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The Release of Soluble Factors Contributing to Endothelial Activation and Damage after Hematopoietic Stem Cell Transplantation Is Not Limited to the Allogeneic Setting and Involves Several Pathogenic Mechanisms

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This study evaluated the relative impact of the intensity of the conditioning regimen and the alloreactivity in the endothelial dysfunction occurring after allogeneic hematopoietic stem cell transplantation (allo-HSCT). It involved a comparative analysis of the effect of incubating human umbilical vein endothelial cells (ECs) with serum samples from patients receiving autologous HSCT (auto-HSCT) or unrelated donor allo-HSCT. In both groups, blood samples were collected through a central line before conditioning (Pre), before transplantation (day 0), and at days 7, 14, and 21 after transplantation. Changes in the expression of EC receptors and adhesion proteins, adhesion of leukocytes and platelets under flow, and signaling pathways were analyzed. Endothelial activation and damage were observed in both groups, but with differing patterns. All markers of endothelial dysfunction demonstrated a progressive increase from day Pre to day 14 in the auto-HSCT group and exhibited 2 peaks of maximal expression (at days 0 and 21) in the allo-HSCT group. Both treatments induced a proinflammatory state (ie, expression of adhesion receptors, leukocyte adhesion, and p38 MAPK activation) and cell proliferation (ie, morphology and activation of ErK42/44). Prothrombotic changes (ie, von Willebrand factor expression and platelet adhesion) predominated after allo-HSCT, and a proapoptotic tendency (ie, activation of SAPK/JNK) was seen only in this group. These findings indicate that endothelial activation and damage after HSCT also occur in the autologous setting and affect macrovascular ECs. After the initial damage induced by the conditioning regimen, other factors, such as granulocyte colony-stimulating factor (G-CSF) toxicity, engraftment, and alloreactivity, may contribute to the endothelial damage seen during HSCT. Further studies are needed to explore the association between this endothelial damage and the vascular complications associated with HSCT.

INTRODUCTION

Allogeneic hematopoietic stem cell transplantation (allo-HSCT) is a well-established treatment for several hematologic malignancies and other nonmalignant disorders [1]. Unfortunately, however, allo-HSCT is associated with several early and late life-threatening complications produced by the toxicity of the conditioning regimen and by alloimmune reactions [2,3]. Several clinical and preclinical studies have suggested that injury to the vascular endothelium may contribute to the initiation and progression of certain early complications after HSCT [4]. The most important of these complications include veno-occlusive disease (VOD) of the liver, capillary leak syndrome, thrombotic microangiopathy, diffuse alveolar hemorrhage,

idiopathic pneumonia syndrome, and acute graft-versus-host disease a (aGVHD), all of which carry significant morbidity and mortality [3,5,6].

Because all of these complications are common in recipients of allo-HSCT, immune reactions are likely crucial for their development. The most common complication in autologous HSCT (auto-HSCT) is the engraftment syndrome (ES) that develops at the time of neutrophil engraftment, likely related to endothelial injury [7].

Endothelial cells (ECs) can be injured during allo-HSCT, first by the chemoradiotherapy included in the conditioning regimen and by the cytokines produced by the injured tissues. Later endothelial damage can result from the translocation of bacterial endotoxins through the damaged gastrointestinal tract and by the calcineurin inhibitors used for GVHD prophylaxis [8,9]. Finally, the vascular endothelium is a target for allogeneic reactions, probably because it is the initial allogeneic barrier separating engrafted donor-derived immune cells from the recipient tissues [6,10,11].

All of these cellular and soluble effectors can induce localized or diffuse proinflammatory, procoagulant, and proapoptotic changes in ECs that result in extravasation of fluid out of the vascular space, recruitment and transmigration of leukocytes, adhesion and aggregation of platelets, activation of procoagulation factors, and, sometimes, microthrombosis. The inflammatory component predominates in capillary leak syndrome, ES, diffuse alveolar hemorrhage, idiopathic pneumonia syndrome, and GVHD, whereas the thrombotic component is more relevant in VOD of the liver and thrombotic microangiopathy.

EC damage during HSCT can be evaluated through the quantification of several soluble markers, including plasma thrombomodulin, von Willebrand factor (vWF), and adhesion molecules [12-16]. Unfortunately, all of these biological makers are unspecific and are seriously hampered by numerous host factors. Recently, endothelial damage has been evaluated by quantifying circulating ECs (CECs) and endothelial microparticles [17,18]. EC cultures offer a unique opportunity to evaluate endothelial dysfunction and damage [19,20]. This approach allows investigators to reproduce different clinical situations, investigate the pathophysiologic mechanisms of these complications, and evaluate different strategies for reducing endothelial toxicity.

The aim of the present study was to analyze and compare the endothelial dysfunction caused by serum from a subgroup of patients at high risk for early complications (ie, recipients of unrelated donor HSCT conditioned with cyclophosphamide and total body irradiation [Cy + TBI]) with another subgroup that usually does not develop these complications—recipients of auto-HSCT conditioned with the BEAM regimen (ie, BCNU 300 mg/m^2 on day−7; etoposide 1600

mg/m^2 on days−6,−5,−4, and−3; cytosine arabinoside 800 mg/m^2 on days−6,−5,−4, and−3; and melphalan (Mel) 140 mg/m^2 on day−2). Both the proinflammatory and prothrombotic effects of the patient sera on human umbilical vein ECs (HUVECs) in culture were explored by analyzing the expression of diverse adhesion molecules, leukocyte adhesion on EC monolayers, changes in composition and reactivity of the extracellular matrix (ECM) generated, and the potential signaling pathways involved. We found that endothelial injury after HSCT was not limited to microvascular ECs. All markers of endothelial damage, excluding activation of the apoptosis pathways, were increased after both allo-HSCT and auto-HSCT, although with differing kinetic profiles, suggesting possibly different pathogenic mechanisms.

