VCAM-1 is Another Ugly Face of IFN-γ in Women with Recurrent Spontaneous Abortion

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ABSTRACT:

BACKGROUND:

The Th-1 cytokines were well implicated in adversely affecting pregnancy and the mechanism by which they cause pregnancy loss was suggested to be mainly vascular by activation of mediators of inflammation like certain adhesion molecules.

OBJECTIVE:

To study the pathological effect of IFN-γ and VCAM-1 in recurrent pregnancy loss.

PATIENTS AND METHODS:

The study included three groups of women; Group A: patients had recurrent abortion (n=24), Group B: patients had spontaneous abortion for the first time (n=10), Group C: women with elective pregnancy termination (n=6). Curate samples obtained from these women were subjected for *in situ* hybridization technique to determine the *in situ* expression of IFN-γ, and immunohistochemistry analysis to detect the expression of VCAM-1.

RESULTS:

The *in situ* expression of IFN-γ was significantly higher in women with RSA as compared with normal pregnant and first abortion groups (p=0.000 and 0.002) respectively, and the expression of VCAM-1 was also significantly higher (p=0.005) in women with RSA as compared with those who had abortion for the first time, with a positive correlation between the expression of IFN-γ and VCAM-1 (r=0.418; p<0.05) in women with RSA.

CONCLUSION:

The data of this study strengthen the possibility that type-1 immune response may have the upper hand in the pathology of RSA on multidirectional bases including up regulation of the surface expression of VCAM-1.

***KEY WORDS*:** RSA (recurrent spontaneous abortion), VCAM-1, IFN-γ

INTRODUCTION:

Originally identified 30 years ago as an agent with antiviral activity, IFN-γ has since been characterized as a homodimeric glycoprotein with pleiotropic immunologic functions [1,2,3]. IFN-γ is primarily secreted by activated T cells and NK cells, and can promote macrophage activation [3,4]. Both autocrine and paracrine IFN-γ signaling might be present in normal utero-placental tissues and receptors for IFN-γ are expressed on many cells that are present at the implantation sites [5].

In 1995 Th1-type cytokine secretion was observed for the first time in women with RSA, when peripheral blood mononuclear cells were activated by a trophoblast cell line [6]. This finding was also supported by other reporters [7,8,9,10]. Th1-type cytokines (IL-2, TNF-α, IFN-γ) can boost, and Th2-type cytokines (IL-3, IL-4, IL-10) can reduce abortion rate in mice [11]. But the inefficiency of NK cell, macrophage, and Th1-type cytokines in killing trophoblasts led to question the mechanism whereby the cytokines produced their effects. A target other than trophoblasts for cytokines was sought; a maternal vascular target was suggested by pathologic specimens of aborted material that showed hemorrhagic necrosis at the trophoblast-decidual interface [12].

Spontaneous abortions (resorptions) in DBA/2-mated CBA/J female (CBA/J X DBA/2) mice is thought to represent a rejection of the semi-allogeneic feto-placental unit by activated NK cells and activated macrophages [13,14], these cells infiltrate maternal mesometrial decidua at the site of implantation, the frequency of implantation sites

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with such an infiltrate is proportional to the percentage of embryos that resorb [14]. Murine resorptions are characterized by focal necrosis at the junction of the fetal trophoblast with the decidua, an infiltrate with polymorphonuclear leukocytes at sites of necrosis and along the walls of large vessels in the decidua, and by thrombosis and hemorrhage [15,16,17]. There are two main sources of this polymorphonuclear infiltration; firstly, when thrombin is generated, it will activate IL-8 secretion by endothelial cells, IL-8 recruits PMNs [5,18]. Secondly, a chemokine, IFN-γ-inducible protein 10 kDa (IP-10) is stimulated by IFN-γ and recruits PMNs in the early pregnant uterus [19]. In addition, pro-inflammatory cytokines like IFN-γ induce leukocyte-endothelial adhesion molecules and increase the transendothelial migration of the recruited leukocytes [20].

