Procoagulant effect of the OKT3 monoclonal antibody: Involvement of tumor necrosis factor

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Procoagulant effect of the OKT3 monoclonal antibody: Involvement of tumor necrosis factor. We recently observed that the prophylactic administration of high doses of OKT3 monoclonal antibody (MoAb) in cadaveric renal transplantation favors the development of thromboses of the grafts' main vessels and of thrombotic microangiopathies. These clinical observations led us to perform sequential determinations of plasma levels of prothrombin fragment 1 and 2 (F 1 + 2) and fibrin degradation products (FDP) after the first injection of 5 or 10 mg OKT3 given as prophylaxis in kidney transplant recipients. The values observed have been compared with those of kidney transplant recipients not treated with OKT3. F 1 + 2 levels peaked four hours after the first injection of 5 mg OKT3 (mean ± SEM: 4.82 ± 0.73 vs. 1.75 ± 0.37 nmol/liter in controls, P < 0.01), indicating activation of the common pathway of the coagulation cascade. FDP levels were already above baseline values at four hours and continued to increase until 24 hours (mean ± SEM at 24 hr, 4729 ± 879 vs. 1038 ± 320 ng/ml in controls, P < 0.05), indicating a fibrinolytic process. The magnitude and the time course of the changes in F 1 + 2 and FDP plasma levels were similar whether the patients received 5 or 10 mg dose of OKT3. The levels of von Willebrand factor (VWF) antigen, a molecule released by activated or damaged endothelial cells, were also significantly increased after injection of OKT3 (mean ± SEM at 24 hr, 3.67 ± 0.18 vs. 2.17 ± 0.11 U/ml in controls, P < 0.05). The procoagulant effects of OKT3 were further investigated in vitro on human umbilical vein endothelial cells (HUVEC). It was found that OKT3 induces peripheral blood mononuclear cells (PBMC) to release soluble mediators that trigger the generation of thrombin at the HUVEC surface by a tissue factor-dependent mechanism. The addition of chimeric anti-TNF-α MoAb to culture supernatants of OKT3-stimulated PBMC strongly inhibited the thrombin generation in this model. We conclude that OKT3 activates the coagulation cascade in vivo and that TNF-α is a mediator of the procoagulant effects of OKT3 at the endothelial cell level.

The first dose reactions observed after the administration of the OKT3 monoclonal antibody (MoAb) in transplant recipients include fever, chills, headaches and digestive symptoms [1]. Occasionally, more severe complications such as pulmonary edema and aseptic meningitis also occur. These adverse reactions appear to be related to the systemic release of cytokines, particularly of tumor necrosis factor-alpha (TNF-α) [2, 3]. We recently observed a new and severe complication of high-dose prophylactic OKT3 (10 mg/day) in kidney transplant recipients, namely the occurrence of intragraft thromboses [4]. In parallel, we found that the first injection of such a high dose of OKT3 is followed by a significant increase in the plasma levels of prothrombin fragment 1 + 2 (F 1 + 2), indicating activation of the common pathway of the coagulation system [4].

The present study was undertaken to further investigate the effects of OKT3 on hemostatic processes. First, we performed sequential determinations of plasma levels of F 1 + 2 and fibrin degradation products (FDP) after injection of a conventional (5 mg) or high (10 mg) dose of OKT3 in kidney transplant recipients. Second, we measured plasma levels of von Willebrand factor (VWF) antigen as a marker of endothelial cell activation. Third, we developed an in vitro model allowing to investigate the role of TNF-α in the procoagulant properties of OKT3 at the endothelial cell level.

Methods

Patients

Adult recipients of cadaveric renal transplants were studied immediately before and during the first four or 24 hours post-transplantation. In the OKT3 groups, patients received an i.v. injection of 5 mg (N = 10) or 10 mg (N = 7) OKT3 (Orthoclone, Ortho Biotech, Raritan, New Jersey, USA) at the initiation of transplant surgery. Other immunosuppressive agents on the day of transplantation included azathioprine 2 mg/kg, and methylprednisolone 8 mg/kg given as an i.v. bolus three hours before surgery. In the control group, patients were immunosuppressed with azathioprine and methylprednisolone only during the first four hours (N = 4) or during the first 24 hours (N = 3) post-transplantation. As shown in Table 1, the immunological characteristics and the ischemia times were similar in the three groups of kidney transplant recipients.

