Distinct Deleterious Effects of Cyclosporine and Tacrolimus and Combined Tacrolimus–Sirolimus on Endothelial Cells: Protective Effect of Defibrotide

Alba Carmona 1, Maribel Díaz–Ricart 1,*, Marta Palomo 1, Patricia Molina 1, Marc Pino 1, Montserrat Rovira 2, Ginés Escolar 1, Enric Carreras 2,3

1 Hemotherapy–Hemostasis Department, Centre de Diagnòstic Biomèdic, Institut d’Investigacions Biomèdiques August Pi i Sunyer, Hospital Clinic, Universitat de Barcelona, Barcelona, Spain
2 Stem Cell Transplantation Unit, Hematology Department, Institut d’Investigacions Biomèdiques August Pi i Sunyer, Hospital Clinic, Universitat de Barcelona, Barcelona, Spain
3 Spanish Bone Marrow Donor Program, José Carreras Foundation, Barcelona, Spain

ABSTRACT

Endothelial dysfunction seems to be a key factor in the development of several complications observed early after hematopoietic stem cell transplantation (HSCT). The conditioning regimen and many other factors associated with the procedure are responsible for this endothelial damage. The effects of immunosuppressive agents on endothelial function have not been explored in detail. We evaluated the effects of 3 drugs commonly used in HSCT: 2 calcineurin inhibitors, cyclosporine A (CSA) and tacrolimus (TAC), and an inhibitor of mTOR, sirolimus (SIR). We also evaluated the effect of the combination of TAC and SIR (TAC+SIR), which is used increasingly in clinical practice. Microvascular endothelial cells (HMEC-1) were exposed to these drugs to evaluate changes in (1) intercellular adhesion molecule (ICAM)-1 expression on the cell surface, assessed by immunofluorescence labeling and expressed as the mean gray value (MGV); (2) reactivity of the extracellular matrix (ECM) toward platelets, upon exposure of the ECM to circulating blood; and (3) whole-blood clot formation, assessed by thromboelastometry. Studies were conducted in the absence and presence of defibrotide (DF) to assess its possible protective effect. The exposure of HMEC-1 to CSA and TAC+SIR significantly increased the expression of ICAM-1 (157.5 ± 11.6 and 153.4 ± 9.5 MGV, respectively, versus 105.7 ± 6.5 MGV in controls [both P < .05]); TAC applied alone increased ICAM-1 slightly (120.3 ± 8.2 MGV), and SIR had no effect (108.9 ± 7.4 MGV). ECM reactivity increased significantly only in response to CSA (surface covered by platelets of 41.2% ± 5.4% versus 30.1% ± 2.0%, P < .05). DF attenuated all these changes. No significant changes in the viscoelastic properties of clot formation were observed in any condition with blood samples incubated in vitro. In conclusion, CSA and TAC+SIR had a proinflammatory effect, but only CSA exhibited an additional prothrombotic effect. Interestingly, DF exerted clear protective anti-inflammatory and antithrombotic effects on the endothelium.

INTRODUCTION

Hematopoietic stem cell transplantation (HSCT) is a well-established therapeutic option for several hematological malignancies and other nonmalignant disorders. HSCT is associated with life-threatening complications that may appear soon after transplantation. Some of these complications, such as veno-occlusive disease (VOD), thrombotic microangiopathy (TMA), engraftment syndrome, and capillary leak syndrome, among others [1], seem to be triggered by endothelial damage.

Using a macrovascular cell model, our group demonstrated the presence of endothelial damage in both allogeneic and autologous HSCT [2,3]. This endothelial damage is characterized by an increase in the expression of adhesion receptors on the cell surface, leukocyte adhesion when cells are exposed to circulating blood, and activation of p38 mitogen-activated protein kinase. Factors released during HSCT stimulate endothelial cells to produce a more prothrombotic extracellular matrix (ECM), enriched with von Willebrand factor and tissue factor, as demonstrated by an increased adhesion of platelets when these surfaces are exposed to blood under flow conditions. These observations were also reproduced in a microvascular cell model that was exposed to sera from patients who received an autologous HSCT. In this particular approach, we were able to demonstrate a protective effect of defibrotide (DF) [4].

