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Low expression of Progesterone Receptor A in intermediate trophoblast of miscarriages

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Summary. Objective: To examine the potential differences in the expression of Progesterone Receptor A and Estrogen Receptor A in intermediate trophoblastic cells at the implantation site in elective abortions and miscarriages by immunohistochemistry.

Study Design: Twenty two (22) samples of miscarriages and eighteen (18) samples of elective abortions were obtained during gestational weeks 6 to 12. Monoclonal antibodies against Cytokeratin 7 and prolactin were used to help discriminate between trophoblastic and decidual cells at the feto-maternal interface on formalin-fixed paraffin-embedded sections. Samples were then stained with ERA and PRA antibodies. Nuclear expression was considered positive. Staining intensity was measured according to a 4 grade scale. Statistical analysis of the results was performed using the Mann-Whitney test and the Wilcoxon signed rank test.

Results: PRA expression in intermediate trophoblastic cells was significantly higher in elective abortions (control group) compared to miscarriages. ERA expression was uniformly negative in both groups.

Conclusion: PRA expression is significantly lower in intermediate trophoblastic cells of miscarriages compared to elective abortion pregnancies. Although this could be solely a result of a secondary event, it is still an important finding in the effort to unravel the complex molecular pathobiology of spontaneous abortions.

Key words: Progesterone receptor A, Estrogen receptor A, Intermediate trophoblast, Miscarriage

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Introduction

There are two types of ovarian sex hormones, the estrogens and the progestins. Estrogen is produced primarily by developing follicles in the ovaries, the corpus luteum, and the placenta. The production of estrogen in the ovaries is regulated by follicle-stimulating hormone and luteinizing hormone. Estrogens constitute a group of steroid compounds, synthesized mainly from cholesterol derived from the blood, which play an important role in the estrous cycle and function as the primary female sex hormone. The human body forms 3 types of estrogens: ß-estradiol, which is the most important and biologically active estrogen, estrone and estriol. These are responsible for the stimulation of endometrial growth, the increase of uterus during the menstrual cycle and a slight increase in production of proteins. They are also responsible for the cellular proliferation and growth of the female sex organs, for the preparation of the female reproductive system regarding the action of progesterone and mainly for the uterine receptivity for implantation (Dey et al., 2004).

Progesterone is a steroid hormone involved in the female menstrual cycle, gestation and embryogenesis. It belongs to a class of hormones called progestins, and is the major naturally occurring human progestin. It is produced in the adrenal glands, the gonads (especially after ovulation in the corpus luteum), the brain and during pregnancy in the placenta (Graham and Clarke, 1997). Progesterone promotes increased secretion by the mucosal lining of the fallopian tubes, which is used to nutrate the fertilized, dividing ovum. Additionally, in the presence of progesterone, the uterine endometrium undergoes decidualization, which involves the hypertrophy of endometrial cells during the latter half of the monthly sexual cycle, thus preparing the uterus for the implantation of the fertilized ovum. It also develops

the lobules and alveoli of the mammary glands and appears to decrease the maternal immune response during implantation and gestation in order to allow the acceptance of pregnancy. Moreover, progesterone appears to decrease the contractility of the uterine smooth muscle. In addition, progesterone inhibits lactation during pregnancy. During term, the beginning of labor is promoted, among other ways, by a sudden drop in progesterone levels. Lactation follows delivery, facilitated by the fall in progesterone levels. Progesterone increases core temperature (thermogenic function) during ovulation, reduces spasm and relaxes smooth muscle, regulates mucus and widens the bronchi. Finally, it appears to prevent endometrial cancer by antagonizing estrogen-mediated cell proliferation (Ito et al., 2007). In general, progesterone prepares the female reproductive system for reception and implantation of the fertilized ovum.

Trophoblast is the outer layer of blastocyst and is diversified in villous and extravillous, which is the interstitial or intrmediate trophoblast. The interstitial trophoblast cells filtrate through the deciduas and the uterine vessels. Extravillous trophoblastic cells invade the uterine wall, called interstitial invasion, the spiral arteries, called endovascular invasion and replace the cells of the vessel wall (Cartwright et al., 2002).

