Epstein–Barr virus latent membrane protein 2A has no growth-altering effects when expressed in differentiating epithelia

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Previous studies using transgenic mice with B-cell expression of LMP2A demonstrated that LMP2A drives B-cell development and survival signal in the absence of normal B-cell receptor (BCR) signal transduction. To determine if LMP2A may have similar effects in epithelial differentiation, six transgenic mouse lines were constructed and analysed with LMP2A expression directed to the epidermis by a keratin 14 (K14) promoter cassette. LMP2A protein expression was verified by immunofluorescence and immunoprecipitation of skin samples using LMP2A-specific antibodies. To evaluate the effects of LMP2A expression on epidermal differentiation, immunofluorescence and histochemistry were performed on tongue and tail samples of transgenic mice and their wild-type littermate controls using differentially expressed keratins. The analysis indicated that LMP2A does not alter the normal epithelial differentiation program in the epithelia of K14–LMP2A transgenic mice.

Epstein–Barr virus (EBV) is one of two human herpes viruses that play an aetiological role in human malignancies (Chang et al., 1994; Rickinson & Kieff, 1996). EBV is the causative agent of infectious mononucleosis and is also associated with Burkitt’s lymphoma, Hodgkin’s lymphoma, AIDS-associated immunoblastic lymphoma, oral hairy leukoplasia and nasopharyngeal carcinoma (NPC) (for review, Rickinson & Kieff, 1996). NPC is an epithelial tumour that occurs worldwide but is characterized by geographical and population differences in incidence (for review, Cruchley et al., 1997; Raab-Traub, 1992; Rickinson & Kieff, 1996). While genetic and environmental factors are associated with NPC, EBV appears to be the strongest and most consistently related factor (for review, Cruchley et al., 1997; Raab-Traub, 1992; Rickinson & Kieff, 1996). In NPC, transcripts for the EBV-encoded genes LMP1, LMP2A, EBNA1, EBERs and BARTs are typically expressed (for review, Cruchley et al., 1997; Raab-Traub, 1992; Rickinson & Kieff, 1996).

Previous studies using in vitro models of EBV latent infections in B lymphocytes established that LMP2A blocks normal B-cell receptor (BCR) signal transduction by the association with Src family protein tyrosine kinases (PTKs) and the Syk PTK (Burkhardt et al., 1992; Fruehling & Longnecker, 1997; Fruehling et al., 1998; Miller et al., 1994, 1995). In vivo studies using transgenic mice that express LMP2A in B lymphocytes have indicated that LMP2A not only blocks BCR signal transduction but also provides a BCR-like signal that allows for B-cell development and survival in the absence of normal BCR signals (Caldwell et al., 1998, 2000). Very little is known in regard to the effects of LMP2A on epithelial biology. A recent study indicates that LMP2A is phosphorylated upon adhesion of epithelial cells expressing LMP2A to extracellular matrices, which suggests that LMP2A may be important in altering normal epithelial signal transduction (Scholle et al., 1999). Signal transduction in epithelial cells uses a similar repertoire of signal-transducing proteins as is used in normal BCR signal transduction. Thus, to determine if LMP2A may also provide an inappropriate developmental or survival signal in epithelial cells which would result in the alteration of normal epithelial biology, transgenic mice with LMP2A expression directed to the basal layer of the epidermis were constructed using a keratin 14 (K14) promoter construct. A total of six murine K14–LMP2A transgenic lines were analysed, all of which showed no alteration in normal epithelial differentiation despite abundant LMP2A expression in the basal layer of the epidermis.

