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Lymphoproliferative disorders in heart transplant recipients: role of hepatitis C virus (HCV) and Epstein-Barr virus (EBV) infection

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Abstract Post-transplant lymphoproliferative disorders (PTLD) are a well known complication after orthotopic heart transplantation (OHT). Although Epstein-Barr virus (EBV) infection has long been implicated in the pathogenesis of such disorders, other factors may play a part. Because of its lymphotropic properties, hepatitis C virus (HCV) may induce clonal expansion of B-lymphocytes and lead to PTLD. The aim of this study was to evaluate the potential association between HCV and EBV infection and PTLD in OHT patients. The retrospective study considered 404 adult patients screened for HCV. EBV serology, histology, and molecular analysis on tissue biopsies were performed in the PTLD patients (10/404, 2.5%). HCV positivity was found in 36/404 (8.9%) patients. The EBV genome was expressed on all neoplastic tissue samples analyzed. A higher proportion of HCV-positive patients developed PTLD than the HCV-negative cases (8% vs 2%, $P = 0.017$). EBV has a demonstrated role in the onset of PTLD, but HCV infection probably has to be considered as well.

Key words Heart transplantation · Post transplant lymphoproliferative disorders · EBV infection · HCV infection

Introduction

As long-term survival after orthotopic heart transplantation (OHT) has improved, neoplastic complications are increasingly being discovered, with a prevalence ranging from 6% to 12% [15]. Immunosuppression clearly exposes patients to a high risk of an unusual spectrum of malignancies, particularly post-transplant lymphoproliferate disorders (PTLD) which accounts for up to 20% of all cancers diagnosed in OHT recipients [16, 21]. These tumors, typically presenting as non-Hodgkin's lymphoma (NHL), are usually of B-cell origin and are often associated with active Epstein-Barr virus (EBV) infection [17].

It has recently been noted that hepatitis C virus (HCV) infection is associated with several extrahepatic disorders, particularly of the immune system [7]. As a result of its lymphotropic properties, HCV may induce clonal expansion of B-lymphocytes and lead to PTLD [5, 18]. HCV infection has been detected in more than 25% of patients with different histological grades of B-cell NHL [4], and it predisposes patients to the onset of type II essential mixed cryoglobulinemia (EMC) [1].

The aim of this study was to evaluate the potential association of HCV and EBV infection with de novo PTLD development in heart-transplant patients.

Patients and methods

OHT was performed in 447 patients from November 1985 to September 1998: 404 adult patients (346 men, 58 women; mean age 59.4 years, range 18–66 years) with at least 1 month of follow-up were retrospectively studied. The immunosuppressive therapy consisted of cyclosporine, azathioprine, and steroids with ATG and OKT3 as the induction regimen. ATG was used in 93% and

OKT3 in 5% of patients, respectively; steroids were not used in 30% of patients.

Stored serum samples from all patients were screened for HCV infection. Anti-HCV antibodies were assessed by third-generation enzyme-linked immunoassay (ELISA III). Qualitative HCV-RNA was assessed by nested-reverse transcriptase polymerase chain reaction (RT PCR) (5' UTR primer). Patients were divided into two groups according to whether they were anti-HCV and/or HCV-RNA positive.

De novo PTLD development

All patients with a diagnosis of PTLD underwent a thorough work-up including chest, abdomen, and brain computed tomography (CT) scans to detect lymphoproliferative sites. Bone marrow biopsy and aspiration were performed where possible. The therapy was multimodal: reduced immunosuppression, chemotherapy, radiotherapy and surgery, as indicated.

Histology and immunohistochemistry

Histological sections of biopsies and postmortem formalin-fixed, paraffin-embedded tissues were examined following routine staining with hematoxylin and eosin. Immunohistochemistry was performed using monoclonal antibodies to the B-cell antigen CD 20 (Dako, Glostrup, Denmark) and T-cell antigen CD45RO (Dako), as described elsewhere [2]. All cases were morphologically classified as reported by Knowles et al. [10].

EBV serological status

Primary EBV infection was diagnosed by the detection of serum anti-EBV immunoglobulin (Ig)M antibodies, while reactivation of EBV infection was diagnosed by the reappearance of anti-EBV IgM antibodies or an increase in IgG.

