REPRODUCTIVE PHYSIOLOGY AND DISEASE

Association between endometrial senescent cells and immune cells in women with repeated implantation failure

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Abstract

Purpose The aim of this study was to compare women with recurrent implantation failure (RIF) and control group in terms of the associations between p16-positive senescent cells and certain types of immune cells in human endometrium during the mid-luteal phase

Methods Immunohistochemical staining was performed in 116 endometrial biopsies taken from 57 women presenting RIF, and control group of 59 women who became pregnant after the first intracytoplasmic sperm injection. Endometrial tissue sections were stained immunohistochemically for p16 (Senescent cells), CD4 (T-helpers), CD8 (T-killers), CD14 (Monocytes), CD68 (Macrophages), CD56 (Natural killers), and CD79α (B-cells). The percentage of positively stained cells for each marker was calculated by HALO image analysis software. The quantity and the relationship between senescent cells and immune cells were assessed and compared between the two groups.

Results The correlation coefficient was highest between senescent cells and CD4+ cells and was lowest between senescent cells and CD14+ cells in RIF women, similarly to the control group. However, most of the observed correlations among senescent and immune cells weaken notably or disappear in the RIF group. When comparing senescent cell-to-immune cell quantitative ratios, only p16+/CD4+ cell ratio was significantly higher in RIF women as compared with patients from the control group.

Conclusion Our study indicates that the quantity of senescent cells in human endometrium during the mid-luteal phase has the strongest association with the amount of T helpers. Moreover, the specificity of this association might have an important impact on the occurrence of RIF.

Keywords (MeSH terms): Cellular senescence · immune cells · endometrium · RIF

Introduction

Nowadays, infertility tends to affect from 9% to 15% of couples of reproductive age worldwide [1, 2]. In Europe, the number of annual treatment cycles in ART clinics has increased from two hundred thousand to more than one million during the last twenty years [3]. The pregnancy rates per transfer have also increased during this period of time from 25% to 35% in the autologous cycles and even to 50% in the egg donation programs [3]. This was possible thanks to the development of assisted reproduction techniques and the improvement of diagnostics and management of infertility [4, 5]. But still, the implantation of the human embryo remains the key limiting factor for successful in-vitro fertilization (IVF) and embryo transfer [6, 7].

Recurrent implantation failure (RIF) refers to cases where women have had three or more consecutive failed in IVF attempts with at least four high-quality embryos in at least three fresh or frozen cycles [8]. It was estimated that 15% of the patients under infertility treatment experience RIF

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[9]. Although the multifactorial pathogenesis of RIF remains unclear, uterine factors are still under investigation [10].

Cellular senescence is a specific state of permanent cell cycle arrest triggered in normal cells in response to various signals [11]. Several studies have suggested a link between endometrial senescence and RIF [12, 13]. Recently, it was found that p16INK4A-positive senescent cells are linked not only to endometrial receptivity and the abundance of uNK cells [11], but also that their relative quantity could be used as a potential biomarker for implantation and miscarriage [12, 13]. An increased number of stromal senescent cells is associated with an increased risk of miscarriage [14], while the decreased number of senescent cells in the luminal epithelium is related to impaired endometrial receptivity [12]. Dysregulation of endometrial immune cells is also strongly associated with implantation failure and miscarriage [15, 16]. In humans, certain changes in the localization, phenotypes or abundance of decidual endometrial macrophages could lead to inappropriate trophoblast invasion [17] and implantation failure [18]. Inadequate uNK cell function, cytokine expression and quantity also have been implicated in recurrent miscarriage (RM) and recurrent implantation failure (RIF) [19]. In addition to innate immune cells, CD4+ and CD8+ T lymphocytes and B cells have changed amounts in patients with RM and RIF [20, 21].

Although the effect of immune profile and cellular senescence on reproduction has been studied in detail, there is scarce data about the quantitative association of senescent and immune cells in human endometrium and its effect on RIF. The purpose of this study was to analyze the association between p16-positive senescent cells and certain types of immune cells in the human endometrial stroma during the mid-luteal phase of the cycle in women with RIF and to assess the impact of this relationship on the implantation outcome by comparison with a fertile control group.

