

# Stress and immune mediators in miscarriage

P.C.Arck<sup>1,3</sup>, M.Rose<sup>1</sup>, K.Hertwig<sup>2</sup>, E.Hagen<sup>1</sup>, M.Hildebrandt<sup>1</sup> and B.F.Klapp<sup>1</sup>

Charité, Campus Virchow, <sup>1</sup>Department of Internal Medicine and <sup>2</sup>Department of Obstetrics, Humboldt University, Berlin, Germany

<sup>3</sup>To whom correspondence should be addressed at: Charité, Campus Virchow Klinik, Biomedizinisches Forschungszentrum, Raum 2.0549, Augustenburger Platz 1, 13353 Berlin, Germany. E-mail: petra.arck@charite.de

**BACKGROUND:** Stress is thought to be abortogenic and psycho-neuro-immunological pathways have been suggested to be involved in triggering miscarriages. From experiments in pregnant mice exposed to stress some insights into the underlying mechanisms have been gained, delineating immunological imbalances as a cause of pregnancy failure. In order to test the validity of the conclusions drawn from murine experiments and the role of stress in human pregnancy loss, the following study was performed. **METHODS:** We used an established perceived stress questionnaire and measured the stress score of women with a confirmed diagnosis of first trimester spontaneous abortion ( $n = 94$ ). Decidual tissue was investigated by immunohistochemistry and in-situ hybridization to detect the presence and distribution of immunocompetent decidual cells [CD56<sup>+</sup> natural killer (NK) cells, CD8<sup>+</sup> and CD3<sup>+</sup> T cells, tryptase<sup>+</sup> mast cells (MCT<sup>+</sup>) and tumour necrosis factor (TNF)- $\alpha$ <sup>+</sup> cells]. The patient cohort was divided into women experiencing low or high levels of stress. **RESULTS:** In the decidua of women with high stress scores we observed significantly higher numbers of MCT<sup>+</sup>, CD8<sup>+</sup> T cells and TNF- $\alpha$ <sup>+</sup> cells per mm<sup>2</sup> tissue ( $P \leq 0.05$ ). No significant differences between individuals with lower or higher stress scores could be observed with respect to decidual CD56<sup>+</sup> NK and CD3<sup>+</sup> T cells. **CONCLUSIONS:** Using a questionnaire to score perceived stress in humans may be a valid approach to assess non-biased stress scores. Stress-triggered abortion in humans, identified by a questionnaire, can be linked to immunological imbalances.

*Key words:* abortion/immunohistochemistry/NK cells/T cells/TNF- $\alpha$

## Introduction

First trimester spontaneous abortion is the most common adverse reproductive outcome. Generally the causes of spontaneous early pregnancy loss are classified as genetic, endocrinologic, anatomic, immunologic and microbiologic (Stray Pedersen and Stray Pedersen, 1984; Klentzeris *et al.*, 1994; Christiansen, 1996). Additionally, the majority of work has focused on potential autoimmune and alloimmune causes (Clark *et al.*, 1999a; King, 2000). Studies relating pregnancy health or loss to social/environmental factors such as stress have recently been performed (O'Hare and Creed, 1995; Neugebauer *et al.*, 1996; Wergerland and Strand, 1998; Wasser, 1999). For the past two decades, experimental contributions have focused on the effect of stress on immune functions in humans and animals, revealing a bidirectional communication between stress and the immune network which might explain how stress affects immunity (Cohen *et al.*, 2001).

Based on the mechanisms identified on stress-immune interactions by our group and others, we investigated psycho-neuro-immunological imbalances as a cause of human miscarriages, analysing decidual tissue of stress-scored miscarriage patients for the presence and distribution pattern of immunocompetent cells and mediators, such as CD3<sup>+</sup> and CD8<sup>+</sup> T cells, CD56<sup>+</sup> NK cells, mast cells and tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ),

all of which have been reported to contribute to pregnancy maintenance or failure (Arck *et al.*, 1995; Lea *et al.*, 1997; Kodama *et al.*, 1998; Vassiliadou and Bulmer, 1998; Clifford *et al.*, 1999; Marx *et al.*, 1999a; Quenby *et al.*, 1999) using immunohistochemical staining and in-situ hybridization. Since a well-balanced immunological environment with low levels of inflammatory Th1 cytokines and high levels of anti-inflammatory Th2 cytokines is mandatory for the maintenance of pregnancy (Lim *et al.*, 1996), our goal was to identify immunological alterations hinting at Th1/Th2 cytokine imbalances in the decidua and to correlate these changes with perceived psychosocial stress in humans.

## Materials and methods

### Subjects

All miscarriage patients ( $n = 94$ ) admitted to the Department of Gynaecology at Charité, were investigated by physical examination and ultrasound. Conditions that provided an obvious reason for the miscarriage, such as uterine abnormalities, infections, or pre-existing medical diseases were excluded from the study. To retrieve enough tissue for the immunological assessment, curettage material exclusively obtained from patients with spontaneous abortion with no vaginal bleeding was used, the diagnosis of abortion was based on transvaginal ultrasound, decreasing  $\beta$ HCG concentrations and absence

of vaginal bleeding ( $n = 56$ ). The study was approved by the local ethics committee.

