabetic rat placentas. However, starting from day 17 of pregnancy, the number of Ki67 positive cells in the labyrinth and spongiotrophoblast regions was higher in diabetic rat placentas as compared to control.

The present results demonstrate that placentas from the diabetic rat model have a significantly higher number of proliferating cells in specific regions of the placenta and at defined developmental stages. It is possible that this increased cell proliferation promotes thickness of the placental barrier consequently affecting the normal maternal-fetal exchanges.

Key words: Trophoblast, Placenta, Cell proliferation, Diabetes, Pregnancy, Rats, Ki67

Introduction

The placenta is a provisional organ that executes endocrine, metabolic and nutritional activities essential for the growth and survival of the fetus and for the control of pregnancy. Diabetes during pregnancy has been associated with some of placental disorders, including increased weight and size of the placenta (Winick and Noble, 1967; Naeye, 1987; Desoys and Shafrir, 1996), and abnormal placental weight / fetal weight ratio (Lao et al., 1997). The strong correlation between placental weight and fetal weight (Molteni et al., 1978; Heinonen et al., 2001) is used as an indicator of fetal health during intrauterine life, since this correlation is affected during pathological conditions such as maternal hypertension and diabetes and fetal intrauterine growth restriction.

The increased placental size in the presence of maternal diabetes, known as placentomegaly, has been associated with an altered placental histology and cellularity. Placentomegaly has been reported in both humans (Diamant, 1991) and rats (Gewolb et al., 1986; Padmanabhan and Al-Zuhair, 1990; Giachini et al., 2008) and has been correlated with increased glycogen content (Robinson et al., 1988; Aerts et al., 1990). In addition, diabetes increases intracellular solutes required to maintain osmotic equilibrium in the presence of maternal hyperglycaemia (Husain et al., 2001), as well as enlargement of spongiotrophoblast area. Moreover,
extracellular matrix proteins, such as fibronectin, are increased in diabetic rat placenta (Giachini et al., 2008).

In vitro studies with trophoblast cell lineages showed that hyperglycemia decreases the rate of cell proliferation (Weiss et al., 2001). Accordingly, under hyperglycemic conditions, angiogenesis is decreased in chicken choioallantoic membrane as a consequence of a low cell proliferation and increased an apoptosis rate in endothelial cells (Larger et al., 2004). Decreased cell proliferation in conditions of high concentrations of glucose was also observed in endothelial cells from human umbilical cord (Curcio and Ceriello, 1992; Varma et al., 2005), in endothelial cells from human pulmonary artery (Liu et al., 2000) and in the human dermis microvasculature (Kamal et al, 1998). According to Varma et al. (2005) hyperglycemia reduces the cell number by affecting cell proliferation rather than apoptosis.

However, other studies suggest that hyperglycemia increases the proliferation of HUVECs (San et al., 1994) and endothelial cells from the retina (Rymaszewski et al., 1992; Morisaki et al., 1999; Kuki et al., 2006). Hyperglycemia also induces a transient increase in the proliferative activity of β-cells from pancreas (Donath et al., 1999; Lange et al., 2006). Furthermore, a higher rate of tumor recurrence and metastasis was observed in diabetic patients with cancer, suggesting that high blood sugar would favor the synthesis of DNA in cancer cells (Suba and Ujpal, 2006).

Considering these contradictory data on the effect of hyperglycemia on cell proliferation, and the possibility that increased proliferation is associated with increased placental weight and size in diabetes, the objective of this study was to determine by immunoperoxidase, the number of Ki67-immunoreactive (proliferating) cells in different regions of the placenta in a rat diabetic model.

Materials and methods

Female Wistar rats were obtained at 14 weeks of age from colonies maintained at the Animal Facilities of the Institute of Biomedical Sciences. The rats were housed in a temperature-controlled environment (21±1°C), on 12-h light/dark cycles, and given free access to tap water and standard rat chow.

Each female was housed with a male for copulation. Vaginal smears were taken daily, and the day on which spermatozoa were found in the vaginal smear was considered day 1 of pregnancy (term, 20 days).

In this study, we used fifteen (n=15) pregnant rats, divided in control (n=8) and diabetic (n=7) groups.

