Antitrophoblast antibodies are associated with recurrent miscarriages

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Objective: To investigate whether antitrophoblast antibodies are associated with unexplained recurrent miscarriages, we used choriocarcinoma cells JEG-3, since these cells are negative for class I and II antigens, but they do express HLA-G, resembling an antigen expression of endovascular and interstitial trophoblasts.

Design: Case-control study.

Setting: Academic research center.

Patient(s): One hundred ninety-four patients with two or more consecutive, idiopathic recurrent miscarriages (RM; <20 weeks of gestation) were compared with 110 controls with normal pregnancies and without pregnancy complications.

Intervention(s): Anti-JEG-3 reactivities were measured by using flow cytometry and comparisons with two in-house standards antibody samples of low and high reactivity.

Main Outcome Measure(s): Anti-JEG-3 reactivities above the 95% confidence interval of controls were defined as positive.

Result(s): Sera of RM patients reacted significantly stronger with JEG-3 cells than that of controls. In addition, RM patients significantly more often had positive anti-JEG-3 reactivities (17.5%) than controls (5%). This difference was markedly increased with a subgroup of 80 RM patients who had three or more miscarriages, as 27 of these women (34%) were anti-JEG-3 positive.

Conclusion(s): Antitrophoblast antibodies show significantly more mean channel shift reactivities, and positive reactivities are significantly more prevalent in RM patients as compared with controls. Such antibodies may be involved in mechanisms affecting pregnancies. (Fertil Steril® 2012;■■■–■■■. ©2012 by American Society for Reproductive Medicine.)

Key Words: Pregnancy loss, recurrent miscarriages, anti-trophoblast antibodies, JEG-3 choriocarcinoma cells
antigens that are cross-reactive on trophoblasts and blood cells. Other studies have used immunohistological or absorption experiments with extraembryonic tissues to establish reactivity patterns (29–34), however, these techniques lack precise quantitation of individual reactivities.

In our studies, we have used the choriocarcinoma cell line JEG-3 because these cells retain many characteristics of normal pregnancy-derived trophoblasts and express a wide range of trophoblast-specific antigens that are candidate targets by maternal antibodies (5, 6, 28, 34–38). In particular, JEG-3 cells are negative for conventional MHC class I and II antigens but do express monomorphic HLA-G (36–41). Therefore, antibodies against paternally inherited HLA antigens should not react with JEG-3 cells. We undertook this case-control study to compare well-characterized RM patients and healthy pregnant and nonpregnant controls for anti-JEG-3 reactivities by flow cytometry. Our results were interpreted by comparison with two in-house standard antibody samples of low and high reactivity, respectively, that were run in parallel for each flow-cytometric analysis.

MATERIALS AND METHODS

Patients and Controls

For this case-control study, we evaluated 194 patients with a history of two or more consecutive miscarriages before 20 weeks of gestation who were seen between 2004 and 2010 at our infertility clinic. The criteria of strengthening the reporting of observational studies in epidemiology were accounted for as far as applicable. All patients had undergone an extensive diagnostic work-up (42) without identifying any potential cause of their RM: infectious diseases, uterine anomalies, endocrinologic dysfunctions (polycystic ovary syndrome according to the Rotterdam criteria (43), hyperprolactinemia, hyperandrogenemia, thyroidal dysfunctions such as hypo-/hyperthyreosis, and thyroid autoantibodies), autoimmune disorders (antinuclear antibodies >1:240, antiphospholipid antibodies IgG and IgM, anti-beta-2-glycoprotein antibodies IgG and IgM, lupus anticoagulant), deficiencies in coagulation factors (protein C, protein S, factor XII, antithrombin III), and fetal and parental chromosomal disorders (numerical aberrations) were excluded. Inherited thrombophilias (factor V–Leiden mutation (FVL), the prothrombin (PT) 20210G → A substitution and the 5,10-methylenetetrahydrofolate reductase (MTHFR) 677C → T exchange) were ruled out (42). An antiphospholipid syndrome was excluded according to the international consensus statement on an update of the classification criteria for definite antiphospholipid syndrome (44). To exclude any immediate interference with gestation, the laboratory evaluation was done at least 2 months after the last pregnancy had ended. At that time, 100-μL serum aliquots were frozen and stored at −20°C for further analyses.

