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ORIGINAL ARTICLE

Small vessel disease after heart transplantation: impact of immunologic and nonimmunologic risk factors*

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Summary

To determine risk factors for small vessel disease after heart transplantation (HTx), characteristics of donors and organ harvesting were evaluated in 246 HTx patients (205 male, 41 female, mean survival 5.4 years). In right ventricular endomyocardial biopsies (EMB, $n = 5421$) evidence of microvascular disease [endothelial cell (EC) swelling/vessel wall thickening] was evaluated by light microscopy (hematoxylin and eosin staining, ×200). Mild EC swelling/vessel wall thickening were found in 204 and 213 patients, severe EC swelling/vessel wall thickening were present in 23 and 142 patients respectively. Evidences of mild and severe acute cellular rejection were found in 2064 and 421 EMB respectively. Microvascular disease was positively correlated with mild acute rejection episodes ($P < 0.05$). EC swelling was more frequent in patients with donors dieing of craniocerebral trauma. No correlations were present to further demographical data. Microvascular alterations after HTx seem to be the result of an immunologic conflict rather than to depend on nonimmunologic factors.

Introduction

Graft vessel disease (GVD) is one of the main limiting factors to long-term survival after heart transplantation (HTx). It is characterized by diffuse lesions of epicardial and intramyocardial blood vessels [1–3]. The proliferation of vascular smooth muscle cells in pre-existing arteries and arterioles and a diminution of capillary vascularization within the terminal vascular system [3–5] leads to diffuse occlusive vasculopathy. Its etiopathogenesis remains unknown. Several immunologic and nonimmunologic risk factors [6–10] have been described as playing a key role in the pathogenesis of epicardial GVD, but risk factors for small vessel disease still have to be illuminated. Therefore, this study tested the role of immunologic and nonimmunologic risk factors in the development of GVD after HTx in right ventricular endomyocardial biopsies (EMB).

Material and methods

The study population consisted of 246 patients of a total of 1334 HTx and heart lung transplantation (HLTx) patients transplanted between 18 April 1986 and 24 February 2000 at our institution, who died beyond the first year after HTx. There were 205 men and 41 women; mean survival time was 5.4 years. Indications for HTx were dilated cardiomyopathy (dCMP) in 137 cases, coronary artery disease (CAD) in 78 cases, primary pulmonary hypertension (PPH) in seven cases and others in 24 cases. Characteristics of donors and organ harvesting were evaluated retrospectively (Tables 1 and 2). Paraffin-embedded EMB ($n = 5421$) harvested from 221 patients within the first 13 years after HTx were strictly classified according to the classification of the International Society for Heart and Lung Transplantation (ISHLT) for acute cellular rejection [11].

Table 1. HTx patients' characteristics.

Parameter	
HTx/HLTx	234/12
Age at HTx/HLTx (years)	47.6 ± 13.1
Survival time (years)	5.4 ± 3.1
Gender (f/m)	41/205
HTx m → m/f → m/m → f/f → f	161/39/21/25
Bretschneider/UW/others	146/26/74
CMV pos./neg./n.d.	104/67/75
Ischemic time (min)	162 ± 47
Reperfusion time (min)	109 ± 73
Indications for HTx	
dCMP	137
CAD	78
PPH	7
Others	24

CAD, coronary artery disease; CMV, cytomegalovirus; dCMP, dilated cardiomyopathy; f, female; HTx, heart transplantation; m, male; n.d., not done; PPH, primary pulmonary hypertension; UW, University of Wisconsin.

Table 2. Donors' characteristics.

Parameter	
Age at time of death (years)	35 ± 13
Gender [f/m/unknown]	64/175/7
CMV pos./neg./n.d.	89/98/59
Brain death to organ harvest (h)	12.4 ± 6.1
Causes of death	
Intracerebral hemorrhage	102
Craniocerebral trauma	107
Cerebral tumor	6
Hypoxia	9
Apoplexia	5
Shooting	11
Encephalitis	2
Unknown causes	4

f, female; m, male; n.d., not done.

At our institution, all grades of acute cellular rejection are treated with an increased immunosuppressive regimen. Treatment of acute cellular rejection grades 01A to 2 consists of an oral steroid scheme for 10 days. All rejection grades 3A or higher or clinical symptomatic acute cellular rejections are treated with polyclonal antibodies and steroids for 3 days. In accordance with our routine post-transplant follow-up, there is no time dependent EMB screening after HTx performed for acute cellular rejection. Routine rejection monitoring is performed noninvasively by echocardiography. However, routine EMB harvesting is carried out for the diagnosis of small vessel GVD at 4 weeks, 1 year and 2 years after HTx and, dependent on the results of this screening, in time intervals of 1–3 years thereafter.

