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Short Communication

Low Expression of CD20 and CD23 in Epstein–Barr Virus–Induced B Cell Tumors in SCID/hu Mice

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Intraperitoneal injection of immunodeficient C.B.-17/scid (SCID) mice with human peripheral blood leukocytes from Epstein-Barr virus (EBV)-seropositive donors or with peripheral blood leukocytes from EBV-seronegative donors followed by an injection of EBV results in the development of human B-cell tumors. EBV-induced oligoclonal SCID/hu tumors closely resemble the EBV-associated lymphoproliferative disorders that are complications in immunosuppressed transplant patients. Previous reports have indicated that SCID/hu tumor cells are phenotypically similar to in vitro–transformed lymphoblastoid cell lines (LCL), which express high levels of mature B-cell lineage/activation antigens (CD19, CD20, CD21, CD23, CD30, CD39). In this study, however, flow cytometric (FACS) analysis showed that expression of CD20 and CD23 by SCID/hu tumor cells was markedly reduced relative to CD20 and CD23 expression by donor-matched, in vitro–transformed LCL. Injection of LCL into SCID mice also produced tumors in which CD20 and CD23 expression was greatly reduced relative to levels expressed by the injected LCL. In addition, tumorigenesis following LCL injection was associated with the production of high levels of human Ig in the sera of SCID mice. Our data thus indicate that EBV-driven tumorigenesis in vivo is associated with significant changes in B-cell phenotype relative to EBV-infected B cells transformed in vitro. (Am J Pathol 1993, 142:353–358)

Epstein–Barr virus (EBV), a human B-lymphotropic herpes virus, is the etiologic agent of infectious mononucleosis and is associated with two malignant disorders, Burkitt's lymphoma and nasopharyngeal carcinoma. EBV is also closely associated with the B-cell lymphoproliferative disorders (LPDs) and lymphomas that arise in patients with iatrogenic or disease-associated immunosuppression, e.g., transplant recipients or AIDS patients, respectively.[1–5] EBV possesses the ability to transform human B cells *in vitro*, thus generating immortalized lymphoblastoid cell lines (LCLs) that express the EBV nuclear antigens EBNA-1, -2, -3A, -3B, -3C, -LP, membrane proteins LMP1 and LMP2, and two small viral RNA species, the EBERs.[6] EBV-transformed LCLs express high levels of B-cell activation antigens such as CD23, CD30, and CD39,[7,8] and LCLs grow in clumps as a result of increased expression of LFA-1, LFA-3, and ICAM-1 adhesion molecules.[9,10]

Analysis of viral and cellular gene expression following EBV infection of B cells *in vivo* has been very limited, as the natural host range is restricted to humans. However, *in vivo* studies have been facilitated by the recent development of the SCID/hu mouse model of EBV infection.[11–14] Aggressive tumors of human B-cell origin develop in SCID mice reconstituted with peripheral blood leukocytes (PBLs) from EBV-seronegative donors and subsequently infected with EBV[12,13] and arise spontaneously in SCID mice reconstituted with PBLs from

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EBV-seropositive donors.[11,13,14] The tumors carry the EBV genome and possess a normal karyotype. Histopathological analysis has shown that the lesions in EBV-infected SCID/hu mice closely resemble the LPDs seen in immunosuppressed individuals.[12–14]

Previous reports have indicated that the phenotype of SCID/hu tumor cells approximates that of *in vitro*–transformed LCLs.[13,14] In this paper, however, we present evidence that the phenotype of biopsy SCID/hu tumor cells differs significantly from *in vitro*–transformed LCLs. Using quantitative flow cytometric analysis, we show that the B-cell antigens CD20 and CD23 are down-regulated *in vivo*. We also demonstrate that tumorigenesis in SCID mice is associated with secretion of high levels of human Ig by EBV-transformed B cells.

Materials and Methods

Human Leukocytes

Blood was obtained by venipuncture from normal adult EBV-seronegative or seropositive donors after informed consent and following protocols approved by the Human Subjects Review Committee of The Scripps Research Institute. PBL were separated over Ficoll-Hypaque gradients. In the experiments described below, donors NT, LG, and JR were EBV-seronegative individuals, whereas donor MM was EBV-seropositive.

Lymphoblastoid Cell Lines

EBV-transformed LCLs were prepared by *in vitro* infection of PBLs with EBV B95–8 (supernatant from PMA-treated B95–8 cell cultures) in the presence of 0.2 μg/ml Cyclosporin A (Sandoz, East Hanover, NJ). All cell lines were maintained in RPMI 1640 plus glutamine, antibiotics, and 10% FCS (Hyclone, Logan, UT) and had been passaged for 3 to 6 months prior to use in these experiments.