PATIENTS AND METHODS

Experimental Design

ECs were obtained from human umbilical veins and cultured with specific growth medium supplemented with pooled sera from healthy donors. Confluent EC monolayers were exposed to growth media containing 20% of pooled sera obtained from patients undergoing auto-HSCT (auto group) or allo-HSCT (allo group) at different times before and after transplantation.

Changes in the following were assessed: (1) endothelial morphology; (2) expression of endothelial adhesion receptors, such as E-selectin, vascular cell adhesion molecule 1 (VCAM-1), and intercellular adhesion molecule 1 (ICAM-1); (3) expression of adhesion proteins from ECM, such as von Willebrand factor (vWF) and tissue factor (TF); (4) adhesion of leukocytes and platelets on EC monolayers and the ECM generated by these cells, respectively, under flow conditions; and (5) intracellular signal transduction through tyrosine phosphorylation of proteins, specifically p38 mitogen-activated protein kinase (MAPK), Erk 42/44, and SAPK/JNK.

Patients

Consecutive patients undergoing HSCT at the Hospital Clinic, Barcelona, between January 2006 and January 2007, were included in the study. Patients undergoing auto-HSCT who received conditioning with the BEAM regimen and those undergoing unrelated allo-HSCT who received conditioning with Cy + TBI were selected. Patients who developed sepsis during the study period were excluded. The auto group comprised 11 patients (4 females and 7 males; median age, 57 years [range, 25 to 68 years]) suffering from either non-Hodgkin lymphoma (NHL; 10 cases) or Hodgkin disease (HD; 1 case). All patients were

treated with the BEAM regimen. The allo group comprised 12 patients (5 females and 7 males; median age, 36 years [range, 19 to 48 years]) with acute myelogenous leukemia (AML; 6 cases), acute lymphoblastic leukemia (ALL; 2 cases), myelodysplastic syndrome (MDS; 2 cases), chronic myelogenous leukemia (CML; 1 case), or chronic lymphocytic leukemia (CLL; 1 case). These patients were treated with Cy (120 mg/kg on days−7 and−6) and TBI (12 Gy in 4 fractions on days−4,−3,−2, and−1). Cyclosporin (CsA; 1 mg/kg/day from day−7 to day−1, followed by 3 mg/kg/day) plus a short course of methotrexate (MTX; 4 doses) was administered for GVHD prophylaxis.

Engraftment ($>5 \times 10^9$/L of neutrophils) occurred after 10.7 ± 1.1 days (mean ± standard error of the mean) in the auto group and 17.5 ± 4.4 days in the group ($P < .001$). Three patients in the auto group developed ES between days 11 and 13, and 2 patients in the allo group developed aGVHD grade II, at days 14 and 18.

Informed consent for blood utilization was obtained from all patients. The study was approved by the Hospital Clinic's Ethical Committee and was carried out in accordance with the principles of the Declaration of Helsinki.

Sample Collection

Blood samples were obtained at different time points of the study: Pre (before the start of the conditioning regimen), day 0 (immediately before HSCT), and days 7, 14, and 21 after HSCT. All samples were obtained through a central line, to avoid sample contamination with EC. Serum samples were obtained by centrifuging blood at $3000 \times g$ for 15 minutes. These samples were stored at $-20°C$ until the day of the analysis, at which time pools of sera for the different time points were prepared.

Human Endothelial Cell Culture

HUVECs were isolated from human umbilical veins as described previously [21]. The cells were maintained and subcultured at 37°C in a 5% CO_2 humidified incubator in Medium 199 (GIBCO/BRL Life Technologies, Paisley, UK), supplemented with 100 U/mL of penicillin, 100 g/mL of streptomycin (GIBCO/BRL Life Technologies), and 20% pooled human sera. Cells were used at a second passage. When required, cells were grown to confluence on 1% gelatin-coated glass coverslips. In some experiments, the composition and reactivity of the ECMs were evaluated. For this, EC monolayers grown on glass coverslips were treated with a 3% solution of ethyleneglycoltetraacetic acid (EGTA) on Hanks' solution for 1 hour at 37°C, to extract the cells and maintain the ECM on the coverslip.

Immunogold Labeling and Silver Enhancement

Glass coverslips coated with ECs incubated with the sera pool for 4 days and ECMs obtained from cells incubated under the same conditions for 7 days were fixed with 4% paraformaldehyde in 0.15 M phosphate-buffered saline (PBS; pH 7.4) for 10 minutes at 4°C. The proteins evaluated were VCAM-1, ICAM-1, and ELAM-1 on ECs and vWF and TF on ECMs. Fixed coverslips were incubated with monoclonal antibodies against VCAM-1 (1/100 dilution; Chemicon, Temecula, CA), ICAM-1 (1/100 dilution; Chemicon), E-selectin (1/100 dilution; Southern Biotechnology, Birmingham, AL), vWF (1/1000 dilution; Dako, Glostrup, Denmark), and TF (1/1000 dilution; American Diagnostica, Stamford, CT) for 45 minutes at room temperature, or with an unspecific IgG (n = 6). After removing the excess of antibody by washing 3 times with PBS, coverslips were incubated with a gold-conjugated goat anti-mouse IgG (1/1000 dilution; Amersham Pharmacia Biotech, Essex, UK) for 1 hour at room temperature. Finally, the samples were treated with an intense silver enhancement reagent (Amersham Pharmacia Biotech) to visualize gold particles bound to the antigen [22,23]. The density of gold particles bound was morphometrically analyzed in a light microscope (Polyvar; Reichert-Jung, Vienna, Austria) equipped with epipolarizing filters. Images were captured by a video camera (ProgRes MF; Jenoptik, Jena, Germany) and transferred to a personal computer with an automated image analysis system (SigmaScan; Jandel Scientific, San Rafael, CA) for the evaluation of the density of gold particles on ECs and ECMs (expressed as gold particles per μm^2).