Biographic and historic data of patients and controls are illustrated in Table 1: 73.2% (n = 142) had primary RM (no infant born either dead or alive after the 20th completed week of gestation and/or weighing more than 500 g before the series of miscarriages; 45), and 26.8% (n = 52) underwent secondary RM (at least one infant born either dead or alive after the 20th completed week of gestation or weighing more than 500 g before the series of abortions; 45). Healthy control individuals with normal pregnancies, one or more normal term deliveries of healthy and normal weight singletons and without gestational pathology, were recruited from our outpatient department between 2004 and 2010. To study the influence of pregnancy on anti-JEG-3 reactivity, non-pregnant (n = 30) and pregnant controls (n = 80) were included. Sera from nonpregnant controls were obtained at least 2 months after their latest deliveries, and sera from pregnant controls were drawn between 15 ± 4 and 37 ± 6 (with a mean of 32 ± 1) weeks of gestation. Neither RM patients nor controls had ever received transfusions of blood or blood products.

Sign-up informed consent was obtained from all participants, allowing analysis of all clinical and laboratory data mentioned in this paper. The Human Investigation Review Board of the Ludwig-Maximilians-University Munich approved the study (no. 238-06).

JEG-3 Cells

JEG-3 cells were grown and processed as described elsewhere (35, 46). In brief, the cells were cultured in complete culture medium RPMI 1640 (Sigma) supplemented with FCS (heat-inactivated, 5% v/v; Dako) and penicillin/streptomycin (100 IE/mL penicillin, 50 g/mL streptomycin; Invitrogen) at 37°C in a humidified atmosphere (with 5% CO2) until 80% subconfluency was reached.

Flow-Cytometric Analysis

JEG-3 cells were prepared for flow-cytometric analysis as follows: cells were detached from the flask by addition of 10 mL ready-to-use Accutase solution containing proteolytic and collagenolytic enzymes in Dulbecco’s phosphate-buffered saline (PBS), pH 7.4 (Sigma, cat. no. A6964) per 75 cm2 surface area and returned to the incubator for 10 minutes. Cell concentration was determined using a Coulter Z1 counter (Coulter Electronics). A suspension of 105 cells was transferred into a 5-mL polystyrene round-bottom tube (BD Bioscience) and washed twice in RPMI supplemented with 20 mg/mL FCS and 0.2 mM Colchicin (Sigma). Subsequently, cells were resuspended and incubated with 20 μL of patient, control, or standard sample sera for 60 minutes at 4°C. The expression of conventional HLA-A/B antigens by JEG-3 cells was analyzed by incubating the cells with a monoclonal mouse anti-HLA-A/B antibody (TP25.99, Sangstat, diluted 1:20 in 20 μL PBS). HLA-G expression was analyzed using a monoclonal mouse anti-HLA-G-1 antibody (MEM-G/9, Abcam; diluted 1:20 in 20 μL PBS) (38–40, 46). Purified mouse IgG (Cedarlane Diagnostics) at identical IgG concentrations served as a control.

After incubation with human sera or primary monoclonal antibodies, the cell suspension was washed twice in RPMI buffer and incubated with FITC-conjugated secondary antibodies diluted 1:10 in 10 μL PBS for 60 minutes at 4°C in the dark. Secondary antibodies were polyclonal goat anti-human IgG/FITC Fab’/2 (Dako), or FITC-conjugated rabbit anti-mouse IgG (Dako), respectively. Details on antibodies...
and sera used for flow cytometry are summarized in Supplemental Table 1.