Hematoxylin and eosin staining were performed according to the following scheme: The biopsy samples (size 1–1.5 mm) were fixed in 4% buffered Lillie formol. After dehydration the tissue sections were embedded in paraffin. Preparation of tissue sections was performed by cooling and cutting the biopsy samples into slices (depth 3 μm) and then drying them on glass slides. The tissue sections were deparaffinated with xylol and rehydrated with alcohol (100%, 90%, 70% and distilled water) and then stained with hemalaun (3 min) and differentiated with HCl alcohol. Finally eosin (2% in alcohol, 30 s) was applied. Grading of vascular reaction, i.e. morphology of microvascular endothelial cells (EC) (normal/prominent/severely swollen) and morphology of microvascular vessel walls (normal/thickened/severely thickened) was carried out at magnification of ×200 [12].

This study focuses on microvascular alterations after HTx *independently* of epicardial GVD. To prevent additional sub-grouping with a consequent decrease in the number of patients for statistical evaluation, coronary angiographic results were disregarded. Intravascular ultrasound investigations and perfusion scans were not performed in the population studied.

Statistics

All data were analyzed using nonparametric tests; a P-value of <0.05 was considered to be significant.

Results

The results concerning donors' and patients' characteristics are shown in Tables 1 and 2. Mean age at HTx was 47.6 ± 13.1 years and mean survival time 5.4 ± 3.1 years. Evidence of positive cytomegalovirus (CMV) status was found in 104 cases. The mean ischemic time was 162 ± 47 min and the mean reperfusion time 109 ± 73 min. Donors consisted of 64 women and 175 men; their mean age at time of death was 35 ± 13 years. Positive CMV status was found in 89 patients and mean time of brain death was 12.4 ± 6.1 h.

Fifty-two percent of EMB investigated here were harvested within the first 6 months after HTx. Between the sixth and 12th month 18%, within the second year 16% and between the third and 13th year 14% of EMB were taken. In 2936 EMB there was no evidence of acute cellular rejection. Of a total of 2485 EMB with signs of acute cellular rejection 2064 showed evidence of mild and 421 of severe acute cellular rejection. During the first year in total 3847 EMB were harvested from 221 patients. Of these, 29 EMB (0.8%) were not representative, 1932 (50.2%) EMB showed no signs of acute cellular rejection according to the ISHLT classification, 1531 (39.8%) EMB

Table 3. Distribution of EMB and rejection episodes per patient within the first year.

Parameter	% of EMB within the first year (mean ± SD)	Absolute *n* [mean ± SD (range)]
Total number of EMB	–	17.4 ± 11.0 (1–48)
Rejection grade 0	53.1 ± 23.4	8.7 ± 6.4 (0–26)
Total number of rejection episodes	46.2 ± 23.6	8.5 ± 7.2 (0–31)
Rejection grades 01A to 2	37.5 ± 20.2	6.9 ± 6.0 (0–28)
Rejection grades ≥3A	8.7 ± 12.0	1.6 ± 2.4 (0–13)
Number of EMB not representative	0.7 ± 3.1	0.1 ± 0.4 (0–3)

EMB, endomyocardial biopsy.

presented rejection grades 01A to 2, and 355 (9.2%) EMB were classified as grade 3A or higher (Table 3). Of a total of 221 patients only eight (with 3.5 ± 2.1 EMB per patient) underwent no acute cellular rejection at all as compared with 213 patients (with 25.3 ± 17.7 EMB per patient) who underwent at least one acute cellular rejection episode ($P < 0.05$). The same sampling bias was found when comparing patients with and without proven severe acute cellular rejection (ISHLT grade 3A and higher). For this reason patients were divided into two groups, one with more ($n = 40$) and the other with less ($n = 181$) than 50% of EMB with evidence of ISHLT grades 01A to 2. Another two groups were determined with more ($n = 79$) or less ($n = 142$) than 50% of EMB with any evidence of acute cellular rejection (Table 4).