Materials and Methods

Experimental design and human endometrial biopsies

It could be hypothesized that the percentage of p16-positive senescent cells in the endometrial stroma could be associated with the percentage of particular types of immune cells in the endometrial stroma, which could have an effect on implantation success or failure. Therefore, study's objective was to analyze and compare the quantity of stromal senescent cells and immune cells in the functional layer of the endometrium in RIF patients and a control group.

The endometrial biopsies were taken from 57 women presenting RIF and a control group of 59 women, using a Pipelle endometrial suction 7 days after luteinizing hormone (LH) surge in a natural cycle. A daily luteinizing hormone urine test (Blue Dream, Wondfo Biotech Ltd., China) was performed from day 9 of the menstrual cycle onward to identify the LH surge. RIF was defined as failure to achieve pregnancy after three or more consecutive IVF attempts in which one to three embryos of high-grade quality were transferred in each cycle [22]. The inclusion criteria were women aged 32-40, with regular ovulatory cycles and no hormonal therapy for at least three months who subsequently underwent embryo transfer with euploid embryos. Only women who had become pregnant within three months after the biopsy and following embryo transfer of good quality day-5 euploid blastocysts were included in the control group. The exclusion criteria for all patients were male factor infertility, postmenopausal status, history of recent inflammatory disease, chronic endometritis diagnosed by CD138 staining, recent antibiotic treatment, endocrinological disorders, genetic disorders, autoimmune diseases, oncological diseases, including endometrial cancer, moderate or severe endometriosis, adenomyosis, uterine hyperplasia, endometrial polyps, and presence of fibroids.

Immunohistochemistry method

Immunohistochemical fixation and staining were performed as previously described (12). The sections were incubated with antibodies against human p16INK4 (MAD-000690QD-7, Master Diagnostica, Granada, Spain) (senescent cell marker), CD4 (Ready-to-use, IS64930-2, Dako) (T-helper marker), CD8 (Ready-to-use, 1-CD040-02, Quartett) (T-killer marker), CD14 (diluted 1:200, E-AB-71017, Elabscience) (monocyte marker), CD68 (Ready-to-use, IS61330-2, Dako) (macrophage marker), CD56 (Ready-to-use, A00121-0007, ScyTek) (uNK cell marker), CD79α (Ready-to-use, IS62130-2, Dako) (B-cell marker), respectively.

Staining was assessed by using Olympus CKX41 inverted microscope (Olympus, Melville, NY, USA), and the images were captured at 200x magnification with an EP50 Microscope Digital Camera (Olympus Europa SE & Co. KG, Hamburg, Germany). The percentage of positive cells and the number of endometrial stromal cells in the selected tissue compartments were assessed using computer-assisted image analysis with HALO Image analysis platform (version 3.4, IndicaLabs, Inc., Corrales, NM) for all images.

The number of positively stained cells was counted in the same 3 tissue sections per slide for all studied immunohistochemical markers. Then the percentage of stained cells from the stromal population was calculated by dividing their number by the number of stromal cells expressing nuclear hematoxylin stain.

Statistical analysis

Data were analyzed using SPSS statistical software, version 21.0 (SPSS, Chicago, IL, USA). Continuous data are expressed as mean and standard deviation (SD) for normally distributed data; otherwise, median and range are reported. The Paired t-test or Wilcoxon signed-rank test were applied according to normal or not normal distribution of data. A Bonferroni correction was applied to the significant levels obtained to show whether the observed significant differences may have occurred due to multiple analysis. The relationship between the percentage of senescent cells in the endometrial stroma and the percentage of immune cells was analyzed with Spearman's rank-order correlation coefficient (ρ). The significance level was set at $P < 0.05$.

Results

Study population and baseline characteristics

Table 1 presents the baseline characteristics of the studied RIF women and control women with good fertility prospects. As shown in Table 1, age, body mass index (BMI), serum progesterone and estradiol levels, and the indication for IVF were similar between women in the two study groups.

