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Original Article

Ultrastructural and immunohistochemical evaluation of endometrial tissues of infertile women with recurrent implantation failure

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Abstract. To evaluate endometrial tissues of fertile women and the women with recurrent implantation failure (RIF) analyzed by using transmission electron microscopy (TEM) and MUC1 immunohistochemistry. In this study, 23 patients with RIF and 11 fertile women participated. Endometrial tissue samples were examined by light and electron microscopes. Additionaly, MUC1 immunoreactivity was evaluated. Electron microscopic examination of the endometrial surface epithelial cells of the recurrent implantation failure revealed lots of cytoplasmic extensions containing secretory vacuoles and irregular microvilli. Furthermore, most of the endometrial cells exhibited secretory vacuoles in the supranuclear region. Immunoreactivity of MUC1 was detected weakly in the luminal epithelium of the control group whereas the staining intensity was conspicuous in RIF group. Significant differences between the groups with respect to glandular epithelium were found. Endometrial tissues of both fertile women and women with RIF were thought to be important in regulating the stage for embryo implantation. Further studies should be done to have a better understanding of factors involved in the establishment of endometrial receptivity.

Key words: Endometrial tissue, endometrial receptivity, recurrent implantation failure, electron microscopy, MUC1

1. Introduction

Implantation occurs after embryo reaches the endometrium following insemination and sheds of zona pellucid. In human, embryo implantation process begins about 6 days after ovulation when it becomes blastocyst. According to the results obtained from in vitro fertilization (IVF) cycles, human embryo gains the implantation ability when it is composed of 6-8 cells unlike other living species. Endometrium and embryo prepare for the implantation by means of endocrine, autocrine and paracrine messages before implantation really takes place (1). In endometrium, series of various structural, cellular and molecular events are controlled by implantation window and as a consequence, these elements are required to achieve endometrial receptivity (2). Molecular events necessary for adhesive interaction between endometrial epithelium and blastocyst and for blastocyst penetration into stroma should emerge concurrently. Blastocysts can interact with endometrial epithelium only in the period of implantation window (3). Determining factors are the effects of progesterone secreted by corpus luteum on endometrium, and slight estrogen peak observed on the 4th day of pregnancy following these events (2,4). Insufficient implantation is one of the important factors that restrict success of in vitro fertilization (IVF). A blastocyst needs to interact with endometrium which gains receptivity for the beginning of implantation (5). Success of IVF depends on the concordant

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occurrence of these events. It is suggested that the underlying causes of the RIF are the problems related to embryo and endometrium (6).

Although the etiology of RIF has not been completely elucidated yet, it is thought that there may be multiple factors causing it. The etiology of RIF can be examined in three different groups such as problems related to uterus, endometrium and tuba uterina, embryo defects, and other factors. RIF can be defined as failure to achieve pregnancy in three consecutive IVF- intra cytoplasmic sperm injection (ICSI) and embryo transfer (ET) cycles or as failure to achieve pregnancy following a total of 10 good quality embryos transfer (7). The studies conducted so far have shown that both endometrium and the quality of oocyst/embryo are among the major factors that affect implantation and pregnancy rates (8). Implantation failure, one of the important unsolved problems of reproductive medicine, is thought to be caused by inadequate endometrial receptivity (9). Preconditions for successful implantation include molecular interactions playing an important role in both blastocyst implantation and preparation of a receptive endometrium and as well as the complicated cascade (10). The related molecules can be listed as cytokines, growth factors, matrix metalloproteinases (MMPs), adhesion molecules, extracellular matrix components, and as well as homeobox element-containing genes (11).

Among these numerous molecules, mucins are considered as one of the important molecules for embryo. Moreover among 14 cloned human mucins, only MUC1 and MUC6 (in relatively few quantities) have been detected in human endometrium (12). MUC1 which extends beyond the glycocalyx is probably the first molecule with which embryo encounters while approaching to the endometrium. The interesting thing is that endometrial MUC1 makes us to think the possibility that it is a molecule repelling the blastocyst until it finds the correct time and place for implantation. However, the distribution and regulation of MUC1 vary through the menstrual cycle and among species (13).

