Cellular immune response and other functional proteins in gestational trophoblastic diseases

Ph.D. Thesis

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Introduction

Gestational trophoblastic diseases are mostly the results of a multispermic conception. The molar pregnancies containing extremely heterogenic mainly paternal chromosomes are characterized by excessive trophoblast proliferation. The complete mole, which contains only paternal chromosomes, has significant invasive potential and in 20% of the cases it persists in the uterus after evacuation. The persistent mole can compromise the life of the patient without effective chemotherapy. The hCG follow up and the possibility of a chemotherapy are significant burdens for the patients not only due to the side effects but also because of social, emotional and financial difficulties. Therefore, there is an increasing demand from the clinicians and patients to develop an alternative therapy, which can be applied at the time of evacuation to prevent persistent diseases. If the development of an effective alternative therapy fails, there is still a need for a reliable marker, which can predict persistence therefore saving 80% of the patient from further follow up. If the marker is specific and sensitive enough to use as a prognostic factor it soon will replace the long hCG follow up in the clinical practice.

According to our understanding, on the mother's side there are 3 distinct mechanisms controlling the invasion and persistence of the trophoblastic tissue in the endometrium and the uterine cavity: The maternal immune system, the protein profile of the decidual cells and the blood supply of the endometrium. None of those is fully understood.

The maternal immune system undergoes a significant change in pregnancy, which has a crucial role in the implantation of the half paternal embryo. During pregnancy, the cellular immune response is suppressed. The

modified and upregulated humoral immune response tries to keep the maternal homeostasis. The primary source of the gestational immunosuppression is the trophoblastic tissue itself expressing high amount of hCG, which stimulates the corpus luteum to produce progesterone. The progesterone in turn increases the production of Th2 cytokines and suppresses the cellular immune response through the Progesterone Induced Blocking Factor (PIBF).

Furthermore, the trophoblast cells utilize several mechanisms to avoid the cellular immune response and control the T cell activation. Addition to the modified cell surface proteins (HLA-G, HLA-E) trophoblast cells express numerous immunosuppressive factors, which inhibit T cell activation and contribute to the conversion of general uterine NK cells to more specialized regulator type NK cells (NKr1, NK1, NK3). In gestational trophoblastic diseases it is not completely defined whether the persistence of the highly allogeneic trophoblastic tissue in the mother is due to the high proliferation rate of the trophoblastic tissue or the immunosuppressive state of the mother caused by the high excess of immunosuppressive factors produced by molar trophoblast. Previous studies have already demonstrated that at the implantation site of the molar pregnancy, the number of the T cells is increased and the number of NK cells is decreased compared to normal pregnancy. However, until now the phenotype of the present cells and the T cell receptor profile of the T lymphocytes have not been described.

The second mechanism, which influences the invasion of the trophoblast cells, is the protein profile of the decidual cells. The investigation of the decidual cell proteins is still in the center of the reproductive biology research. The decidual cells control the invasion of the trophoblast cells by the expression of cell adhesion molecules and different proteins modifying the

structure of the extracellular matrix. A microarray study demonstrated that the expression of the laminin receptor 1 molecule in molar pregnancy is higher compared to normal placenta. Until now the confirmation of this finding by RT-PCR and histologic localization of the molecule have not been done.

The third mechanism, which might have a role in the proliferation and persistence of the trophoblastic tissue, is the modification of the spiral arteries and the angiogenesis at the implantation site. Like solid tumors, the growing trophoblast tissue needs increasing blood supply for further proliferation in gestational trophoblastic diseases. As in several premalignant diseases, the intratumoral or stromal microvessel density was able to predict malignant progression, therefore raising the question that microvessel density might have the same prognostic role in the diagnosis of persistent trophoblastic diseases.

Aims

In my Ph.D. work, I focused on the placental site. I comprehensively investigated all three areas (cellular immunity, protein profile of decidual cells and angiogenesis at the implantation site) to better understand the invasion controlling mechanisms.

By investigating the cellular immune response, I attempted to answer the following question: Does the proliferation of the trophoblast cells or the stronger immunosuppression have the primary role in the persistence of the mostly paternal trophoblastic tissue in the maternal uterus?

Furthermore, for developing an alternative immunotherapy it is necessary to identify a specific antigen, which activates the immune cells at the implantation site. Until now all attempt failed to find one specific antigen on the surface of the trophoblast cells. My aim was to investigate the T cell

receptor variable beta chain profile instead of the trophoblast surface itself to learn more about the activating antigen or antigens.

I also attempted to identify a reliable and more comfortable marker for clinical persistence than hCG follow up. With the angiogenesis studies next to validating a histologic marker, I also attempted to support the application of an already approved anti-VEGF therapy (Avastin) in gestational trophoblastic diseases.

Methods

On histologic slides with immunostaining method, I investigated the number and the function of the immune cells at the implantation site of normal and molar pregnancies. I counted the regulator (Treg), effector T cells and the Granzyme B positive NK cells, using monoclonal antibodies against FoxP3, Granzyme B and CD8. Furthermore, I investigated the distribution of immune cells in and around intraplacental and postmolar choriocarcinomas. From fresh frozen normal placenta and molar tissues, I characterized the T cell receptor variable beta chain usage utilizing RT-PCR method.

Again, on fresh frozen tissues and paraffin fixed slides I measured the expression of laminin receptor 1 by RT-PCR and immunostaining coupled with digital image analysis. I also used immunostaining method with CD31, VEGF Angiopoietin 1 and 2 monoclonal antibodies and digital image analysis to determine the microvessel density at the implantation site and the expression level of VEGF and angiopoietin 1 and 2 in normal placenta persistent and non-persistent partial and complete moles.