Quantity of senescent and immune cells in endometrial stroma

The percentage of senescent cells and immune cells did not differ significantly between the studied groups (p>0.05) (Table 2).

Table 1. Baseline characteristics of the patient groups.

Variables	RIF group (n = 57)	Control group (n = 59)	P value
Age, years	36.2 (6.1)	34.6 (4.2)	0.18
Body mass index (BMI), kg/m^2	23.9 (2.3)	22.8 (3.1)	0.66
Serum progesterone, ng/mL	17.2 (9.7)	15.2 (8.3)	0.39
Serum estradiol, pg/mL	151.3 (74.5)	144.9 (72.8)	0.55
Indication of IVF			
Tubal factor infertility	42%	44%	
Unexplained infertility	58%	56%	
Number of previous IVF attempts	4.1 (1.8)	0	<0.01
Number of implantation failure	4.1 (1.8)	0	<0.01

Data are presented as mean (standard deviation).

RIF, recurrent implantation failure.

Senescent to immune cells coefficients

The analysis of senescent to immune cell coefficients revealed that in both groups of women p16/CD79a and p16/CD4 have significantly higher values than the other senescent/immune cell ratios (p<0.05). Only the p16/CD4 ratio in endometrial stroma was significantly lower in the control group as compared with the RIF women (Table 2). In addition, the range was nearly twice lower in the group with successful implantation. There were no significant differences in the other senescent to immune cell ratios between the women with RIF and the control group (p>0.05). However, the range in most senescent to immune cells ratios was wider in the RIF women group. An exception from this tendency was p16+ to CD8+ ratio and p16+ to CD79a+ ratio, which showed a higher range in the control group (Table 2).

Relationship between p16-positive senescent cells and immune cells

The correlation analysis was performed separately for the two studied groups of patients. In the control group, a significant positive correlation was found between the percentage of stromal senescent p16-positive cells and all of the studied seven types of immune cells (P<0.05). The strongest correlation was found between p16-positive cells and CD4-positive cells (r=0.51), followed by CD8-positive cells (r=0.47), CD56-positive cells (r=0.33), CD79α-positive cells (r=0.33), and CD68-positive cells (r=0.34). Furthermore, relatively low positive correlation coefficients were observed between p-16 positive cells and CD14-positive cells (r=0.27).

The correlations between senescent cells and immune cells were weaker in the RIF group than in the control group (Figure 1). Moreover, we did not find a significant correlation between p16+ cells and CD68+ macrophages, CD14+ monocytes, and CD79a+ B cells in the patients with RIF. An exception was the correlation coefficient between senescent cells and CD56+ NK cells which was lower in the control group.

Discussion

For clinicians and patients, another implantation failure in women with RIF, especially when euploid, high-quality embryos are transferred, leads to negative effects or even could cause pathological conditions [23]. The abnormal immune and senescent cellular profile observed in RIF women is characterized by the presence of specific pro-inflammatory cytokines (IL-1A, IL-6, IFNg and others) as a result of the impaired balance of cytotoxic immune cell types and senescent cells and a decrease in the regulatory

Table 2. Percentage of senescent and immune cells and senescent/immune cell ratios in the endometrial stroma in the studied groups of women

Study population	RIF group (n = 57)	Control group (n = 59)	P value
p16+ cells (Senescent cells), %	0.36 (0.04-5.57)	0.24 (0-12.65)	0.33
CD4+ cells (T-helpers), %	0.15 (0.01-2.36)	0.21 (0–4.05)	0.14
CD8+ cells (T-killers), %	0.37 (0.10-9.85)	0.41 (0.02-7.62)	0.77
CD56+ cells (NK cells), %	0.47 (0.11-13.15)	0.43 (0.11-9.41)	0.95
CD14+ cells (Monocytes), %	0.59 (0.04-17.14)	0.79 (0.25-12.75)	0.07
CD68+ cells (Macrophages), %	0.65 (0.01-12.46)	0.53 (0.08-14.44)	0.32
CD79α+ cells (B-cells), %	0.06 (0.01-5.57)	0.05 (0 – 3.07)	0.17
Senescent / immune cell ratios			
p16+/CD4+	2.29(0.21-25.51)	1.25(0.04-11.81)	0.04*
p16+/CD8+	0.91(0.06-3.69)	0.73(0-5.98)	0.77
p16+/CD14+	0.48(0.05-8.56)	0.34(0-3.47)	0.11
p16+/CD68+	0.46(0.04-118.43)	0.60(0-4.66)	0.90
p16+/CD56+	0.78(0.06-4.11)	0.53(0-11)	0.24
p16+/CD79α+	4.65(0.15-51.81)	4.77(0-119.27)	0.32