#### **Validation of the German perceived stress questionnaire (PSQ) version**

From October 1998 until December 1999, the study participants ( $n = 94$ ), (mean age  $\pm$  SD) ( $30.2 \pm 7.7$ ), gestational age 8.9 weeks, suffering from first trimester spontaneous abortion, were asked to fill out the questionnaires upon confirmation of the spontaneous abortion and prior to curettage at the Department of Obstetrics at Charité. Women suffering from recurrent spontaneous abortions (three or more consecutive miscarriages) were not included in this study. Since stress questionnaires similar to the PSQ are not validated in German, we chose the internationally established WHO-Quality of Life (QoL) instrument for validating the German PSQ, assuming that the insufficient personal coping capacity of demands (stressors) will reduce the QoL. Further, as high social support is thought to be a stress buffer, we included a questionnaire measuring social support (SOZU K 22) for validation of the PSQ. For the WHO-QoL and the SOZU K 22 instruments, large databases of representative German cohorts have been published (Fydrich *et al.*, 1988; Angermeyer *et al.*, 1999). The PSQ was originally published in English, it had been translated into German, retranslated into English by an independent native speaker and again, translated into German by another translator. The final version was approved by all individuals involved in the translations. The result of each scale yields to the PSQ score, which ranges from 0 (lowest possible level of stress) to 1 (highest possible level of stress). To obtain stress scores of a healthy young female reference population we asked female students ( $n = 115$ , mean age 24.2 years) who were enrolled at the Medical Faculty to fill out the questionnaire shortly before a major examination.

#### **Biopsies**

We exclusively used fresh decidual tissue samples and were careful to avoid any differences in the timing of sample collection. Samples that showed tissue damage histologically, such as necrosis, old blood clots or infections, were excluded from the study. For this reason, decidual samples from only 50 patients were suitable for further immunohistochemistry (IHC), based on the H&E tissue evaluation by a pathologist. All tissues had been routinely fixed in 5% formalin and embedded in paraffin. For each patient we examined 2–4 different sections of tissue. Sections of 2  $\mu$ m were cut and IHC stained with a monoclonal antibody against pan cytokeratin (CK) to confirm the presence or absence of invasive trophoblast and proliferating endometrial glands. On this basis, the decidua was classified as decidua basalis (invasive trophoblast present, Figure 2A) or decidua parietalis (no trophoblast or proliferating glands). We only focused on tissue in which invasive fetal cells had been detected by CK-IHC since only in this case is trophoblast in contact with maternal immunocompetent cells and can be a target of rejection. This subgroup of 46 patients with spontaneous abortion did not differ significantly in their stress scores compared with the complete cohort of 94 patients (0.38 versus 0.39).

#### **Karyotyped decidual specimen**

To exclude the possibility that populations of immunocompetent cells increased due to influences other than perceived stress, especially in the presence of abnormal karyotype trophoblast, we immunohistochemically quantitated mast cells tryptase<sup>+</sup> cells and CD56<sup>+</sup> cells in decidual biopsies ( $n = 94$ ) that had previously been karyotyped using standard G banding techniques (Stern *et al.*, 1996). The tissue was kindly provided by Dr C.Coulam, Chicago, IL, USA. Immunohistochemistry for CK revealed invasive trophoblast cells as a marker for

decidua basalis specimens in 31 cases, which were used in the present study.

#### **Immunohistochemical staining for CK, CD56, CD3, CD8 and mast cell tryptase**

Consecutive slides were stained with monoclonal antibody against mast cell tryptase, CD3, CD8 (Dako, Hamburg, Germany), CD56 (Novo Castra, Newcastle, UK) or CK (Immunotec, Marseille, France) respectively, following our standard protocol for IHC and double staining IHC as previously performed (Marx *et al.*, 1999b).

#### **TNF- $\alpha$ in situ hybridization**

Probes specific for human TNF- $\alpha$  mRNA were kindly provided by Dr Richard Lea, Rowett Research Institute, Aberdeen, UK, and stored at  $-70^{\circ}$  C until use (Lea *et al.*, 1997). In brief, 5  $\mu$ m paraffin sections were dewaxed and rehydrated, then washed in diethylpyrocarbonate (DEPC) treated water, immersed in 0.1 mol/l HCL followed by two washes in standard sodium citrate (SSC) buffer at room temperature. Sections were then exposed to 10  $\mu$ g/ml proteinase K and post-fixed in 0.4% paraformaldehyde at  $4^{\circ}$ C. Hybridization was carried out at  $59^{\circ}$ C overnight using S<sup>35</sup> UTP-labelled cRNA. Following hybridization, sections were washed in  $4\times$  SSC and treated with RNase A (20  $\mu$ l/ml) for 30 min. The slides were desalted, dehydrated, air dried and dipped into M-1 emulsion (M-1; Amersham Pharmacia Biotech, Bucks, UK) and developed after 2 weeks by using D-19 developer (Kodak, Chalon-sur-Saone, France). The sections were counterstained with hemalaun. All chemicals were purchased from Sigma, unless otherwise stated. Tissue of an inflamed bowel was used as a positive control. A sense probe was used as a negative control.

#### **Microscopic evaluation**

At the time of microscopic evaluation, the investigators were blinded to the outcome of the perceived stress score as well as to the karyotype. The number of positive cells per mm<sup>2</sup> of tissue was evaluated by two independent observers using a light microscope (Axiophot, Zeiss, Jena, Germany) with scaled eye pieces pre-calibrated with a slide micrometer, at a magnification of 200 (20 $\times$  objective, with a 10 $\times$  ocular), without knowledge of the patient's stress levels. Ten high powered fields were counted per slide. The mean count per patient was calculated for each cell subset.

#### **Statistics**

Correlation between PSQ and WHO-QoL-Bref or SOZU K 22 and between mast cell counts and CD8 cell counts were tested using the Pearson's product moment correlation coefficient. Statistical data analysis was performed using SPSS statistical software (SPSS Inc.Chicago, IL, USA).

The correlations between immunological (cell counts) and psychological parameters (PSQ stress score) were calculated using the mean of each cell subset count per patient, splitting the group of miscarriage patients by the median of the stress score (0.39), which resulted in a group with lower levels of perceived stress ( $n = 23$ ) and a group with higher levels of perceived stress ( $n = 23$ ). Statistical comparisons for mean cell counts of these two groups were calculated using a non-parametric Mann-Whitney test, significance was set at  $P < 0.05$ .