The cell suspensions were analyzed on a Beckton Dickinson flow cytometer (FACScan) equipped with a 2.4-mW argon ion laser at an excitation wavelength of 488 nm. The green signals of FITC on a log scale of each analyzed cell fraction were collected using a 590-nm band pass filter. A marker was set in the FITC histogram as the cutoff between the background signals and positive staining, which was determined by comparison with negative control samples. A minimum of 15,000 cells was analyzed in each run. Reactivities were measured in mean channel shifts (MCS), and anti-JEG-3 reactivity was expressed as a percentage of the difference between the reactivities of two in-house standard samples that were included in each assay. The standard sample with high reactivity consisted of a pool of 10 patient sera, identified as highly reactive in preliminary experiments. These patients had experienced a mean number of 4 (minimal-maximal: 3–8) unexplained miscarriages. Although they met the criteria of RM patients, we did not include them in our study group. Identical serum aliquots of each of these individuals were pooled to obtain a positive standard sample, and this respective anti-JEG-3 reactivity was arbitrarily defined as 100%. The standard sample with low reactivity consisted of a blood group AB standard sample provided by our local blood bank (registration number 505110), and this anti-JEG-3 reactivity was arbitrarily defined as 0%. Anti-JEG-3 reactivity of individual test sera was calculated by using the following formula:

\[
\text{Activity of test serum (MCS)} - \text{activity of low reacting standard (MCS)} \\
\text{Activity of high reacting standard (MCS)} - \text{activity of low reacting standard (MCS)} \times 100 = \% 
\]

Cell viability was investigated by propidium iodide staining: parallel with each experiment, the cells were washed twice and incubated with propidium iodide (1 mg/mL; Sigma). The red signals of propidium iodide were quantified flow-cytometrically by using a 650-nm band pass filter; the propidium iodide–positive cells were considered dead, and propidium iodide–negative cells were considered alive. Only samples with at least 90% viability were included for the analysis.

The intra-assay coefficient of variation (intra-assay CV) was calculated from five independent measurements of a given serum within one measurement and calculated to be 8.9%. The interassay CV was calculated from five different measurements of a given serum at five different dates and calculated to be 13.3%.

Statistical Analyses

Statistical analyses were performed with the Statistical Package for Social Sciences (SPSS for Windows 19.0, SPSS Inc.). Comparisons of pregnancy histories and biographic data of JEG-3-positive and JEG-3-negative patients as well comparison of RM patients and control groups were performed by standard t-test and Mann-Whitney U-test.

Positive reactivity with JEG-3 cells was defined to be above the 95% confidence range of the control group. This was calculated according to the formula above. \( P < .05 \) was considered statistically significant.

RESULTS

JEG-3 Cell Expression of HLA

When JEG-3 cells were analyzed flow-cytometrically with mAbs MEM-G9, specific for HLA-G, and TP 25.99, specific for MHC class I antigens HLA-A and -B, results confirmed this trophoblast cell line to express HLA-G but to be negative for HLA-A and -B. This expression pattern resembles that of extravillous trophoblasts that are exposed to decidua and maternal blood. In addition, these experiments confirm that JEG-3 cells do not serve as targets for maternal antibodies to paternally inherited MHC class I antigens that have been shown not to be detrimental for gestation (14, 22–25).

| TABLE 1 | Biographic and historic data of patients and controls. |
|-----------------|-----------------|-----------------|-----------------|-----------------|
|                 | Patients (n = 194) | Pregnant controls (n = 80) | Nonpregnant controls (n = 30) | \( P \) value |
| Mean age (range) | 33 (21–40) | 31 (21–41) | 31 (20–45) | NS |
| Mean pregnancies (range) | 4 (2–20) | 2 (1–5) | 2 (1–5) | NS |
| Mean deliveries (range) | 0 (0–2) | 2 (1–5) | 2 (1–5) | NS |
| Weeks of gestation | 32 + 1 (15 + 4 to 37 + 6) | 0 (0) | 0 (0) | .000001 |
| Mean miscarriages (range) | 4 (2–20) | Primary RM (%) | 142 (73.2) | Secondary RM (%) | 52 (26.8) | No. of miscarriages (%): | 2 | 70 (36.1) | 3 | 68 (35.1) | 4 | 31 (15.9) | 5–8 | 20 (10.3) | 9–20 | 5 (2.6) |
| 2 | 70 (36.1) | 3 | 68 (35.1) | 4 | 31 (15.9) | 5–8 | 20 (10.3) | 9–20 | 5 (2.6) |