Perfusion Studies

Leukocyte Adhesion on EC Monolayers

Leukocyte adhesion on ECs was explored using a parallel-plate perfusion chamber [22,24,25]. EC monolayers, grown for 4 days with medium supplemented with sera of patients from both groups, were perfused with citrated blood at a shear rate of 300 s^{-1} for 10 minutes. After perfusion, the coverslips were carefully washed with PBS and stained with May-Grünwald and Giemsa stains. The coverslips were examined with a light microscope (magnification ×320), and the number of adherent leukocytes was expressed as a percentage with respect to the number of ECs (number of leukocytes per 100 ECs).

Platelet Adhesion on ECMs

Perfusion studies to evaluate platelet adhesion on ECMs were carried out in a parallel-plate perfusion chamber at a shear rate of 800 s^{-1} for 5 minutes [24,26]. For each perfusion, two coverslips were inserted in separate receptacles of a parallel-plate

perfusion chamber and perfused with 25-mL aliquots of whole blood anticoagulated with citrate (100 mM sodium citrate, 16 mM citric acid, 18 mM sodium hydrogen phosphate, and 130 mM dextrose; final citrate concentration, 19 mM) from a single healthy donor. After perfusion, 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 in the perfused surface was measured in 10 microscopic fields per coverslip (magnification ×320), and evaluated en face by means of an automated method, as described previously [27]. The surface covered by platelets was expressed as a percentage with respect to the total area of the coverslip screened (%SC).

Evaluation of Phosphorylation Kinetics of p38 MAPK, Erk42/44, and SAPK/JNK

To evaluate the effect of patient sera on p38 MAPK, Erk 42/44, and SAPK/JNK, ECs were starved 24 hours before experimentation. Before and after being exposed to a sera pool (for durations of 30 seconds to 5 minutes), the ECs were lysed with Laemmli's buffer (68.5 mM Tris, 2% sodium dodecyl sulfate, 10% glycerol, 5 mM β-mercaptoethanol, and 0.003% bromophenol blue) containing 2 mM sodium ortho-vanadate and 0.625 mg/mL N-ethylmaleimide as inhibitors. Samples were resolved by 8% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes (BioRad, Hercules, CA), which were probed with specific antibodies to phosphorylated proteins: p38 MAPK, Erk42/44, and SAPK/JNK (Cell Signaling Technology, Danvers, MA) [28,29]. The presence of proteins was confirmed using specific antibodies.

Statistical Analysis

Results are expressed as mean ± standard error of the mean (SEM) obtained from replicate experiments (n = 6 for each approach). Evaluation of the experiments was always blinded and verified by a second (senior) investigator. Statistical analyses were performed using the Student *t*-test for paired samples, and results were considered significant when $P < .05$. The SPSS statistical package (SPSS Inc, Chicago, IL) was used for all analyses.

RESULTS

Cell Growth and Morphological Changes

Exposure of ECs to media supplemented with sera from patients from either the auto group or the allo group resulted in alterations in both cell morphology and growth kinetics. Proliferation was accelerated in ECs exposed to sera from both groups, which achieved confluence earlier compared with control ECs. Moreover, in both experimental situations, the cells exhibited variable sizes, irregular shapes, abundant vacuoles, and the presence of detritus. These phenomena were more noticeable at day 14 in the auto group (Figure 1B), whereas in the allo group, they were detected mainly at days 0 and 21 (Figure 1C).

Expression of Adhesion Molecules in EC Monolayers

Changes in the expression of VCAM-1, ICAM-1, and E-selectin on the surface of ECs in culture were explored by immunocytochemistry using gold staining. In control experiments, VCAM-1 and E-selectin were not detected, and ICAM-1 expression was 7 ± 4.1 pixels/μm^2. Increased expression of the 3 adhesion molecules, especially at gap junctions, was detected in cells grown in the presence of sera from patients in both the auto and allo groups (see Figure 2D–F for VCAM-1 expression).

In the auto group, VCAM-1 expression increased progressively from day Pre (35 ± 14.1 pixels/μm^2) to day 14, the day on which it reached a peak of maximal expression (89 ± 8.2 pixels/μm^2) ($P < .05$ vs Pre), after which it decreased up to day 21 (58.3 ± 10 pixels/μm^2) (Figure 2E). ICAM-1 expression was similar to
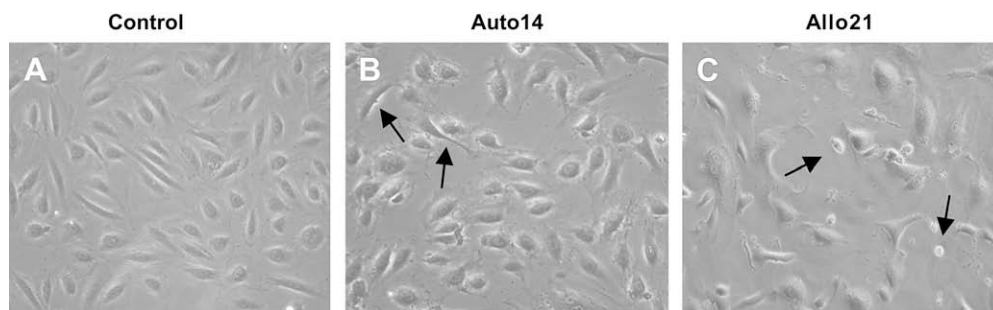