## **Results**

### **Psychometric assessment and PSQ validation**

As presented in Table I, we observed, according to our hypothesis that higher stress scores were associated with lower QoL, an inverse association between the PSQ and the WHO-

**Table I.** Correlations between PSQ and WHO-QoL/SOZU

|                                | WHO-QoL-Bref domains ( <i>n</i> = 168) |          |        |          | SOZU ( <i>n</i> = 168) |                |
|--------------------------------|--|----------|--------|----------|------------------------|----------------|
|                                | physical                               | psychol. | social | environ. | QoL-global             | social support |
| PSQ Score<br>( <i>n</i> = 650) | -,617                                  | -,788    | -,593  | -,572    | -,577                  | -,606          |

Pearson correlation coefficients; all correlations are significant at  $P < 0.01$  level.

**Table II.** Absolute cell number (mean  $\pm$  SD) and cell relations

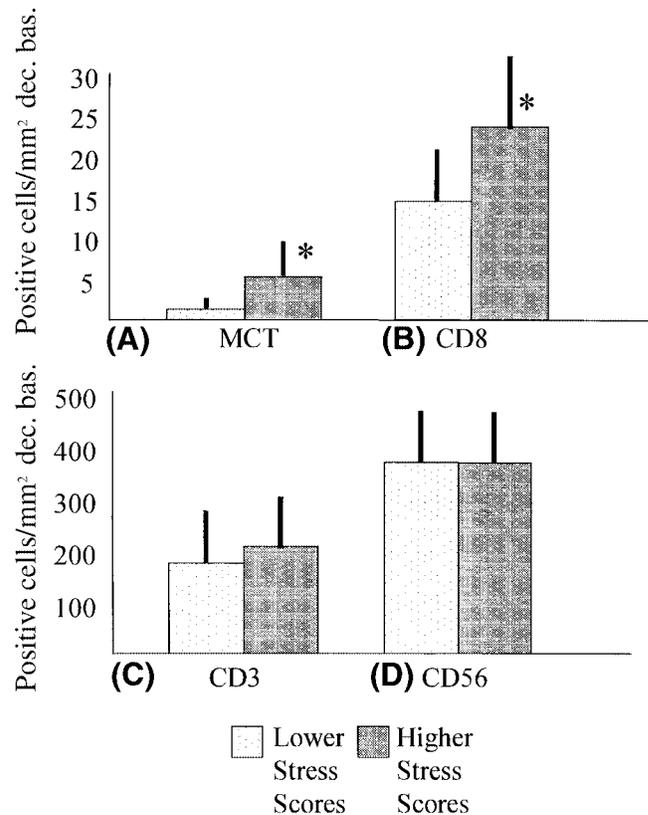
|               | Low stress       | High stress      | Mann-Whitney test |
|---------------|------------------|------------------|-------------------|
| MCT           | 1.3 $\pm$ 1.1    | 5.1 $\pm$ 2.5    | *                 |
| CD8           | 14.4 $\pm$ 3.2   | 23.8 $\pm$ 3.7   | *                 |
| CD3           | 182.9 $\pm$ 9.7  | 210.7 $\pm$ 10.5 | NS                |
| CD56          | 368.3 $\pm$ 14.2 | 366.3 $\pm$ 15.3 | NS                |
| TNF- $\alpha$ | 3.4 $\pm$ 2.5    | 12.2 $\pm$ 2.8   | *                 |
| CD8/CD3       | 0.08             | 0.11             | *                 |
| MCT/CD3       | 0.007            | 0.02             | *                 |

\* $P < 0.05$ .

QoL-Bref, with the highest co-variation among the psychological domain of the WHO questionnaire. Confirming the assumption that high social support buffers the perception of stress, we further obtained a negative correlation ( $P < 0.01$ ) between the PSQ and the questionnaire describing the social support (SOZU K 22). Further, testing the PSQ versus age, marital, educational or employment status in the group of miscarriage patients revealed no significant correlation (data not shown). Using the median stress score calculated for the miscarriage patients (0.39) as a cut-off, we considered all patients with  $<0.39$  as experiencing lower levels of stress ( $n = 23$ ) and all patients scoring  $\geq 0.39$  as more stressed ( $n = 23$ ). This approach is supported by the finding that female students shortly before a major examination, which can be considered as an extreme stressor, scored the PSQ with a median of 0.36 and were therefore almost identical to the miscarriage group.