In order to drive expression of LMP2A to differentiating epithelia, a previously described K14 cassette was obtained from E. Fuchs (University of Chicago, USA). The K14 construct containing the K14 promoter, K14 polyA tail and betaglobin...
Fig. 1. Generation and analysis of K14–LMP2A transgenic mice. (A) Schematic representation of construct used for generation of K14–LMP2A mice. To generate the K14–LMP2A transgene construct the EcoRI fragment of pRL192 (Caldwell et al., 1998, 2000) was cloned into the BamHI site of the pG3Z-K14 plasmid. The pG3Z-K14 expression plasmid consists of the keratin 14 (K14) promoter, a betaglobin intron, and the K14 polyA tail. A chimeric LMP2A gene was used consisting of both genomic and cDNA sequences. The K14–LMP2A sequences were excised from the parental vector and used for injections into fertilized mouse embryos. (B) Identification of K14–LMP2A transgenic mice. Genomic DNA from the parental vector and used for injections into fertilized mouse embryos. Twenty-six mice were produced. At 3 weeks of age, DNA was prepared from mice tail snips, digested with BamHI, subjected to gel electrophoresis in 0.8% agarose, and transferred to Gene Screen Plus (NEN Life Science) as previously described (Caldwell et al., 2000). Southern blot analysis of genomic tail DNA using an LMP2A-specific probe identified eight founder mice (Fig. 1B). Seven of the eight lines contained the unit length 5100 bp band resulting from head to tail concatamers of the transgene (Fig. 1B, lines 101, 102, 103, 104, 105, 107 and 108). Line 106 lacked this band and was not analysed further. The additional bands in each line which hybridize to the LMP2A-specific probe probably represent fusion bands of the LMP2A transgene with murine DNA sequences or rearranged LMP2A transgenes. The transgenic mice were bred into a CD1 wild-type background purchased from Jackson Laboratories. Transgenic line 108 did not breed and therefore was not analysed. Lines 101–105 and 107 were analysed as described below.

To verify expression of LMP2A in epithelial tissue, a 1 cm² piece of dorsal back skin was isolated from euthanized transgenic mice (lines 101, 102 and 107) and littermate controls. The samples were placed in 2 ml 1% Triton X-100 lysis buffer (Fruehling et al., 1996). The skin sample was then homogenized using a Polytron PT 2100 (Kinematica). Homogenates were incubated at room temperature for 25 min and then centrifuged to pellet insoluble debris. LMP2A was then immunoprecipitated using the LMP2A rat monoclonal antibody 14B7 (Fruehling et al., 1996) for 1 h at 4 °C. Immune complexes were then isolated with protein G-Sepharose. The beads were washed four times in 1% Triton X-100 lysis buffer. An equal volume of 2 × SDS loading buffer was then added to each sample followed by incubation at 70 °C for 10 min. The samples were then subjected to 8% SDS–PAGE as previously described (Fruehling et al., 1996). The separated proteins were transferred to nitrocellulose, blocked with 4% milk for 1 h at room temperature, immunoblotted with biotinylated 14B7 in Tris-buffered saline–Tween, incubated with horseradish peroxidase-linked neutravidin, and detected by enhanced chemiluminescence. Fig. 1(C) shows a Western blot of immunoprecipitations from a litter resulting from the breeding of K14–LMP2A mice from line 102 with CD1 wild-type mice. The two transgenic mice resulting from this mating show a clear LMP2A band, while the two wild-type littermate controls resulting from the same mating do not (Fig. 1C). An EBV⁺LMP2A⁺ lymphoblastoid cell line (LCL) and an
Fig. 2. Normal differentiation program and LMP2A expression in the epidermis of K14–LMP2A transgenic mice. (A) Schematic representation of epithelial differentiation (left) compared to a representative photomicrograph of an H&E-stained mouse tail skin section taken from a wild-type control mouse (right). The epidermis can be divided into four layers. Each layer is characterized by the position, morphology and biochemical markers of the cells within (for review, Fuchs & Byrne, 1994). Cells in the innermost layer (basal) are replicating and express keratin 5 (K5) and K14. As cells leave this layer and commit to differentiation they move upward into the stratum spinosum. In this layer cells flatten, down regulate the expression of K5 and K14, and express K1 and K10. Cells continue upward into the stratum granulosum where they start to lose their nuclei, begin to express filagrin and are eventually sloughed off the surface (stratum corneum). (B, C) Immunolocalization of K14 and LMP2A expression in K14–LMP2A transgenic lines in tail samples (B) or tongue samples (C) using indirect immunofluorescence. H&E staining on a 5 µm paraffin-embedded section showing the different layers and morphology in detail.