SCID/hu Chimeric Mice

C.B.-17/scid (SCID) mice were from a breeding colony maintained at The Scripps Research Institute. This colony is free of mouse hepatitis virus and Sendai virus. SCID mice were intraperitoneally (i.p.) injected with 1 to 3 $\times$ 10^7 PBLs from EBV-seronegative donors, or 5 $\times$ 10^7 PBLs from EBV-seropositive donors. Mice that received PBLs from EBV-seronegative donors were subsequently i.p. injected 7 to 14 days later with 0.2 to 0.5 ml EBV B95–8. SCID mice reconstituted with human PBLs are hereafter described as SCID/hu mice. In some experiments, mice were i.p. injected with 5 $\times$ 10^6 *in vitro*–transformed LCLs.

Preparation of Tumor Cells

SCID/hu mice that developed EBV-induced human B-cell tumors were sacrificed when clinical signs of disease (hunching, ruffled fur, lethargy, and tachypnea) were observed. Tumors were predominantly located in the peritoneal cavity and excised at autopsy. Tumor cells were recovered by treatment of minced tissue with collagenase (700 U/ml, 15 to 30 minutes at 37C; Sigma Chemical Co., St. Louis, MO). Collagenase treatment of control LCLs had no effect on levels of CD20 or CD23, as detected by flow cytometry.

Flow Cytometry

The following monoclonal antibodies against human B-cell antigens were used: anti-CD19 (B4, Coulter, Hialeah, FL); anti-CD20 (Leu 16, Becton-Dickinson, Mountain View, CA); anti-CD23 (BU38, Binding Site, Birmingham, UK). Goat anti-mouse IgG (H + L) FITC conjugate (Sigma), preabsorbed against human serum proteins, was used as the second antibody for indirect staining. Tumor cells stained strongly with MAb W6/32 (ATCC), specific for human HLA class I molecules, but failed to label with MAb M1/42 (ATCC), specific for mouse H-2 molecules. Propidium iodide staining permitted dead cells to be gated out during analysis with either a FACSTAR or FACSCAN (Becton-Dickinson).

ELISA for Human Immunoglobulins

Human Ig in the sera of SCID/hu mice was quantified in a microwell ELISA, using polyclonal goat anti-human Ig (Sigma) as the first layer, followed by blocking of nonspecific binding with 2% bovine serum albumin (Sigma) and 2% dried milk (Carnation) in PBS. The plates were then incubated with serial dilutions (in PBS) of SCID/hu sera, prior to incubation with HRP-conjugated goat anti-human Ig. The plates were washed with PBS plus 0.02% Tween 20 between each step. The enzyme substrate was o-phenylene diamine in citrate/phosphate buffer, pH 5.0, and absorbance was measured with a Titertek plate reader after stopping the reaction with 2 mol/L sulfuric acid.

Results

Expression of CD19, CD20, and CD23 by SCID/hu Tumors and Autologous In Vitro*–Transformed LCLs*

Tumor biopsy cells were recovered from SCID mice reconstituted with PBLs from EBV-seronegative donors and subsequently infected with EBV B95–8 (the EBV infection SCID/hu tumor model) or from SCID mice reconstituted with PBLs from EBV-seropositive donors (the spontaneous SCID/hu tumor model). Comparative flow cytometric analysis of human B-cell antigen expression showed that CD19 is expressed at comparably high levels by SCID/hu tumor biopsy cells of the EBV infection model and by donor-matched, *in vitro*–transformed LCLs (Figure 1). In contrast, it was found that CD20 and CD23 expression by tumor cells was dramatically reduced relative to the autologous LCLs (Figure 1). Donor MM's spontaneous tumor cells also expressed high levels of CD19, but markedly reduced levels of CD20 and CD23, relative to autologous MM.LCL (Figure 2). Donor MM's spontaneous tumor cells carry an endogenous strain of EBV, rather than B95–8, and thus the observed reduction in CD20 and CD23 expression relative to B95–8-transformed MM.LCL may be a consequence of EBV strain differences; however, analysis of B95–8 infection tumors gave essentially the same results (see above), indicating that tumor phenotype is not related to EBV strain differences.