Figure 1. Cell growth and morphological changes. Light micrographs showing EC monlayers grown in the presence of control sera (A) and sera collected from patients in the auto group at day 14 (B) and from patients in the allo group at day 21 (C). The images correspond to semiconfluent monolayers. The arrows point out differences in cell morphology and the presence of detritus in (B) and (C), respectively. These micrographs are representative of 6 different experiments.
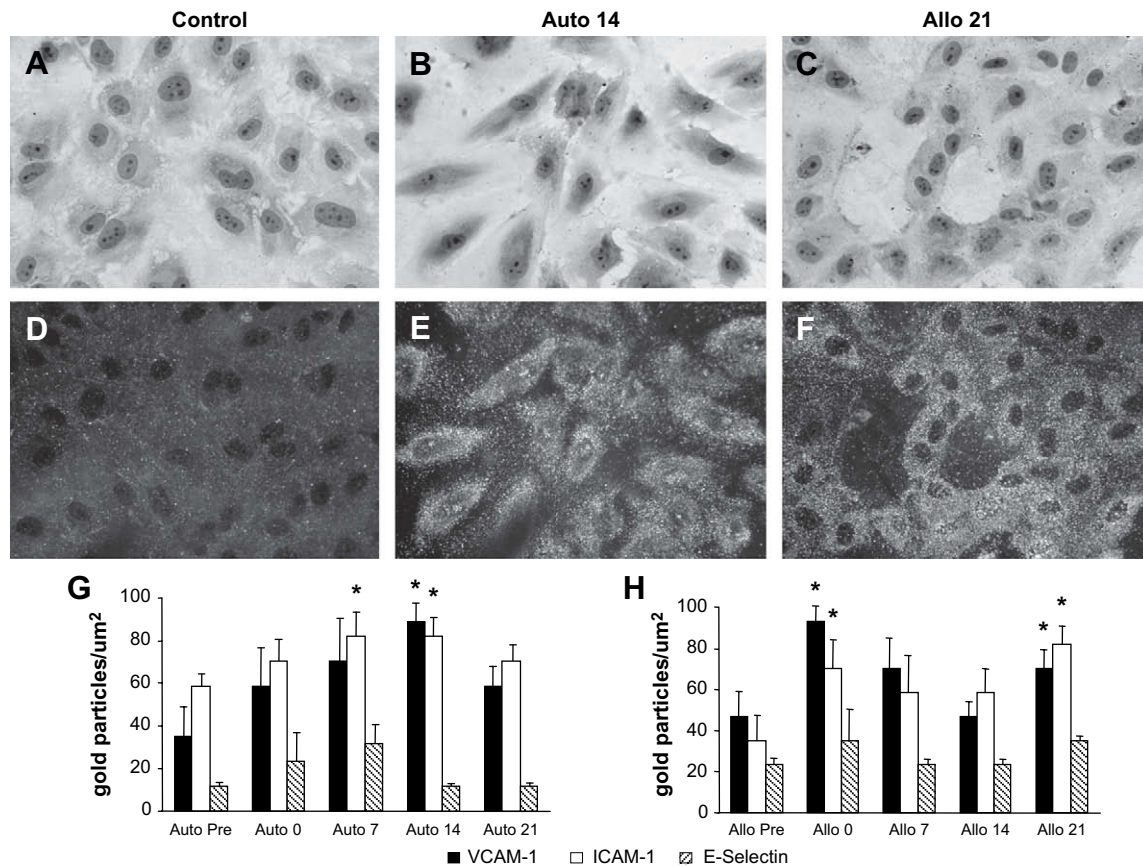
Figure 2. Expression of VCAM-1, ICAM-1, and E-selectin on the surface of ECs. Light micrographs (original magnification, $\times320$ for all micrographs) show EC monolayers grown in the presence of sera from controls (A and D), from patients in the auto group at day 14 (B and E), and from patients in the allo group at day 21 (C and F). Micrographs (D), (E), and (F) correspond to images captured with the epi-polarizing filter to visualize VCAM-1 expression and are representative of 6 different experiments. Bar diagrams represent the density of gold labeling (pixels/μm^2; mean $\pm$ SEM; n = 6), corresponding to the expression of the adhesion receptors VCAM-1, ICAM-1, and E-selectin in cells grown with sera from patients in the auto group (G) and the allo group (H). *$P < .05$ versus Pre condition.

VCAM-1 expression, although the increase occurred earlier (Figure 2G). Expression of E-selectin was not as significant as that for the other receptors (Figure 2G).

In the allo group, changes in the expression of the 3 molecules were more noticeable and appeared earlier, reaching 2 maximal peaks of expression at day 0 (93.3 ± 7 pixels/μm^2; $P < .05$ vs day Pre) and day 21 (70 ± 9.2 pixels/μm^2; $P < .05$ vs day Pre) (Figure 2H). Because the most notable changes were always detected at day 14 in the auto group and at day 21 in the allo group, these figures consistently show images corresponding to the data obtained at these time points.