Evidence of mild small vessel disease (mild EC swelling/vessel wall thickening) was found in 204 and 213 patients respectively. Severe EC swelling and vessel wall thickening were present in 23 and 142 patients respectively. The distribution of small vessel disease in EMB is shown in Table 5. Microvascular GVD was positively correlated with mild but not with severe acute cellular rejection episodes (Figs 1 and 2; $P < 0.05$). Statistical analysis showed significant correlations between the total number of acute cellular rejection episodes, as well as between moderate and severe acute cellular rejection episodes within the first year after HTx and the evidence of small vessel GVD. But these results were less pronounced as compared with the overall incidence of acute cellular rejections in relationship to microvascular GVD (Table 6). Vessel wall thickening was correlated positively with EC swelling (Fig. 3). Cumulative survival analysis showed decreased survival in patients with evidence of microvascular GVD in ≥60% of their EMB (Fig. 4). One-third of the studied patients showing <60% of EMB with evidence of small vessel GVD died because of cardiac related causes (Table 7). EC swelling was more frequent in patients with craniocerebral trauma but no correlations were found to other causes of death of organ donors. There were no significant differences in the number of EMB per patient when they were grouped according to

Table 4. EMB in patients with rejection compared with those without.

Parameter	Number of patients	Number of EMB per patient (mean ± SD)
Overall	221	24.5 ± 17.8
No ACR at all	8	3.5 ± 2.1***
At least one ACR	213	25.3 ± 17.7***
≤50% of EMB with ISHLT grades 01A to 2	181	23.5 ± 16.6**
>50% of EMB with ISHLT grades 01A to 2	40	29.3 ± 22.2**
≤50% of EMB with any grade of ACR	142	23.3 ± 16.4*
>50% of EMB with any grade of ACR	79	26.7 ± 20.1*

Mann–Whitney test *$P = 0.233$; **$P = 0.145$; ***$P < 0.05$.
ACR, acute cellular rejection; EMB, endomyocardial biopsy; ISHLT, International Society for Heart and Lung Transplantation.

Table 5. Distribution of small vessel disease (number of EMB).

Parameter	No	Mild	Severe
Endothelial cell swelling	4107	1194	37
Vessel wall thickening	2738	2143	444

EMB, endomyocardial biopsies.

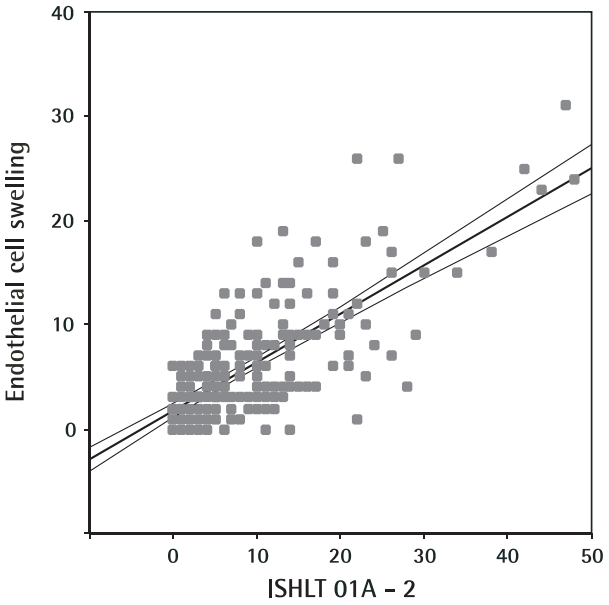

Figure 1 Correlation of endothelial cell swelling with mild acute cellular rejection episodes ($r = 0.745$, $P < 0.05$).

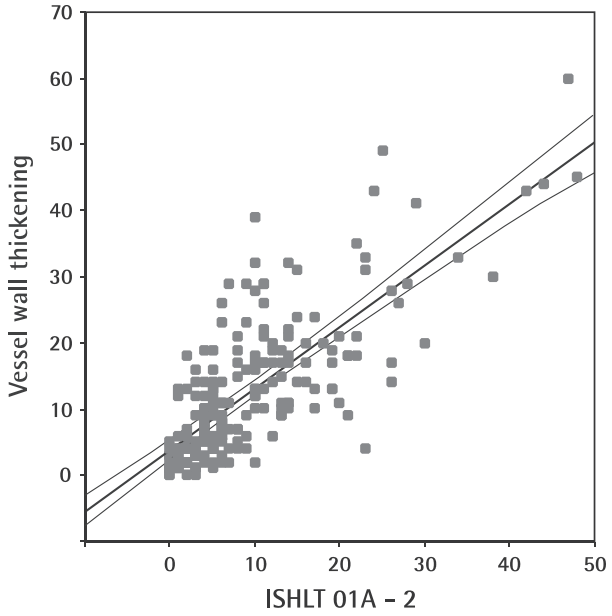

Figure 2 Correlation of vessel wall thickening with mild acute cellular rejection episodes ($r = 0.772$, $P < 0.05$).