VCAM-1 is a 110 kDa protein binding to the integrin α4β1 very late antigen (VLA)-4 cell surface heterodimer, but its also known to interact weakly with the closely related integrin α4β7 [21,22]. VCAM-1 is a type 1 transmembrane protein belonging to the immunoglobulin superfamily [23]. Structurally, the extracellular amino-terminal portion of the molecule consists of seven immunoglobulin-(Ig) like domains. These Ig-like domains appear to have arisen by internal duplication, as domains I, II, and III are highly homologous to domains IV, V, and VI [24]. Both Ig-domains I and IV are capable of independently interacting with α4 integrins[25]. The binding to domain IV may require activation of the leukocyte integrins [26].

VCAM-1 was originally identified as a cytokine inducible surface protein that mediate adhesion of a number of leukocytes including lymphocytes, monocytes, mast cells, eosinophils, and tumor cells to umbilical vein endothelial cells [27.28]. Proteins for integrin subunits (α4, αv, β1, and β3) and VCAM-1 are expressed within the developing murine placenta, and mRNAs for these specific integrin subunits and VCAM-1 are present in the utero-placental units and placentas [29].

PATIENTS, MATERIALS AND METHODS:

Patients were collected from Al-Kadhmya and Al-Ulwiya teaching hospitals in Baghdad, and were divided into three groups; Group A: 24 pregnant ladies presented with incomplete first trimester abortion, all of whom gave a history of previous 3-6 consecutive first trimester abortions, with no medical diseases, family history of genetic diseases or uterine anatomical anomaly, also all of them were negative for acute infection with rubella, HCMV and toxoplasmosis. Group B: 10 pregnant ladies presented with incomplete first trimester abortion and had at least three previous normal pregnancies with no previous abortion, and no history of any medical illness. And Group C: 6 pregnant ladies with elective termination of pregnancy in the first trimester for a maternal indication under approved consent of two senior gynecologists and a physician. Curate samples of the materno-fetal interface were taken from all these women at the end of evacuation curate operation, samples were embedded in paraffin and subjected for *in situ* hybridization technique.

***In situ* Hybridization:** For *in situ* hybridization technique (ISH), DNA Probe Hybridization/Detection System In situ kit (Maxim Biotech, Inc., USA) was used. Kit contents included: biotinylated housekeeping gene probe, hybridization solution (ready to use), protein block, detergent wash buffer, RNase A (15 µg/ ml), streptavidin-AP conjugate, substrate (BCIP/NBT), and lyophilized proteinase K (4 mg); which is dissolved in a 2 ml DNase and RNase free dilution buffer to form 10X proteinase K, then diluted by deionized water to 1X proteinase K. The probe was biotin-labeled DNA probe for human IFN-γ (249 bp), (Maxim Biotech, Inc., USA).

Tissue sections were deparaffinized in xylene and rehydrated through a series of ethanol dilutions. After digestion with 1X proteinase K at 37°C,hybridization was carried out by applying 10 µl hybridization mixture (0.8 µl of biotin-labeled DNA probe diluted in 9.2 µl hybridization solution) per slide. After overnight incubation, the slides were soaked detergent wash. The biotin-labeled hybrids were detected with streptavidin-alkaline-phosphatase conjugate, and an enzyme-substrate chromogen (bromo-chloro-indolyl-phosphate/ in nitro-blue-tetrazolium salt) BCIP/NBT, yielding an intense blue-black signal appears at the specific site of the hybridized probe. The slides were counterstained with nuclear fast red stain, for more details refer to reference [30].

Evaluation of ISH signal:

The expression of IFN-γ mRNA was measured by counting the number of positive decidual and trophoblastic cells, which gave a blue-black (BCIP/NBT) nuclear staining under the light microscope. The extent of the ISH signal in the villi was determined in 10 fields (X100 magnification). In each field the total number of villi were counted and the extent of nuclear staining of the cytotrophoblast and syncytiotrophoblast

in a given villous was graded as 3, (75–100%); 2, (25–75%); or 1, (<25%). The total staining score was divided by the number of whole villi per field in 10 fields. These scores (between 1 and 3) were added for each field, and a score between 10 and 30 was gained for each sample [30]. To compare with VCAM-1 immunostaining; the percentage of positively stained villi was calculated for each case by taking the mean of the percentages of the positively stained villi in the 10 fields.