Progesterone receptors are members of a large family of structurally related gene products known as the nuclear receptor superfamily (Conneely et al., 2003). They consist of two proteins encoded by the same gene, termed PRA and PRB, which are identical except for an additional 165 amino acid sequence, present only in the N terminus of human PRB. Progesterone receptors are expressed in the epithelial, stromal and myometrial compartments of the uterus, and their spatiotemporal expression within these compartments is regulated by both estrogen and progesterone (Zhao et al., 2008). Although PRB shares many important structural domains with PRA, they are in fact two functionally distinct transcription factors, mediating their own response genes and physiological effects with little overlap (Kastner et al., 1990; De Vivo et al., 2002).

The estrogen receptor is a member of the nuclear hormone family of intracellular receptors which is activated by the hormone 17ß-estradiol secreted by growing follicles during the follicular phase (Paige et al., 1999). There are two different forms of the estrogen receptor, ERA and ERB, each one encoded by a separate gene (Macklon et al., 2006). They are both widely expressed in different tissue types; however there are some notable differences in their expression patterns. ERA and ERB are coexpressed in glands and stroma with ERA being the dominant receptor, whereas ERB is only expressed in endothelium (Koehler et al., 2005). ERA is expressed primarily in the uterus, liver, kidney, and heart, whereas ERB is expressed primarily in the ovary, prostate, lung, gastrointestinal tract, bladder, and hematopoietic and central nervous systems (Matthews et al., 2003).

Materials and methods

The study group was obtained from 22 women who miscarried between the ages of 20-47 years and during gestational weeks 6-12 [week 6 (n=4), week 7 (n=6), week 8 (n=6), week 9 (n=3), week 10 (n=1), week 11 (n=1) and week 12 (n=1)]. Controls consisted of 18 healthy women between the ages 27-39 years, who had electively terminated their pregnancies during gestational weeks 6-12 [week 6 (n=4), week 7 (n=4), week 8 (n=7), week 10 (n=1), week 11 (n=1), week 12 (n=1)]. The gestational age was calculated from the last normal period to the date of curettage.

Pathology-examination

Formalin-fixed, paraffin-embedded blocks of placental material were cut in 5 μm sections and stained with hematoxylin and eosin (H/E). In all specimens, the implantation site was identified by cytokeratin (CK7) immunohistochemistry, which is positive in trophoblastic cells (Figs. 1, 2). Furthermore, for the discrimination between decidual and trophoblastic cells at the fetomaternal interface, duplicate sections were stained with a monoclonal antibody against prolactin for the visualization of decidual cells (Figs. 3, 4).

From a representative block of formalin-fixed, paraffin-embedded tissues from each case, 5 μm sections were transferred to positive charged and properly prepared glass plates, which were kept in an oven, at 65°C for over 60 min. Deparaffinization was performed in xylene. Afterwards, sections were immersed in absolute alcohol, 96%, 70% for 10 min each and were rinsed in distilled water. In a vessel containing buffer citric Na 0.01 (ph 6.0), the slides were covered in tape with holes for ventilation and the vessel was subjected to microwave radiation by a microwave oven at 650-800W in 3 cycles for 5 min each. The sections stayed for 20 min more in the microwave oven which was switched off and were rinsed in PBS buffer (ph 7.6) for 5 min. Thereafter, they were incubated in H_2O_2 0.3% for 10 min, to quench endogenous peroxidase activity, and were double rinsed in PBS buffer.

Then, the sections were placed in normal horse serum (concogene US) for 20 min, and the excess of the reagent was whipped. Following this procedure, the sections were covered with 1:40 dilution of the primary tonic monoclonal antibody PRA (NCL-PGR NovoCastra-UK) and with 1:40 dilution of the primary tonic monoclonal antibody ERA (NCL-ER-6F11 NovoCastra-UK), overnight in a humid environment in a refrigerator.