The activation of and damage to endothelial cells during HSCT are caused by different factors, including chemoradiotherapy used as a conditioning regimen, cytokines produced by the injured tissues, bacterial endotoxins translocated through the damaged gastrointestinal tract, the complex process of engraftment, and allogeneic reactions with the donor-derived immune cells. Some drugs used during the procedure, such as granulocyte colony-stimulating factor [5] and immunosuppressant therapy, can contribute to this damage. Although there is evidence of the toxic or deleterious effect on the endothelium caused by cyclosporine A (CSA) [6–8], it is still the immunosuppressive drug of choice in HSCT. There is little information on the effect on the endothelium of other immunosuppressive drugs used in HSCT [9,10]. The knowledge acquired from other clinical situations, such as kidney transplantation, indicates that some agents are more toxic to the endothelium than others [11]. The long-term follow-up of patients...
receiving HSCT has shown a very high incidence of vascular complications (eg, cerebral stroke, ischemia, lacunar defects, coronary angiina, infarction, peripheral claudication, ischemia, pain, and gangrene) [12]. It is thus reasonable to question whether these agents, administered for months or years after transplantation, play a role in such pathogenesis.

The aim of the present study was to evaluate and compare the effects of the calcineurin inhibitors, CSA and tacrolimus (TAC), and the mTOR (mammalian Target of Rapamycin) inhibitor sirolimus (SIR) on the endothelium in a cell culture model. The combined use of TAC and SIR (TAC + SIR) was also assessed because it seems to be associated with a higher incidence of TMA [13-15] and VOD [16,17], depending on the conditioning regimen. The potential protective effect of DF was also explored in this setting. To do this, we applied the experimental approach used in our previous studies aimed at evaluating changes related to inflammation and thrombosis.

METHODS
Experimental Design
An immortalized human microvascular endothelial cell line was independently exposed in culture to CSA and TAC, 2 calcineurin inhibitors, and SIR, an mTOR inhibitor. In some experiments, cells were exposed simultaneously to TAC + SIR. Changes in both the expression of the intercellular adhesion molecule (ICAM)-1 at the cell surface and the adhesion of platelets on the ECM generated by these cells under flow conditions were assessed. The effect of DF was explored by incubating the cells with 100 µg/mL DF for 24 hours before exposing the cells to the immunosuppressive drugs and during the exposure to these drugs. The effect of the drugs included in the study was also explored by measuring changes in the thromboelastometric properties of blood.

Human Endothelial Cell Culture
The human microvascular endothelial cell line CDC/EU-HMEC-1 (henceforth referred to as HMEC-1 cells) [18] was grown at 37°C in a 5% CO2 humidified incubator in MDCB131 medium (Gibco BRL–Life Technologies, Barcelona, Spain) supplemented with 4% fetal bovine serum (FBS) and 1% penicillin–streptomycin (Gibco BRL–Life Technologies), 2 µg/mL hydrocortisone (Sigma-Aldrich Quimica SA, Madrid, Spain); 10 ng/mL epidermal growth factor (BD Biosciences, Erembodegem, Belgium), and 10% FBS (Gibco BRL–Life Technologies). Culture medium was replaced every day to study the effect of the different compounds, CSA, TAC, or SIR were added at a final concentration of 200 ng/mL, 10 ng/mL, or 10 ng/mL, respectively. In the experiments with TAC + SIR, the same final concentration for each substance was used. These concentrations were chosen based on levels achieved in clinical practice. Cells were finally cultured on 1% gelatin-coated glass coverslips (18 x 18 mm²).

HMEC-1 cultures were grown in the absence or presence of DF added every 24 hours. The DF concentration was based on the dose used in vivo for therapy [19] and after review of the literature on in vitro studies [20,21].

Immunofluorescence Detection of ICAM-1 on the Endothelial Cell Surface
Cells were seeded into 6-well plates containing 18 x 18 mm² coverslips and exposed to the immunosuppressive drugs for 24 hours. In conditions in which DF was included, the cells were preincubated with DF for 24 hours before exposure to the immunosuppressant. Cells were fixed with 4% paraformaldehyde in 0.15 M PBS (pH 7.4) for 10 minutes at 4°C. The fixed coverslips were then incubated with a specific monoclonal antibody (MAB2146; Chemicon International, Temecula, CA) (dilution 1:200) for 1 hour at room temperature. The excess antibody was removed by washing 3 times with PBS, and the coverslips were incubated with a donkey anti–goat IgG conjugated with Alexa Fluor 555 (Molecular Probes, New York, NY) (dilution 1:2000) for 1 hour at room temperature. Finally, the samples were evaluated by light microscopy (Leica DM4000 B, Barcelona, Spain) using appropriate filters, and images were captured using a video camera (Leica DFC310 FX, Barcelona, Spain). Fluorescence micrographs were analyzed densitometrically using Image J software (version 1.43 m; National Institutes of Health, Bethesda, MD, http://rsb.info.nih.gov/nih-image/ manual/tech.html#analyze). This software automatically analyzes the gray density of each pixel in a scale ranging from 0 (black) to 255 (white). Cultured cells were selected from the background with the threshold tool, and the fluorescence intensity was measured only in the selected area. Results are expressed as mean gray value (MGV).