Results

Both in partial mole and complete mole the immune cell infiltrated area was significantly greater than in normal placenta (p=0,035, P=0,008). The number of both the effector and the Granzyme B positive NK cells have significantly increased in complete mole and postmolar choriocarcinoma ($p<0.05$). 92-96% of the CD8 positive cells produced Granzyme B therefore most of the cytotoxic T cells were so called effector cells in all groups in both normal and molar (partial and complete) pregnancy and also in the adjacent tissue of the postmolar choriocarcinomas. As a result of the increased T cell activation Treg cells appeared at molar implantation site and the number of Treg cells significantly correlated with the number of CD8 positive cells (p=0,032). The number of the Treg cells did not show association with the effector ratio (CD8+/GrB+ effector cells/ all CD8 cells). Surprisingly with FoxP3 immunostaining we did not find any Treg cells at the implantation site of the normal placenta.

There was no immune cell infiltration neither in the intraplacental choriocarcinoma nor in the adjacent villi. We found normal immune cell infiltration at the implantation site of the placenta. On the contrary, a vigorous immune cell infiltration could be seen in the adjacent tissue of postmolar choriocarcinoma, but in 6 out of 7 cases, we could not see immune cell infiltration in the tumor tissue. A sharp infiltration border could be seen at the edge of the choriocarcinoma tumor tissue.

In normal pregnancy, the T cell receptor (TCR) analysis of the infiltrating T lymphocytes showed predominance of the variable beta chain

gene number 2 and number 4. In the TCR analysis of the T cells at complete mole implantation site, only variable beta chain gene number 4 was significantly up regulated. The TCR variable beta chain profiles were very homogenous in each group and did not show substantial case-to-case variability.

The decidual cells in both normal placenta and molar pregnancy express the laminin receptor 1 molecule. The decidual cells in molar pregnancy (especially in partial mole) express laminin receptor 1 significantly greater than normal pregnancy both in mRNA and protein level.

At the implantation site of persistent partial and persistent complete moles the microvessel density was significantly higher than is normal pregnancy. In complete mole the microvessel density was also higher in the persistent group compared to the non-persistent moles, but the difference did not reach significance due to the small number in our study ($p=0.115$). Across all mole types MVD strongly correlated with post evacuation hCG level. The VEGF production of the trophoblast cells did not show significant differences among groups. The angiopoietin 1 with immunostaining method did not show expression on the gestational tissues; however, the angiopoietin 2 was expressed by the trophoblast cells and showed a significantly lower expression in moles with a high villus-to-villus variability.

Conclusions

1. In gestational trophoblastic diseases as the allogenicity and agressivity of the tissue increases, the T cell immune response becomes more and more vigorous and plays a more important role in controlling the trophoblastic tissue proliferation. Parallel to these changes the total NK pool is decreasing, but the number of the Granzyme B producing NK cells increasing. The regulator T cells cannot be found in normal pregnancies, but they appear in the molar pregnancies associated with a more vigorous T cell response. Tregs probably play a feedback suppression role on T cells at complete mole implantation site, but they are not able to change the phenotype of the present activated T cells, only attenuate the T cell activation. The present method is not able to investigate the immunosuppressive characteristics of the Treg cells.
2. The T cell receptor variable beta chain analysis implies that there is a specific ongoing T cell activation at the implantation site, which requires the expression of either variable beta chain gene number 2 or number 4 within the TCR. As the TCR profiles did not show case-to-case variability, we can conclude that the activating antigen/antigens are conservative paternal placental antigens.
3. The maternal immune system does not recognize the intraplacental choriocarcinoma; therefore, vigorous antitumoral or anti-placental immune response cannot be seen. On the contrary, in postmolar choriocarcinoma cases vigorous immune cell response could be seen in the adjacent tissue, probably as the result of the increased antigenicity of the tissue (complete allograft). The immune cells do not infiltrate the tumor in spite of the vigorous immune cell activation in the adjacent tissue implying that avoiding the trophoblast- immune cell interaction has

higher importance in the survival of the trophoblastic tissue than immunosuppressive factors.

4. The decidual cells express the laminin receptor 1, which might have a role in the implantation of the trophoblast cells. Laminin receptor 1 generally has very important role in the migration and metastasis of cancers by influencing the conformation of the laminin in the extracellular matrix. The increased expression of laminin receptor 1 on decidua cells might explain the more invasive potential of the molar pregnancy. The hormonal treatment (estrogen (E), progesterone (P), E+P and hCG) of the immortalized human endometrial stromal cells did not influence the expression of the laminin receptor 1, therefore the underlying mechanism of this finding remains unknown.
5. At the implantation site of persistent partial and persistent complete moles the microvessel density is higher than in normal pregnancy. The increased microvessel density and the morphology imply an ongoing intensive angiogenesis at the complete molar implantation site. Persistent complete mole had a tendency to an increased microvessel density therefore the microvessel density analysis together with pathologic analysis might be a useful factor to predict persistent disease. MVD correlated with post evacuation hCG levels in molar pregnancies but not with the persistence in general which supports the hypothesis that hCG is a potent angiogenic factor in pregnancy and its high level contributes to an increased MVD at the implantation site. The other examined angiogenic factors (VEGF, Angiopoietin 1 and 2) did not correlate with MVD and the clinical data.

*6. **Publication list***

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