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Short communication

Stress increases VCAM-1 expression at the fetomaternal interface in an abortion-prone mouse model

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ABSTRACT

Sound stress exposure increases fetal loss via inflammatory pathways. Inflammation is known to up-regulate cell adhesion molecules, such as vascular cell adhesion molecule-1 (VCAM-1), which mediates the adhesion of leukocytes to the vascular endothelium. In this work, we studied the frequency of VCAM-1$^+$ vessels at the fetomaternal interface in stressed and non-stressed pregnant CBA/J female mice mated with DBA/2J (high fetal loss model) or BALB/c (low fetal loss model) males. The high fetal loss model had fewer large vessels on gestation day 6.5, and stress reduced the frequency of large vessels to a similar number in both high and low fetal loss models. In the high fetal loss model, however, the frequency of VCAM-1+ vessels was dramatically increased. This study shows that VCAM-1 expression is modulated by stress at the fetomaternal interface in abortion-prone cross-breeding.

1. Introduction

During human and mouse decidualization, maternal immune cells are recruited to the decidua to exert an immunomodulatory effect on the placenta. Leukocyte homing into the decidua is regulated through a specialized mechanism at the maternal endothelium of local vessels. This mechanism allows the entry of only specific leukocyte subsets. Vascular cell adhesion molecule-1 (VCAM-1) is expressed on the endothelium of some vessels in the decidua basalis of early human and mouse pregnancy (Kruse et al., 2002). VCAM-1 is an immunoglobulin-like adhesion molecule expressed on the surface of activated endothelial cells that binds to $\alpha_4\beta_1$ integrin, an integrin constitutively expressed on lymphocytes, monocytes, and eosinophils. VCAM-1 can mediate both rolling-type adhesion and firm adhesion, depending on the avidity status of $\alpha_4\beta_1$ integrin. It was observed that the specialized uterine NK cells, which are $\alpha_4\beta_1$-positive, constitute a defined cluster located around the VCAM-1 positive vessels at the implantation site (Burrows et al., 1993; Kruse et al., 2002). VCAM-1 also enhances extravasation of polymorphonuclear leukocytes that play a role in subsequent resorptions/abortions (Clark et al., 1998; Lee et al., 2008; Waugh and Lomakina, 2009).

Pregnancy failure usually takes place as spontaneous abortion during the first trimester in humans; it is known that stress increases fetal loss during the peri-implantation period. Over the last few years, the mechanisms involved in fetal loss were studied employing the murine model of DBA/2J-mated CBA/J females, which provides an established experimental approach, particularly in pregnancies challenged by stress (Arck et al., 1995; Blois et al., 2004a). These allogenically pregnant mice subjected to ultrasonic sound stress early in gestation showed a significant

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increase in the abortion rate (Arck et al., 1995). Adhesion molecules participate in stress-triggered abortion in this model by facilitating the recruitment of inflammatory cells to the fetomaternal interface (Blois et al., 2005).

In this work, we studied the expression pattern of VCAM-1 in implantation sites from gestation day 6.5 from stressed and non-stressed pregnant CBA/J mice. We compared the results from DBA/2J (high fetal loss model) and BALB/c mating (low fetal loss model).

2. Materials and methods

2.1. Animals

Two sets of experiments were carried out. Mice were purchased from Charles River Breeding Laboratories (Germany) and from Comisión Nacional de Energía Atómica (Argentina). Animal care and experimental procedures were followed according to each institution's guidelines (LaGeSo in Germany and CICUAL, Facultad de Medicina, Universidad de Buenos Aires in Argentina). DBA/2J- and BALB/c-mated CBA/J female mice were randomized into a control and a stressed group (each one $n = 10$). The observation of a vaginal plug was denoted on 0.5 day of pregnancy. The mice were sacrificed on gestation day (gd) 6.5; implantation sites were removed, counted and cryo-sectioned. By this gd, viable and resorbing sites cannot be distinguished and the implantation sites are easily visible externally as spherical swellings measuring 3×4 mm approximately. Three implantation sites were analyzed per mouse. Simultaneously, additional control and stressed groups of both cross-breeding (each one, $n = 10$) were sacrificed at day 13.5 to record the abortion rate, which was calculated as reported previously (Blois et al., 2004a).

2.2. Stress induction

Stress induction was performed according to Blois et al. (2004a). The CBA/J mice were exposed to sound stress for a single 24-h period on day 5.5 of pregnancy. The sound was emitted by a rodent repellent device (Conrad Electronics, Germany) at 300 Hz in intervals of 15 s. The device was placed into the mice's cage so that they could not escape the sound perception.