Double-rinse of the sections with PBS, 10 min each, was followed by incubation in the secondary antibody (bionylated antibody ongogenus) for 30 min at room temperature. After being double-rinsed with PBS 5 min

each, they were incubated in A.B.C. complex (Concogenous Avidin-Biotin HRP Complex), followed by another double-rinse cycle with PBS, 5 min each. For visualization, a solution of chromogen diaminobenzidine DAB (Sigma USA) in PBS containing a solution of hydrogen peroxide 0.3% at room temperature was used.

The sections were rinsed in tap water, immersed in aqueous solution of ammonia (97 ml distilled water and 3 ml ammonia) and counterstained with hematoxylin Harris for 10 seconds. After dehydration in escalating densities of a series of alcohols and carboxylol, the sections were covered with Canada balsam.

Microscopic evaluation was performed to the cells of the intermediate trophoblast and the decidua basalis on the implantation site of miscarriage and elective abortion material. The intensity of the nuclear staining of PRA and ERA was evaluated in a scale of 4 grades: negative, low, moderate and strong.

Statistical Analysis

Statistical analysis was performed using the SPSS software v.17. A *P* value of <0.05 was considered statistically significant as determined by the Mann-Whitney test for two independent samples. In order to take into consideration gestational week, samples from the group of miscarriages were paired with matching samples of elective abortion group. The Wilcoxon signed rank test was performed to analyze the results. A *p* value of <0.05 was considered statistically significant.

Results

The mean age of patients belonging to the miscarriage group was 34, 9 years (range 20-47),