Frozen mouse tail sections (5 µm) from transgenic and wild-type littermate controls were analysed for the presence of K14 by indirect immunofluorescence. K14 expression was the same in both the transgenic and the wild-type, being localized to the basal level of developing epidermis. LMP2A expression was similar to K14, being confined to the basal layer of the epidermis, consistent with the K14 promoter construct used to construct the transgenic mice.

EBV+LMP2A− LCL were used as positive and negative controls respectively. A background band is evident in both the wild-type control and transgenic mice. Lines 101 and 107 were tested in a similar fashion and also demonstrated LMP2A expression in the epithelial samples (data not shown).

To determine if the expression of LMP2A altered the normal morphology and/or differentiation of the epithelium, tail and tongue sections were analysed from 6-week-old K14–LMP2A transgenic mice and littermate controls. Fig. 2(A) shows a schematic representation (left) of the four stages of epithelial development and the expression of the differentiation markers associated with each stage lined up with a representative photomicrograph (right) of an H&E-stained tail section from a wild-type mouse control for reference purposes. H&E-stained paraffin-embedded sections were prepared by placing mouse tail or tongue samples in buffered Formalde-Fresh (Fisher), embedding in paraffin, and staining 5–6 µm sections with H&E. As observed in Fig. 2(B) (tail sections) or Fig. 2(C) (tongue sections), there was no apparent change in thickness or epithelial differentiation in the LMP2A transgenic lines 101, 102 and 107 when compared to littermate controls. This result was also observed in the 103, 104 and 105 K14–LMP2A transgenic lines (data not shown).

To further investigate epithelial differentiation and confirm LMP2A expression in the basal layer of the epithelium, immunofluorescence was performed using specific antibodies on mouse tongue and tail samples that were quick-frozen in liquid nitrogen using OCT (Sakura Finetek). Following freezing, 6 µm sections were cut, placed on super-frost plus slides (Fisher) and immediately fixed in acetone at −20 °C for 10 min. The sections were then air-dried and stored at −20 °C until used. Sections were treated for 10 min with 20% goat serum, incubated with primary monoclonal antibodies against K14 (CKB1, obtained from Sigma) and LMP2A (14B7). Slides were then washed in PBS, followed by incubation with the appropriate species-specific secondary antibodies directly conjugated with a chromophore. The slides were then washed five times in PBS prior to viewing by fluorescence microscopy. K14 expression was limited to the basal layer of epidermis in both K14–LMP2A transgenic mice as well as littermate controls in both tongue and tail samples (Figs 2B and 3C). LMP2A expression was also readily observed with its expression limited to the basal layer in both tail and tongue samples as expected, exhibiting K14-like regulated expression (Fig. 2B, C). No staining for LMP2A was observed in wild-type littermate controls (Fig. 2B, C). All K14–LMP2A transgenic lines were examined and no differences were observed. Only lines 101, 102 and 107 are shown in Fig. 2.

To further investigate any LMP2A-specific effects on epithelial differentiation, expression of K10, K6 and filagrin in
Fig. 2. For legend see page 2247.
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epithelial samples from the K14–LMP2A transgenic mice was investigated. Antibodies were obtained from Sigma. K10 and filagrin are markers of the differentiated suprabasal layers (Fig. 2A). Like K14, reduced or disordered expression of K10 can occur in oral squamous cell carcinomas (for review, Morgan & Su, 1994). K6 is normally expressed in the skin only in hair follicles and in the proliferating epidermis of wounds, but is aberrantly expressed in the suprabasal layers in hyperplastic, neoplastic or psoriatic skin (Knapp et al., 1982; Moll et al., 1984). Immunofluorescence was performed in the same way as for Fig. 2. Expression of K10 and filagrin was normal in the K14–LMP2A transgenic mice when compared to littermate controls (Fig. 3). K10 expression, in both the transgenic and littermate controls, was limited to the spinosum and granulosum layers, while filagrin expression was readily observed in the granulosum and corneum layers as expected (for review, Fuchs & Byrne, 1994). Consistent with a complete absence of any LMP2A-induced changes in epithelial differentiation, there was no expression of K6 detected in the LMP2A transgenic lines and littermate controls (data not shown). Results with the K14–LMP2A transgenic lines 101 and 107 were indistinguishable (data not shown) from the results shown for the K14–LMP2A 102 transgenic line (Fig. 3).