*Bonferroni correction was used when the results were significantly different (p<0.05)

Data are presented as median and range (Min-Max).

RIF, recurrent implantation failure.

cells [24–27]. Because RIF is a condition associated with quantitative changes of senescent and immune cells in human endometrium, the association among these cell populations could also be altered by RIF. The ability to identify correctly and subsequently to treat and modify the observed abnormal endometrial cell–to-cell balance and relationship could improve assisted reproduction technologies and lead to an increased pregnancy rate in this problematic group of patients.

The quantitative analysis revealed that the number of stromal senescent cells and immune cells in the functional layer of the endometrium of RIF women is similar to that of women with implantation success. In addition, except for the p16/CD4 cell ratio, the other senescent-to-immune cell ratios did not differ significantly between the studied groups. It could be suggested that the endometrial immune and senescent profile change in the RIF patients concerns mainly the physiological state of immune and senescent cells, such as their activation and maturation rather than their relative quantities [28]. However, the existing balance between the quantities of senescent cells and immune cells becomes more unstable (with higher fluctuations) in the RIF group and shows a significant change in the equilibrium between T helpers and senescent cells (p16/CD4 cell ratio). That means that T helpers could become insufficient in patients with unsuccessful implantation. This might not ensure proper macrophage and T-killer-dependent elimination of senescent cells during the process of implantation and especially during the invasion of endometrial stroma by trophoblast cells. Moreover, CD4+ T helper cells retain the strongest correlation with senescent cells

in comparison to the other immune cells in RIF women. It might be suggested that T helpers are the main link between the senescent cells and the other immune cell subtypes. Subpopulations of T helpers produce IL-4, IL-5, IL-13, IL-10, and promote the recruitment of immune cells to the target areas [29], while other subpopulations secrete IFN-gamma, IL-2, and TNF-alpha, and promote activation of T killers and macrophages, which supports viral and probably senescent clearance [30]. A previous study showed that proper macrophage elimination of senescent cells relies on CD4+ T helper cells [31]. It has been demonstrated that the depletion of T helpers results in a defect in the recruitment of CD8+ T killers to sites of infection and delayed viral clearance [32]. Recently, it was hypothesized that senescent cells and their secretome, that includes TGF-beta, are one of the main contributors to age-related abnormal T helpers' differentiation, migration and expansion [33]. It might be suggested that in RIF women with relatively high amounts of p16+ senescent cells, not only the functionality but also the quantity of T helpers is reduced and thus becomes insufficient to ensure a normal immune cell profile.

