Pericapillary hemorrhage
as criterion of
severe human digestive graft-versus-host disease

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Abstract
In an experimental model we demonstrated that endothelial cells of all organs are targets of the alloimmune reaction. Here, in 68 digestive biopsies, we found endothelial lesions by immunohistochemistry and ultrastructure in patients with severe acute graft-versus-host disease (GVHD). In contrast, no such endothelial cell alterations were found either in patients without GVHD or in non-grafted controls. In the biopsies with severe GVHD lesions, ultrastructure showed rupture of the capillary basal membrane and extravasated red blood cells. These pericapillary hemorrhages were highly correlated with GVHD severity. In a separate cohort of 39 patients who underwent an allogeneic transplantation after a non-myeloablative conditioning, 8 patients had intestinal biopsies. Three of these later patients had both severe pathological lesions of GVHD and similar endothelial lesions, thus, strengthening the concept that endothelial lesions are linked to GVHD severity and not to the intensity of the conditioning regimen.

Introduction
Graft-versus-host reaction, with damage of skin, liver and gut epithelial cells, is the main complication of allogeneic hematopoietic stem cell transplantation (HSCT).

Endothelial cell involvement following HSCT is suggested by clinical and biological data. Thrombotic microangiopathies and capillary leak syndrome as increased blood levels of thrombomodulin, PAI-1, and von Willebrand factor have been reported after HSCT. Radiation and LPS experimentally-induced endothelial damage involving TNF-α, a known effector of acute GVHD lesions. In an animal model using allogeneic lymphocytes transfer in non irradiated immuno-deficient mice, we demonstrated that endothelial cells of all organs are targets of acute GVHD. In humans, endothelial cell injury and microvessel loss have been recently found in chronic cutaneous GVHD.

Here, we systematically assessed microvessel damage in human duodenal biopsies performed before Day 100.

Patients and methods.
Between 1996 and 1998, 68 out of 258 patients who underwent HSCT developed digestive symptoms requiring an upper digestive endoscopy with biopsies for GVHD diagnosis before Day 100. The male/female ratio was 41/27, median age was 35 years (range: 7-55). Patient-, disease and transplant characteristics of these 68 patients are summarized in Table 1. Conditioning regimen consisted in chemotherapy alone in 30 cases or chemotherapy and total body irradiation in 38 patients. GVHD in skin, liver, and gut was clinically graded at the time of digestive biopsy, according to Seattle criteria. Clinical acute GVHD was found in 56 patients (82.3%), [grade IV: 16, grade III: 15, grade II: 22, grade I: 3]. Each patient had 4 duodenal biopsies whether or not there was a mucosal lesion detectable by endoscopic examination. Two biopsies were embedded in paraffin and 2 processed for ultrastructure. As controls, normal digestive specimen for surgical pieces of 10 non transplant patients were taken. One additional biopsy was systematically screened for microbial and/or viral infections (none of the patients with pericapillary hemorrhage had evidence of digestive infection). Biopsies were taken with a “multibite” biopsy device (Boston scientific microvasive, reference 1012). Biopsies for pathological examination were fixed in AFA (alcohol, formalin and acetic acid ) for 2 hours and further processed for paraffin embedding, or fixed in glutaraldehyde 4 % and processed for ultra-structural examination. Patients underwent biopsies in their individual sterile room, if white blood cell counts were less than 0.5 G/L, and in a surgical room if the counts were higher. If platelet counts were below 50 G/L, systematic transfusion was performed before biopsy. The patients gave their informed consent according to the declaration of Helsinki.
Histological assessment of digestive GVHD was performed blindly by 2 different pathologists according to criteria described by Sale\textsuperscript{12} and modified by Epstein\textsuperscript{13}: grade 1, crypt cell degeneration or epithelial cell apoptosis, without crypt loss; grade 2, loss of up to 3 contiguous crypts; grade 3, loss of 4 or more crypts without sloughing; grade 4, total sloughing. For the 68 patients, results were: grade 4: 3, grade 3: 10, grade 2: 15, grade 1: 22, no GVHD: 18.