On day 2 of pregnancy, diabetes was induced by intravenous injection of 40 mg/kg of alloxan (Sigma, St. Louis, MO, USA) freshly prepared in saline solution (pH 7.0), as previously described (Fortes et al. 1989). Follow-up was performed 48 h subsequent to induction in order to confirm per photometric estimation of blood sugar levels (digital glycosimeter, Accu-Chek Active®, Roche Ltd, Basel, Switzerland) the presence or absence of diabetes which was defined as glycaemia >200 mg dl⁻¹. The rats also received subcutaneous injections of 1 IU of NPH insulin (neutral protamine Hagedorn insulin; Iolin; Biobras, Montes Carlos, Brazil) every 48 h. Control rats received injections of vehicle (physiological saline solution).

Control and diabetic pregnant rats were weighed and sacrificed on days 14, 17 and 20 of pregnancy. Foetuses and placentas were quickly extracted from the uterus and weighed.

Tissue collection

The placentas were removed and fixed in paraformaldehyde at 4% in phosphate buffer saline solution (PBS) 0.1M pH 7.2 for 12 h at 4°C, and embedded in Paraplast (Oxford, St. Louis, MO, USA) at 60°C. Sections of 5 μm in thickness were cut and adhered to glass slides using 0.1% poly-L-Lysine (Sigma) and then dried at room temperature (25°C). Prior to the immunoreaction, some samples were stained with haematoxylin and eosin for morphological studies.

Immunoperoxidase procedures

Immunohistochemistry was performed according to a previously established protocol (Giachini et al., 2008). Briefly, sections were treated with 3% (v/v) H₂O₂ in PBS for 30 min to block endogenous peroxidase activity. Each of the succeeding steps was followed by a thorough rinse with PBS. All steps were performed in a humid chamber under care to avoid dehydration of the sections. Antigenic retrieval was performed by immersing the slide in citrate sodium solution (10mM, pH 6.0) for 15 min at 95°C. Non-specific staining was blocked by incubating the slide with Cas-Block solution (Zymed Laboratories, South San Francisco, CA, USA) for 10 min.

Incubation with the primary antibody was achieved by subecting the sections to monoclonal anti mouse-Ki67 host rabbit (LabVision, USA), diluted 1:200 in PBS containing 0.3% (v/v) Tween 20, overnight at 4°C. After rinsing in PBS, all sections were incubated for 1 h at room temperature with biotin-conjugated goat anti-rabbit IgG (Rockland, Gilbertsville, PA, USA), diluted 1:1000 in PBS. After rinsing in PBS, sections were incubated with Vectastain ABC kit (Vector) for 1 h at room temperature. Peroxidase reaction was visualised using NovaRED kit (Vector, Burlingame, CA, USA). After immunostaining, sections were lightly stained with Mayer’s haematoxylin (Merck, Darmstadt, Germany).

For each immunohistochemical reaction, controls were performed by incubating the sections with normal rabbit serum or by omitting the primary antibody. Sections were examined in a Zeiss Axioskop 2 microscope, and the images were captured using a digital camera (Canon) and KS 100 3.0 software (Zeiss).
Cell proliferation evaluation procedures

At least three sections (5-µm thick) obtained from different placental blocks of control and diabetic rats, were obtained near the midline of placentas. The different regions of the placenta (labyrinth, spongiotrophoblast and giant trophoblasts cells), were viewed and captured in 541 fields at 400X magnification, using a Zeiss Axioskop 2 microscope (Carl Zeiss, Jena, Germany) with a digital camera Canon Power Shot G5 (Canon USA, Lake Success, NY, USA) and KS 100 3.0 software (Carl Zeiss). The numbers of Ki67 positive nuclei observed were directly counted in each captured fields using the Image-Pro Plus software (Media Cybernetics, Silver Spring, MD, USA).

Statistical analysis

Statistical analysis was performed using the Stata 11.0 software (StataCorp, USA). Parametrical statistics were used. Analysis of the counted nuclei in each experimental group is shown as means and standard error (SE). Statistical significance was accepted when p<0.05, using the T-test or one way analysis of variance.

Ethical aspects

All experiments were conducted in accordance with the ethical principles of animal research adopted by the Brazilian College of Animal Experimentation and in accordance with the “Principles of Laboratory Animal Care” formulated by the National Society for Medical Research and published by the National Institutes of Health (NIH Publication No. 86–23, revised 1996). The procedures involving animal experiments were approved by the joint Ethics in Animal Research Committee of the Institute of Biomedical Sciences of the University of Sao Paulo, Brazil (authorisation no. 115/2000-107/2000) and Universidad de Valparaiso.

