Expansion in scid mice of Epstein–Barr virus-associated post-transplantation lymphoproliferative disease biopsy material

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Post-transplant lymphoproliferative disease (PTLD) biopsy material is rarely available in adequate quantity for research. Therefore, the present study was designed to expand biopsy material in scid mice. Epstein–Barr virus (EBV)+ve PTLD samples from five transplant patients were established in scid mice. PCR analysis of immunoglobulin gene rearrangements demonstrated that four of the five biopsies (80%) gave rise to scid tumours which represented the original tumour cell clones. Immunophenotyping showed that these four biopsies (and all scid tumours) expressed all EBV latent genes and a B lymphoblast phenotype; ≥ 26% T cells were found in the biopsy material whereas scid tumours showed a paucity of T lymphocytes. RT–PCR analysis revealed expression of IL-2, -4, -6, -10 and IFN-γ in all tumour material, suggesting key roles for these factors in tumour growth. The results show that EBV+ve PTLD material can be expanded in scid mice giving rise to quantities of homogeneous malignant tissue sufficient for research studies.

Post-transplant lymphoproliferative disease (PTLD) develops in up to 10% of solid organ transplant recipients and carries up to 70% mortality despite treatment (Armitage et al., 1991; Opelz & Henderson, 1993). The majority of PTLD cases are associated with Epstein–Barr virus (EBV). Main risk factors are primary EBV infection in previously seronegative organ recipients, and high-level immunosuppressive drug therapy (Ho et al., 1985; Thomas et al., 1990). Typically, tumour cells display an activated B lymphoblastic phenotype and express all EBV latent genes [six EBV nuclear antigens (EBNAs); three latent membrane proteins (LMPs)] and two EBV-encoded small non-polyadenylated RNAs (EBERs), although more restricted viral gene expression has been described (Cen et al., 1993; Oudejans et al., 1995). A minority of cells support lytic virus infection. PTLDs range from diffuse polymorphic lesions with polyclonal immunoglobulin (Ig) gene expression to malignant monoclonal lymphomas. The latter generally consist of large immunoblasts but include Hodgkin’s disease (HD) and plasmacytoma (reviewed in Crawford, 2001).

PTLD biopsy material is scant and does not generally grow in vitro. The few cell lines available do not reflect initial tumour phenotype (Cen et al., 1993; Itoh et al., 1993). Additionally, biopsies generally contain heavy infiltrates of non-malignant T cells (Perera et al., 1998) which can limit their use in molecular studies. The aim of this study was to expand original PTLD biopsies in scid mice, which readily accept human xenografts due to lack of functional B and T cells (Bosma et al., 1983; Johannessen & Crawford, 1999).

PTLD biopsies were obtained from five solid organ graft patients (designated ‘patient 1–5’; for patient details, see Table 1). For each biopsy, 25 × 10⁶–50 × 10⁶ cells (denoted ‘biopsy’) were injected intraperitoneally (i.p.) into a scid mouse within 12 h of biopsy (sample from patient 2 was inoculated into two animals). Tumours (denoted ‘scid tumour’) formed in all mice. It was possible to passage material from patient 3. Patients 3 and 5 experienced primary EBV infection following transplantation whereas patients 1, 2 and 4 were persistently infected. All five biopsies gave rise to i.p. tumours in scid mice. EBER in situ hybridization was performed on all sample material using standard methods (Howe & Steitz, 1986). Similar to biopsies, all scid tumours were EBER+ve [for an assessment of the relative proportion of EBV+ve cells in the sample material, see ‘(2) Cell phenotype’ below].

Our tissue panel of five sets of biopsy and scid tumours was analysed for (1) Ig and EBV clonality, (2) cell surface phenotype, (3) in vitro proliferation and (4) cytokine expression. Sample material was limited, and a full complement of tests could not always be carried out.

(1) Clonality. In order to ascertain whether scid tumours represented the corresponding biopsy, samples were assessed for Ig and EBV clonality. Ig clonality was analysed by PCR...
indicating virus clonality (data not shown). Each set of samples analysed with this assay. All lesions contained a single band.