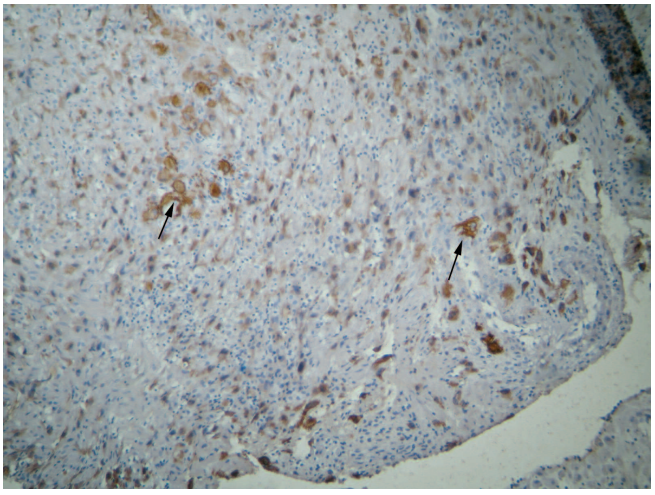

Fig. 1. Implantation site – CK7. Detection of trophoblastic cells. x 40

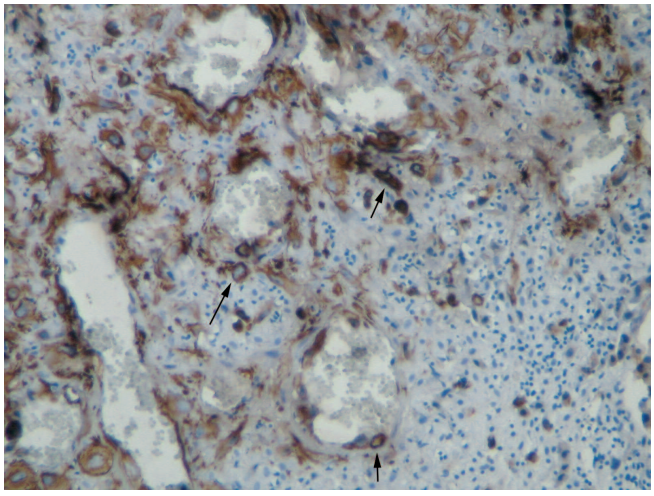

Fig. 2. CK7. Detection of trophoblastic cells. x 71

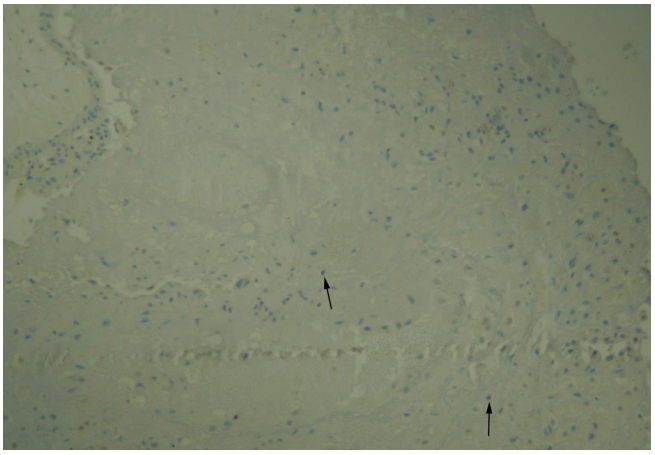

Fig. 3. Implantation site –Prolactin. Detection of decidual cells. x 16

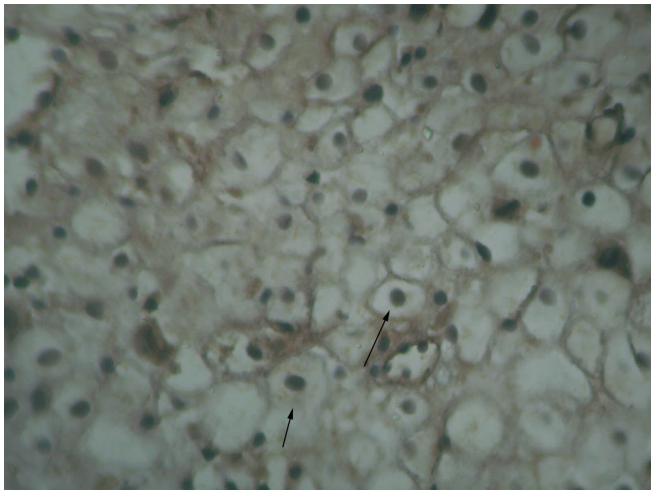

Fig. 4. Implantation site –Prolactin. Detection of decidual cells. x 160

relatively older than the mean age of patients that underwent elective abortion was 33, 1 years (range 27-39). Both patient groups presented with the similar estimated gestational age at the time of the abortion (median age: 8 weeks).

In miscarriage specimens, no ERA expression was detected in either trophoblastic or decidual cells. Similarly, ERA was uniformly negative in both trophoblastic as well as decidual cells of elective abortions (Figs. 5, 6). Immunohistochemistry for PRA on the contrary showed varying results depending on specimen and cell type. In the trophoblastic cells of miscarriage specimens, negative expression was observed in 13 cases, low expression in 4 and moderate expression in 5 (Figs. 7, 8). On the contrary, all 18 elective abortions showed positive staining, in most cases of moderate intensity ($p<0.001$). Strong PRA immunostaining was only observed in elective abortion

Table 1. PRA staining intensity in trophoblastic cells, n(%).

	NEGATIVE	LIGHT	MODERATE	STRONG
Miscarriage	13(59.1)	4(18.2)	5(22.7)	0
Elective abortion	0	2(11.1)	12(66.7)	4(22.2)