LMP1, EBNA1, BARTs, EBERs and LMP2A are the EBV latency-associated genes most consistently detected in NPC tumour biopsies (for review, Raab-Traub, 1992; Cruchley et al., 1997; Rickinson & Kieff, 1996). Work previously done on LMP1 has shown it to have transforming effects in rodent fibroblasts, growth-altering effects in B lymphocytes, and it is absolutely required for transformation of primary B lymphocytes with EBV (for review, Kieff, 1996; Longnecker, 1998). In transgenic mice, LMP1 induces epithelial hyperplasia and aberrant expression of keratin when expressed in the epithelium and induces lymphoproliferation when expressed in lymphocytes (Kulwichit et al., 1998; Wilson et al., 1990). There is little information in regard to the effects of EBNA1 on normal epithelial biology. Transgenic murine studies have indicated that EBNA1 may predispose lymphocytes to malignant transformation (Wilson et al., 1996). Despite these observations, EBNA1 does not dramatically alter B-lymphocyte phenotype in vitro. The exact role of the BARTs and EBERs in EBV-mediated transformation is yet to be determined. Results with LMP2A transgenic mice with lymphocyte-directed expression indicated a putative role for LMP2A in EBV latency and transformation that was not previously appreciated. In these studies, LMP2A was shown to alter normal B-cell development and provide a survival signal to B lymphocytes (Caldwell et al., 1998, 2000). In this study, we investigated the effect of LMP2A expression on normal epithelial differentiation also utilizing a transgenic approach.

Work in vitro has shown that LMP2A specifically binds to and regulates the activity of the Src family PTKs and the Syk PTK. The Syk PTK accumulates in most haematopoietic cell types, including B cells, mast cells, platelets and immature T cells (for review, Weiss & Littman, 1994). Zap-70, a related PTK, is expressed only in T cells (for review, Weiss & Littman, 1994). There is no indication that either of these PTKs are expressed in epithelial-derived cell lines. In contrast, at least four Src family PTKs (Thomas & Brugge, 1997), two of which have been shown to bind LMP2A, are expressed in epithelial cells (Burkhardt et al., 1992). Src has been shown to be activated following integrin engagement following attachment of epithelial cells to a fibronectin matrix (Kaplan et al., 1995). Other signal transduction cascades present in epithelial cells in which Src family kinases are important include signalling induced by the cadherins and receptor protein tyrosine kinases. In addition to the role of Src family PTKs in epithelial signal transduction, there are additional signalling proteins, many of which are shared with B-lymphocyte signal transduction, which may be affected by the expression of LMP2A. Despite the central role of Src family PTKs in normal epithelial signal transduction and the ability of LMP2A to alter normal Src family PTK function in B lymphocytes, there was no observable effect of LMP2A expression on epithelial cells in the K14–LMP2A transgenic mice.

Given the dramatic effects that LMP2A can have on B-lymphocyte differentiation and survival, it is surprising that LMP2A appears to have little effect on epithelial differentiation despite the importance of Src family PTK signalling in epithelial cells. LMP2A is phosphorylated by Csk upon adhesion to extracellular matrixes in cell lines grown in tissue culture. Since LMP2A is consistently detected in most NPC samples, it is likely that LMP2A will have a role in the development of EBV-associated epithelial malignancies. LMP2A may not be the primary initiating event, but may act in concert with other viral
proteins such as EBNA1 and LMP1, which are also typically expressed in NPC biopsies. Alternatively, the alteration of a specific cell protein may be required for LMP2A-specific effects in epithelial cells. Future studies utilizing the LMP2A transgenic mice constructed in this study will entail the mating of the LMP2A transgenic lines with transgenic lines expressing other EBV proteins expressed in NPC to determine if they may act in concert. In addition, studies to determine if LMP2A may have more subtle effects on epithelial cells, which may be evident by analysing wound healing and chemical carcinogenesis in the LMP2A transgenic mice, will be pursued. These studies may shed light on the role of LMP2A in EBV-associated epithelial cancers.

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References


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