Note: NS = nonsignificant.
Anti-JEG-3 Reactivities in RM Patients and Controls

When anti-JEG-3 activities of RM patients (median, 19.8%; interquartile range [IQR], 9.2%–33.9%) and all control individuals (median, 15.2%; IQR, 6.3%–23.5%) were compared, RM patients had significantly stronger reactivities ($P= .004$). Interestingly, nonpregnant (median, 13.3%; IQR, 0.5%–26.2%) and pregnant controls (median, 15.1%; IQR, 7.6%–22.7%) were not different ($P = .5$), suggesting that normal pregnancies are not associated with altered anti-JEG-3 activities. Accordingly, for further statistical analyses, nonpregnant and pregnant controls were analyzed together. While a large proportion of RM patients and controls had comparable anti-JEG-3 reactivities, a substantial number of RM patient sera (17.5%) had very strong anti-JEG-3 activities that were well above the 95% confidence interval (CI) of normal patients (Fig. 1A). When comparing anti-JEG-3 reactivities in RM patients and controls as a function of number of pregnancies, there was no correlation between JEG activities and number of pregnancies in patients or controls (Fig. 1B). Also, we did not observe a difference between RM patients who lost their pregnancies solely in the first trimester ($\leq$ 12 weeks of gestation; $n = 138$; 19.9% median JEG-3 activity; IQR, 6.8%–35.2%), solely in the second trimester ($n = 9$; 28.7% median JEG-3 activity; IQR, 17.7%–88.1%; >12 weeks of gestation), or in the first as well as in the second trimesters ($n = 47$; 16.4% median JEG-3 activity; IQR, 9.2%–29.1%).

Positive Anti-JEG-3 Reactivities

We defined positive anti-JEG-3 reactivities above the 95% CI of controls (39% anti-JEG-3 reactivity) as significant. With this definition, 5% ($n = 6$) of controls were anti-JEG-3 positive and this was significantly less than the 17.5% ($n = 34$) of patients with two or more miscarriages ($P = .002$) and the 34% ($n = 27$) of patients with three or more miscarriages ($P = .000001$; Table 2).

When JEG-3-positive and JEG-3-negative patients were compared for historical and pregnancy data, we did not find significant differences in age, numbers of deliveries, or pregnancies or in primary or secondary RM. Furthermore, the time span between the most recent miscarriage and serum collection did not differ between the JEG-3-positive and JEG-3-negative patients (Table 3). Interestingly, JEG-3-positive patients showed a trend ($P = .07$) of having three or more miscarriages (77%) as compared with the JEG-3-negative group, 61% of which had three or more miscarriages (Table 3).

DISCUSSION

Our flow-cytometric analyses confirmed the choriocarcinoma cell line JEG-3 to react with monoclonal antibody MEM-G-9 that is specific for the monomorphic MHC class I antigen HLA-G (34–41). In addition, we have shown JEG-3 to be

| TABLE 2 |

| Prevalence of positive anti-JEG-3 reactivities in controls as compared with patients with two or more and three or more miscarriages. |
|-----------------|-----------------|-----------------|-----------------|-----------------|
|                 | JEG-3 positive  | JEG-3 negative  | Total            | $P$ value       |
| Two or more miscarriages | 34 (17.5)       | 160 (82.4)      | 194 (100)        | .002            |
| Three or more miscarriages | 27 (34)         | 53 (66)         | 80 (100)         | .000001         |
| Controls         | 6 (5)           | 104 (95)        | 110 (100)        |                 |

Note: Numbers in parentheses are percents.
negative with monoclonal antibody TP25.99 that reacts with HLA-A and -B. This pattern of expression of MHC antigens resembles that of extravillous trophoblasts that are exposed to maternal blood and tissues. This appears to make JEG-3 cells an interesting model for studying antitrophoblast antibodies.