Perfusion Studies
HMEC-1 monolayers were grown on glass coverslips for 7 days and then extracted by treatment with 6X ECTA (1 hour at 37°C) to expose the ECM on the coverslips. Perfusion studies to evaluate platelet adhesion were performed in a parallel-plate perfusion chamber at a shear rate of 800/s for 5 minutes [22-24], in which the ECM-coated coverslips were exposed to 25-ml aliquots of citrated whole blood (at a final citrate concentration of 19 mM) from a healthy donor. After perfusion, the coverslips were rinsed with 0.15 M PBS, fixed with 0.5% glutaraldehyde in 0.15 M PBS at 4°C for 24 hours, and stained with 0.02% toluidine blue. The degree of platelet deposition was measured in 10 microscopic fields per coverslip and was evaluated en face using an automated method [22]. The surface covered by platelets is expressed as a percentage relative to the total area of the coverslip screened (TSC).

Thromboelastometry Studies
To evaluate in vitro the effects of the immunosuppressive drugs in the absence and presence of DF on whole-blood clot formation, we used a ROTEM thromboelastometry analyzer (Pentapharm GmbH, Munich, Germany) to perform dynamic thromboelastometry of whole-blood coagulation [25] according to the manufacturer’s instructions. We used the commercial test EXTEN (Pentapharm GmbH, Munich, Germany) to measure changes in the extrinsic pathway of coagulation, fibrinogen and fibrin polymerization, and platelet function. Clots obtained in the EXTEN comprised platelets and fibrin. We recorded the clotting time and clot formation time (CFT), which indicated the dynamics of clot formation, and maximum clot firmness, which gave information about clot strength and stability, and was largely dependent on fibrinogen and platelets. The clotting time was defined as the time when the forming clot reached 2 mm; the CFT was defined as the time when the clot reached 20 mm.

To evaluate the effects of CSA, TAC, SIR, and TAC + SIR on the thromboelastometric properties of blood, we incubated whole blood with the immunosuppressive drugs at the concentrations indicated above. In conditions in which DF was added, the blood samples were preincubated with 100 µg/mL DF for 30 minutes.

Statistical Analysis
Results are expressed as means ± SEM. Statistical analysis was performed with raw data using Student’s t-tests for paired and unpaired samples. The results were considered significant at P < .05.

RESULTS
Proliferation of HMEC-1 in the Presence of Immunosuppressive Drugs
In the HMEC-1 monolayers, microscopic observation showed no changes in the proliferation kinetics in the presence of CSA or TAC. However, in the presence of SIR, HMEC-1 monolayers were rarely confluent and fewer dividing cells were seen. The percentage of dividing cells relative to the total number of cells present was calculated in each microscopic field, which comprised 10 fields per experiment. The percentages were 0.1% ± 0.2% in response to SIR versus 1.0% ± 0.1% in control experiments (P < .001) (Figure 1). Interestingly, preincubation with DF minimized the inhibitory effect of SIR on cell proliferation.

Changes in the Expression of ICAM-1 in Cell Monolayers
Expression of ICAM-1 increased significantly in HMEC-1 monolayers treated with CSA (157.5 ± 11.6 MGV in treated cells versus 105.7 ± 6.5 MGV in control monolayers, P < .05; expressed as mean ± SEM measured by densitometry; n = 5). Only a small increase in ICAM-1 expression was observed in cells exposed to TAC (120.3 ± 8.2 MGV, n = 5), and almost no effect was observed in response to SIR (108.9 ± 7.4 MGV, n = 5). The combination TAC-SIR showed a markedly increased ICAM-1 expression (153.4 ± 9.5 MGV, n = 5, P < .05 versus control) to a level greater than that obtained for each immunosuppressive drug separately (Figure 2). Preincubation of cells with DF attenuated the increase in ICAM-1 expression in cells treated with the immunosuppressive drugs: CSA (124.9 ± 10.0 MGV, n = 5), TAC (102.2 ± 8.8 MGV, n = 5).
Reactivity of the ECM Generated by HMEC-1 Exposed to Immunosuppressant Drugs

The reactivity toward circulating platelets by the ECM generated by HMEC-1 cells was evaluated before and after exposure to the immunosuppressive drugs (Figure 3). In control experiments, the %SC was 30.1 ± 2.0% (n = 11). Exposure to CSA increased the %SC to 41.2 ± 5.4% (P < .05 versus control). The %SC was lower than the control percentage after incubation with TAC (23.2% ± 3.0%), SIR (21.7% ± 2.1%), or TAC+SIR (26.9 ± 4.8%) (n = 5 for each).