2. Materials and methods

Twenty-three patients with RIF and 11 fertile women were included in our study. 23 patients consulting to Centre of Assisted Reproductive Techniques (ART) and Preimplantation Genetic Diagnosis (PGD) of Istanbul Memorial Hospital and diagnosed with RIF indication were defined as case group and endometrial biopsy samples were taken from these individuals. In addition, 11 people consulting to Centre of ART and PGD of Istanbul Memorial Hospital for various complaints were defined as the control group and biopsy samples of these individuals were also taken. Before taking these samples, individuals were clearly and completely informed and their consents were taken.

2. 1. Tissue Processing for Electron Microscopy

Tissues for electron microscopic examination were sensitively washed several times with Early's Balanced Salt Solution (EBSS) including 100 IU/ml penicillin, 0.1 mg/ml streptomycin and 0.25µg/ml amphotericin so as to be cleared from blood, mucus and exudate. After that the tissues were immediately placed in 2.5% glutaraldehyde buffered at pH 7.4 with Millonig phosphate buffer for three hours. The tissue samples were subsequently fixed in 1% osmic acid for two hours. The tissue pieces were then dehydrated in graded ethanols, embedded in araldite and processed for electron microscopy using conventional methods. The thin sections were taken by using ultramicrotome (Leica Ultracut), stained with uranyl acetate and lead citrate and examined with the JEOL 1011 Transmission Electron Microscope (Japan).

2. 2. Immunohistochemistry for MUC1

MUC1 was used for the immunohistochemical assessment of the tissues. Tissue sections put into microscope slides were kept at 55°C for a night and were subjected to deparaffinization and dehydration processes and then were washed with distilled water and phosphate salt buffer (PSB; pH: 7.2 - 7.4). Tissue sections in sodium citrate buffer (2.94 gram of trisodium citrate and 22 ml of HCI in 978 ml of dH2O; 0.01M, pH: 6.0) were processed in a microwave oven at 750 W (2 X 3 min) in order to avoid antigenic masking. Hydrogen peroxide (Biogenex HK 111-5K, 3%) was applied after being passed through PSB. The IGF-1 (NHP) and bFGF (F3393, Sigma) sections washed in PSB were incubated with primary antibodies for one night (in humid environment; at room temperature). Both secondary antibody with biotin (Anti-MUC1/episialin 0.5-652, Millipore) and streptavidin-peroxidase complex (Anti-MUC1/episialin, 0.5-652, Millipore) were respectively applied to the sections passed through PSB and each process lasted for 30 min. The sections that were re-passed through PSB were treated with 3.3' diaminobenzidine tetrahydrochloride (DAB-Kit, 00-2020, Zymed) for 2 to 5 minutes. The sections washed with distilled water were monitored. And then immunohistochemical control sections were only waited in PSB without treating with primary

antibody. Finally the sections were examined with a light microscop (Nikon Eclipse 80i).

Immunoreactivity intensity detected and assessed (half quantitatively) by the same author was classified into groups such as 0.5 (very low), 1 (low), 2 (moderate), 3 (high). Immunoreactive cell percentage was calculated by taking 300 cells into account. The scoring system was as follows: no immunoreactive cell, score: 0; 10% positive cell, score: 1; between 10-50 % score: 2; between 51-80 %, score: 3; 80 % and over, score: 4. According to the literature, Immuno-histochemical Scoring (IHS) was obtained by multiplying the values "the immunoreactive cell number score" and half quantitative immunoreactive intensity (14,15).

Kolmogorov-Smirnov Test was used for the compliance of the groups with the normal distribution while Student t test was used for two groups comparison.

3. Results

3. 1. Electron Microscopy of Endometrial Tissues of Fertile Group and RIF Group

Endometrial surface epithelial cells of the fertile group were disclosed cytoplasmic extensions (called pinopodes) containing secretory vacuoles. As a secretory material, pinopode pieces unconnected with the cells were found in lumen. It was observed that secretory vacuoles existed more intensely in the supranuclear regions. It was remarkable that vacuoles in supranuclear regions and as well as in pinopodes were larger compared to the others and that these vacuoles were combined to form huge vacuoles (Fig. 1a).