Material from cases 2, 4 and 5 was studied using the Gardella gel technique (Hurley & Traub, 1986). The number of reiterated 500 bp EBV genome terminal direct repeats involved in forming the virus episome following infection of a target B cell is characteristic of any infection event. Progeny EBV +ve cells retain the same episome thus giving rise to an EBV clonal population which can be studied using the Gardella gel technique (Hurley & Thorley-Lawson, 1988). Material from cases 2, 4 and 5 was analysed with this assay. All lesions contained a single band indicating virus clonality (data not shown). Each set of samples from patients 2 and 5 contained bands of identical size, indicating a common infection event in each case, whereas material from patient 4 contained different EBV clones, suggesting that the scid tumour arose from a latently infected, non-malignant B cell.

(2) Cell phenotype. Primary antibodies against human and EBV antigens were applied to tumour sections (see Table 2). Bound antibody was detected as previously described (Reedman & Klein, 1973; Van Noorden, 1986). Immunophenotyping studies on biopsy and scid tumours from patients 2, 3, 4 and 5 demonstrated that all lesions expressed B cell surface antigens CD19 and CD23, indicating an activated, lymphoblastoid phenotype (Table 2). Biopsies from patients 2, 3 and 5 showed full EBV latent gene expression (EBNA-1, LMP-1). Similarly, all scid tumours demonstrated full latent gene expression. In biopsies, low level (< 5% of cells) expression of lytic cycle antigens BZLF1, early antigen [EA(D)], viral capsid antigen (VCA) and membrane antigen (MA) was detected. Increased lytic cycle antigen expression (5–10%) was observed in corresponding scid tumours. The patient 4 biopsy showed a restricted pattern of EBV gene expression typical of HD (Deacon et al., 1993): only LMP-1 was visualized by immunostaining, although EBNA-1 transcripts were detected by RT–PCR (data not shown). No lytic cycle antigens were expressed. Conversely, patient 4 scid tumour showed full EBV latent gene expression with lytic antigen expression similar to other scid tumours tested, further emphasizing differences in tumour cell type of the biopsy and scid lesion in this case.

(3) In vitro proliferation. In order to determine in vitro growth characteristics of biopsy and scid tumours, lesions from patients 1 and 3 were teased out into single cell suspensions and placed in culture. The proliferative response was assessed by [3H]thymidine incorporation using standard methods (data not shown; Taylor et al., 1957; Rubini et al., 1960). Whilst scid-derived material grew continuously in vitro for up to 56 days

### Table 1. Patient details

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Age* (years)</th>
<th>Sex</th>
<th>Transplanted organ</th>
<th>Onset of 1° tumour (months)*</th>
<th>EBV-serostatus at time of 1° tumour</th>
<th>Site of 1° tumour</th>
<th>Pathological diagnosis of 1° tumour</th>
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<td>Lymph node</td>
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<tr>
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<td>7</td>
<td>Primary infection</td>
<td>Lymph node</td>
<td>BLPD</td>
</tr>
</tbody>
</table>

* At time of transplantation.
† Time from graft to tumour.
‡ Recurrence; original lesion arose 2 months post-graft.
§ Recurrence; original lesion arose 29 months post-graft.
Expansion of EBV+ve PTLD in scid mice