### Immunological assessment

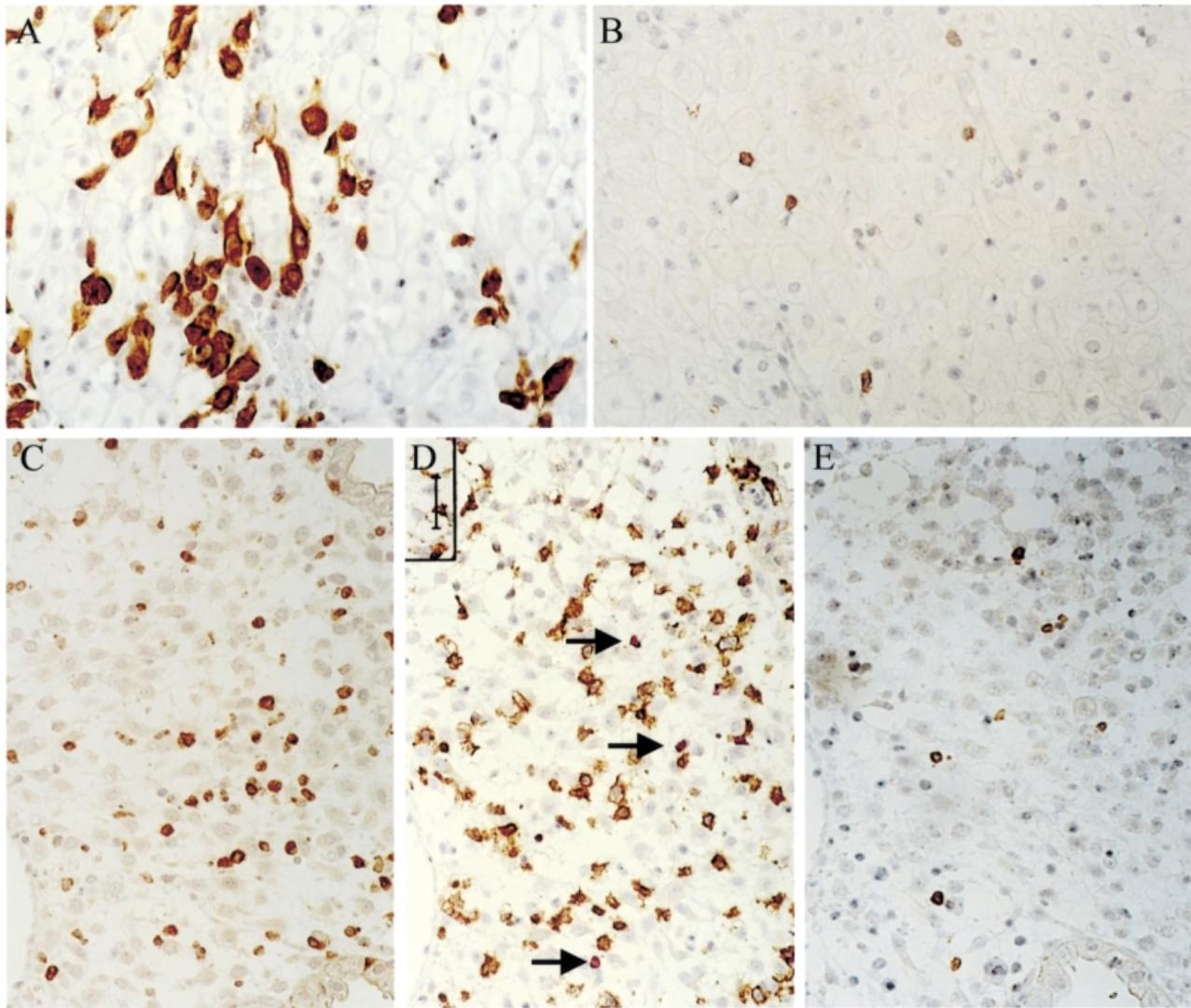
The mean cell counts for mast cells, CD8<sup>+</sup>, CD3<sup>+</sup>, CD56<sup>+</sup> and TNF- $\alpha$ <sup>+</sup> cells of the two groups (low stress versus high stress in patients suffering from abortion) were compared (Table II). As shown in Figure 1A we observed significantly higher numbers of tryptase<sup>+</sup> mast cells/mm<sup>2</sup> decidua basalis in more stressed patients having a spontaneous abortion (for IHC see Figure 2B). In the group of miscarriage patients with higher stress levels we further observed a significantly higher amount of CD8<sup>+</sup> cells/mm<sup>2</sup> decidua basalis tissue (Figure 1B; Figure 2E for IHC). Correlating these two cell populations (mast cells and CD8<sup>+</sup> cells in decidua basalis) with each other revealed a positive correlation coefficient of  $r = 0.26$  (NS). The results for CD3 quantification did not differ between the non-stressed and stressed miscarriage patients (Figure 1C). We observed a slight tendency of higher numbers of CD3<sup>+</sup> cells in the stressed group, although this failed to reach statistical



**Figure 1.** (A) Number of tryptase<sup>+</sup> mast cells (MCT)/mm<sup>2</sup> decidua basalis (dec. bas.) of patients with low stress scores ( $<0.39$ ) and high stress scores ( $\geq 0.39$ ), as determined by the median stress score as threshold. The data presented were obtained after counting the positive cells under the microscope using a scaled eye-piece, the means and SD for the two groups of patients were calculated. (B) Results of CD8 cell counts, procedure as above. (C) Results of CD3 cell counts (D) Results of CD56 cell counts. The higher numbers of mast cells and CD8<sup>+</sup> cells in decidua basalis from patients with abortion and higher stress scores versus patients with low stress scores was statistically significant ( $P \leq 0.05$ , depicted by \*).

significance. The increase in CD3<sup>+</sup> cells might derive from the increase in CD8<sup>+</sup> cells, since double staining showed that cells marked CD8<sup>+</sup> were exclusively of CD3 phenotype (Figure 2C). The fourth population of immunocompetent cells investigated, i.e. CD56<sup>+</sup> cells in decidua basalis, revealed no significant differences between individuals experiencing lower or higher levels of stress (Figure 1D).

As shown in Figure 3, we observed a significant increase in TNF- $\alpha$ <sup>+</sup> cells/mm<sup>2</sup> decidua basalis of more stressed miscarriage patients compared with patients experiencing lower levels



**Figure 2.** Immunohistochemical analysis of human decidua cells. (A) Cyokeratine staining of tissue obtained from a patient suffering from abortion, showing decidua basalis, as classified by invasive trophoblast cells. Original magnification  $\times 20$ . (B) Mast cell tryptase IHC, positive mast cells are depicted by brown staining. (C) CD3/CD8 double staining. (D) CD56/CD8 double staining. Due to the difficulty of distinguishing between double/single staining, arrows indicate CD8 single<sup>+</sup> cells (purple). (E) CD8<sup>+</sup> cells appear in brown. Scale: 1 cm on figures A–E is 50  $\mu$ m in-situ.

of stress. An example for the in-situ hybridization is shown in Figure 4A,B for the sense probe and at lower magnification in Figure 4C for the antisense probe.