2.3. Immunohistochemistry

Acetone fixed-cryo sections (8-μm thickness) from implantation sites were rinsed in PBS and then incubated for 30 min at room temperature with 0.3% H_2O_2 in MeOH. Sections were blocked with 10% goat normal serum for 15 min at 37 °C and incubated afterwards overnight at 4 °C with rat anti-VCAM-1 (BD Biosciences), anti-PECAM (platelet endothelial cell adhesion molecule, BD Biosciences) or the respective control rat isotypes. A biotin-conjugated secondary antibody was then added for 30 min at 37 °C. The Vectastain® ABC peroxidase kit (Vector Labs) was used for PECAM staining while the alkaline phosphatase one was used for VCAM-1 detection. Diaminobenzidine and H_2O_2 were employed as a substrate for PECAM, while VCAM-1 was detected with New-fuchsin. Finally, the sections were counterstained with Mayer's hematoxylin and mounted using Crystal/Mount™ (Biomeda).

2.4. Vascular detection

PECAM staining was used for determining the number of vessels in cryosections (Fong et al., 1995). The mean number of blood vessels in each implantation site was calculated from three non-serial cryosections. Vessels were counted in the entire section and classified according to their size in: small ($<55 \times 55$ μm); medium (55×55 μm–110×110 μm) and large ($>110 \times 110$ μm).

2.5. Statistical analysis

The histochemical labeling was assessed in a double-blinded manner by two independent observers. Data were transformed with square root function for PECAM (number of vessels per section) and number of implantation sites and with arcsine function for VCAM-1 prior to statistical analysis. One-way analysis of variance (ANOVA) and Newman–Keuls multiple comparison test were performed to compare mean differences using GraphPad Prism 5-Graphics Software. Differences at $p < 0.05$ were considered statistically significant.

3. Results and discussion

In order to check the effect of sound stress on the resorption rate of the high fetal loss and low fetal loss mouse models (CBA/J × DBA/2J and CBA/J × BALB/c respectively), female pregnant mice exposed or not exposed to sound stress were sacrificed at gd 13.5. This work was carried out in two different facilities. In Germany (GE), the abortion rate of the CBA/J × DBA/2J cross-breeding was around $10 \pm 2\%$ ($n = 10$), as previously reported (Blois et al., 2004a) and in Argentina (AR) this value increased to $23 \pm 2\%$ ($n = 10$), in agreement with previous results (Miranda et al., 1998; Blois et al., 2004b). Flora and endogenous stress levels appear to explain baseline loss rate differences among different animal colonies (Clark et al., 2004, 2008). Nevertheless, both in the GE and in the AR colonies, sound stress increased this value to $44 \pm 3\%$ ($n = 10$). On the other hand, independently of the animal facility the abortion rate of the low fetal loss model was around 5–8% and was not affected by stress, in agreement with Arck et al., 1995. Therefore, DBA/2J-mated CBA/J females are more susceptible to stress and microenvironment conditions than BALB/c-mated CBA/J females.

Events leading to spontaneous abortion/resorption in the CBA/J × DBA/2J model occur prior to gestation day 9.5 (Clark et al., 1999, 2001; Girardi et al., 2006). In the present study, we focused on changes in the maternal uterine lining at the implantation site on gd 6.5, 2 days after implantation, with or without sound stress delivered on gd 5.5. In the implantation sites, endothelial cells were identified by PECAM immunohistochemistry and vessels were classified according to their size into small, medium or large vessels (Fig. 1A). It can be seen that the number of small and medium-sized vessels was similar in both models (Fig. 1B), but the number of large sized vessels was reduced