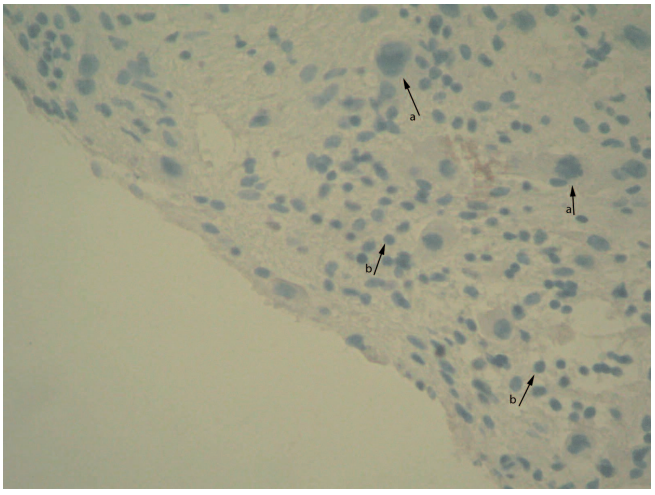

Fig. 5. Implantation site – estrogen (ERA). Evaluation of ERA expression. a: trophoblastic cells ERA (-), b: decidual cells ERA (-). x 71

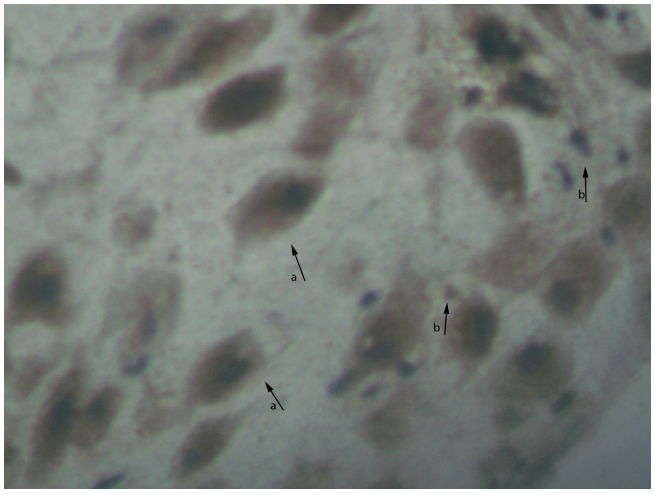

Fig. 6. Implantation site – estrogen (ERA). Evaluation of ERA expression. a: trophoblastic cells ERA (-), b: decidual cells ERA (-). x 284

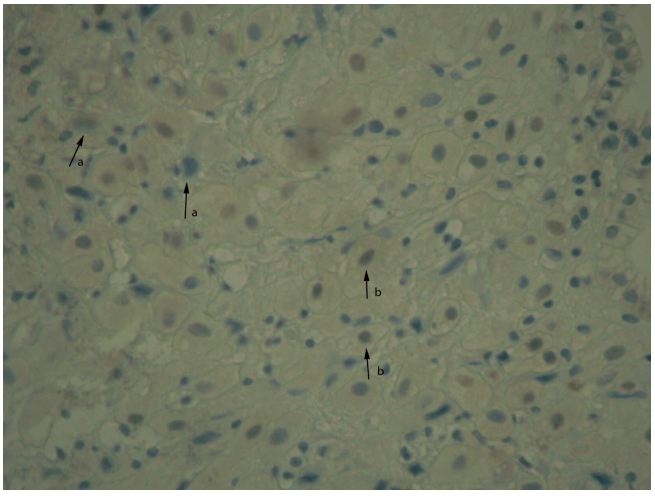

Fig. 7. Implantation site – Progesteron (PRA). Evaluation of progesteron expression. a: trophoblastic cells PRA (-), b: decidual cells PRA (+). x 40

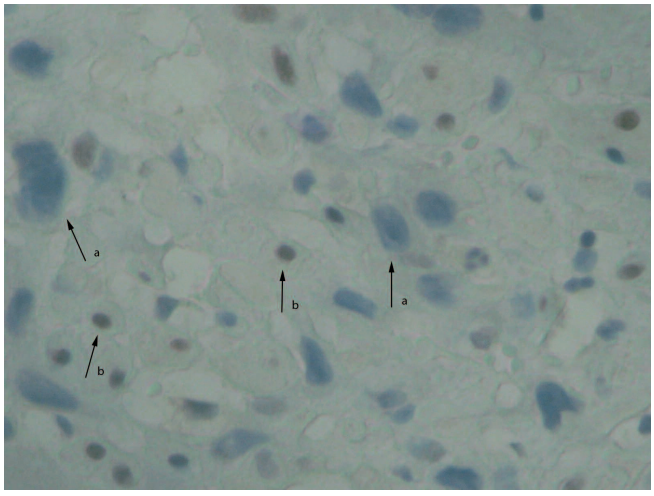

Fig. 8. Implantation site –Progesteron (PRA). Evaluation of progesteron expression. a: trophoblastic cells PRA (-), b: decidual cells PRA (++). x 284