Leukocyte Adhesion on ECs Exposed to Blood under Flow Conditions

Adhesion of leucocytes to EC monolayers was explored under flow conditions with citrated blood, using a parallel-plate perfusion chamber (300 s^{-1}, 10 minutes) (Figure 3). Under the experimental conditions, some degree of leukocyte adhesion on control

EC monolayers was observed ($1.5\% \pm 0.5\%$; n = 6) (Figure 3A). Interestingly, adhesion of leukocytes on ECs grown in the presence of sera from both groups of patients paralleled the findings for the expression of VCAM-1 and ICAM-1 receptors.

On monolayers grown with sera from the auto group, the percentage of leucocyte adhesion increased progressively from day Pre to day 14 (Figure 3B), day on which it reached its maximum value ($6.13\% \pm 0.4\%$; n = 6; $P < .05$ vs Pre condition) (Figure 3D).

In the allo group, the percentage of leukocyte adhesion showed 2 peaks, at day 0 ($4.7\% > \pm 0.8\%$; n = 6) and day 21 ($4.9\% \pm 0.3\%$; n = 6) (Figure 3C). Both of these values were significantly higher ($P < .05$) than those observed at day Pre ($2.5\% \pm 0.5\%$; n = 6) (Figure 3E).

Platelet Adhesion on ECM under Flow Conditions

Changes in the reactivity of the ECMs generated by the ECs grown in the presence of sera from the various study groups were evaluated by exposing the
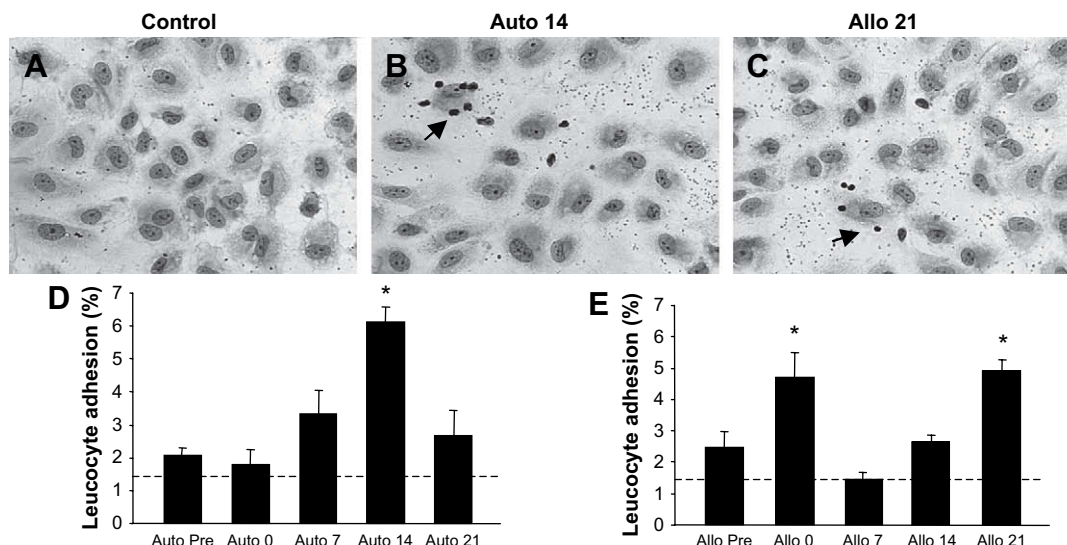
Figure 3. Adhesion of leukocytes on ECs exposed to blood under flow conditions. Light micrographs (original magnification, ×320 for all micrographs) showing leukocytes (arrows) adhered to ECMs grown in the presence of control sera (A), sera from patients in the auto group at day 14 (B), and sera from patients in the allo group at day 21 (C). The bar diagrams represent data corresponding to the adhesion of leucocytes, expressed as a percentage with respect to the number of ECs (number of leukocytes/100 ECs; mean ± SEM; n = 6) grown with sera from patients in the auto group (D) and the allo group (E). The dotted line represents the mean value for control results. *$P < .05$ versus Pre condition.

ECMs to blood anticoagulated with citrate under flow conditions (800 s^{-1}, 5 minutes). In control experiments, the percentage of surface covered by platelets (%SC) was 20.1% ± 1.3% (n = 12) (Figure 4A).

After the perfusion of ECMs from ECs grown with media supplemented with sera from the auto group, the %SC at day Pre was slightly superior to that found in control experiments (23.4% ± 1.5% vs 20.1% ± 2.4%; n = 6; $P < .05$). A statistically significant increase was measured only at day 14 (31.5% ± 1.7%; n = 6; $P < .05$ vs day Pre) (Figure 4B), and the values returned to control values at day 21 (20.47% ± 2.9%; n = 6) (Figure 4D).

The %SC values were significantly higher in the allo group than in the auto group and in the controls. In the allo group, the %SC was 26.8% ± 0.9% (n = 6) at day Pre ($P < .05$ vs control) and it increased significantly, to 37% ± 1.3% at day 0 and 35.5% ± 1.4% at day 21 ($P < .05$ vs day Pre) (Figure 4E).