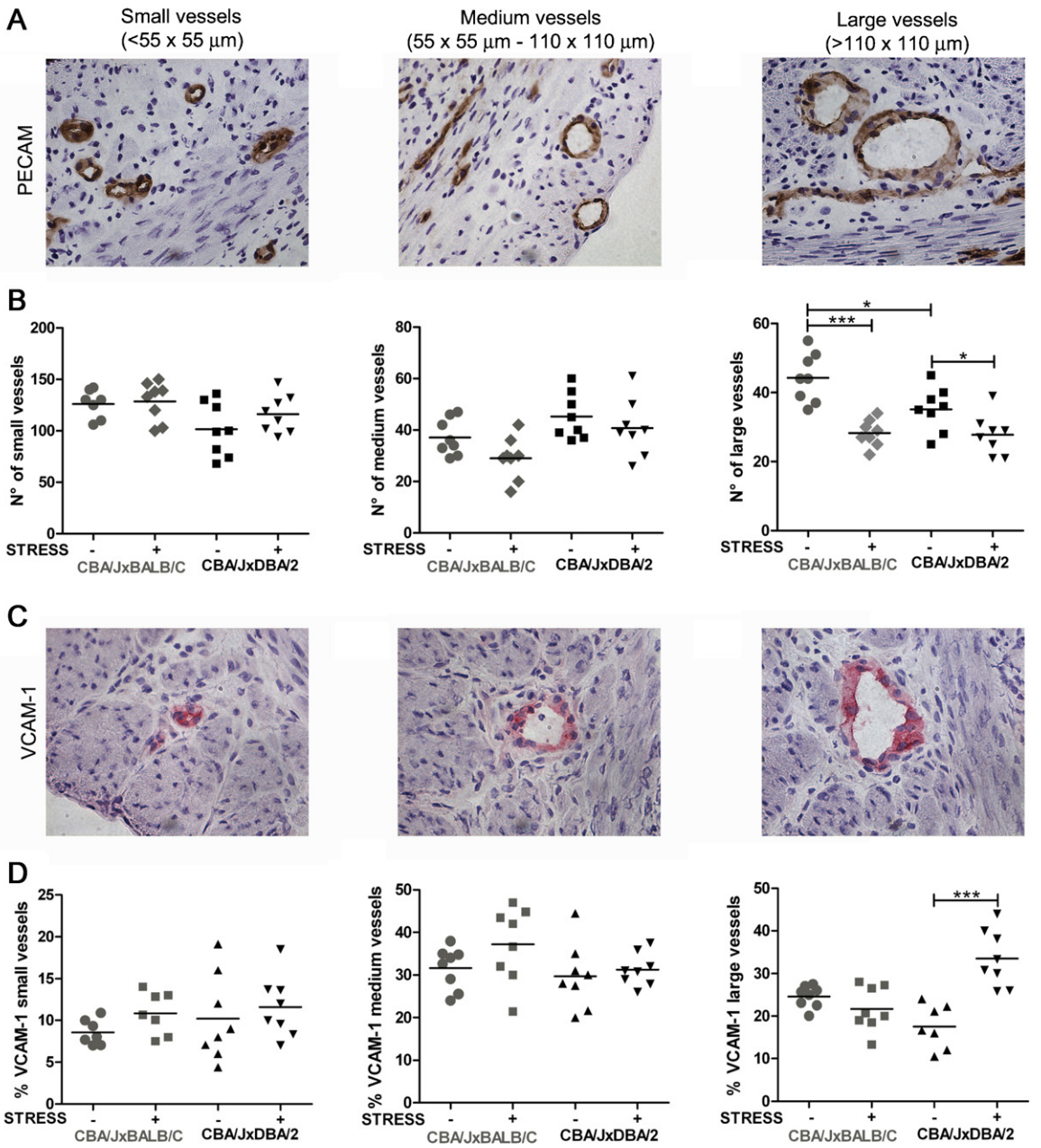

Fig. 1. (A) Representative pictures of PECAM staining, which allowed the classification of vessels into small, medium and large vessels in CBA/J (BALB/c or DBA/2J mating) fetomaternal interface at gestation day 6.5 (400× magnification). (B) The number of small, medium and large vessels was calculated as the mean obtained from the analysis of PECAM staining of three cryosections from a single implantation site. Three implantation sites were analyzed per mouse and the mean value was calculated and is shown in each graph. (C) Representative pictures of VCAM-1 staining of small, medium and large vessels in CBA/J (BALB/c or DBA/2J mating) fetomaternal interface at gestation day 6.5 (400× magnification). (D) Proportion of VCAM-1 positive small, medium and large vessels in both cross-breedings. Three implantation sites were analyzed per mouse and the mean value was calculated and is shown in each graph. Each symbol represents an individual mouse (mean of three implantation sites). Data are a mixture from German and Argentinian animal facilities. Pregnant mice were stressed ($n = 8$ for each cross-breeding) or not ($n = 8$ for each cross-breeding) by ultrasonic sound at gd 5.5 for 24 h. $^{*}p < 0.05$; $^{**}p < 0.01$.

in the high fetal loss rate model (Fig. 1B). Stress significantly reduced the frequency of large vessels in both models, but curiously, only in the high fetal loss rate model were abortions subsequently increased, as described above.