EBV molecular analysis

PCR for EBNA-1 and gp220. High-molecular-weight DNA was extracted from frozen and/or formalin-fixed tissue using the standard procedure [13]. DNA was amplified to detect EBV sequences coding for EBNA-1 and gp220 (BLLF1) based on the procedure of Talenti et al. [20]. DNA (1 μg) in PCR buffer containing 2.5 U Taq polymerase (Perkin-Elmer Cetus Corporation, Norwalk, Conn.), 200 μM of each dNTP, and 0.1 μM 5' and 3' primers was amplified in a thermocycler (Perkin-Elmer) using the following program: denaturation at 94°C, 45 s at 60°C, and 2 min at 70°C. Human b-globin gene amplification was used as a positive control for DNA. DNA extracted from Daudi cell lines was used as a positive control for EBNA and gp 220 gene amplification. Negative controls were provided by the reaction mixture without DNA. Previously published primer sequences for human b-globin, EBNA, and gp220 genes were used [20].

ISH for EBER. ISH for EBER was done on 4-μg paraffin sections in all cases, using the fluorescein-conjugated EBER oligonucleotide probe for EBER RNA (Epstein-Barr encoded RNAs, Biogenex, San Ramon, Calif.). The χ^2 test was used to evaluate the association of HCV infection with PTLD.

Table 1 Types of cancer after heart transplantation (*CNS* central nervous system, *PTLD* post-transplant lymphoproliferative disorder)

No.	Type	Site
1	Adenocarcinoma	Lung
2	PTLD	Large bowel
3	Carcinoma	Salivary glands
4	Carcinoma	Prostate
5	PTLD	CNS, large bowel
6	Adenocarcinoma	Lung
7	PTLD	Lung
8	Kaposi	Legs
9	Squamous carcinoma	Larynx
10	Sarcoma	Retroperitoneum
11	Carcinoma	Pancreas
12	Melanoma	Shoulder
13	Oat cell carcinoma	Lung
14	Epidermoid carcinoma	Lung
15	Adenocarcinoma	Lung
16	PTLD	Nodal
17	PTLD	Large bowel
18	PTLD	Larynx
19	PTLD	Larynx
20	Carcinoma	Breast
21	PTLD	Nodal
22	Carcinoma	Bladder
23	PTLD	Thigh

Results

Of the 404 (5.7%) OHT patients, 23 developed a de novo non-cutaneous malignant tumor during a mean follow-up of 60 months. The different types and sites of the cancers are listed in Table 1.

PTLD occurred in 10 patients (9 men, 1 woman; 8 ATG and 1 OKT3 induction immunosuppression therapy), 2.5% of OHT patients. PTLD represented 41% of the de novo cancers.

Out of 404 (8.9%) OHT patients, 36 were positive for anti-HCV or HCV-RNA, or both: 25 were anti-HCV + /HCV-RNA + , 5 anti-HCV + /HCV-RNA–, 6 anti-HCV-/HCV-RNA + .

De novo PTLD evolution

The diagnosis of PTLD was made 4–79 months after OHT (mean 42.8 months). Two patients developed the disease in the 1st year after transplantation. In 9 patients the diagnosis was made by neoplastic tissue biopsy, while in the others it was made at the post-mortem examination. In 2 patients, the tumor had only nodal development. In 7, extranodal sites were found (large bowel involvement in 2, large bowel and brain in 1, larynx in 2, thigh in 1, and lung in 1). Immunosuppression was reduced in all patients. Surgical resection was performed in 4 patients, gancyclovir and chemotherapy

were administered in 4 patients (1 from the surgical group). Radiotherapy was performed only in 1 patient. During follow-up 5 patients died, while 2 had disease regression.

HCV infection was detected in 3 PTLD patients, i.e., anti-HCV-/HCV-RNA + in 1 and anti-HCV + /HCV-RNA + in 2. A significantly higher rate of PTLD was seen in the group of HCV-positive patients than in those who were HCV-negative (8% vs 2%, $P = 0.017$).