Studies have shown that, in response to inflammatory signals released by decidual cells and even by senescent cells, NK cells are recruited to tissue regions with senescent cells [11, 34]. NKG2D ligands, available on the senescent cell surface, bind to NKG2D receptors, which are present on the NK cells [35], which leads to senescent cell death by the release of perforin, granzymes, and proteoglycans [36]. However, the inhibitory presence of NKG2A receptors in NK cells may compromise the elimination of senescent cells
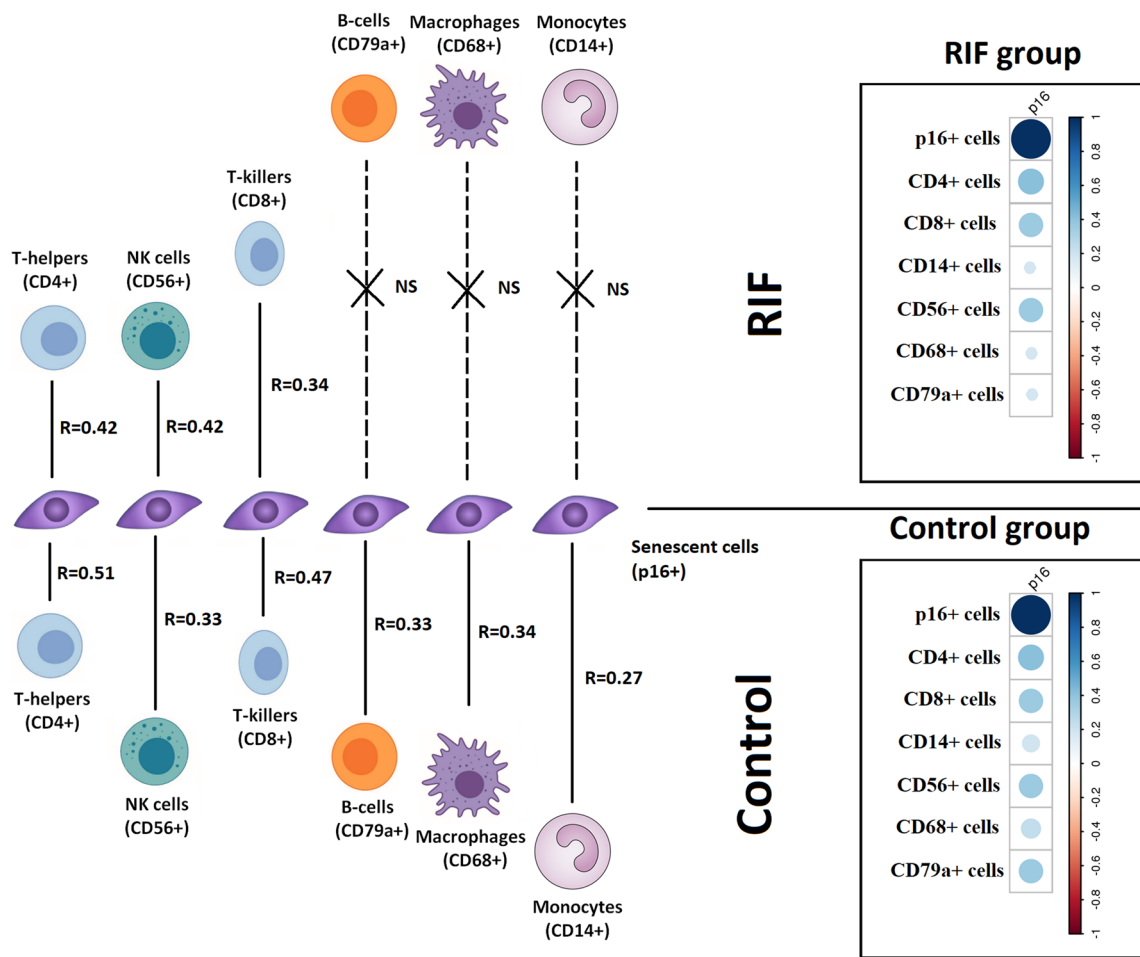
Fig. 1 Correlograms and schematic presentation of correlation between p16-positive cells and immune cells in the functional layer of human endometrium during the mid-luteal phase of RIF patients (top half of the figure) and control group (bottom half of the figure). The length of the lines connecting senescent and immune cells corresponds to the correlation strength (shorter line represents higher correlation coefficient)

* Positive correlations in the correlograms are displayed in blue and negative correlations in red. The color intensity and the size of the circle are proportional to the correlation coefficients. Next to the correlogram, the legend color shows the correlation coefficients and the corresponding colors.

* R – correlation coefficients
* NS – non significant correlation (P>0.05)
* RIF, recurrent implantation failure.

[37] and could be responsible for the observed change in the associations between senescent cells and NK cells in the studied RIF women. Interestingly, in contrast to the other associations, the relationship between NK cells and senescent cells was strengthened in this women group. This finding suggests the establishment of a specific balance between senescent cells and NK cells in the endometrium of women with RIF.