Assessment of capillary damage was based on: i. presence of pericapillary hemorrhages on paraffin sections; ii. rupture of capillary basal membrane on ultrastructure; iii. alterations of endothelial cell on ultrastructure and immunohistochemistry.

Results for pericapillary hemorrhages were expressed by the presence or absence of hemorrhage in one of the 3 fields systematically studied at magnification 400 for each biopsy.

For ultrastructure, 4 resin-embedded blocks were obtained from 2 biopsies for each patient. The analysis was carried out on a minimum of 4 capillaries on each resin block, focusing on both endothelial cells and basal membranes.

Immunohistochemistry, using anti-human CD31 (Novocastra, Newcastle, UK) was performed on paraffin sections, with controls by omitting the first antibody and by using an irrelevant antibody of identical isotype. Counts of damaged endothelial cells were performed on 3 fields at magnification 400 on an Olympus AX 70 microscope, with wide-field eyepiece number 26.5, providing a field size of 0.344 mm\textsuperscript{2} at magnification 400. Endothelial cell lesions were considered when endothelial damage was found at least on 5 of the 50 capillary sections systematically studied per biopsy.

Controls and differential diagnoses. The best control group would have been patients who were without signs and symptoms following myeloablative therapy and allogeneic HSCT. However, for obvious ethical reasons such patients did not had endoscopy and biopsies. Thus, we decided to examine two groups of patients as controls: i) patients who underwent autologous stem cell transplantation and who had biopsies for diagnostic purpose, and ii) patients who received a non-myeloablative (NMA) conditioning regimen followed by allogeneic HSCT. GVHD-associated thrombotic thrombocytopenic purpura (TTP) was systematically searched. Criteria for TTP included red cell fragmentation, laboratory findings of hemolysis, and de novo or persistent thrombocytopenia caused by consumption, in the absence of disseminated intra-vascular coagulation. Additional criteria (not mandatory) for TTP were hypertension and renal insufficiency.

Statistical analyses. Two-sided Chi-2 test was used to compare the grades of clinical and of histological digestive GVHD, with the presence of pericapillary hemorrhages and of endothelial damage. Correlations were statistically significant for $P<5\times10^{-2}$.

Results and discussion

Only one of the 68 patients of this series had intestinal bleeding as an indication for endoscopy. Endoscopic examinations were performed as soon as diarrhea or nausea, vomiting occurred, thus intestinal biopsies were taken very early in the course of GVHD. Pericapillary hemorrhages were found in biopsies of 13 patients, preferentially in the upper part of duodenal villi (Figure 1A). Ultrastructure showed ruptures of capillary basal membrane with extravased red blood cells (Figure 1C). However, capillary lesions were heterogeneous, with, in the same area (Figure 1C), intact capillaries, capillaries with damaged endothelial cells only, and capillaries with damaged endothelial cells and rupture of basal membrane.

Among these 13 patients, 7 underwent transplantation after a total body irradiation-based conditioning and 6 after chemotherapy only. None of these patients had evidence at the time of biopsy of TTP or evidence of veno-occlusive disease of the liver. The clinical
presentation of the patients with pericapillary hemorrhages differed from the presentation of
the patients without pericapillary hemorrhage, since the patients with pericapillary
hemorrhage had signs and symptoms of GVHD that were more severe than the patients
without pericapillary hemorrhage (table). Major changes (including mucosal ulceration) were
found in 4 patients (33%) and minor changes such as edema in 5 (41%).

Pericapillary hemorrhages were only found in grade 4 (3/3), and grade 3 (10/10)
histological grades, with a highly significant correlation between hemorrhages and severity of
histological GVHD, [grade (0-2) versus (3-4), p<4.7x10^{-15}, \chi^2=61.66, ddl=1]. Considering
clinical grade, pericapillary hemorrhages were found in 8/16 grade IV, 3/15 grade III, 2/22
grade II, but not in grade I or in controls (p<4x10^{-3}). Twelve out of the 13 patients with
pericapillary hemorrhages died in 33 days, as a mean (range; 7-65) after the biopsy. Thus, as
previously reported, digestive GVHD is an important factor of morbidity and mortality. Its
incidence and severity may be underestimated if patients with digestive symptoms following
HSCT are not systematically biopsied^{14,15}.