In vitro culture of biopsy cells from both the spontaneous and EBV infection SCID/hu tumor models

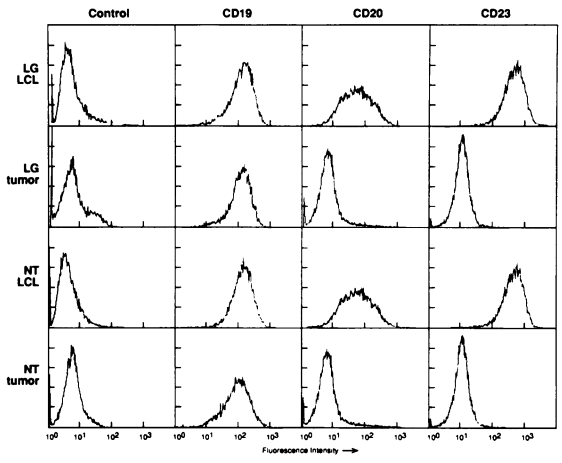

Figure 1. *Flow cytometric analysis of B-cell antigen expression by tumor cells from SCID mice reconstituted with PBL from EBV-seronegative donors and subsequently infected with EBV B95-8 (the EBV infection SCID/hu tumor model). Expression of CD19, CD20, and CD23 by tumor cells is compared with levels expressed by donor-matched,* in vitro*–transformed LCLs.*

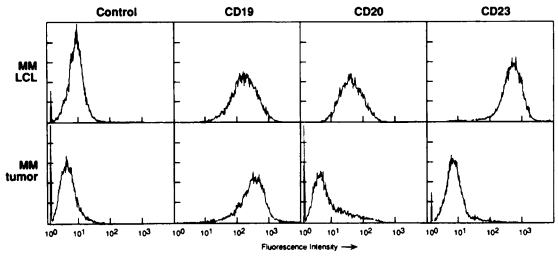

Figure 2. *Flow cytometric analysis of B-cell antigen expression by tumor cells from SCID mice reconstituted with PBL from an EBV-seropositive donor (the spontaneous SCID/hu tumor model). Expression of CD19, CD20, and CD23 is compared with levels expressed by the donor-matched,* in vitro*–transformed LCLs.*

results in gradual increased expression of CD20 and CD23 to levels comparable with *in vitro*–transformed LCLs (data not shown). This observation raises the possibility that only a minority of the tumor cells are EBV-infected, with the majority representing a reactive (uninfected) B-cell population that expresses low levels of CD20 and CD23; resultant outgrowth of EBV-transformed cells may thus account for the apparent up-regulation *in vitro*. We addressed this question directly by examining expression of CD20 and CD23 in tumors resulting from i.p. injection of established *in vitro*–transformed LCLs. These LCLs had been passaged for 3 to 6 months, and thus it can reasonably be assumed that all the injected cells carry the EBV genome. We found that such tumor cells also expressed reduced levels of CD23 relative to the injected JR.LCL and LG.LCL (Figure 3). CD20 was also markedly reduced in LG.LCL-derived tumor cells, but much less so in tumors resulting from injection of JR.LCL (Figure 3); however, we noted that JR.LCL expressed atypically low levels of CD20 *in vitro*.

Taken together, these results clearly show that EBV-infected cells express low levels of CD20 and CD23 *in vivo*, although they do not exclude the possible presence of uninfected B cells in the EBV infection or spontaneous SCID/hu tumor models. However, *in situ* hybridization with an EBV *Bam*W probe on SCID/hu (infection or spontaneous) tumor sections has indicated that the majority of tumor cells are EBV-infected.[15]

Human Immunoglobulin Levels in the Sera of Tumor-Bearing SCID Mice

It has previously been reported that high levels of human Ig (>20 mg/ml) can be detected in the sera of SCID/hu mice with EBV-induced human B-cell LPDs and tumors.[15–17] In these experiments, we find that tumors induced by injection of LCLs are also associated with the appearance of high levels of human