Our studies show that sera from RM patients react significantly stronger with JEG-3 choriocarcinoma cells than sera of healthy controls without pregnancy pathology. Furthermore, very strong anti-JEG-3 reactivities that were above the 95% CI of the control group and that we defined as “positive” were significantly more prevalent in RM patients than in controls. We suggest that the suppression of antitrophoblast activity might be impeded in a subgroup of patients with unexplained RM compared with women with normal pregnancies and normal deliveries. A lack of such an adequate suppression resulting in excessive anti-JEG-3 activities could activate immunologic reactions that interfere with pregnancy progress. Nevertheless, a small percentage of normal controls also have high anti-JEG-3 activities without apparent harm for pregnancies in this small subgroup of individuals. We suggest that highly reactive anti-JEG-3 antibodies identified in RM patients and controls might differ in respect to downstream mechanisms such as binding of complement and/or of cellular Fc-receptors causing different effects in the placentas of the two groups studied. Also, there might be differences in binding to extravillous trophoblasts as well as characteristic immunopathological features in the placentas of respective individuals. Such important questions need to be addressed in further studies.

The concept of a physiological regulation of humoral antitrophoblastic activities is supported by our observation that healthy controls do not appear to initiate or increase anti-JEG-3 activities during normal pregnancies. It should prove interesting to study longitudinally anti-JEG-3 reactivities during normal and abnormal pregnancies to ascertain whether differential reaction patterns and/or different iso-types of antibodies are involved.

Our data do not show any association of positive anti-JEG-3 reactivity with age, primary or secondary miscarriages, pregnancies, or deliveries. Furthermore, we could not find any significant clinical or historic difference between anti-JEG-3-positive and anti-JEG-3-negative RM patients. These findings fail to provide a pattern of historical data or clinical signs or symptoms associated with excessive anti-JEG-3 reactivity within the group of patients with unexplained RM. Neither do these findings point to a traditionally defined mechanism of pathology that might result from antitrophoblast antibodies. However, trophoblast pathology may resemble other types of antibodies such as those found in patients with antiphospholipid antibody syndrome, wherein the antibodies are associated with a broad spectrum of pregnancy pathologies (2, 18, 47, 48). Likewise, anti-JEG-3 antibodies also might recognize a spectrum of specificities to allo- and/or autoantigens that could cause various pathological effects on the course and outcome of pregnancy. Despite the proposed divergent effects of antitrophoblast antibodies, strong anti-JEG-3 reactivity is a significant laboratory finding in 17.5% of patients with two or more and even 34% of patients with three or more unexplained RMs. Indeed, 77% of our anti-JEG-3-positive RM patients had experienced three or more miscarriages as compared with 61% in our anti-JEG-3-negative RM patients ($P$ = .07). Patients experiencing three or more consecutive miscarriages are more likely than patients with only two miscarriages to have some kind of defined systematic pathology resulting in loss of euploid concepti. In conclusion, our data suggest that antitrophoblast antibodies as detected by our anti-JEG-3 assay may participate in the immunopathology of pregnancy.

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REFERENCES

### SUPPLEMENTAL TABLE 1

**Antibodies and sera**

<table>
<thead>
<tr>
<th>Antibodies and sera</th>
<th>Specificity</th>
<th>Description</th>
<th>Secondary antibodies for flow cytometry analysis:</th>
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</thead>
<tbody>
<tr>
<td>AB sera (low reacting control)</td>
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<td>Blood group AB-sera; defined as 0%</td>
<td>FITC-anti-human IgG</td>
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<tr>
<td>Pool of highly reactive sera</td>
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<td>Pool of 10 patient sera of high reactivity; defined as 100%</td>
<td>FITC-anti-human IgG</td>
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<tr>
<td>Sera of patients and controls</td>
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<td>–</td>
<td>FITC-anti-human IgG</td>
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<tr>
<td>Mouse IgG1 isotype-control</td>
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<td>Monoclonal antibody, mouse IgG1</td>
<td>FITC-anti-mouse IgG</td>
</tr>
<tr>
<td>TP25.99</td>
<td>HLA-G-1 on cell surface, soluble HLA-G-5 isoforms in B2 microglobulin associated forms</td>
<td>Monoclonal antibody, mouse IgG1</td>
<td>FITC-anti-mouse IgG</td>
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<tr>
<td>MEM-G/9</td>
<td>–</td>
<td>–</td>
<td>FITC-anti-mouse IgG</td>
</tr>
</tbody>
</table>

**Description**

- FITC-anti-human IgG: Human IgG
- FITC-anti-mouse IgG: Mouse IgG

**References**