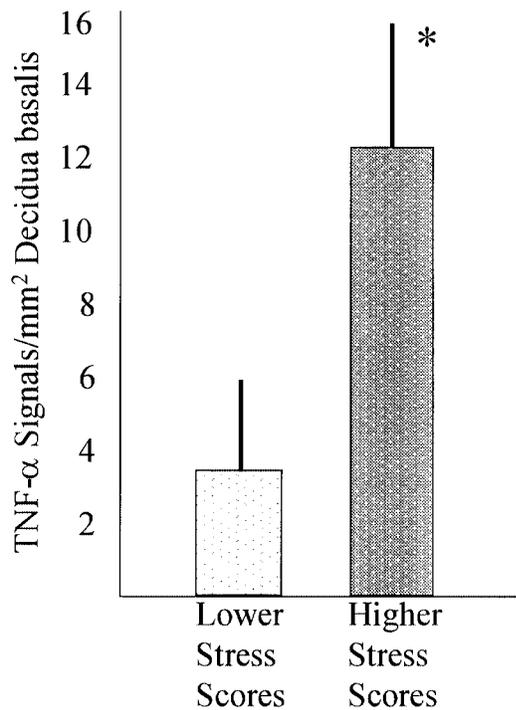
As summarized in Table III, we observed no significant differences of mast cell counts with respect to the trophoblast karyotype in the tissue pool obtained from Chicago. Interestingly, staining for CD56<sup>+</sup> decidua cells revealed a significant decrease ( $P < 0.02$ ) when an abnormal trophoblast karyotype was detected.

**Discussion**

Defining stress using the PSQ in a cohort of patients suffering from spontaneous first trimester miscarriages revealed a positive correlation of higher stress scores and decidua basalis mast cells, CD8<sup>+</sup> T cells and TNF- $\alpha$  expression, which is a potent Th1 inflammatory cytokine family member. The Th1/Th2 balance has been suggested to determine pregnancy outcome:

a bias towards Th1 is strongly correlated with pregnancy failure in mice and humans (Hill *et al.*, 1995; Lim *et al.*, 2000). Successful pregnancy might be associated with a Th2 cytokine serum phenotype and progesterone-stimulated production of suppressor factors, such as transforming growth factor- (TGF-) $\beta$ 2, interleukin(IL)-4 and IL-10, by CD8<sup>+</sup> T cells may be responsible (Lin *et al.*, 1993; Szekeres-Bartho and Wegmann, 1996). Interestingly, there is published data available that does not indicate a systemic shift in the general balance between Th1 and Th2-type cytokine pattern, suggesting a local shift at the fetomaternal interface as more probable (Palfi *et al.*, 1999). However, from experiments in mice we know that abortion is triggered by release of TNF- $\alpha$ , interferon-(IFN-) $\gamma$ , and IL-1, which cause abortion via a novel prothrombinase, Fgl2 (Clark *et al.*, 1999b). External dangers in the form of stress and Th1 cytokine responses upregulate Fgl2 prothrombinase in trophoblast as well as in decidua, resulting in placental hemorrhage and necrosis.

In the present study we observed higher numbers of mast cells in the decidua basalis of women with higher levels of perceived stress, compared with the group reporting lower levels of stress. Recently, mast cells have been described as



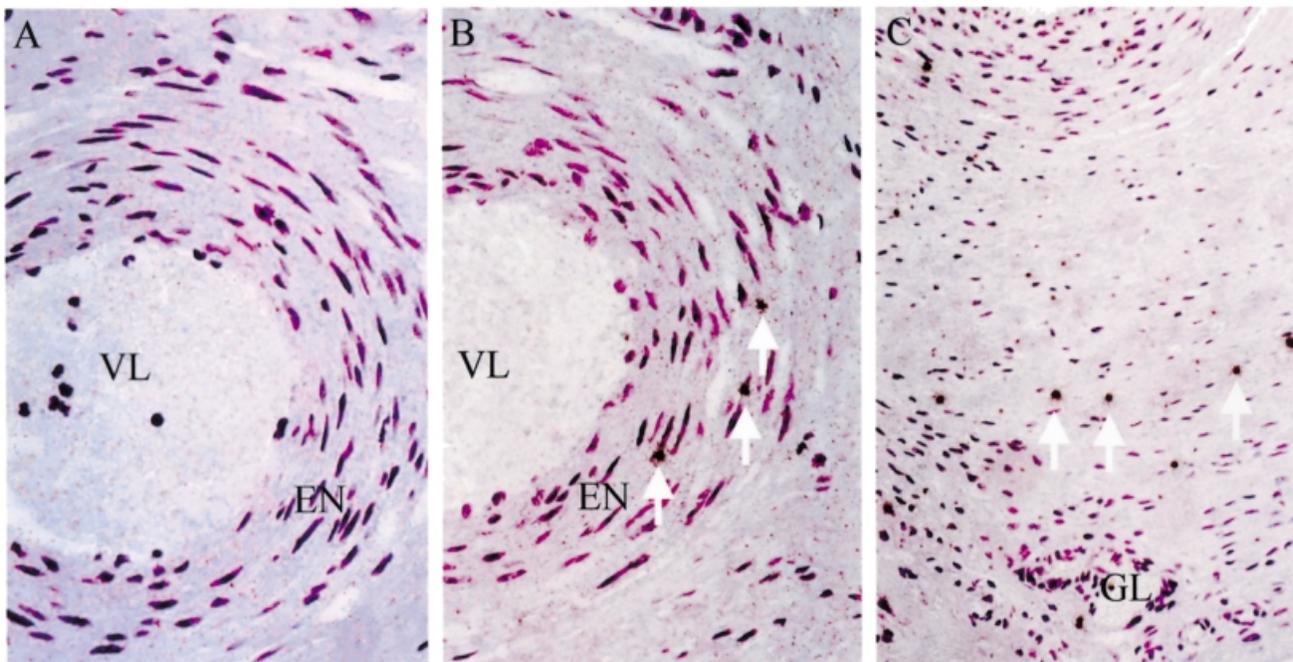
**Figure 3.** TNF- $\alpha$  mRNA<sup>+</sup> cells/mm<sup>2</sup> decidua basalis of miscarriage patients with low versus high stress scores. The higher levels of TNF- $\alpha$  mRNA<sup>+</sup> cells/mm<sup>2</sup> tissue in from patients with higher versus lower stress scores was statistically significant ( $P \leq 0.05$ , depicted by \*).