Immunohistochemistry: Using DakoCytomation LSAB2 System- HRP code KO673 (DakoCytomation, USA), Immunohistochemistry detection kit, contains: Peroxidase Block, Biotinylated Link, Strepavidin-HRP (horseradish peroxidase), and Substrate DAB Chromogen (3,3`-diaminobenzidine in a chromogen solution). The monoclonal antibodies are listed in the table.

Tissue sections were deparaffinized in xylene for 5 minutes and rehydrated through a series of ethanol dilutions, after retrieval of the tissue antigen in a citrate buffer under 121°C in the autoclave for two minutes, the sections were washed in distilled water, and then endogenous peroxidase was blocked using peroxidase block for 30 minutes [23], 100µl of the diluted primary (monoclonal) antibody was applied onto the sections and incubated overnight at 37°C, then washed in a phosphate buffer saline (PBS) bath for 5 minutes, followed by applying the biotinylated link (secondary antibody) and incubation at 37°C for one hour, and then the same washing in PBS was performed, followed by placing 1-2 drops of the Strepavidin-HRP conjugate onto the tissue sections and incubation at 37°C for one hour then washing, and then the DAB substrate chromogen was applied for 10-20 minutes yielding a brown signal at the antigenic site, followed by counterstaining with Mayer's hematoxylin. Negative controls were obtained by omitting the monoclonal antibody and using antibody diluent alone to verify the signal specificity.

Monoclonal antibodies included in the study

Antibody type	Dilution	Source	Specificity
VCAM-1	1:50	DAKO Denmark	Antibody clustered as anti-CD106 molecule
Von Willebrand Factor (vWF)	1:50	DAKO Denmark	Endothelial cells Megacaryocytes and Megacaryoblasts

The expression of VCAM-1 on endothelial cells was evaluated by counting the total number of vWF positive blood vessels, then counting the total number of VCAM-1 positive blood vessels for each case using (X100 magnification), and then the percentage VCAM-1 positive vessels in each case was calculated as follows:

$$\text{The percentage of VCAM-1 positive vessels} = \frac{\text{The total number of VCAM-1 positive vessels X 100}}{\text{Total number of vWF positive vessels}}$$

For each slide, the blood vessels were counted in the entire tissue section, and any brown-staining endothelial cell or endothelial cell cluster that was clearly separated from adjacent vessels was considered as a single, countable vessel. Vessel lumens were not necessary for a structure to be defined as a vessel, and red blood cells were not used to define a vessel lumen. Branching structures were counted as a single vessel [31].

Statistics:

ANOVA test was used to determine the difference in the expression of IFN-γ or VCAM-1 among the three groups, and the relationship between these two parameters was measured using the correlation coefficient (r). Values of $p<0.05$ were considered as statistically significant.

RESULTS:

The expression of IFN-γ was detected by ISH technique, while VCAM-1 expression was studied using immunohistochemistry analysis, tables (1) and (2) show the percentages of the *in situ* expression of IFN-γ and VCAM-1 surface expression respectively in terms of mean ± SE, median, minimum and maximum values of the three groups. Tables (3) and (4) show the difference in the expression of IFN-γ and VCAM-1 among the three groups and within the groups respectively using ANOVA analysis.

The study demonstrates a highly significant positive correlation between the *in* situ expression of IFN-γ and VCAM-1 in the RSA group ($r=0.418$, $p<0.05$), while there was no significant correlation

between the two in those who had abortion for the first time or in normal pregnancy (r=0.493, p>0.05; r=0.925, p>0.05) respectively.

The expression of IFN-γ was heterogenous blue-black nuclear staining, involving both decidual and trophoblastic cells, as shown in figure (1, A&B), while VCAM-1 positive endothelial cells displayed granular cytoplasmic staining pattern as shown in figure (1, D).