Interestingly, the high fetal loss mouse model showed fewer implantation sites than the low fetal loss model (6.50 ± 0.75 and 11.00 ± 1.25 respectively, each one $n = 10$). It is possible that the reduced frequency of large vessels was related to a reduced number of implantations, but a cause–effect relationship cannot be inferred without additional data, such as number of ovulated oocytes. Stress did not modify the number of implantation sites in

any cross-breeding. This is probably because implantation occurs approximately at gd 4.0 and stress was induced at gd 5.5.

VCAM-1 is an important adhesion molecule that facilitates emigration of leukocytes that participate in the abortion process. VCAM-1 immunoreactivity localized in the vascular endothelium of the implantation sites (Fig. 1C). The number of small, medium and large VCAM-1-expressing vessels was similar in non-stressed mice from both models. However, stress reduced the number of large VCAM-1 expressing vessels (−50%) in the low fetal loss model. Since the number of large vessels was reduced by stress to the same extent in this model, the frequency of large VCAM-1 expressing vessels was not altered. Therefore, stress does probably not alter VCAM-1 expression in this model. On the contrary, stress increased the number of VCAM-1 expressing vessels in the high fetal loss model (+25%). Considering the diminished number of large vessels upon stress exposure in the implantation sites of these females, the frequency of large VCAM-1 expression vessels was greatly increased (Fig. 1D).

Pregnancy outcome has been associated with a Th1/Th2 cytokine balance: the predominance of anti-inflammatory Th2 cytokines over pro-inflammatory Th1 cytokines in the decidua correlates with fetomaternal tolerance in mice and humans (Arck et al., 1999; Raghupathy, 2001). Employing the CBA/J × DBA/2J murine model, it was demonstrated that exposure to sound stress during early gestation inhibits protective suppressor mechanisms and promotes secretion of abortogenic cytokines such as TNFα, IFNγ, and IL-1 (Arck et al., 1995; Blois et al., 2005; Clark et al., 1999). These pro-inflammatory cytokines were shown to up-regulate VCAM-1 in vascular endothelium (Lechleitner et al., 1998). Therefore, the enriched Th1 cytokine profile present in the fetomaternal interface in the high fetal loss model could explain the increased VCAM-1 expression observed in this work upon stress exposure. Th1 polarization of decidual immune cells depends on adhesion molecules such as intercellular adhesion molecule 1 (ICAM1) that recruit Th1 cells into the uterus (Blois et al., 2005). Our results suggest that VCAM-1 might also be implicated.

In conclusion, we propose that the high number of vessels expressing VCAM-1 in the high fetal loss model fetomaternal interface in conjunction with the already described events that follow stress perception might be implicated in fetal loss. Redecha et al. showed fibrin deposition along maternal blood vessels on gestation day 6.5 in the CBA/J × DBA/2J model (Redecha et al., 2009). Fibrin deposition is part of the pathogenesis of abortions/resorptions that depends on thrombin/C5a/neutrophils. Taking into account our observations, the upregulation of large VCAM-1-positive vessels could be involved in this process and blocking VCAM-1 should prevent stress-triggered abortions.

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References

Arck, P.C., Merali, F.S., Manuel, J., Chaouat, G., Clark, D.A., 1995. Stress-triggered abortion: inhibition of protective suppression and promotion of tumor necrosis factor-alpha (TNF-alpha) release as a mechanism triggering resorptions in mice. Am. J. Reprod. Immunol. 33, 74–80.

Arck, P.C., Ferrick, D.A., Steele-Norwood, D., Egan, P.J., Croitoru, K., Carding, S.R., Dietl, J., Clark, D.A., 1999. Murine T cell determination of pregnancy outcome. Cell Immunol. 196, 71.

Blois, S., Joachim, R., Kandil, J., Margni, R., Tometten, M., Klapp, B.F., Arck, P.C., 2004a. Depletion of CD8+ cells abolishes the pregnancy protective effect of progesterone substitution with dydrogesterone in mice by altering the Th1/Th2 cytokine profile. J. Immunol. 172 (10), 5893–5899.

Blois, S., Alba Soto, C.D., Olmos, S., Chuluyan, E., Gentile, T., Arck, P.C., Margni, R.A., 2004b. Therapy with dendritic cells influences the spontaneous resorption rate in the CBA/J × DBA/2J mouse model. Am. J. Reprod. Immunol. 51 (1), 40–48.