being present in the uterus and to be associated with human pregnancy failure (Massey *et al.*, 1991; Marx *et al.*, 1999a). The dramatic increase of mast cells in the case of human abortion must be assumed to result in changes of cytokine production. It has been proposed that mast cells produce IL-4, IL-10 and TNF- $\alpha$ , which represents a Th1/Th2 cytokine response (Okayama *et al.*, 1995). It remains to be identified whether the decidual mast cells we observed are of the Th1 or Th2 phenotype. In experimental observations of murine pregnancies a striking increase in percentage of activated, TNF- $\alpha$  producing mast cells in the uteri of stressed animals could be observed (Markert *et al.*, 1997). Blocking of neurotransmitter substance P (SP) has been proven to decrease decidual TNF- $\alpha$  levels in stressed mice (Arck *et al.*, 1995). Therefore mast cells may represent the cellular link between the neurotransmitter SP and an increase in decidual TNF- $\alpha$  release leading to abortion. From the accounts of greater numbers of mast cells and TNF- $\alpha$  signals in decidual basalis in miscarriage patients with higher stress scores, one can assume that these cells are of an abortogenic phenotype,

**Table III.** Absolute cell number (mean  $\pm$  SD) dependent on trophoblast karyotype

| Decidua basalis | Normal karyotype trophoblast (n = 5) | Abnormal karyotype trophoblast (n = 26) | Mann-Whitney test |
|-----------------|--------------------------------------|---|-------------------|
| MCT             | 2.8 $\pm$ 0.1                        | 2.5 $\pm$ 0.4                           | NS                |
| CD56            | 553.3 $\pm$ 15.1                     | 453.3 $\pm$ 33.9                        | *                 |

\* $P < 0.02$ .



**Figure 4.** (A) TNF- $\alpha$  hybridization sense control. VL = vessel lumen, EN = endothelial cells. (B) Corresponding area as in A, antisense hybridization of TNF- $\alpha$  mRNA, as depicted by the black dot clusters, original magnification  $\times 40$ . (C) TNF- $\alpha$  mRNA antisense hybridization, GL = gland, original magnification  $\times 20$ . For scale: 1 cm on A and B is 30  $\mu$ m in-situ, 1 cm on C is 50  $\mu$ m in-situ.

because increased detectability of TNF- $\alpha$  mRNA and co-occurrence of SP in human abortion decidua has been observed (Marx *et al.*, 1999a). An investigation of the presence or absence of SP receptors on human decidual mast cells and their cytokine profile will be mandatory to confirm the suggested role of mast cells in cytokine-mediated abortion.

Although cytokine-producing Th1 cells can be identified within the CD4<sup>+</sup> population, the present findings confirm an involvement of CD8<sup>+</sup> cells in altered cytokine production (Ouellette *et al.*, 1999). Recent literature revealed that an immunomodulatory protein known as progesterone induced blocking factor (PIBF) is produced by peripheral CD8<sup>+</sup> T lymphocytes of pregnant females. Additional data indicate that the PIBF affects the Th1/Th2 balance, and contributes to decreased cell-mediated responses during pregnancy via altered cytokine ratios (Szekeres-Bartho and Wegmann, 1996). One might conclude from these observations that CD8<sup>+</sup> cells, peripherally and locally, are very likely to be pregnancy-protective, and we assume that the increase in CD8<sup>+</sup> decidual cells observed in miscarriage patients with higher stress scores may reflect an attempt to abrogate the imminent or present failure of pregnancy. However, recent published data obtained from murine pregnancies revealed the presence of a decidual CD8<sup>+</sup> cell population producing TNF- $\alpha$  in stress-triggered abortion, hence suggesting a rather abortogenic function for this population (Joachim *et al.*, 2001).

In the present study we did not observe significant differences on NK and CD3<sup>+</sup> cell numbers in the decidua. This was surprising, especially since NK cells have been described to be upregulated with stress (Schedlowski *et al.*, 1993). However, data on NK cells underlying the influence of stress as published by various groups are still contradictory, whereby most of the results were acquired by flow cytometry using peripheral blood lymphocytes. Decidual NK cells have an unusual phenotype, CD3<sup>-</sup> CD16<sup>-</sup> CD56<sup>+++</sup>, distinguishing them from peripheral blood NK cells. Decidual NK cells may control trophoblast migration and placentation (Verma *et al.*, 1997; Vassiliadou and Bulmer, 1998). In addition, decidual CD56<sup>+++</sup>16<sup>-</sup>3<sup>-</sup> NK cells have been shown to release the Th1 cytokine  $\gamma$ -IFN after stimulation with IL-12 and IL-2 (Marzusch *et al.*, 1997). The number of decidual CD56<sup>+++</sup>16<sup>-</sup>3<sup>-</sup> NK cells in spontaneous abortions might be downregulated with abnormal placental chromosomes, as suggested by our data. Unfortunately, we do not have any patient stress scores for the karyotyped tissue pool, since we obtained these samples from a different group. Interestingly, published data have shown an increase of CD56<sup>+</sup> decidual cells with an abnormal karyotype (Yamamoto *et al.*, 1999). However, one has to consider the methodology of evaluation, which might lead to different results, especially in rather low sample numbers. Since we used immunohistochemistry and Yamamoto and co-workers (1999) isolated decidual cells and investigated the cell suspensions by flow cytometry (Yamamoto *et al.*, 1999), we suggest that additional research on a large number of samples is needed to characterize the abnormal CD56 immune response with an abnormal karyotype.