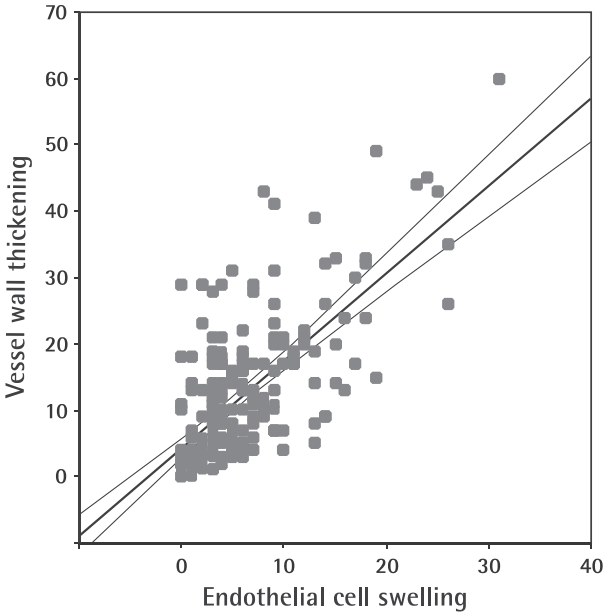

Figure 3 Correlation of endothelial cell swelling with vessel wall thickening ($r = 0.772$, $P < 0.05$).

Table 6. Spearman's correlation analysis: acute cellular rejection within the first year after HTx and small vessel GVD.

Parameter	Endothelial cell swelling	Vessel wall thickening
Total number of rejection episodes	$r = 0.446$ ($P < 0.05$)	$r = 0.534$ ($P < 0.05$)
Rejection grades 01A to 2	$r = 0.442$ ($P < 0.05$)	$r = 0.517$ ($P < 0.05$)
Rejection grades ≥3A	$r = 0.256$ ($P < 0.05$)	$r = 0.349$ ($P < 0.05$)

HTx, heart transplantation; GVD, graft vessel disease.

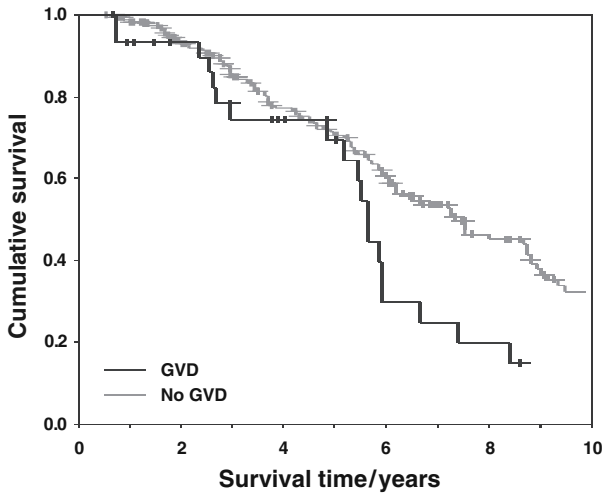

Figure 4 Survival analysis in patients with microvascular graft vessel disease in ≥60% of their endomyocardial biopsies ($P = 0.05$).

the donors' causes of death (Fig. 5; Mann–Whitney test $P = 0.220$). No correlations were present to donor and recipient age, CMV status, gender and gender mismatch, ischemic time, reperfusion time, or duration of brain death.

Discussion

While infection and rejection are the most life-threatening complications during the early postoperative period after thoracic organ transplantation, GVD remains the leading long-term cause of death after HTx [2,3]. Diffuse stenotic lesions affect the entire vascular tree [1–3] and are characterized by intimal proliferation in epicardial coronary arteries and, within the terminal vascular system, proliferation of smooth muscle cells (SMCs) in pre-existing arteries and arterioles and diminution of capillary vascularization [3–5]. The role of immunologic and nonimmunologic risk factors in the etiopathogenesis of GVD is still controversial. Several nonimmunologic risk factors, such as higher donor age [7,13] a dysfunction in coagulation [14,15], increased activity of the complement system [16] and an increased reperfusion and ischemic time [6,17] have been identified. Even an association of University of Wisconsin intracellular solution with an increased incidence of vasculopathy has been suggested [18]. Data concerning the duration of brain death as a promoter of vascular alterations are rare.

Table 7. Causes of death in patients with ≥ or <60% of EMB with evidence of small vessel GVD.

Causes of death	No small vessel GVD ($n = 190$)	Small vessel GVD ($n = 31$)
Cardiac	60	10
Acute cellular rejection	8	–
Solid tumor	25	3
Lymphoma	8	4
Sepsis	36	4
Cerebral	9	1
Pulmonary	6	1
Unknown	25	6
Others	13	2

EMB, endomyocardial biopsies; GVD, graft vessel disease.