specimens (Table 1). When adjusting for gestational week, this parameter did not affect the difference in PRA expression between the two groups, although it seemed to influence statistical significance in a negative manner (p=0.02<0.05 as determined be the Wilcoxon signed rank test). No significant difference was found in the expression of PRA in decidual cells, strong staining intensity being observed in most cases, i.e. in 17 out of 22 cases of miscarriage and 14 out of 18 abortions. The remaining 5 and 4 cases accordingly showed moderate staining. PRA immunostaining was independent of advancing gestational week or age of the patients.

Discussion

Many studies have been contacted in the last two decades concerning the conception and maintenance of gestation and the affinity of different hormones and their receptors in this procedure. It is known that ovulation, one of the major female reproductive events, appears to involve the expression of a variety of specific genes. The functional role of PR, which is the main concern in our study, and its isoforms associated with ovulation, has been well delineated with PR gene-deficient mice (Shao et al., 2003).

In our study, we have observed that very low expression of PRA in interstitial trophoblast, a finding previously reported, is possibly correlated with high risk of miscarriage of the implanted and growing fetus. However, there are also some variables that have not been taken into consideration, such as the history of previous spontaneous abortions as well as the history of full-term pregnancies. This conclusion is also suggested by other studies, contacted in different species, mainly rodents. For example, PR is claimed to be the intercessor for the effectuation of decidualization, the period of rapid proliferation the uterine stromal fibroblast cells undergo during implantation (Ogle et al., 1998). Progesterone Receptors also play an important role in thymic involution during pregnancy (Tibbetts et al., 1999) by regulating the endothelial and vascular smooth muscle cell proliferation and response to vascular injury. There is a variation of the ratio of PR-positive cells in the endothelium of vessels in different organs, but the cycling endometrium shows the higher proportion of endothelial PR-positive cells, thus proving the role of PR in the regulation of angiogenesis in the endometrium and corpus luteum during the menstrual cycle (Vasquez et al., 1999).

Very few studies investigating progesterone receptors in association with gestation have been conducted with human material. According to these studies, the expression of PRA in intermediate trophoblastic cells is significantly lower in pregnancies resulting in spontaneous abortions, leading to the conclusion that the lack of an adequate number of PRA may play a role in defective implantation and subsequent abortion (Hickman et al., 2002).

As for the expression of ERA, we did not find any in either material. It is known that the expression of ERA and ERB is correlated with trophoblast differentiation (Bukovsky et al., 2003a,b). Interestingly, ER expression has been associated with inhibition of angiogenesis in cancer cells (Ali et al., 2000), but it remains unknown whether this is also the case with human pregnancy (Ali et al., 2000; Schiessl et al., 2005). ERA, generates the terminal differentiation of mononucleated trophoblastic cells to syncytiotrophoblast and promotes placental function through the significant increase of estrogen production occurring with pregnancy progress. Unexpectedly, no ERA immunoreactivity was found in interstitial trophoblast cells and adjacent decidual cells (Bukovsky et al., 2003a,b). Our failure to detect the ER does not entirely preclude the presence of this receptor in human trophoblasts, but might be attributed to a relatively low number and density of ER on these cells.

Concluding, although the main finding of this study could be solely the result of a secondary event affecting the profile of the trophoblast progesterone receptors, it is still valuable information in the effort of revealing the complex molecular pathology of spontaneous abortions.

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