The data presented in this paper may provide an explanation for the cause of pregnancy failure in the group of our patients experiencing high levels of stress. However, since all patients

suffered from a miscarriage, one might wonder at the causes for miscarriage in the group with lower stress scores. Since we mainly focused on increases of abortogenic factors, one might conclude that in the less stressed patients, a decrease of pregnancy protective factors might have caused the failure. Further, we did not examine the hormonal status, i.e. cortisol, ACTH or progesterone, although the hormonal alterations induced by stress are very likely to be responsible for changes in cytokine concentrations. Since hormonal levels are influenced by various factors, i.e. the circadian pacemaker (Czeisler and Klerman, 1999), we refrained from measuring hormonal levels. Further the expression of the oestrogen and progesterone receptors on uterine mucosal leukocytes has been examined by dual immunohistology. Neither the oestrogen receptor nor the progesterone receptor was expressed by lymphocytes, macrophages or CD56 cells. It has been suggested that although the accumulation and survival of these NK cells appears to be hormonally dependent, these effects appear to be indirect (King *et al.*, 1996).

Screening first trimester pregnant women for their PSQ score and correlating it to onset of abortion would have been an alternative study design. However, we had to refrain from a prospective clinical trial for ethical reasons, to avoid increasing anxiety in pregnant women and risking them suffering a miscarriage due to stress. However, in the present study we were able to delineate a correlation between increased stress scores and immunological dysregulations in the uterus, therefore we feel encouraged for a prospective approach, which is currently being pursued.

Interestingly, psychotherapy has been reported in one study to result in a successful pregnancy outcome in patients with a history of recurrent spontaneous abortion (Stray-Pedersen and Stray-Pedersen, 1984). Improvement of individual stress-managing capabilities may now seem more plausible to account for these otherwise unexplained observations. Women with recurrent pregnancy loss experience considerable stress. Offering putatively effective treatments to reduce stress might result in a decrease in the levels of Th1 cytokines, such as TNF- $\alpha$ . Information on the impact of stress-induced changes on the immunological balance and the risk for the onset of diseases is very limited. However, our data support the assumption that the down-regulation or dysregulation of different components of the immune response associated with psychological stressors may have implications with regard to the onset of abortion.

## Conclusions

We propose that stress-triggered abortion in humans may be linked to immunological imbalances and could be identified using a questionnaire. Future research is needed, enrolling a large number of pregnant women in a prospective study. However, our recent data may encourage physicians to use the Levenstein perceived stress questionnaire (Levenstein *et al.*, 1993) which might help in understanding the role of stress in the pathogenesis of other immune-mediated diseases.

## Acknowledgments

This work was supported by grants from the Deutsche Forschungsgemeinschaft (Ar 232/8–1) and UFF Charité (99–648). We thank P. Busse and J. Oder for their technical support and E. Mattheis and A. Mierke for their excellent assistance in documentation. Discussions with Dr S. Levenstein are also gratefully acknowledged.