On the other hand, it was observed in the endometrial tissue samples of RIF group that luminal epithelium of endometrium is comprised of lower cylindrical cells. These cells, which were contrasted with uranyl acetate and lead citrate, were recognized to be relatively darkly stained. A great number of irregular microvilli were detected in the apical surface of most of the cells. Moreover, among these cells, cells with cilia were also detected but they were less in numbers. In some of the cells with microvilli, the formation of cytoplasmic extensions towards lumen was monitored. It was observed that healthy pinopod structure could not be achieved, although secretory vacuoles existed in the areas close to the lumen. Furthermore, pinopod-like structures were detected in the lumen. It was also found that secretory vacuoles were slightly stained. Randomly distributed secretory vacuoles were detected in the cytoplasms of surface epithelial cells. Expansions close to the basal surface were observed between the cells (Fig. 1b).

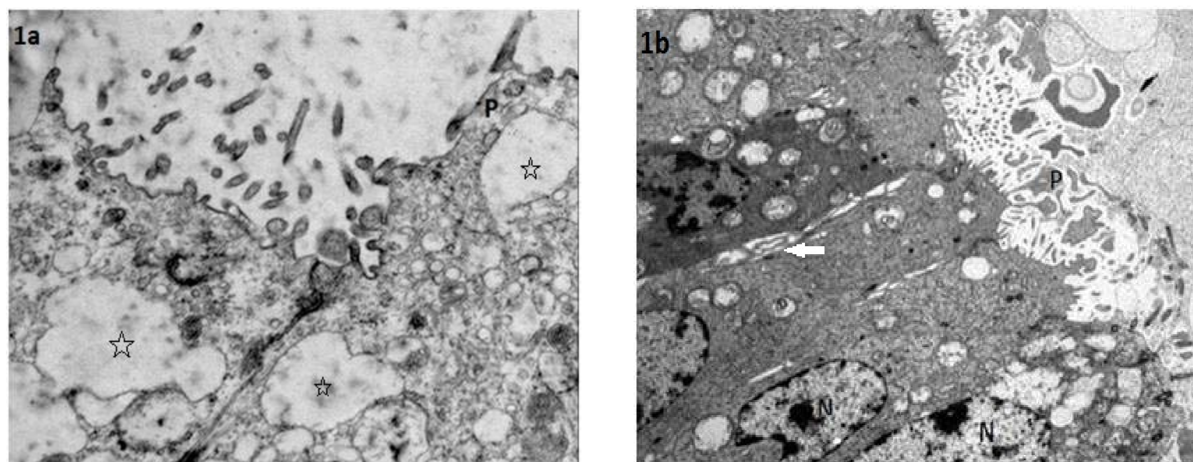

Fig. 1. Electron microscopy of the endometrial tissue samples of fertile (a) and the RIF group (b). In the epithelium of fertile group, a portion of pinopod was seen in the endometrium lumen (X10,000). Pinopod parts of the epithelium in the RIF group was seen in the endometrium lumen. Additionly, low cylindrical surface epithelium cells with heterochromatic nuclei (N) were seen in the RIF group (X6000). White arrow; interdigitations between cells, P; pinopod and parts of the pinopod, star; vacuole.

3. 2. Immunohistochemistry of Endometrial Tissues of Fertile Group and RIF Group

During the light microscopic assessment of these groups, in the endometrial epithelial cells of the fertile group, the immunoreactivity of MUC1 was immunohistochemically scored and according to this scoring, the immunoreactivity was assessed. Although, low to moderate staining was observed in the luminal epithelium of endometrial tissue of fertile group according to

MUC1 and IHS scoring (Fig. 2a), staining scores obtained from the glandular epithelium were rather high (Fig. 2b). It was detected in the MUC1 immunoreactivity assessment of RIF that the luminal epithelium of endometrium was highly stained while the glandular epithelium was poorly stained (Fig. 3b).

3. 3. Statistical Analyses

While the low staining was observed in the luminal epithelium of control group, the staining rate detected in the RIF group was relatively higher. In addition to the staining results of the luminal epithelium, staining results obtained from the glandular epithelium lumen also showed difference between the groups. Kolmogorov-Smirnov Test was used to detect the compliance of the groups with the normal distribution and Student t test was used for two groups comparison. When the groups were compared in terms of gland epithelium and lumen epithelium, the p value was detected as $p < 0.05$.