Pericapillary hemorrhages are the ultimate result of capillary wall damage, and are
easy to detect on paraffin sections. However, lesions limited to endothelial cells, without basal
membrane rupture, were also observed with anti CD-31 antibody (Figure 1B) and
ultrastructure (Figure 1C). Neither pericapillary hemorrhages nor endothelial damage we
detected at the ultrastructural level were associated with thromboses or fibromuscular
hyperplasia in vessels of adjacent lamina propria. When compared with histological grade of
GVHD, endothelial lesions were found in all grade 4 (3/3), grade 3 (10/10), as in 5/15 grade 2
(p<2.4x10^{-10}, \chi^2=40.09, ddl=1). When compared with clinical grade of GVHD, they were
found in 11/16 grade IV, 4/15 grade III, and 3/22 grade II (p<5x10^{-4}).

Endothelial cells are targets of alloimmune reactions in solid organ rejection^{16}. Endothelial lesions have also been reported in acute GVHD in skin^{17} or liver^{18}. We have
demonstrated widespread endothelial lesions following allogeneic splenocytes transfer in
immuno-deficient mice. In this experimental model of pure allogeneic reaction, endothelial
lesions were massive and synchronous in all organs^{8}. However, in humans, biopsies can only
be performed for diagnostic purpose. At the time of biopsy, lesions in target organs associate,
damages linked to conditioning regimen and GVHD but also signs of wound healing^{19}. In this
series, a striking ultrastructural feature was the coexistence, in the same area, of severely
damaged capillary sections with extravased red blood cells, and of normal capillary sections.
This suggests that both acute damage and reparation process exist in digestive biopsies
performed before Day 100. This is in accordance with the recently proposed concept^{8, 9} that
microvessel loss follows endothelial injury of acute GVHD disease.

Vascular ectasia have been described in patients who received autografts and in
radiation-induced endothelial lesions. Thus our findings raised the question of the specificity
of endothelial damage in the context of severe intestinal GVHD. We do believe on this
specificity for the following reasons: 1) Lack of these findings in grade I and II GVHD, 2)
Lack of such of such endothelial lesions in intestinal biopsies following autologous stem cell
transplantation (n=3), and most importantly 3) Among a cohort of 39 patients who underwent
an allogeneic transplant after a NMA conditioning [fludarabine and 2 Gy TBI (n=34)], 8
patients had intestinal biopsies. Among these 8 patients, 3 had no evidence of pathological
GVHD, 2 had pathological evidence of GVHD without endothelial lesions, but 3 patients had
both severe pathological lesions of GVHD and endothelial lesions, thus strengthening the
concept that endothelial lesions are linked to the severity of the GVHD process and not to the
intensity of the regimen used.

Therefore, it seems important to assess endothelial cell lesions of acute GVHD. This
systematic study of digestive biopsies of patients with acute GVHD showed that pericapillary
hemorrhages, which are easy to detect on paraffin sections, can be proposed to characterize the severity of microvessel damage during the acute phase of GVHD.
Legend to the Table figures.

**Figure 1.** Pathological features of capillary damage in human digestive graft-versus-host disease.

a. Blood suffusion (arrow) around capillary loops (white broken lines) in the upper part of a duodenal villi. Paraffin section hematoxylin-eosin stain x 300.

b. High magnification of a capillary, with partial detachment of an endothelial cell (*). Indirect immunoperoxidase with an antibody directed against CD-31 x 1200.

c. Ultra-structural aspect of close capillary sections in the lamina propria of a duodenal biopsy. 1 = capillary section with intact endothelium and basal membrane. 2 = capillary section with endothelial cytoplasm of irregular thickness around the capillary lumen, without basal membrane rupture. 3 = capillary section with discontinuity in the basal membrane (—is-) and endothelial cytoplasmic cover. Insert = higher magnification of basal membrane rupture. 4 = extravased red blood cells