Blois, S., Tometten, M., Kandil, J., Hagen, E., Klapp, B.F., Margni, R.A., Arck, P.C., 2005. Intercellular adhesion molecule-1/LFA-1 cross talk is a proximate mediator capable of disrupting immune integration and tolerance mechanism at the fetomaternal interface in murine pregnancies. J. Immunol. 174, 1820–1829.

Burrows, T.D., King, A., Loke, Y.W., 1993. Expression of adhesion molecules by human decidual large granular lymphocytes. Cell Immunol. 147 (1), 81–94.

Clark, D., Chaouat, G., Arck, P., Nittnuecker, W., Levy, G., 1998. Cytokine-dependent abortion in CBA/J × DBA/2J mice is mediated by the procoagulant fgl2 prothrombinase. J. Immunol. 160, 545–549.

Clark, D., Ding, J., Chaouat, G., et al., 1999. The emerging role of immunoregulation of fibrinogen-related procoagulant Fgl2 in the success or spontaneous abortion of early pregnancy in mice and humans. Am. J. Reprod. Immunol. 42, 37–43.

Clark, D., Ding, J., Yu, G., Levy, G., Gorczynski, R., 2001. Fgl2 prothrombinase expression in mouse trophoblast and decidua triggers abortion but may be countered by OX-2. Mol. Hum. Reprod. 7 (2), 185–194.

Clark, D., Manuel, J., Lee, L., Chaouat, G., Gorczynski, R., Levy, G., 2004. Ecology of danger-dependent cytokine-boosted spontaneous abortion in the CBA × DBA/2 mouse model. I. Synergistic effect of LPS and (TNF-alpha + IFN-gamma) on pregnancy loss. Am. J. Reprod. Immunol. 52 (6), 370–378.

Clark D.A., Chaouat G., Banwatt D., Friebe A., Arck P.C., 2008. Ecology of danger-dependent cytokine-boosted spontaneous abortion in the CBA × DBA/2 mouse model: II. Fecal LPS levels in colonies with different basal abortion rates. Am. J. Reprod. Immunol. 60(6) 529–533.

Fong, G.-H., Rossant, J., Gertsenstein, M., Breitman, M.L., 1995. Role of the Flt-1 receptor tyrosine kinase in regulating the assembly of vascular endothelium. Nature 376, 66–70.

Girardi, G., Yarilin, D., Thurman, J., Holers, V., Salmon, J., 2006. Complement activation induces dysregulation of angiogenic factors and causes fetal rejection and growth restriction. J. Exp. Med. 203, 2165–2175.

Kruse, A., Martens, N., Fernekorn, U., Hallmann, R., Butcher, E.C., 2002. Alterations in the expression of homing-associated molecules at the maternal/fetal interface during the course of pregnancy. Biol. Reprod. 66, 333–345.

Lechleitner, S., Gille, J., Johnson, D.R., Petzelbauer, P., 1998. Interferon enhances tumor necrosis factor-induced vascular cell adhesion molecule 1 (CD106) expression in human endothelial cells by an interferon-related factor 1-dependent pathway. J. Exp. Med. 187, 2023–2030.

Lee, C., Lin, C., Luo, S., Lee, H., Lee, I., Aird, W., Hwang, T., Yang, C., 2008. Tumor necrosis factor-α enhances neutrophil adhesiveness: induction of vascular cell adhesion molecule-1 via activation of Akt and CaM kinase II and modifications of histone acetyltransferase and histone deacetylase 4 in human tracheal smooth muscle cells. Mol. Pharmacol. 73 (5), 1454–1464.

Miranda, S., Malán Borel, I., Margni, R., 1998. Altered modulation of the in vitro antibody synthesis by placental factors from the CBA/J × DBA/2 abortion-prone mating combination. Am. J. Reprod. Immunol. 39 (5), 341–349.

Raghupathy, R., 2001. Pregnancy: success and failure within the Th1/Th2/Th3 paradigm. Semin. Immunol. 13, 219.
Redecha, P., van Rooijen, N., Torry, D., Girardi, G., 2009. Pravastatin prevents miscarriages in mice: role of tissue factor in placental and fetal injury. Blood 113 (17), 4101–4109.
Waugh, R.E., Lomakina, E.B., 2009. Active site formation, not bond kinetics, limits adhesion rate between human neutrophils and immobilized vascular cell adhesion molecule 1. Biophys. J. 96 (1), 268–275.