The increased reactivity of the ECM in response to CSA was attenuated in the presence of DF (25.6% ± 4.5%, n = 5, P < .05). The adhesion of platelets to the ECM generated by HMEC-1 was not affected by exposure to TAC, SIR, or TAC+SIR in the presence of DF (23.7% ± 3.1%, 22.5% ± 1.7%, and 24.8% ± 4.9%, respectively). No significant effect of DF itself on the reactivity of the ECM generated by control cells was observed (27.3 ± 3.5 versus 30.1% ± 2.0% in controls).

Modifications of the Viscoelastic Properties of Clots: Thromboelastometry Studies

The lack of effect on the reactivity of the ECM in response to TAC, SIR, and TAC+SIR prompted us to evaluate the effects of these drugs on clot formation with samples incubated in vitro with the drugs under study. The study of the extrinsic pathway with the EXTEM test showed no significant differences in the viscoelastic properties of clots (measured as the clotting time, CFT, and maximum clot firmness) between the different conditions (Table 1 and Figure 4). In whole blood preincubated with DF alone or with the immunosuppressive drugs, CFT increased slightly although not significantly (Figure 4), indicating a slight delay in the formation of clots, although this was always within the normal range of 34 to 159 seconds.

DISCUSSION

Our group demonstrated previously that endothelial damage occurs in association with allogeneic or autologous HSCT. The degree of endothelial damage observed both in vivo and in vitro is related directly to the intensity of the conditioning treatment, among other factors [2,3]. These factors include substances released by damaged cells, such as cytokines and chemokines, and treatments given before and during the HSCT process. The deleterious effects on the endothelium of CSA, an agent used frequently in allogeneic HSCT, were reported previously [6-8]. The present work was performed to compare the effects of 3 different immunosuppressive drugs, 2 calcineurin inhibitors, CSA and TAC, and the mTOR inhibitor SIR. Our results indicate that both calcineurin inhibitors have a proinflammatory effect, although only CSA exhibited significant proinflammatory and prothrombotic effects on the endothelium. By contrast, although these effects were not observed with SIR, this mTOR inhibitor changed the proliferation kinetics and decreased the reactivity of the ECM. Interestingly, DF attenuated the changes caused by the immunosuppressive drugs evaluated.

Many early complications associated with HSCT seem to be ascribed to endothelial injury occurring in the microvasculature [1]. There is evidence of different behaviors between immunosuppressants, as shown by analyzing aspects of cell proliferation, oxidative stress, side effects, and renal function. Most studies have been performed in animal models or with solid transplants [9,10].

Our comparison of the effects of CSA, TAC, and SIR on microvascular endothelial cells indicates that these cells respond differently to different immunosuppressive strategies and to the 2 different calcineurin inhibitors tested;
this is consistent with previously reported clinical evidence [11]. Although both calcineurin inhibitors had a proinflammatory effect, which was moderate in the case of TAC, only CSA exhibited significant proinflammatory and prothrombotic effects on the endothelium. By contrast, exposure of cells to SIR did not significantly change the expression of adhesion receptors on the cell surface or generate an ECM that was more reactive toward platelets. Our results are consistent with previous data demonstrating that SIR has few damaging effects on the endothelium. SIR decreases vascular endothelial growth factor synthesis [26] and seems to prevent or limit intimal thickening [27]. SIR is the immunosuppressant of choice for renal transplantation and is being used more frequently in the HSCT setting. It is also interesting that SIR-eluting stents produced with different coating strategies are now used to prevent coronary restenosis because of the ability of SIR to inhibit cell proliferation [28,29]. The microscopic observations of cells grown in the presence of SIR in our study confirmed the effect of this drug on cell division and proliferation.