In addition, we studied three patients who received a first injection of 10 mg OKT3 during the first trimester posttransplantation as anti-rejection therapy while they were under low dose prednisolone (0.3 to 0.5 mg/kg) and azathioprine (1 to 2 mg/kg), together with (N = 1) or without (N = 2) cyclosporin A (4 mg/kg). As in the prophylactic protocol, the OKT3 injection was preceded by an i.v. bolus of methylprednisolone (8 mg/kg).
Table 1. Immunological parameters and ischemia times

<table>
<thead>
<tr>
<th></th>
<th>Controls (N = 7)</th>
<th>OKT3 5 mg (N = 10)</th>
<th>OKT3 10 mg (N = 7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of recipients with previous grafts</td>
<td>0/7</td>
<td>1/10</td>
<td>1/7</td>
</tr>
<tr>
<td>No. of recipients with anti-HLA antibodies</td>
<td>2/7</td>
<td>3/10</td>
<td>1/7</td>
</tr>
<tr>
<td>HLA incompatibilities</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>0.57 ± 0.37</td>
<td>0.70 ± 0.21</td>
<td>0.57 ± 0.20</td>
</tr>
<tr>
<td>B</td>
<td>0.86 ± 0.34</td>
<td>0.30 ± 0.15</td>
<td>0.29 ± 0.18</td>
</tr>
<tr>
<td>DR</td>
<td>0.14 ± 0.14</td>
<td>0 ± 0</td>
<td>0.14 ± 0.14</td>
</tr>
<tr>
<td>Ischemia times (hours)</td>
<td>26.1 ± 2.2</td>
<td>22.5 ± 1.8</td>
<td>26.3 ± 3.2</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SEM; differences between the groups are not statistically significant.

Blood collection

Blood samples were collected through a central venous catheter immediately before and 2, 4, 8 and 24 hours after the first injection of OKT3. In control patients, blood sampling was started at the initiation of surgery and followed the same timing as in OKT3 patients, except that four patients of this group could only be investigated during the first four hours because they subsequently received OKT3. After discarding the first 20 ml, blood was collected into vacuum tubes containing CTAD or trisodium citrate 3.8% as the anticoagulant (Diatube, Stago, Terumo Europe, Leuven, Belgium). Samples were kept on ice, centrifuged at 4000 rpm and plasma were stored at −70°C until assayed.

Hemostatic assays on plasma samples

Commercially available ELISA kits were used for determination of F 1 + 2 (Behringwerke, Marburg, Germany), FDP (Fibrinostika, Organon Teknika, Boxtel, Holland) [5] and VWF antigen (Stago, Franconville, France).

In vitro stimulation of peripheral blood mononuclear cells with OKT3

Peripheral blood mononuclear cells were isolated from blood obtained from healthy volunteers by Ficoll-Hypaque density gradient centrifugation and cultured in 5% CO2 atmosphere in M199 medium (Flow, Irvine, Scotland, UK) supplemented with 20% human serum, in the presence or absence of OKT3 10 ng/ml. Culture supernatants were collected after 24 hours and stored at −70°C until used in the assays described below.

Generation of thrombin at the surface of human umbilical vein endothelial cells

Human umbilical vein endothelial cells (HUVEC) isolated according to the method of Jaffé [6] were cultured using M199 medium supplemented with 20% human serum, essential amino acids, endothelial cell growth factor (ECGF) (40 µg/ml), heparin (100 µg/ml), penicillin and streptomycin. Cells from the second passage (2 10^6 cells/ml) were transferred into 96 well plates and used at confluence after 24 hours in the absence of heparin and ECGF. After washing, 100 µl culture supernatants of PBMC were added, and the thrombin generated at the surface of HUVEC was determined by addition of calcium (100 µl, 30 mM, 2 min at 37°C) followed by normal human citrated plasma (45 sec at 37°C) before incubation with the chromogenic substrate (S2238, Kabi Vitrum, 0.7 mM, 100 µl, 20 min at 37°C). The reaction was stopped with acetic acid (50 µl) and the absorbance read at 405 nm [7]. The amount of thrombin generated in this system was calculated using a standard curve obtained with purified thrombin and was expressed in mUnits of thrombin per 10^6 HUVEC. In some experiments, supernatants of OKT3-stimulated PBMC were incubated for two hours with different amounts of chimeric (human-mouse) anti-human TNF-α MoAb (provided by Centocor, Malvern, USA) or of an isotype-matched MoAb before to be incubated with HUVEC.

Statistical analysis

Differences between groups were analyzed by Student's t-test.

Results

In vivo activation of the common pathway of coagulation by 5 mg OKT3

We measured F 1 + 2 and FDP plasma levels after the first injection of 5 mg OKT3 given prophylactically during transplantation surgery. As shown in Figure 1, a major increase in F 1 + 2 levels, reflecting activation of the common coagulation pathway, was observed at four hours (mean ± SEM at 4 hr, 4.82 ± 0.73 vs. 1.75 ± 0.37 nmol/liter in controls, P < 0.01).