Table 1:The expression of IFN-γ among the studied groups

IFN-γ	n	Mean ± S.E.Ψ	Median	Min Value	Max Value
Group 1	24	69.8 ± 2.96	69.4	45	93.8
Group 2	10	49.5 ± 5.07	61.4	34.7	88
Group 3	6	40.1 ± 5.6	43.7	25	62.4

Ψ Standard error

Table 2:The expression of VCAM-1 among the studied groups

IL-10	n	Mean ± S.E.Ψ	Median	Min Value	Max Value
Group 1	24	55.9 ± 2.74	55.6	28.2	83
Group 2	10	32.4 ± 3.9	32.3	10.3	54.2
Group 3	6	42.5 ± 1.99	44.4	38.8	50

Ψ Standard error

Table 3:The significance of difference in the expression of IFN-γ in between the groups

IFN-γ	*P* Value
Among the groups	0.000
Between group A and B	0.002
Between group A and C	0.000
Between group B and C	0.645

Table 4:The significance of difference in the expression of VCAM-1 in between the groups

VCAM-1	*P* Value
Among the groups	0.000
Between group A and B	0.000
Between group A and C	0.064
Between group B and C	0.374

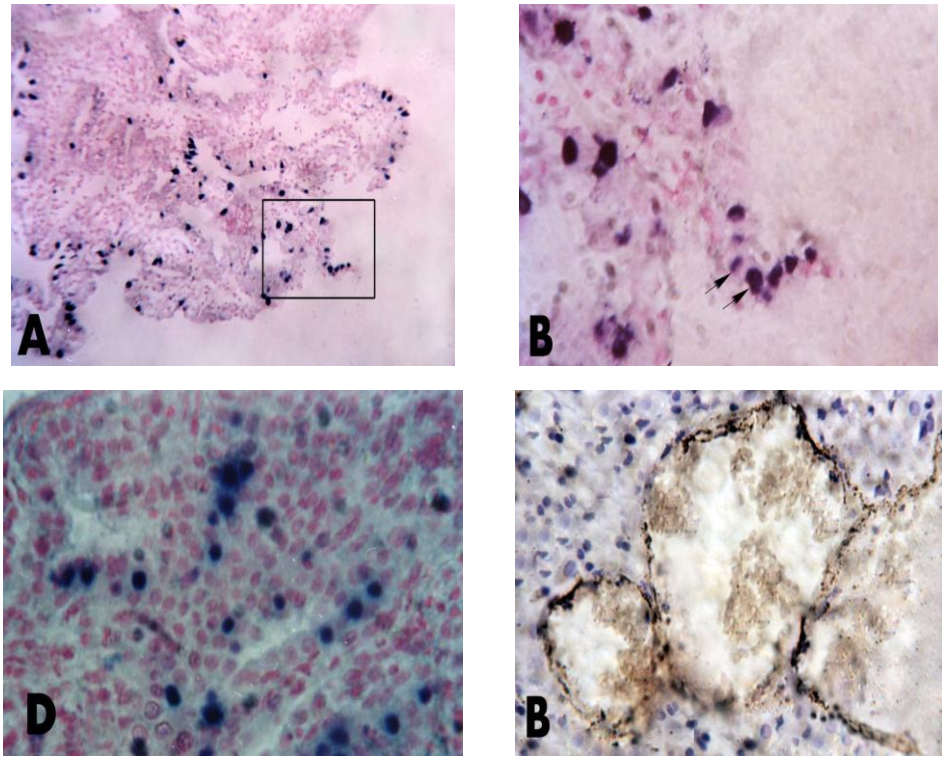

Figure 1: Detection of IFNγ by *in situ* hybridization and VCAM-1 by immunohistochemistry in women with abortion. Staining of IFN-γ mRNA in the nuclei of the decidua and trophoblasts by BCIP/NBT (blue-black) counterstained with nuclear fast red. (A) Tissue from patient with RSA shows positive IFNγ hybridization signals. (B) Higher magnification of (A) demonstrates the heterogenous nuclear staining pattern (arrows). (C) Positive control (housekeeping gene) probe. (D) Staining of VCAM-1 by DAB chromogen (dark brown) counterstained with Mayer's heamatoxylin, patient with RSA shows positive VCAM-1 immunostaining. Magnification power of A (X100), B-D (X400).