Detection of vWF and TF on ECM

We measured changes in the expression of vWF and TF in the ECMs of ECs grown in the presence of the sera under study. The ECMs grown with media supplemented with sera from the auto group exhibited increased vWF expression (Figure 5A), with the difference reaching statistical significance at day 14 with respect to day Pre (53.7 ± 6.2 vs 28.6 ± 8 gold particles/ μm^2; n = 6; $P < .05$). In the allo group, the density of labeling for vWF on ECMs exceeded that in the control experiments even at day Pre (34.7 ± 5.4 vs 30 ± 2 gold particles/μm^2), with the difference reaching statistical significance at days 0 and 21 (59 ± 7.3 and 48.1

± 5.9 gold particles/μm^2, respectively; $P < .05$ vs day Pre) (Figure 5B).

TF expression was low in control ECMs and remained unchanged in ECMs from both the auto and groups.

Phosphorylation Kinetics of p38 MAPK, Erk 42/44, and SAPK/JNK

Phosphorylation of the MAP kinases p38 MAPK, Erk 42/44, and SAPK/JNK was evaluated using the same experimental design (n = 6). Activation of p38 MAPK occurred after day 14 in the auto group and after day 0, with peaks of intense phosphorylation at days 0 and 21, in the allo group. Rapid phosphorylation (30 seconds) was seen in both the allo and auto groups. Phosphorylation was heavier in the allo group (Figure 6A) and lasted for at least 15 minutes.

The phosphorylation kinetics of Erk 42/44 followed the same tendency seen for the markers of endothelial dysfunction (Figure 6B). The auto group demonstrated a progressive activation in the days after transplantation. In the allo group, Erk 42/44 appeared to be phosphorylated especially at days 0 and 21. Phosphorylation was rapid (within 30 seconds) and persisted for at least 5 minutes after exposure to the patient sera.

Phosphorylation of SAPK/JNK was not detected in ECs exposed to sera from the auto group at any time point. In the allo group, activation of SAPK/JNK followed the same pattern as seen for p38 MAPK and Erk 42/44, appearing phosphorylated especially at days 0 and 21. Phosphorylation of SAPK/JNK was
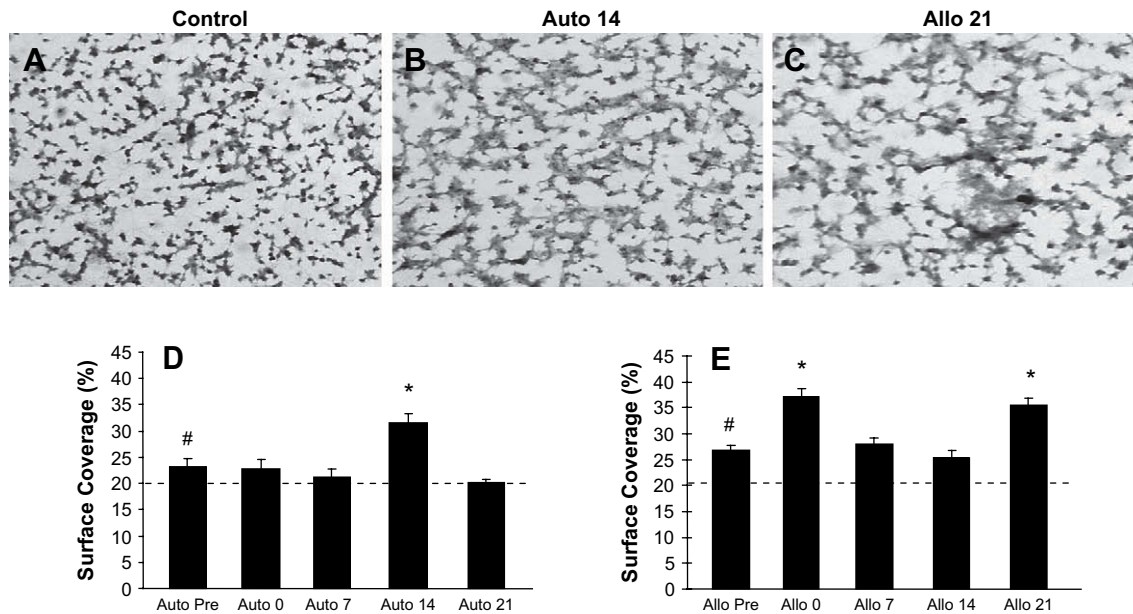
Figure 4. Platelet adhesion on ECMs generated by ECs. Light micrographs (original magnification, ×320 for all) show platelet interaction with ECM from cells grown in the presence of control sera (A) and sera from patients in the auto group at day 14 (B) and from those in the allo group at day 21 (C). Images are representative of 6 different experiments. Perfusion experiments were performed with citrated blood at 800 s^{-1} for 5 minutes. Bar diagrams represent the percentage of surface covered by platelets (%SC) expressed as mean ± SEM (n = 6 on the ECM generated by EC grown with sera from patients in the auto group (D) and the allo group (E)). The dotted line represents the mean value for control results. *P <.05 versus Pre condition #P <.05 versus control.

rapid (30 seconds) and persisted for at least 5 minutes after exposure to the patient sera (Figure 6A).

The presence of the 3 proteins under study was confirmed to be homogeneous in all samples evaluated by incubating the immunoblots with specific antibodies (Figure 6).

DISCUSSION

Endothelial damage has been associated with allo-HSCT, an established method for treating hematologic malignancies and other nonmalignant diseases. To evaluate the relative impacts of the intensity of the conditioning regimen and of alloreactivity, we conducted a comparative analysis of the effects of incubating macrovascular ECs in vitro with sera samples from patients undergoing auto-HSCT and allo-HSCT. Our findings indicate that an inflammatory reaction of ECs occurs in both groups. This reaction is characterized by increased expression of adhesion receptors on the surface of ECs in culture, with leukocyte adhesion when cells are exposed to circulating blood, and activation of p38 MAPK. In addition, we observed a prothrombotic effect that was much more pronounced in the allo group, with ECs producing ECMs enriched with vWF, with increased platelet adhesion when exposed to blood under flow conditions. Both the allo and the auto groups exhibited cell proliferation, as measured by activation of Erk 42/44. Interestingly, when evaluated by measuring activation of the SAPK/JNK pathway, apoptosis was seen only the allo group.