Fig. 1. Analysis of immunoglobulin gene rearrangements. All samples were analysed for Ig clonality in order to ascertain whether scid tumours represented the corresponding biopsy. PCR was performed with 1 μg of DNA using primers specific for a conserved region of the V segment of Fr1 or Fr3 and J segments of IgH gene locus. PCR product (30 μl) was purified using a QIAquick 8 PCR purification kit (Qiagen), eluted in 60 μl of buffer, and 3 μl of purified product run on an ABI PRISM 310 Genetic Analyser with 0.5 μl of GS350 marker and 10 μl of formamide. Results were analysed using GeneScan software. (A) Electropherogram of Fr1 PCR from patient 2. Top panel: biopsy result. Middle and bottom panels: results from two scid tumours derived from biopsy. VH3 rearrangement of 328 bp is indicated in each panel. (B) Electropherogram of Fr3 PCR products from patient 3. Top panel: biopsy result showing oligoclonal distribution. Second panel from top: scid tumour showing oligoclonal distribution with three dominant rearrangements. Third panel from top: scid tumour (passage 1) showing one dominant rearrangement. Bottom panel: scid tumour (passage 2) showing a single rearrangement. (C) Electropherogram of Fr3 PCR products from patient 4. Top panel: biopsy result. Bottom panel: scid tumour result. Dominant clones were detected at 109 and 91 bp, respectively, as indicated. The arrowheads (↓) indicate GeneScan 350 ROX labelled size standard. →, 97 bp; →, 100 bp; ↓, 109 bp.

(when the experiment was terminated), the biopsies failed to expand, which is in agreement with the experience of others (Itoh et al., 1993; unpublished observations from our laboratory). This is unexpected since the majority of PTLD express all EBV growth-promoting latent viral genes. However, this phenomenon may be due to lack of necessary growth factors in vitro resulting in programmed cell death.

(4) Cytokine expression. Extensive T cell infiltrates (>26% of all cells; see Table 2) were apparent in all biopsies as previously described (Perera et al., 1998). Conversely, only occasional T cells were identified in corresponding scid tumours. To address the possible role of T cell-derived factors in tumour growth, human cytokine gene expression of all material from patients 1–5 was analysed by RT–PCR (Fig. 2). Analysis of human cytokine mRNAs [interleukin (IL)-2, -4, -6, -10 and interferon (IFN)-γ] was carried out using published conditions (Yamamura et al., 1991, 1992). Primers for IL-10 did not cross-amplify EBV-encoded viral IL-10. Biopsy and scid lesions demonstrated a similar cytokine profile with expression of the B cell growth factors IL-2, -4, -6, -10 and IFN-γ suggesting a key role in tumour growth. In situ hybridization studies in our laboratory have demonstrated that these growth factors are expressed by PTLD cells themselves (unpublished observations). The results suggest that T cells may contribute growth factors in the original malignancy whilst lack of T cells in scid tumours indicates that tumour cells supply themselves with necessary factors in an autocrine fashion supporting autonomous growth. This is in line with our previously proposed model of PTLD pathogenesis (Johannessen et al., 2000).

The results demonstrate that biopsies from four out of five (80%) patients (numbers 1, 2, 3 and 5) were successfully
Table 2. Immunophenotypic comparison of biopsy and scid tumours

<table>
<thead>
<tr>
<th>Antibody specificity</th>
<th>Pt 2</th>
<th>scid 2</th>
<th>Pt 3</th>
<th>scid 3</th>
<th>Pt 4</th>
<th>scid 4</th>
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<td>—</td>
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<td>oc</td>
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</table>

* EBNA1 +ve on RT–PCR.

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expanded in scid mice (we have previously reported on in vivo outgrowth of PTLD biopsy from patient 5; see Perera et al., 1996). Conversely, scid tumour from patient 4 probably represents a non-malignant EBV + ve B cell in the HD biopsy. This finding has been reported by others (Meggetto et al., 1996).
To our knowledge, this is the first report demonstrating outgrowth of a panel of PTLD biopsies in scid mice and contrasts with an earlier study by Randhawa et al. (1997) suggesting that biopsies did not grow in vivo. Analysis of passaged scid tumours from patient 3 showed progression from oligoclonal to monoclonal tumour (Fig. 1B), thus providing possible insight into PTLD clonal progression.

Our study provides an adequate quantity of homogeneous PTLD material from four graft recipients which is uncontaminated with infiltrating non-malignant cells, thus providing opportunity for further molecular studies on PTLD pathogenesis.

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References


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