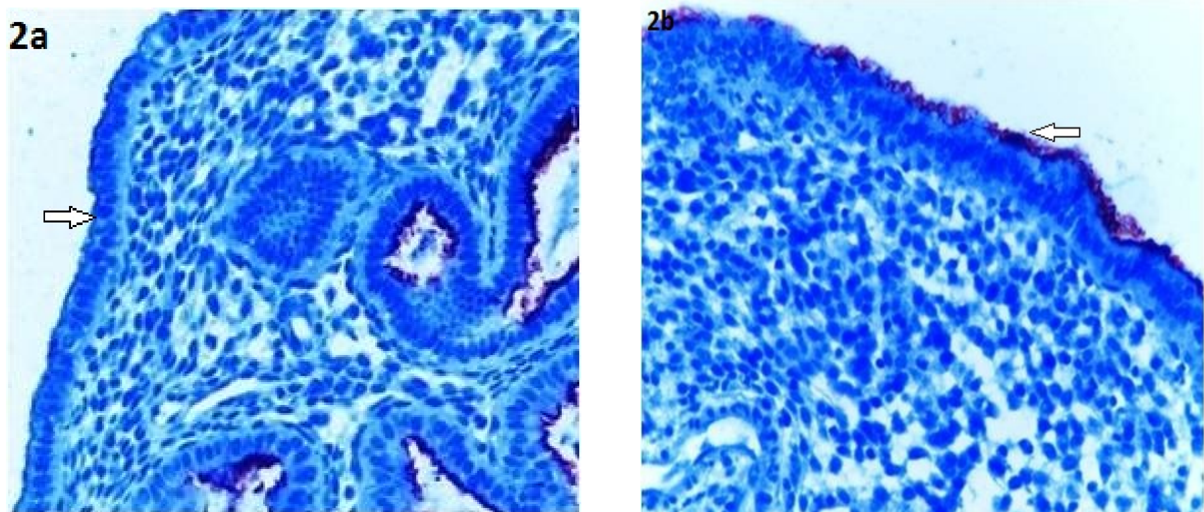

Fig. 2. Endometrial tissue samples from the fertile and RIF group immunohistochemically stained with MUC1 (a). Less staining with MUC1 was observed in the luminal epithelium of the fertile group (b). Luminal epithelium of RIF group was significantly stained. White arrow; Luminal epithelium. X20, Nikon 80i.