F 1 + 2 plasma levels returned to basal values within 24 hours except in two OKT3 patients. In one of them, thrombosis of the graft vein became apparent on the second postoperative day.
while the other developed a thrombotic microangiopathy diagnosed on the fifth post-operative day. The increase in F 1 + 2 levels was only observed after the first injection of OKT3. No significant changes were observed after the subsequent injections of the MoAb (data not shown).

As compared with controls, OKT3 patients also displayed increased FDP levels, indicating the occurrence of a fibrinolytic process (Fig. 2). This change was already apparent at four hours, but was more pronounced at 24 hours (mean ± SEM, 4729 ± 879 vs. 1038 ± 1038 ng/ml in controls, P < 0.05).

As shown in Table 2, the changes in hemostatic parameters were essentially independent of the OKT3 dose. Peak plasma levels of F 1 + 2 were slightly higher in the patients who received 10 mg OKT3 but the difference did not reach statistical significance (P = 0.411).

Activation of coagulation was also evident when OKT3 was given as anti-rejection therapy. As shown in Figure 3, F 1 + 2 and FDP levels increased in the three patients studied with a kinetic similar to that observed under prophylactic OKT3.

**Table 2. Peak plasma levels of F 1 + 2 and FDP levels according to OKT3 dosage**

<table>
<thead>
<tr>
<th>OKT3 dose</th>
<th>F 1 + 2 nmol/liter</th>
<th>FDP ng/ml</th>
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<tbody>
<tr>
<td>5 mg (N = 10)</td>
<td>4.82 ± 0.73</td>
<td>4729 ± 879</td>
</tr>
<tr>
<td>10 mg (N = 7)</td>
<td>5.88 ± 0.75</td>
<td>4422 ± 595</td>
</tr>
</tbody>
</table>

Increased plasma levels of von Willebrand factor after injection of OKT3

As several cytokines released after the first injection of OKT3 might affect endothelial cells, we monitored plasma levels of VWF antigen [6]. Data represented in Figure 4 show that VWF plasma levels rose significantly following the injection of 5 mg prophylactic OKT3 (mean ± SEM at 24 hr, 3.67 ± 0.18 vs. 2.17 ± 0.11 U/ml in controls, P < 0.05).

**OKT3 generates procoagulant activity at the surface of HUVEC**

We next investigated the ability of OKT3 to induce thrombin generation at the surface of endothelial cells. For this purpose, HUVEC were incubated with OKT3 or with supernatants of PBMC stimulated by 10 ng/ml OKT3 before measurement of thrombin activity. We first observed that culturing HUVEC either with OKT3 alone (10 ng/ml) or with supernatants of unstimulated PBMC did not result in significant thrombin generation (Fig. 5). On the other hand, supernatants of OKT3-stimulated PBMC induce a massive generation of thrombin at the HUVEC surface. The magnitude and the time-course of this process was similar to that induced by recombinant TNF-α (10 ng/ml). The same experiments were repeated using factor VII-deficient plasma. Under this condition, supernatants of OKT3-stimulated PBMC did not induce any detectable thrombin generation, suggesting that this phenomenon was dependent on tissue factor expression (data not shown).

**TNF-α is a mediator of the procoagulant effect of OKT3 at the endothelial cell level**

Since supernatants of OKT3-stimulated PBMC contain high levels of TNF-α and because of the well-known procoagulant properties of this mediator, we investigated the role of TNF-α in the effects of OKT3 on HUVEC. As shown in Figure 6, the addition of a neutralizing chimeric anti-TNF-α MoAb to a culture supernatant of OKT3-stimulated PBMC (containing 1.9 ng/ml TNF-α) resulted in a dose-dependent inhibition of the thrombin generation. A 80% inhibition was achieved with 1 μg/ml chimeric anti-TNF-α MoAb whereas an isotype-matched control MoAb at the same concentration had no effect.

**Discussion**

The first observation of this study is that the first dose of 5 mg OKT3 in transplant recipients induces the activation of the common pathway of coagulation, as indicated by a significant rise in F 1 + 2 plasma level four hours after the injection of the MoAb. F 1 + 2 fragment is released during conversion of prothrombin to thrombin so that its measurement directly reflects activation of prothrombin [8]. In addition, the increase in FDP levels indicates that a fibrinolytic process also occurs after injection of OKT3. Although parameters of primary fibrinolysis were not measured, the late increase in FDP levels suggests that fibrinolysis after injection of OKT3 might, at least in part, be secondary to activation of the coagulation cascade.

Since our first observations of thrombotic complications consecutive to prophylactic OKT3 were made in patients receiving a high (10 mg) daily dose of the MoAb [4], we analyzed the impact of OKT3 dosage on hemostatic parameters. F 1 + 2 and FDP peak levels were similar whether the patients received the usual 5 mg dose or the high 10 mg dose of OKT3. This is in agreement with our most recent clinical data suggesting that, in kidney transplant recipients, 5 mg OKT3 treatment also carries
an increased risk of thrombotic complications, as illustrated in two patients of the present study.