Using our experimental approach, markers of endothelial dysfunction were increased in macrovascular EC cultures. These alterations were not limited to the apoptosis observed by Eissner et al. [30] in HUVECs receiving ionizing radiation, suggesting silent damage of the whole endothelium in addition to the microvascular damage responsible for the clinical manifestations occurred after HSCT. Whether this systemic macrovascular damage is implicated in the early vascular events and in the high incidence of cerebral, coronary, and peripheral cardiovascular events seen even in young persons long after HSCT remains to be elucidated [31,32].

The endothelial dysfunction observed in both the allo and auto groups had a different profile in the first weeks after HSCT. A kinetic analysis of our findings revealed some degree of endothelial activation even before the conditioning regimen in both the auto and allo groups, being more evident in the latter. Moreover, endothelial dysfunction was found in both groups at the time of transplantation (day 0), to a much more intense degree in the allo group. Clearly, this must be attributed to the toxicity of the conditioning regimen, especially to the use of TBI in the allo-HSCT setting [30]. It should be noted that the conditioning used in the auto-HSCT setting, despite being less intensive than that used in allo-HSCT, cannot be considered reduced intensity conditioning, (RIC) because it included a higher dose of etoposide than that included in the classical BEAM regimen. This fact could explain why some authors
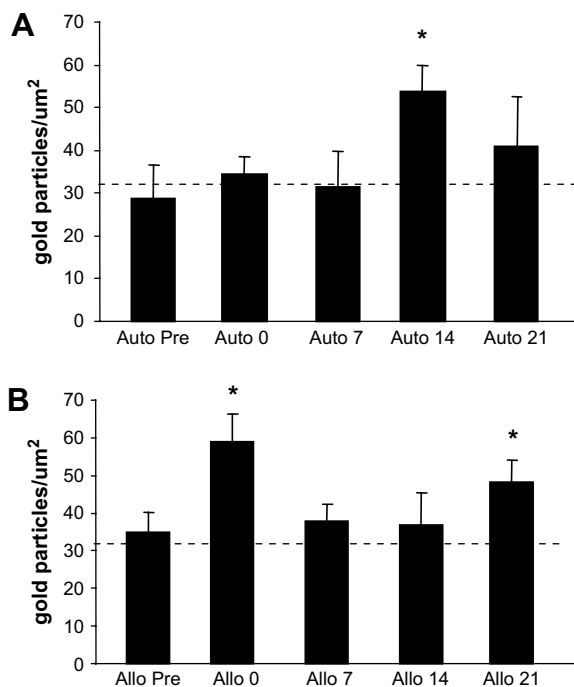
Figure 5. vWF concentrations on ECMs from EC monolayers evaluated by immunocytochemistry using gold staining. The bar diagrams represent the expression of vWF (gold particles/μm^2) on ECMs generated by EC monolayers grown in the presence of sera from patients in the auto group (A) and the allo group (B). Values are mean $\pm$ SEM (n = 6). *P <.05. The dotted line represents the mean value for control results.

who investigated patients undergoing auto-HSCT with true RIC regimens found no endothelial dysfunction [14].

After transplantation, the degree of endothelial damage exhibited a different profile in the allo and auto groups. In the auto group, markers of endothelial damage increased progressively up to day 14 and decreased thereafter. The values found on day 7 cannot be attributed to the use of granulocyte colony-stimulating factor (G-CSF), a well-known proinflammatory agent [33], because G-CSF was given to all of our patients on day 7, just after blood samples were drawn for this study. Because the time to engraftment (> 5 × 10^9/L neutrophils) was 10.7 $\pm$ 1.1 days in the auto group, it is plausible that the progressive endothelial dysfunction observed in this setting may be related to changes associated with the engraftment process. Evidently, both engraftment and G-CSF could play roles in the damage seen on day 14. The extreme dysfunction observed at that time also could explain why 3 of the 11 patients in the auto group developed ES between days 7 and 14.

We found a completely different pattern in the allo group. Markers of endothelial dysfunction tended to decrease at days 7 and 14. This tendency practically excludes the possibility of a role of CsA, which was administered at high doses starting at day−1.

Interestingly, this same evolutive pattern was reported by Woywodt et al. [18] when quantifying circulating ECs in a subgroup of patients receiving TBI. Because these patients had not received G-CSF, the second peak occurring at day 21 could be attributed only to the first alloreactivity phenomenon or, as in the autologous setting, to the engraftment of donor cells occurring after 17.5 $\pm$ 4.4 days in this group. These 2 peaks of endothelial dysfunction also were noted by Salat et al. [15] when analyzing vWF levels in patients undergoing allo-HSCT with or without GVHD, suggesting a possible mechanism linked to engraftment. A similar conclusion was reached by Ganster et al. [34] in a study of apoptosis in patients undergoing allo-HSCT. More studies of endothelial function on day 28 after HSCT are needed to elucidate the actual impact of each of these factors.