The similar positive associations revealed between the quantities of senescent cells and macrophages, and between the percentage of senescent cells and B cells in endometrial stroma of patients in the control group, disappeared in the RIF group. Macrophages and B cells also have an important cooperative role in senescent cell elimination [31, 38, 39] that might be adversely affected in the endometrium of RIF women. In mouse models, macrophages clear stromal senescent cells located close to the implantation site [40], while B cells activate a macrophage-dependent mechanism of clearance of senescent cells [31]. Senescent cells also participate in this cell-to-cell communication with the secretion of specific cytokines that attract macrophages and could activate their proliferation [41] and/or regulate their maturation from M1 to M2 subtype [42, 43].

The observed loss of correlation between senescent cells and certain types of immune cells in RIF patients might be explained by increased quantitative differences between senescent cells and these immune cells from woman to woman and the increased variation in the senescent/immune cells ratio in patients with implantation failure. The reason for this tendency might be the impaired

balance between senescent cells and immune cells and their altered secretion of pro- and anti-inflammatory cytokines that could play a prominent role in the establishment of normal endometrial receptivity. It was hypothesized that most subtypes of immune cells could recognize and clear senescent cells only in a particular period of time [34]. However, senescent cells could possibly influence immune cell recruitment [34] and attraction [44] and could escape immune clearance by a change in their specific senescence-associated secretory phenotype (SASP) [35, 37]. The existing mechanisms by which senescent cells could change the recruitment of immune cells and escape clearance in the endometrial microenvironment are not fully understood. However, activating such mechanisms would probably lead to an imbalance between senescent cells and immune cells in RIF women that might also be the cause of the observed loss of association.

An inherent limitation of the immunohistochemical approach is the inability to elucidate a clear relation between cell quantities and their in vivo action. Nonetheless, the presence of larger number of cells of a specific type is indicative of their involvement in a physiological process. The function of these cells can be demonstrated indirectly by molecular methods for analysis of their protein secretion profile. Two such well developed methods are enzyme-linked immunospot (ELISpot) assays and multiplex cytometry-based techniques [45]. However, they are not appropriate for the assessment of tissue spatial distribution of immune and senescent cell types nor their cytokine expression. These limitations might be overcome by the application of immunohistochemical and immunofluorescent methods to visualise the cytokine expression but they also have certain drawbacks [46, 47]. These techniques are poorly quantitative, and require indirect immunostaining with multiple labeling steps by using different chromogens in sequential incubations, and careful selection of cytokine-specific antibodies [48].

Numerous challenges in reproductive immunology still exist for an adequate translation of the discussed findings to clinically feasible therapies for treatment of RIF patients. Understanding the existing mechanisms for spatio-temporal balance between senescent cells and immune cells and the relation with their physiological state could provide novel insights into the unexplained reasons for implantation failure. Moreover, the acquired information would offer a variety of new therapeutic avenues for treatment of recurrent implantation failure, based on the improvement of immune-to-senescent cells ratios. Hypothetically, the observed change of relation between senescent cells and T helpers in RIF women and the decreased p16/CD4 ratio in endometrial stroma could be restored by intrauterine administration of activated peripheral blood mononuclear cells (PBMC) [49, 50] in combination with senolytics or SASP inhibitors [51,

52]. However, the effect of this complex treatment has not yet been applied and warrant further examination.

Conclusion

The successful implantation depends on the existence of specific senescent-to-immune cells association and balance that is essential for the establishment of appropriate endometrial receptivity. In the endometrial stroma of women with RIF during the mid-secretory phase, we found a significant change in this balance, expressed as a decreased association between senescent cells and most types of immune cells. In addition, the strongest change in cell-to-cell association was found between senescent cells and T-helpers.

The results from our study support the hypothesis that the imbalance of senescent and immune cell numbers contributes to the heretofore unexplained causes for unsuccessful embryo implantation. The altered relationship between senescent cells and T helpers might be the reason for implantation failure in women with RIF.

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