It is likely that cytokines released in the circulation after the first injection of OKT3 play a central role in the procoagulant effect of the MoAb, as previously suggested by Kanfer et al [9]. Thus, interferon-γ has been shown to be involved in the induction of monocyte procoagulant activity by OKT3 in vitro [10]. Moreover, TNF-α is known to promote fibrin deposition and intravascular coagulation in vivo [11]. Interestingly, the magnitude and the time course of the changes in F1 + 2 plasma levels in our patients were very similar to those reported by van der Poll et al in healthy volunteers injected with recombinant TNF-α [12]. In this setting, it is clear that the extrinsic route and not the contact system of coagulation was activated [12]. Endothelial cells could play an important role in this phenomenon since TNF-α stimulates the expression of tissue factor at their surface [13].

Our previous clinicopathological observations of thrombotic microangiopathy mimicking hemolytic-uremic syndrome as a complication of prophylactic OKT3 already suggested that endothelial cells are important targets of OKT3 toxicity [4]. The twofold increase in VWF levels 24 hours after injection of OKT3 is consistent with the hypothesis that endothelial cells are activated and/or damaged as a consequence of the administration of the MoAb. In vitro experiments on HUVEC allowed to determine more precisely the procoagulant effects of OKT3 at the endothelial cell level. While the MoAb has no direct effect on HUVEC, it induces PBMC to release soluble factors which trigger thrombin generation at the HUVEC surface by a tissue factor-dependent mechanism. The blocking effect of a chimeric anti-TNF-α MoAb revealed that TNF-α is a crucial mediator of the procoagulant effect of OKT3 in this
system. This does not exclude the involvement of other cytokines, that is, interferon-γ, which are known to act in synergy with TNF-α in the induction of endothelial cell changes [11].

As far as the clinical relevance of our findings is concerned, one should first stress that the activation of coagulation is very transient and is only observed after the first injection of the MoAb. Interestingly, the release of cytokines also only occurs after the first injection OKT3 [2, 3]. In fact, the changes in hemostatic parameters parallel the release of cytokines which is maximal two hours after the first OKT3 injection. Since the main thrombotic complications in our experience occurred in patients receiving prophylactic OKT3 and involved intragraft vessels, it is possible that endothelial damages induced by ischemia and/or surgery contribute to the development of thromboses. Although thrombotic complications have not been reported as yet in patients receiving OKT3 as anti-rejection therapy, one should be aware that activation of coagulation also occurs in this setting.

Since TNF-α appears as an essential mediator of the procoagulant activity of OKT3, the irreversible thrombotic complications induced by prophylactic OKT3 could possibly be prevented by neutralization of TNF-α or abrogation of its release. This might represent the rationale for a trial of anti-TNF-α MoAb in patients receiving OKT3 as well as for the clinical development of non-activating OKT3-like MoAb [14].

Acknowledgments

This work was supported by the Fonds de la Recherche Scientifique Médicale (Belgium) and by Cilag Benelux. We thank Dr. Wendy Dixon (Centocore, Malvern, USA) for providing the chimeric anti-TNF-α MoAb. The technical assistance of Claude Habran and Alain Crusiaux (Centocore, Malvern, USA) for providing the chimeric anti-TNF-α MoAb is gratefully acknowledged.

Reprint requests to Michel Goldman, M.D., Hôpital Erasme, Department of Immunology, Hematology, Transfusion, 808 route de Lennik, B-1070 Brussels, Belgium.

Note added in proof

Since the submission of this manuscript, another study has demonstrated activation of coagulation and fibrinolysis following OKT3 administration [RAASVELD MH, HACK CE, TEN BERGE IJ: Thromb Haemostas (in press)].

Appendix. Abbreviations

<table>
<thead>
<tr>
<th>A</th>
<th>B Thrombin, mIU/10⁶ HUVEC</th>
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<tbody>
<tr>
<td>OKT3 Sn</td>
<td>MoAb ng/ml</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>+</td>
<td>Control 1000</td>
</tr>
<tr>
<td>+</td>
<td>Anti-TNF 2</td>
</tr>
<tr>
<td>+</td>
<td>Anti-TNF 40</td>
</tr>
<tr>
<td>+</td>
<td>Anti-TNF 1000</td>
</tr>
</tbody>
</table>

Fig. 6. Role of TNF-α in the generation of thrombin at the HUVEC surface. HUVEC were cultured with a supernatant of OKT3-stimulated PBMC in the presence of various concentrations of chimeric anti-TNF-α MoAb or of an isotype-matched control MoAb, and the generation of thrombin (mean ± SEM of triplicate wells) was measured after 4 hours.

References