Results

Placental morphology

As previously reported by our group (Giachini et al., 2008), placetas harvested from diabetic rats presented...
various morphological alterations over the course of pregnancy, mainly represented by an increased area of the spongiotrophoblast layer and changes in the classical pattern of trophoblast giant cell distribution. Accordingly, compared with placentas from the control group, the weight of the placentas from the diabetic

![Immunoperoxidase staining for Ki67. Placentas of control and diabetic rats on day 17 of pregnancy. Immunoreaction is observed in the nucleus of endothelial and trophoblast cells in the labyrinth region (arrows), spongiotrophoblast cells (arrows) and giant trophoblast cells (arrows). Bar: 100 µm.](image1)

![Immunoperoxidase staining for Ki67. Placentas of control and diabetic rats on day 20 of pregnancy. Immunoreaction is observed in the nucleus of endothelial cells in the labyrinth region (arrow), spongiotrophoblast cells (arrows) and giant trophoblast cells (arrow). Bar: 100 µm.](image2)
animals was significantly higher on day 20, the last period of pregnancy (Fig. 1).

**Immunolocalization and quantification of Ki67**

The Ki67 antigen was detected in the nucleus of the different cell types of all placentas. However, the number of immunoreactive cells displayed differences visible between the defined time line, between particular compartment and between experimental condition.

**14 day of pregnancy**

Ki67 immunolocalization was observed in trophoblast and endothelial fetal cells of the labyrinth region; however, no reactivity was observed in mononuclear trophoblast cells in both control and diabetic rats. Furthermore, the spongiotrophoblast region and giant trophoblast cells showed Ki67 immunolabeling. The distribution of Ki67 signal within specific placental regions did not differ between the placentas of control and diabetic rats. In terms of signal number, however, the placentas of diabetic rats have a lower count of immunoreactive cells, mainly attributed to a significantly decreased count within the spongiotrophoblast region on this day. A similar tendency was observed within the giant trophoblast cells, but this result did not achieve significance. No difference was observed between the labyrinth regions at this time (Fig. 2).

**17 day of pregnancy**

In the control group placentas of this period were few Ki67 positive endothelial cells in the labyrinth region, however, in diabetic group, this region showed a higher number of positive trophoblast and endothelial cells. Ki67 positive cell number drastically decreased

---

**Fig. 5.** Cell proliferation on total placenta (a), in labyrinth region (b), in spongiotrophoblast region (c), in giant cells region (d), determined by the number of nuclei stained by Ki67, in total placental tissue on days 14, 17 and 20 of pregnancy. Bar graph showing the number of Ki67-positive cells. Results are presented as mean ± SE. * P<0.05 vs. control.
Maternal diabetes affects placental cell proliferation

almost to the level of indetectability in the spongiotrophoblast region and giant trophoblast cells of control group placentas in comparison to day 14. However in the diabetic group, cells of both regions still maintained measurable reactivity to Ki67 (Fig. 3).

20 day of pregnancy

Only endothelial cells of fetal blood vessels were Ki67 positive in labyrinth section of both control and diabetic placentas. In the same manner, only very few cells were found positive within both control and diabetic placentas (Fig. 3).

General Timeline

Along the timeline of gestation, there was significant and gradual decrease in the overall and region specific count of immunoreactive cells of all placental segments regardless of origin (control/diabetic groups). By the final day of pregnancy none of trophoblast subsets displayed any sign of proliferation, indicating that this organ have completed its growth to meet the demands of the fetus. However, the rate of decrease was much less pronounced, especially within the labyrinth region, but also in all other placental compartment of the diabetic animals in comparison of control (Fig. 5a-d).

Discussion

Qualitative and quantitative parameters have demonstrated that placental development in rodents, especially in rats, is completed around days 12 to 14 of pregnancy (de Rijk et al., 2002). During this period, the placenta undergoes extensive growth to meet the demands of the fetus. In mice, this growth is associated with intense cell proliferation (Iguchi et al., 1993). At day 14 of pregnancy, we showed higher total cell proliferation attributed to higher proliferation levels within the spongiotrophoblast section of control placentas as compared to diabetic placentas. Disturbed cell cycle pathways with the participation of different mitotic regulators was proposed by Acar et al. (2008) as a mechanism that may explain the small placenta that is seen in the early stages of pregnancy in diabetic rats.

Accordingly, we observed that following this growth period, the number of proliferating cells decrease in placentas of both control and diabetic animals during pregnancy.