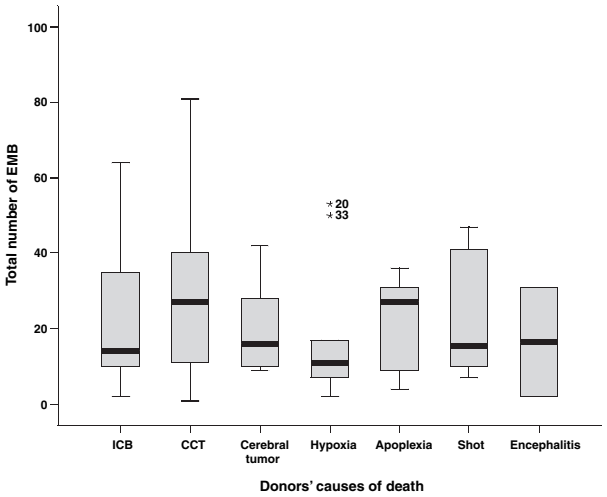

Figure 5 Number of endomyocardial biopsies (EMB) per patients grouped according to donors' causes of death. CCT, cranio-cerebral trauma; ICB, intracerebral bleeding ($P = 0.220$); *extreme cases.

Novitzky *et al.* [19] suggested that brain death induces major histopathologic changes in the myocardium and induces contraction band necrosis of smooth muscle cells of the coronary arteries in the baboon. Biswas *et al.* [20] demonstrated that brain death further promotes ischemic reperfusion injury of the rabbit myocardium. In analogy to Ross [21] who described the development of CAD in non-transplanted hearts as a result of an excessive inflammatory-fibroproliferative response to various forms of insult to the endothelium and smooth muscle cells of the artery wall, Deng *et al.* and Weis and von Scheidt [2,22] postulated 'response to injury' as a major factor for the development of GVD. This may be reflected by the correlation of EC swelling with vessel wall thickening (see Fig. 3).

With respect to the results in this study, there was a lack of correlation between patients' and donors' demographic characteristics (see Tables 1 and 2) and the development of microvascular GVD, which has also been found by others [23,24]. Only patients who had been transplanted with hearts from donors dying from cranio-cerebral trauma showed a tendency towards a more pronounced frequency of EC swelling in EMB.

Evidence of microvascular GVD in myocardial tissue sections has been described by Palmer *et al.* [25], who declared the presence of proliferative arteriolar occlusion in EMB as predictive of poor heart graft survival. Decreased survival in patients with evidence of microvascular disease was also found in this study (see Fig. 4). Additionally, the feasibility of diagnosis of microvascular GVD in EMB has been demonstrated by us and others [4,26]. In this study the majority of patients displayed signs of mild small vessel disease (see Table 3), which may reflect physiological adaptation [5] or initial stages of progressive microvasculopathy.

Acute cellular rejection is one of the predominant lethal complications during the first year after HTx [22,27]. Evaluation of EMB is the gold standard for diagnosis of acute cellular rejection [28,29]. All EMB were strictly graded according to the working formulation of the ISHLT as standard graduation system [11]. As well as the role of CMV infection in the development of GVD [8,30] also the association with the incidence, time point after HTx, and duration and severity of acute cellular rejection episodes are controversial [10,31], probably because of the fact that the main parts of these investigations were carried out retrospectively in patients with different immunosuppressive regimens. In this study, in 54% of EMB there were no signs of acute cellular rejection; in EMB with signs of acute cellular rejection ($n = 2485$) 83% displayed evidence of mild (ISHLT grade 1A, 1B, 2) and 17% of severe acute cellular rejection. Correlations to the development of microvascular GVD could only be demonstrated for mild acute cellular rejection episodes (see Figs 1 and 2) and thus our findings conform to those of Kobashigawa *et al.* [31] and Winters *et al.* [10].

According to the results presented here, one-third of the studied patients showing <60% of EMB with evidence of small vessel GVD died because of cardiac related causes. This may suggest (i) relevant GVD of epicardial coronary arteries, (ii) both ('relevant'?) epicardial and microvascular GVD or (iii) underscored small vessel GVD. Although (facultative) co-occurrence of epicardial and small vessel GVD is undisputed, the time course of both diseases as well as the question of reciprocal influence still has to be elucidated in *prospective* clinical trials.

Conclusion

In summary, microvascular alterations represent a common phenomenon after HTx. This seems to reflect the

result of an immunologic conflict rather than to depend on nonimmunologic factors.

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