HCV-related liver disease was observed in 21 (58.3%) of the 36 HCV-positive patients, but none of the patients who developed PTLD with HCV infection had liver dysfunction.

Histology and immunohistochemistry

All PTLD patients expressed immunological markers of B-cell lineage (CD20 positive). The tumors were classified as immunoblastic lymphoma in 9 cases and as plasmacytic hyperplasia in 1.

EBV serological status

One patient was found positive for EBV IgM antibodies; 4 patients had a significant 4-fold elevation in EBV-IgG titer at the time of PTLD diagnosis. Five patients presented no EBV IgG antibody variations.

EBV molecular analysis

EBNA and gp220 gene sequences were detected in 5/5 tested samples, as well as in the positive controls used. In situ hybridization to the EBV-encoded small RNA (EBER) was strongly positive in all cases, and it was strongly expressed in the neoplastic B-cells.

Discussion

In the setting of OHT, problems of rejection and infection are usually reported in terms of outcome. However, the development of de novo malignancies represents a leading cause of death in the long-term follow-up after transplantation. Patients who have received solid organ transplants have a 28- to 49-fold higher incidence of non-Hodgkin's lymphoma (NHL) than the general population [15]. The incidence of lymphoproliferative disease varies, depending on the different organs transplanted and the amount of immunosuppression administered. The highest rate is observed in heart and heart-lung transplantation, and when OKT3 or ATG are used in the immunosuppressant therapy. PTLD have a characteristically aggressive behavior and a tropism for extranodal sites; the clinical course and outcome are unpredictable, with partial or complete regression after reducing or withdrawing immunosuppressant therapy in some cases, and disease progression and death despite therapeutic measures in others [14, 19]. We found a prevalence of de novo non-cutaneous malignancies comparable with the one previously described by other authors, whereas our prevalence of PTLD was lower [6]. Although in the literature up to 12% of cases have reportedly been of T-cell origin, all lymphomas diagnosed in our patients were of B-cell origin, and 90% were classified as high grade.

EBV infection has long been implicated in the pathogenesis of PTLD [17], and our results confirm its role. Heavy immunosuppression may allow EBV-infected cells to express latency proteins and expand without selective pressure from cytotoxic T-cells. EBV facilitates the expansion of multiple EBV-infected and immortalized B-cell clones. However, not all lymphomas in transplant recipients are associated with EBV. A careful search for the EBV genome has yielded negative results in several lymphomas in organ allograft recipients [9, 11]. It may be that EBV does not have a primary role in lymphoma genesis in some cases, but that it subsequently infects the malignant cells. We believe that other factors may have a role in the onset of PTLD. HCV infection has been associated with several nonhepatic disorders, including cryoglobulinemia, Waldenstrom's macroglobulinemia and Sjogren's syndrome, all of which are associated with clonal proliferation of lymphocytes – considered to be a form of low-grade NHL [8]. Moreover, recent studies from Italy have reported HCV infection in 38% of patients with low-grade NHL [3]. It has been speculated that the long-term stimulation of B-cells by HCV, either directly or in combination with other (infectious, environmental and/or genetic) factors, might result in mono- or polyclonal B-cell expansion, followed by malignant transformation into lymphoma [4, 5]. In our OHT population, a significant association was found between HCV infection and PTLD. More anti-HCV- and/or HCV-RNA-positive patients developed lymphoma than did those who were HCV-negative. HCV is considered to be both hepatotropic and lymphotropic, but it is still not clear whether there is a definite sequence in the kind of tissue involvement. We have not observed HCV-related liver disease in anti-HCV PTLD patients, suggesting that the two pathologies are independent, and the evolution of one into the other is unlikely.

It seems that some primarily lymphotropic strains exist [12]; in transplant recipients, lymphotropic paths are probably favored by immunosuppression, leading to a higher risk of developing lymphomas. Moreover, the hepatotoxic role of HCV may be hidden by immunosuppression, preventing hepatocellular damage. Although EBV remains the most important etiologic factor for

the onset of PTLD, it is unlikely to be the only element responsible for the development of lymphomas in transplant recipients, and some other factors are probably involved. HCV infection should be considered – especially in cases, as reported by some authors [11], in which EBV cannot be found.

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