The present data also demonstrate that diabetes promotes maintains cell proliferation rate in the labyrinth, spongiotrophoblast and giant trophoblast cell regions as compared to control placentas, especially visible in the last periods of pregnancy (day 17), which may explain the placentomegaly observed in diabetic placentas on day 20 of pregnancy. The higher number of proliferative cells in this period are probably due to the effect of diabetes in the placenta, since all trophoblast subsets at this time are still subject to maternal hyperglycaemia.

Kim et al. (2010) showed that high glucose increases fibronectin synthesis through the angiotensin II or TGF-beta pathways, which in part mediates proliferation of mouse embryonic stem cells.

In our previous study, we showed the higher presence of fibronectin, which is an extracellular matrix molecule, in diabetic placentas as compared to control placentas (Giachini et al., 2008).

Moreover, we observed that experimentally induced diabetes in rats promotes the higher presence of various growth factors in placentas, probably related with cell proliferation signaling (data not shown).

Increase in placental size in diabetic rats has been previously demonstrated by quantification of dry and wet placental weight on day 20 of pregnancy (Husain et al., 2001; Giachini et al., 2008).

In rodents the placenta is partially syncytial. An increased rate of cell proliferation may directly affect the normal development of the maternal-fetal unit by creating a thick barrier that disturbs the normal interchange between the mother and the fetus.

Placentas from diabetic mothers showed immaturity of the villi and hypertrophy of the capillaries (Younes et al., 1996; Evers et al., 2003; Daskalakis et al., 2008). In addition, Gewolb et al. (1986) could demonstrate that placentas from diabetic rats have a number of features that are consistent with immaturity, suggesting that this condition provides a stimulus for continuous growth and cell division-delayed maturation.

Acar et al. (2008) showed that the intensity of PCNA immunoreaction decreases on day 13 of pregnancy in the placentas of diabetic and normoglycemic rats. Subsequently, on day 17 of pregnancy, the immunointensity and the quantity of the PCNA protein was higher in the placentas of the diabetic group than in the placentas of normoglicemic animals. Using Ki67 antigen as a marker of proliferating cells, the present data add new and important information, by showing that diabetes promotes spatial and temporal alterations of cell proliferation in the placenta.

The labyrinth region is the major site of fetomaternal exchange (Davies and Glasser, 1968), whereas the basal zone (spongiotrophoblast region) is the major site of placental hormone production over the late period of pregnancy (Matt and McDonald, 1985). These regions of the placenta were the most affected in our model.

Normal fetal growth and development depend on a normal placental function. Some types of intrauterine growth restriction in human and animal fetuses have been related to disturbances in placental blood flow and/or defective transport of nutrients from the mother to the fetus (Cassady, 1981). The increased number of cells in the labyrinth and spongiotrophoblast zones on 17 day of pregnancy, in diabetic condition, is expected to produce a thickened placental barrier consequently reducing the maternal-fetal exchanges. This hypothesis is supported by the study of Chartrel et al. (1990) showing that the uteroplacental hemodynamic...
disturbances that occur in diabetic rats are the major cause of fetal growth restriction.

This study shows that in the rat, placental cell proliferation is down regulated during normal pregnancy. In diabetic conditions however, cell proliferation is increased in a spatial and temporal specific pattern until the last period of pregnancy. These data suggest that augmented cell proliferation in placental tissues from diabetic animals may not be only mechanism that regulates the placental growth, but also may lead to disturbances in placental blood flow and/or defective transport of nutrients from the mother to the fetus.

In conclusion, our and others’ data showed that impaired placental morphology may be related with the frequency of fetal complications in diabetic pregnancies in humans and animal models.

Acknowledgements. This work was supported by grants from DIPUV (Universidad de Valparaíso, Valparaíso, Chile; grant no. 12/2006, 07/2008 and CI 05/2006), Programa de Investigación Interdisciplinario (PIA) from Comisión Nacional de Investigación en Ciencia y Tecnología (CONICYT) (Anillos ACT-73), Chile; and FAPESP (Sao Paulo, Brazil; grant nos. 04/05472-9 and 05/58074-3). We gratefully acknowledge the linguistic assistance provided by Dr. Justine S. Fitzgerald and the technical assistance provided by Makarena Gonzalez.

References


Morisaki N., Watanabe S., Fukuda K. and Saito Y. (1999). Angiogenic interaction between retinal endothelial cells and pericytes from...

Accepted March 7, 2011