Our morphological findings indicate changes in growth and morphology in ECs exposed to sera from recipients of auto-HSCT and allo-HSCT. In both groups, these ECs reached confluency more rapidly than control cells, with activation of Erk 42/44, although the timing was different in the 2 groups. Interestingly, despite the accelerated proliferation, these cells readily detached when monolayers were exposed to circulating anticoagulated blood. This observation correlates with previous reports of increased numbers of circulating ECs [15,18]. In addition, the possible apoptosis (as evidenced by increased presence of cell detritus in the growth media) was confirmed by activation of SAPK/JNK in the allo group, but not in the auto group.

Our findings indicate that the HSCT environment is proinflammatory. Although this observation has been reported for allo-HSCT [4,15], little direct evidence of it has been found in auto-HSCT. In the present study, the expression of VCAM-1 and ICAM-1 as markers of endothelial damage followed the kinetics seen for both types of HSCT, with excellent correlation with the interaction of circulating leukocytes on EC monolayers exposed to flowing blood. ECs do not have storage forms of endothelial adhesion receptors [15,35] and their expression depends largely on new mRNA synthesis. In fact, we found that the increased VCAM-1 and ICAM-1 expression was closely correlated with p38 MAPK phosphorylation.

Our findings indicate that along with a proinflammatory effect, allo-HSCT leads to a higher and more persistent prothrombotic state. The ECMs generated by cells grown in the presence of sera from patients in both groups were more reactive to platelets compared with control ECMs when exposed to circulating blood. This reactivity was much more notable in ECMs from the allo group and was significant only at day 14 in the auto group, in accordance with the changes seen in vWF expression. These findings are in agreement with the higher incidence of VOD and
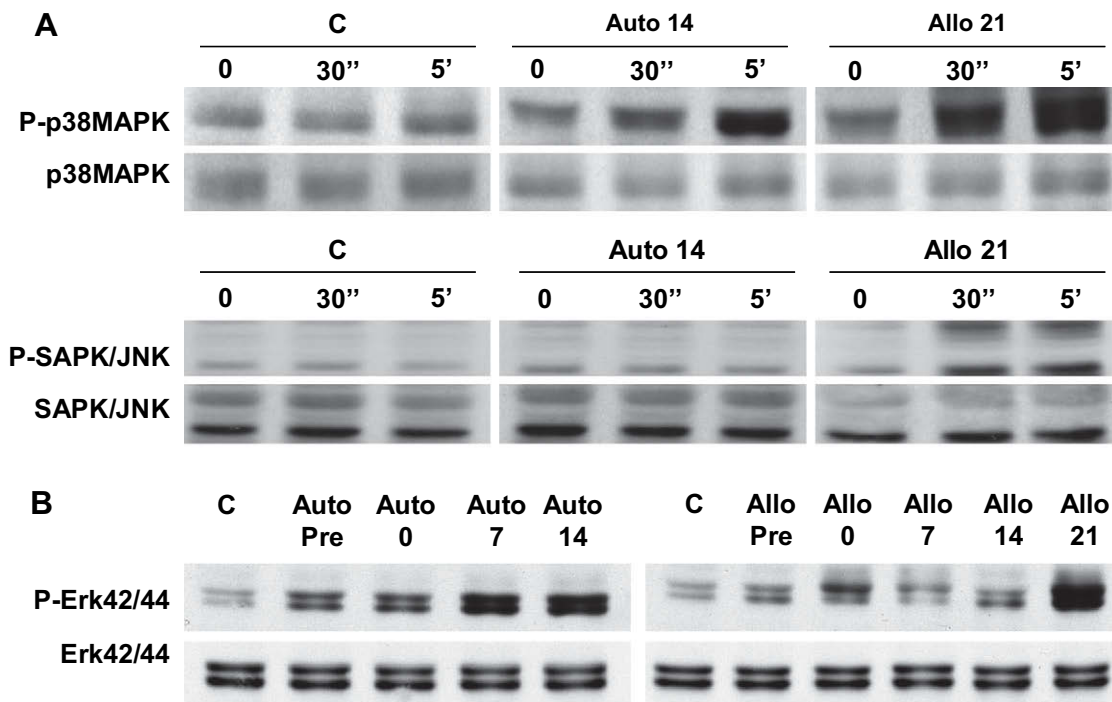
Figure 6. Phosphorylation kinetics of p38 MAPK, SAPK/JNK, and Erk 42/44. (A) Phosphorylation kinetics of p38MAPK and SAPK/JNK in cells exposed to control sera and sera from patients in the auto group at day 14 and from patients in the allo group at day 21, for 0 seconds, 30 seconds, and 5 minutes. (B) Phosphorylation kinetics of Erk42/44 after 30-second exposure of cells to the sera samples collected at the different time points, as indicated. Blots showing equal amounts of p38MAPK, SAPK/JNK, and Erk42/44 are presented under each phosphorylated protein. The blots are representative of 6 different experiments.

thrombotic microangiopathies described in allo-HSCT recipients [5,15].

The absence of SAPK/JNK pathway activation in the auto group likely explains the lower incidence and severity of the endothelial-related complications in this group. The findings in the allo group on day 0 can be explained by a potentially proapoptotic effect of the sera of patients receiving TBI, as has been observed in vitro previously [15,30]. The proapoptotic status observed in the second peak of endothelial dysfunction can be attributed solely to alloreactivity, because it was not observed in the auto group.

In conclusion, our findings provide insight into the endothelial damage that occurs after HSCT. Our methodology allows us to reproduce different clinical situations and to evaluate various strategies aimed at reducing endothelial toxicity and early (and perhaps also late) complications of HSCT.

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