**Table:** Patient-, disease-, and transplant characteristics; clinical and pathological correlations.

* According to a severity index described in 20: Both TNF expression and number of apoptotic epithelial cells correlated with grade 3-4 pathological grade and thus with endothelial damages (p<.01).** In patients with pathological grade 3-4 (n=13), Major changes (including ulceration of the mucosal) was found in 4 patients (33%) and minor changes such as edematous mucosal in 5 (41%).
<table>
<thead>
<tr>
<th>Parameter</th>
<th>Number of patients (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Initial diagnosis</strong></td>
<td></td>
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<tr>
<td>- Acute leukemia</td>
<td>22 (32%)</td>
</tr>
<tr>
<td>- Myelodysplastic syndrome</td>
<td>6 (9%)</td>
</tr>
<tr>
<td>- Chronic myelogenous leukemia</td>
<td>26 (38%)</td>
</tr>
<tr>
<td>- Aplastic anemia</td>
<td>10 (15%)</td>
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<tr>
<td>- Lymphoma</td>
<td>4 (6%)</td>
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<tr>
<td><strong>Donor type</strong></td>
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<tr>
<td>- HLA-identical sibling donor</td>
<td>47 (69%)</td>
</tr>
<tr>
<td>- Mismatched related donor</td>
<td>2 (3%)</td>
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<tr>
<td>- Unrelated donor</td>
<td>19 (28%)</td>
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<tr>
<td><strong>Conditioning regimen</strong></td>
<td></td>
</tr>
<tr>
<td>- Including total body irradiation</td>
<td>38 (56%)</td>
</tr>
<tr>
<td>- Without total body irradiation</td>
<td>30 (44%)</td>
</tr>
<tr>
<td><strong>Graft-versus-host disease prophylaxis</strong></td>
<td></td>
</tr>
<tr>
<td>- Including cyclosporine</td>
<td>66 (98%)</td>
</tr>
<tr>
<td>- Cyclosporine and methotrexate</td>
<td>52 (78%)</td>
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<td><strong>Source of transplanted cells</strong></td>
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</tr>
<tr>
<td>- bone marrow</td>
<td>48 (70%)</td>
</tr>
<tr>
<td>- peripheral stem cells</td>
<td>14 (21%)</td>
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<tr>
<td>- cord blood</td>
<td>6 (9%)</td>
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<tr>
<td><strong>Digestive graft-versus-host disease</strong></td>
<td></td>
</tr>
<tr>
<td>- Stage 0-1</td>
<td>49 (72%)</td>
</tr>
<tr>
<td>- Stage 2 or more</td>
<td>19 (28%)</td>
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<tr>
<td><strong>Pathological grade</strong></td>
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<tr>
<td>- grade 0</td>
<td>18 (26%)</td>
</tr>
<tr>
<td>- grade 1</td>
<td>22 (32%)</td>
</tr>
<tr>
<td>- grade 2</td>
<td>15 (22%)</td>
</tr>
<tr>
<td>- grade 3**</td>
<td>10 (15%)</td>
</tr>
<tr>
<td>- grade 4**</td>
<td>3 (4%)</td>
</tr>
<tr>
<td><strong>More than 20 cells per field expressing</strong></td>
<td></td>
</tr>
<tr>
<td>TNFα, according to pathological grade*</td>
<td></td>
</tr>
<tr>
<td>- patients with grade 3-4</td>
<td>10/13 (77%)</td>
</tr>
<tr>
<td>- patients with grade 2</td>
<td>3/15 (20%)</td>
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<tr>
<td><strong>More than 5 apoptotic cells per field, within the cellular infiltrate, according to pathological grade</strong></td>
<td></td>
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<tr>
<td>- patients with grade 3-4</td>
<td>3/13 (23%)</td>
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<tr>
<td>- patients with grade 2</td>
<td>1/15 (7%)</td>
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<td><strong>More than 10 apoptotic epithelial cells per field, according to pathological grade</strong></td>
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<tr>
<td>- patients with grade 3-4</td>
<td>12/13 (92%)</td>
</tr>
<tr>
<td>- patients with grade 2</td>
<td>7/15 (47%)</td>
</tr>
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</table>
REFERENCES


Figure 1
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