Numerous clinical trials have shown TAC to be superior to CSA in the prevention of acute rejection, and trials have demonstrated the superiority of TAC over CSA in terms of allograft survival [30,31]. In bone marrow transplantation, the incidence of grades II to IV graft-versus-host disease was significantly lower with TAC than with CSA [32,33]. However, administration of CSA seems to be more established than TAC in the setting of HSCT, and only some centers have replaced CSA with TAC. The results from our in vitro studies showing a more deleterious effect of CSA than TAC on endothelial cells are consistent with previously published observations. In addition to our results demonstrating both proinflammatory and prothrombotic effects of CSA, another study reported a stronger induction of oxidative stress after exposure of HMEC-1 cells to CSA compared with TAC, an effect that correlated with metabolic activity and apoptosis [10]. In addition, CSA alters endothelial cell morphology, whereas TAC does not. The effects of CSA may relate, at least partly, to increased endothelin release by endothelial cells [34]. Taken together, these findings, along with the high incidence of long-term vascular complications observed after allogeneic HSCT, lead us to consider abandoning CSA as the immunosuppressant of choice for HSCT.

After the introduction of SIR in clinical practice, several authors reported a higher incidence of transplant-associated TMA [13–15]. Similarly, an increased incidence of VOD was observed in both busulfan or cyclophosphamide plus total body irradiation–containing regimens [16,17]. Our present observations suggest that SIR is not the cause of the increased incidence of TMA and VOD but rather the
combined use of TACþSIR, which explains why some cases of TMA observed in patients who receive TAC improve when TAC is substituted by SIR [35]. The combined use of TACþSIR has provided favorable results by reducing the incidence of graft-versus-host disease [15,36,37]. Studies comparing different administration schedules and combinations could be performed to circumvent the endothelial injury produced by the combined use of these drugs. Another possible approach may be to abandon the calcineurin inhibitors and to substitute them with other combinations of immunosuppressive agents such as mycophenolate mofetil plus SIR, despite the fact that initial good results observed after kidney and heart transplantation have not been reproduced in SCT [38,39]. Finally, it may be possible to attenuate or prevent endothelial injury by administrating DF during the early phases of HSCT, because this drug prevents endothelial injury and additionally seems to decrease the risk of graft-versus-host disease [40]. DF is a well-tolerated drug that is used successfully for the treatment [19,41-43] and prophylaxis [40] of VOD. Our previous in vitro studies demonstrated that DF may be useful as a protective agent in both allogeneic and autologous HSCT [4]. Our present results indicate that DF can attenuate the damaging proinflammatory and prothrombotic effects of CSA and TACþSIR. In addition, DF shows a consistent although very mild effect of delaying the time of clot formation when using whole blood, although these results have been obtained with blood from healthy donors incubated in vitro with the drugs under study and should not be over-interpreted.

In conclusion, the calcineurin inhibitors studied here and the combination of TAC and the mTOR inhibitor SIR exerted

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Modifications on the Viscoelastic Properties of Clots in the Presence of Immunosuppressants and Effect of DF</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>CT Mean</td>
<td>57.3</td>
</tr>
<tr>
<td>SEM</td>
<td>6.7</td>
</tr>
<tr>
<td>CFT Mean</td>
<td>86.3</td>
</tr>
<tr>
<td>SEM</td>
<td>8.9</td>
</tr>
<tr>
<td>MCF Mean</td>
<td>63.7</td>
</tr>
<tr>
<td>SEM</td>
<td>1.5</td>
</tr>
</tbody>
</table>

N = 5. Clotting time (CT) is the time when the forming clot reached 2 mm; CFT is the time when the clot reached 20 mm; and the maximum clot firmness (MCF) relates to clot strength and stability.
proinflammatory action on the endothelium. Only CSA exhibited both significant proinflammatory and prothrombotic actions. By contrast, these effects were not observed in response to SIR. Interestingly, DF exhibited reproducible protective effects to counteract the endothelial aggression induced by the immunosuppressant compounds.

ACKNOWLEDGMENTS

The authors acknowledge Dr. Edwin Ades and Mr. Francisco J. Candal of the Centers for Disease Control and Prevention and Dr. Thomas Lawley of Emory University for providing the HMEC-1 cells.

Financial disclosure: Supported partially by grants from the German José Carreras Leukemia Foundation (R 07/41V), SAF2011-28214 (Ministerio de Ciencia y Tecnología), and RD06/0009/1003 (Red HERACLES, Instituto de Salud Carlos III). A.C. received a grant from Gentium S.p.A.

REFERENCES