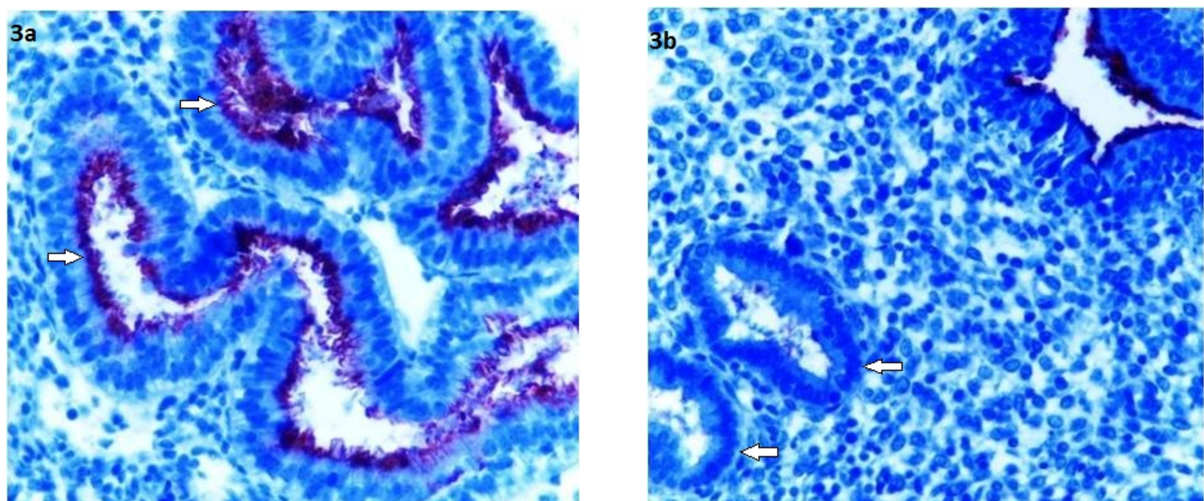

Fig. 3. In fertile and RIF groups, endometrial gland epithelium was observed (a). The gland epithelium of the fertile group was significantly stained with MUC1 (b). Gland epithelium of RIF group in (b) is observed, but there was not staining in the epithelium. White arrow; Gland epithelium. X20, X40. Nikon 80i.

4. Discussion

Recently, experts in Assisted Reproductive Techniques increasingly tend to associate the underlying reason of IVF failures with poor embryo quality whereas in addition to embryologic factors, a number of factors such as poor endometrial receptivity causing implantation

problem can also affect the success of IVF (16). Although implantation is a dynamic process occurring spontaneously between blastocyst and endometrial layers, the priming of endometrium towards the implantation window is of maternal origin. On the contrary, the main reason of the infertility in healthy women is implantation failure, the currently unsolved problem of reproductive medicine. Inadequate endometrial receptivity is responsible for about two thirds of the implantation failure (9). In IVF, RIF is a complex condition and has not been thoroughly and completely understood yet (17). Decline of some anti-adhesive factors such as MUC1 can contribute to the adhesion of these cell lines (3). Masking of adhesive ligands can be taken into account for the blastocyst. A study conducted by Gokcimen and coworkers noted that according to the primer sources of the factors playing roles in the implantation and the implantation stages in which they are most effective, MUC1 existed in endometrium and shows its effect in the first stage of the implantation (18). It was thought that it not only ensured maintaining normal tissue homeostasis but also prevented the infection (3,19). As indicated in the literature by Susan and coworkers MUC1 staining detected in the endometrial luminal epithelium of fertile group was relatively lower (3). When these results were compared with the results obtained from RIF group, p value was detected as $p < 0.05$. Staining rate detected in the luminal epithelium of RIF group was higher. Heavily glycosylated mucins such as MUC1 normally perform the masking function. In this case, real function of MUC1 comes to light. It has been suggested that MUC1 acts as a selective barrier to prevent adhesion of substandard blastocysts to luminal epithelium (20,21). The correlations between MUC1 and MUC4 (one of other mucins) were observed. The appearance of MUC1 is under the control of ovarian steroids as seen in MUC4 (3,20,21). In women, carbohydrate structures such as keratan sulfate, associated with implantation success carried by MUC1. CD44 isoforms are present in peri-implantation of human embryos. CD44 isoforms could form bridging ligands interacting with the abundant sialylated and sulfated carbohydrates on the apical surface of human and murine luminal epithelium (22).

As the blastocysts were examined, it was reported that ICAM-1 existed in human embryo and adhered to MUC1. Human MUC1 has the characteristic of turning into an adhesion molecule via carbohydrate epitopes. At the same time it might block interaction with other cell adhesion molecules on substandard blastocysts lacking lectin type receptors. In addition to this inhibiting function, adhesion increases as MUC1 is removed from the cell surface. Co-culture of human blastocyst with endometrial epithelium shows that MUC1 locally disappears next to the blastocyst connecting to the cell surface (3). Considering the endometrium of fertile group, we observed a decline of MUC1 in luminal epithelium in our study. However, the observations made in RIF group showed that the presence of MUC1 in this area could be clearly seen. Contrary to our findings, some researchers claim that MUC1 exists at high levels during the period of peri-implantation (21).

As embryo approaches to luminal epithelial surface, it encounters mucinous and glycocalyx layer (23). Among the mucins which form anti-adhesive molecules in this layer, MUC1 is the most important. Mucins are a glycoprotein family existing in the surfaces of human epithelial cells. In human, they are present at high levels during the peri-implantation period (21,24). High levels of MUC1 were detected in the blastocyst during the peri-implantation period. Moreover, MUC1 is thought to play a role in protecting blastocyst from other preventive factors. Alternatively, it can include a structure that has the ability to identify the embryo. It can also be understood from the decreased MUC1 levels of women who have experienced recurrent miscarriage that this molecule also plays an important role in the period of early pregnancy (25).