DISCUSSION:

This study demonstrates that the *in situ* expression of IFN-γ is significantly higher in women with RSA than that in women with first abortion or normal pregnancy (p= 0.002, 0.000) respectively, notifying that the three groups in this study were comparable in age and gestational age at the time of abortion. A part from the causes of this significant increase in the expression of IFN-γ in women with recurrent abortion, revision was made for the previous studies that examined the association between Th1 type cytokines and recurrent abortion, first studies in Hill's laboratory [6] have shown that peripheral blood mononuclear cells (PBMC) of women with a history of RSA when stimulated with a trophoblast antigen extract produced significantly higher concentrations of the Th1 cytokines, IFN-γ and TNF-α, as compared with normal pregnancy. Moreover, it has been demonstrated that stimulation of the maternal PBMC with autologous placental cells *in vitro* results in a Th1-biased production of cytokines in women undergoing unexplained RSA [7,9]. This was mirrored by the situation at the feto-maternal interface shown by other studies [5,30,32,33].

Although this study, like many of the studies on human pregnancy failure, has not addressed a direct cause-and-effect relationship between Th1-type reactivity and recurrent pregnancy loss, considerable amount of evidence suggest that Th1 cytokines might well be implicated in adversely affecting pregnancy, directly by interfering with trophoblast survival and function, and indirectly by activating cell-mediated immune effectors. Evidences supporting this suggestion are that, the administration of one of the Th1 cytokines like IFN-γ, TNF-α or IL-2 to normal pregnant mice

causes abortion [34]. IFN-γ and TNF-α inhibit the proliferation of human trophoblast cells *in vitro* [35] and are toxic to human trophoblast cells [36]. Uterine resorption sites in a murine model of recurrent abortion were infiltrated by NK cells [37]; given the fact that the activation of NK cells has been shown to be detrimental to murine pregnancy and that NK cells are activated by the Th1 cytokine; IFN-γ [38], the relevance of these data to pregnancy failure is obvious. In addition, other studies stated that, the concentrations of uNK cells were abnormally high in women with recurrent miscarriage [39], activated macrophages were present in the decidua of resorbing murine embryos [40] and embryo loss in mice has been shown to be associated with local production of nitric oxide [41]. Furthermore, strong Th1-dominant responses against pathogens compromise pregnancy; for example infection by *Leishmania major* results in resorptions, with a concurrent increase in the concentrations of IFN-γ in the placenta [42].

As the target of pro-inflammatory cytokines in triggering abortion was found to be mainly vascular [43], another aspect carried in this study, focusing on investigating the possible role of VCAM-1, that was seen in the literature playing a role in pregnancy, and to solidify the possible role of IFN-γ as a pro-inflammatory cytokine, since the pro-inflammatory cytokines cause up-regulation of VCAM-1 [20]. Interestingly, INF-γ and VCAM-1 expressions were significantly correlated in this study in women with RSA (r=0.418, p<0.05), which could be explained by the previous studies on VCAM-1 which showed that it is an inducible molecule, and pro-inflammatory cytokines, especially IFN-γ can cause a profound increase in VCAM-1 density on endothelial cells within 5-9 hours after stimulation and promote trans-endothelial migration [20,44,45].

In addition, multiple VCAM-1 isoforms has been identified that are encoded by alternatively spliced mRNAs, a unique isoform VCAM-1^{GPI}, has been identified which contains only the first three Ig domains and is anchored to the cell membrane via glycosylphosphatidylinositol. VCAM-1^{GPI} is encoded by an alternatively spliced mRNA that is preferentially induced by proinflammatory cytokines and LPS. [46]

Moreover, the current study showed that the expression of VCAM-1 is significantly higher in women with RSA than that of women with first abortion (p=0.000) and also higher than that in normal pregnancy (p=0.064), which strengthens our suggestion that VCAM-1 could play a role in the pathology of recurrent miscarriage by enhancement of trans-endothelial migration of recruited leukocytes in the decidual blood vessels, participating in the inflammatory process, also it mediate stimulation of the adjacent endothelial cells by the adhering leukocytes increasing the inflammatory process [47,48].

CONCLUSION:

This study strengthen the possibility that type-1 immune response may have the upper hand in the pathology of RSA on multidirectional bases including up regulation of the surface expression of VCAM-1.

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