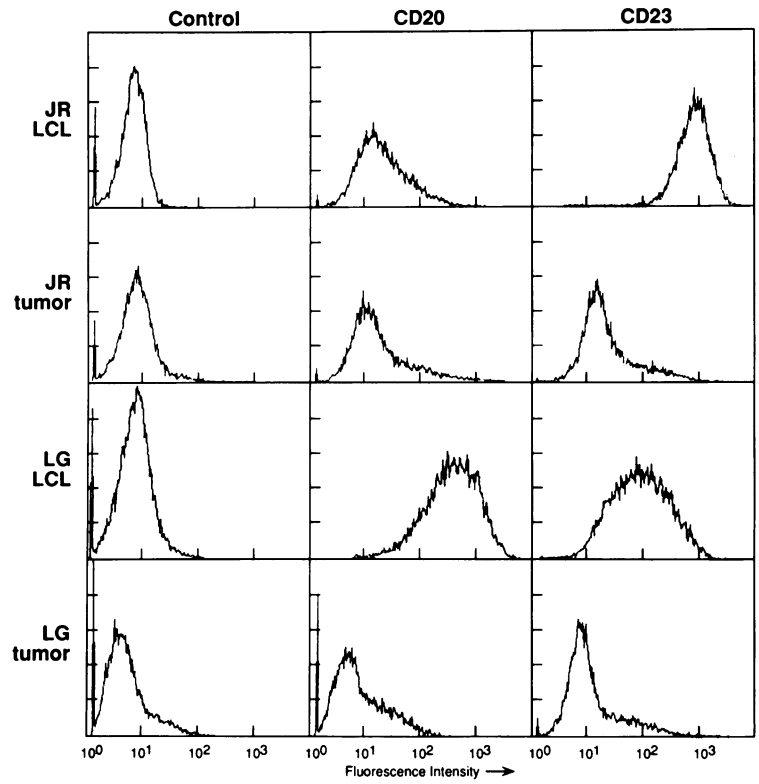

Figure 3. *Flow cytometric analysis of CD20 and CD23 expression by tumors resulting from injection of SCID mice with JR.LCL and LG.LCL, compared with levels expressed* in vitro *by the injected LCLs.*

Ig in the sera of SCID mice. Injection of JR.LCL (described above; see Figure 3) resulted in serum Ig levels in excess of 17 mg/ml, although human Ig could not be detected in the supernatants of JR.LCL cultured *in vitro*. However, lower levels of human Ig (1 to 7 mg/ml) were usually observed in the sera of tumor-bearing SCID mice injected with LCLs. Human Ig was not detected in supernatants from 4 of 5 LCL cultures tested. These data strongly suggest that Ig secretion by EBV-transformed tumorigenic LCLs is markedly increased *in vivo*.

Discussion

In this paper, we have used quantitative FACS analyses to examine the expression of CD19, CD20, and CD23 B-cell antigens in the SCID/hu model of EBV-induced B-cell LPD and in autologous donor-matched, *in vitro*–transformed LCLs. We have found that CD19 is expressed at comparable levels *in vivo* and *in vitro* but that expression of CD20 and CD23 is dramatically reduced in SCID/hu tumor cells. These observations contrast with a report of strong CD23 staining on SCID/hu tumor sections[14] but are supported by the immunohistochemical analysis of SCID/hu tumor phenotype by Nakamine et al, who found that CD23 was expressed by tumor cells from only three of eight mice.[18]

Low expression of CD23 in SCID/hu tumors may be a consequence of reduced expression of EBV EBNA-2 and LMP, both of which are involved in induction of CD23 expression in LCLs.[19] Alternatively, reduced CD23 expression may be a consequence of plasmacytoid B-cell differentiation *in vivo*; this proposal may additionally explain the observed reduction in CD20 expression, as CD20 and CD23 are not normally expressed by plasma cells.[20] Furthermore, the finding that tumorigenesis is invariably associated with the appearance of high levels of human Ig in SCID sera may also be indicative of plasmacytoid differentiation. We have previously noted that SCID/hu tumors of the EBV infection model display conspicuous plasmacytoid atypia.[12] In this context, it is notable that Thomas and colleagues found that CD23 was not expressed in 5 of 9 LPD lesions from posttransplant patients and individuals with X-linked B-cell proliferative syndrome and that CD38, a plasma cell antigen, was expressed by 3 of the 5 CD23-negative lesions[21]; furthermore, Nalesnik et al have reported that the majority of posttransplant LPDs show histopathological evidence of plasmacytic or plasmacytoid differentiation.[22] In contrast, though, Young et al

found that CD23 was expressed in 3 out of 3 lesions from immunocompromised patients with EBV-associated LPD, suggesting that the *in vivo* phenotype of LPD cells resembles *in vitro*–transformed LCLs.[23]

In conclusion, our results indicate that the phenotype of EBV-induced tumors in SCID/hu mice differs significantly from the phenotype of *in vitro*–transformed LCLs and may be a consequence of *in vivo* differentiation of EBV-infected B cells. However, the relationship of B-cell phenotype and EBV gene expression to the pathogenesis of EBV-induced LPD in immunocompromised individuals clearly merits further study. Detailed analysis of EBV-induced B-cell LPD in SCID/hu mice, as well as its utilization as a model for therapeutic strategies, is currently in progress in this laboratory.

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