## References

- Angermeyer, M.C., Kilian, R. and Meils, H. (1999) WHOQOL 100 – Handbook for the German translation of the WHO instrument to assess quality of life. Göttingen: Hogrefe, pp. 1–139.
- Arck, P., Merali, F., Stanisz, A. *et al.* (1995) Stress-induced murine abortion associated with substance P-dependent alteration in cytokines in maternal uterine decidua. *Biol. Reprod.*, **53**, 814–819.
- Christiansen, O.B. (1996) A fresh look at the causes and treatments of recurrent miscarriage, especially its immunological aspects. *Hum. Reprod. Update*, **2**, 271–279.
- Clark, D.A., Arck, P.C. and Chauat, G. (1999a) Why did your mother reject you? Immunogenetic determinants of the response to environmental selective pressure expressed at the uterine level. *Am. J. Reprod. Immunol.*, **41**, 5–22.
- Clark, D.A., Ding, J.W., Chauat, G. *et al.* (1999b) 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.
- Clifford, K., Flanagan, A.M. and Regan, L. (1999) Endometrial CD56+ natural killer cells in women with recurrent miscarriage: a histomorphometric study. *Hum. Reprod.*, **14**, 2727–2730.
- Cohen, S., Miller, G.E. and Rabin, B.S. (2001) Psychological stress and antibody response to immunization, a critical review of the human literature. *Psychosom. Med.*, **63**, 7–18.
- Czeisler, C.A. and Klerman, E.B. (1999) Circadian and sleep-dependent regulation of hormone release in humans. *Recent Prog. Horm. Res.*, **54**, 97–130.
- Fydrich, T., Scheib, P. and Sommer, G. (1988) Social support and stresses in patients with duodenal ulcer. *Psychother. Psychosom. Med. Psychol.*, **38**, 159–166.
- Hill, J.A., Polgar, K. and Anderson, D.J. (1995) T-helper 1-type immunity to trophoblast in women with recurrent spontaneous abortion. *JAMA*, **273**, 1933–1936.
- Joachim, R.A., Hildebrandt, M., Oder, J. *et al.* (2001) Murine stress-triggered abortion is mediated by increase of CD8+ TNF-alpha+ decidual cells via Substance P. *Am. J. Reprod. Immunol.*, **45**, 303–309.
- Lea, R.G., Tulppala, M. and Critchley, H.O. (1997) Deficient syncytiotrophoblast tumour necrosis factor-alpha characterizes failing first trimester pregnancies in a subgroup of recurrent miscarriage patients. *Hum. Reprod.*, **12**, 1313–1320.
- King, A. (2000) Uterine leukocytes and decidualization. *Hum. Reprod. Update*, **6**, 28–36.
- King, A., Gardner, L. and Loke, Y.W. (1996) Evaluation of oestrogen and progesterone receptor expression in uterine mucosal lymphocytes. *Hum. Reprod.*, **11**, 1079–1082.
- Klentzeris, L.D., Bulmer, J.N., Warren, M.A. *et al.* (1994) Lymphoid tissue in the endometrium of women with unexplained infertility: morphometric and immunohistochemical aspects. *Hum. Reprod.*, **9**, 646–652.
- Kodama, T., Hara, T., Okamoto, E. *et al.* (1998) Characteristic changes of large granular lymphocytes that strongly express CD56 in endometrium during the menstrual cycle and early pregnancy. *Hum. Reprod.*, **13**, 1036–1043.
- Levenstein, S., Prantera, C., Varvo, V. *et al.* (1993) Development of the perceived stress questionnaire: a new tool for psychosomatic research. *J. Psychosom. Res.*, **37**, 19–32.
- Lim, K.J.H., Odukoya, O.A., Li, T.C. *et al.* (1996) Cytokines and immunendocrine factors in recurrent miscarriage. *Hum. Reprod. Update*, **2**, 459–468.
- Lim, K.J.H., Odukoya, O.A., Ajjan, R.A. *et al.* (2000) The role of T-helper cytokines in human reproduction. *Fertil. Steril.*, **73**, 136–142.
- Lin, H., Mosmann, T.R., Guilbert, L. *et al.* (1993) Synthesis of T helper 2-type cytokines at the maternal-fetal interface. *J. Immunol.*, **151**, 4562–4573.
- Massey, W.A., Goo, C.B., Dvorak, A.M. *et al.* (1991) Human uterine mast cells. Isolation, purification, characterization, ultrastructure and pharmacology. *J. Immunol.*, **147**, 1621–1621.
- Markert, U.R., Arck, P.C., McBey, B.A. *et al.* (1997) Stress triggered abortions are associated with alterations of granulated cells in the decidua. *Am. J. Reprod. Immunol.*, **37**, 94–100.
- Marx, L., Arck, P., Kieslich, C. *et al.* (1999a) Decidual mast cells might be involved in the onset of human first-trimester abortion. *Am. J. Reprod. Immunol.*, **41**, 34–40.
- Marx, L., Arck, P., Kapp, M. *et al.* (1999b) Leukocyte populations, hormone receptors and apoptosis in eutopic and ectopic first trimester human pregnancies. *Hum. Reprod.*, **14**, 1111–1117.
- Marzusch, K., Buchholz, F., Ruck, P. *et al.* (1997) Interleukin-12 and interleukin-2 stimulated release of interferon- $\gamma$  by uterine CD56<sup>++</sup> large granular lymphocytes is amplified by decidual macrophages. *Hum. Reprod.*, **12**, 921–924.
- Neugebauer, R., Kline, J., Stein, Z. *et al.* (1996) Association of stressful life events with chromosomally normal spontaneous abortion. *Am. J. Epidemiol.*, **143**, 588–596.
- Palfi, M., Jablonowska, B., Matthiesen, L. *et al.* (1999) Circulating interferon-gamma- and interleukin-4-secreting cells in recurrent spontaneous abortions. *Am. J. Reprod. Immunol.*, **41**, 257–263.
- O'Hare, T. and Creed, F. (1995) Life events and miscarriage. *Br. J. Psychiatry*, **167**, 799–805.
- Okayama, Y., Bradding, P., Tunon-de-Lara, J.M. *et al.* (1995) Cytokine production by human mast cells. *Chem. Immunol.*, **61**, 114–134.
- Ouellette, M.J., St-Jacques, S. and Lambert, R.D. (1999) CD8 membrane expression is down-regulated by transforming growth factor (TGF)-beta, 1, TGF-beta, 2, and prostaglandin E2. *Am. J. Reprod. Immunol.*, **41**, 183–191.
- Quenby, S., Bates, M., Doig, T. *et al.* (1999) Pre-implantation endometrial leukocytes in women with recurrent miscarriage. *Hum. Reprod.*, **14**, 2386–2391.
- Schedlowski, M., Jacobs, R., Stratmann, G. *et al.* (1993) Changes of natural killer cells during acute psychological stress. *J. Clin. Immunol.*, **13**, 119–126.
- Stern, J.J., Dorfmann, A.D., Gutierrez-Najar, A.J. *et al.* (1996) Frequency of abnormal karyotypes among abortuses from women with and without a history of recurrent spontaneous abortion. *Fertil. Steril.*, **65**, 250–253.
- Stray-Pedersen, B. and Stray-Pedersen, M. (1984) Etiologic factors and subsequent reproductive performance in 195 couples with a prior history of habitual abortion. *Am. J. Obstet. Gynecol.*, **148**, 140–146.
- Szekeres-Bartho, J. and Wegmann, T.G. (1996) A progesterone-dependent immunomodulatory protein alters the Th1/Th2 balance. *J. Reprod. Immunol.*, **31**, 81–95.
- Vassiliadou, N. and Bulmer, J.N. (1998) Characterization of endometrial T lymphocyte subpopulations in spontaneous early pregnancy loss. *Hum. Reprod.*, **13**, 44–47.
- Verma, S., King, A. and Loke, Y.W. (1997) Expression of killer cell inhibitory receptors on human uterine natural killer cells. *Eur. J. Immunol.*, **27**, 979–983.
- Wasser, S.K. (1999) Stress and reproductive failure: an evolutionary approach with applications to premature labor. *Am. J. Obstet. Gynecol.*, **180**, 272–274.
- Wergeland, E. and Strand, K. (1998) Work pace control and pregnancy health in a population-based sample of employed women in Norway. *Scand. J. Work. Environ. Health.*, **3**, 206–212.
- Yamamoto, T., Takahashi, Y., Kase, N. *et al.* (1999) Role of decidual natural killer (NK) cells in patients with missed abortion: differences between cases with normal and abnormal chromosome. *Clin. Exp. Immunol.*, **116**, 449–452.

Received on January 24, 2001; accepted on April 4, 2001