In women, during the receptive phase, MUC1 is strongly expressed at the epithelial apical cell surface and in uterine secretions, although the pattern of its glycosylation changes during the menstrual cycle (26-28). It has been suggested to act as a selective barrier to prevent adhesion of substandard blastocysts to luminal epithelium (20). In support of this idea, when human embryos are allowed to attach to endometrial epithelial cell monolayers, MUC1 expression disappears from the area around the attached blastocyst, suggesting that the human embryo may play an active role in MUC1 removal at implantation (21).

The results achieved in our study also supports this information obtained from the literature. While decreased MUC1 levels was observed in endometrial luminal epithelium of fertile women, MUC1 levels observed in RIF group were evident (Figs. 2a and 2b). Human endometrial MUC1 was found to be up-regulated during the peri-implantation period (26,29). Indeed both MUC1 mRNA and protein show and several fold increase from the proliferative to the mid-secretory phase (30). This finding presents a

paradox; one would expect inhibitory factors to decrease during implantation, as was described in other species. It was suggested that human require a locally acting mechanism for the removal of the MUC1 barrier to the implanting embryo. Immunohistochemistry on human endometrium, using monoclonal antibodies against the MUC1 ectodomain, could not detect noticeable variations in its localization on the apical surface of epithelial cells (31). Nevertheless, scanning electron microscopy combined with immunohistochemistry has succeeded in precisely consigning the MUC1 epitope only to ciliated cells. In contrast, MUC1 was missing from the surface of non-ciliated cells and from uterine pinopods (32). Meseguer et al. (33) suggest that the importance of pinopods is to supply an area, free of the widespread MUC1 inhibition to embryo–endometrial interaction. Indeed, human in vitro implantation models indicate that MUC1 is lost at the site of embryo attachment. As a result of examination of tissue sections with TEM, we can conclude that pinopode structures were evident in fertile group and that pinopode formation did not occur but pinopode-like structures were present in RIF group (Figs. 1a and 1b). The p value was found to be <0.05. Moreover, the unevaluated parameters in literature such as MUC1 levels in glandular epithelia and immunoreactivity were evaluated and these parameters of fertile and RIF groups were compared. In the comparison made between the two groups in terms of luminal epithelium and glandular epithelium, a paradox result was achieved. In the glandular epithelium of fertile group MUC1 could be clearly seen contrary to the luminal epithelium. The staining detected in the glandular epithelium of RIF group was unobvious and poor (Figs. 3a and 3b). By means of statistical analysis of data obtained with immune scoring, a significant p value such as < 0.05 was detected. According to the result obtained from the immunohistochemical assessment in glandular epithelium, we may conclude that endometrial tissues of fertile group get ready for the implantation by maintaining or increasing the level of MUC1 in its glandular structure. On the other hand it was found in the RIF group that the anti-adhesive molecule MUC1 only poorly existed in glandular epithelium despite its intense presence in luminal epithelium of the same group. This makes possible to detect the localization of MUC1 by means of immunohistochemical stainings. It should be kept in mind that there may be other factors that can impact the cell MUC1 stability except for MUC (34). Another factor affecting the implantation success can be that human MUC1 gene shows polymorphism including various numbers of tandem repeat regions (35).

We speculated that MUC1 extends beyond the glycocalyx covering the endometrium and is the first molecule that meets the blastocyst entering the endometrial epithelium. As detailed above, MUC1 is widely expressed throughout the endometrium and, surprisingly, even increases before implantation. Furthermore to prevent the blastocyst from adhering to an area with poor chances of implantation, an important role is played by the repellent activity of MUC1. MUC1 appears to be a negative factor for embryo implantation. Indeed, in the area where implantation takes place, MUC1 disappears and endometrial MUC1 increases at the time of implantation, this factor has a crucial role to direct the embryo temporally and spatially to effective implantation. The mechanism by which MUC1 is removed is not entirely clear. Further research will better define its precise role in human embryo implantation failure or success.

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