Examination of B cell and virus interactions through transformation by a pathogen (EBV) and transduction by a lentiviral vector (HIV-1)

Krisztián Kvell MD

PhD thesis

Department of Immunology and Biotechnology
Faculty of Medicine, University of Pécs, Hungary

2007

Examination of B cell and virus interactions through transformation by a pathogen (EBV) and transduction by a lentiviral vector (HIV-1)

Krisztián Kvell MD

PhD thesis

Program leader: Prof. Péter Németh MD PhD

Project leaders: Dr. Péter Balogh MD PhD[1], Prof. Rudolf H. Zubler MD PhD[2] and Prof. Péter Németh MD PhD[1]

1: Department of Immunology and Biotechnology
Faculty of Medicine, University of Pécs, Hungary

2: Research and Development Unit, Division of Hematology
Department of Internal Medicine, Geneva University Hospitals, Switzerland

INTRODUCTION

The Epstein-Barr virus (EBV) is a member of the herpes virus family, human pathogenic ds DNA virus with vast genetic material (172 kb) and programme (85 genes). EBV has expressed human B-cell tropism. Over 90% of the adult population harbours the virus. The virus has a biphasic life-cycle: Following quick replication during the initial lytic cycle it resides in long-lived memory B-cells during latency. During the first phase there is extensive viral gene activity that becomes rather restricted during latency programmes. EBV is responsible for various diseases: Mononucleosis, Burkitt's lymphoma, nasopharyngeal carcinoma, Hodgkin's disease and lymphoproliferative disease seen in transplanted- and AIDS-patients (PTLD). PTLD is the result of EBV reactivation seen in the cases of strong and prolonged immune-suppression. PTLD may not only be triggered by the reactivation of endogenous EBV, but also by exogenous EBV superinfection. Gene expression patterns seen in various diseases or during the lytic and latent programmes are significantly different and characteristic to the pathologies. The utilisation of EBV-based viral vectors is an existing, but to date technically demanding method. Moreover, even a technically advanced EBV-based viral vector is unlikely to be an inert vector for experiments examining individual EBV genes or complete EBV gene clusters when assayed in human primary B-cells.

The human immune-deficiency virus-1 (HIV-1) is a human pathogen retrovirus that belongs to the family of lentiviruses. Via biotechnological methods HIV-1 based lentiviral vectors may be prepared starting from the wild-type virus. Vectors created by the removal of genes from the original HIV-1 genome responsible for virulence and replication may be securely used under standard P2 laboratory conditions. If the original viral envelope protein (gp120) is pseudotyped by a ubiquitous ligand (VSV-G or vesicular stomatitis virus G-protein), then vector tropism is significantly broadened, allowing for the infection of practically any cell type of any given mammalian species. The above modifications yield a

very flexible and yet safe viral vector that may be used for the stable delivery of any given transgene into the nuclei of both dividing and resting cells of cell-lines or primary cells. The research-oriented and therapeutic applications of lentiviral vectors *in vitro* and *in vivo* are all very promising fields. If one would create a human primary B-cell monoculture system suitable for the efficient use of lentiviral vectors, that would also allow for the examination of individual EBV genes, complete EBV gene clusters, and even entire (lytic and latent) EBV gene-expression programmes affecting B-cells via the use of an inert carrier (HIV-1 based lentiviral) vector.

AIMS

1. Development of a human-mouse (hu-SCID) chimera system as an animal model for human primary B-cell-dominant PTLD disease triggered by endogenous EBV reactivation.

2. Standardisation of the animal model of the PTLD disease by performing *in vivo* superinfection utilising a known EBV strain, proving its presence and dominance.

3. Development of a human primary B-cell monoculture system that allows for the efficient use of lentiviral vectors, assay of gene delivery efficiency through the use of marker gene (GFP).

4. The introduction of functionally active intracellular (vFLIP) and secreted (IL-4) transgenes along with GFP and the assay of their functionality in primary B-cells (proof of principle).

MATERIALS AND METHODS

Isolation of human cells

Buffy-coats derived from healthy blood donors were further purified by Ficoll-gradient centrifugation. From the resulting leukocyte suspension (PBMC) then adherent cells were removed (PBL) followed by anti-CD19-beads separation to yield a highly pure (>97%) B-cell suspension.

SCID mice

C. B. scid/scid homozygous mice were kept under SPF conditions in continuous antibiotic prophylaxis. Animals were regularly checked for leaky phenotypes that were.

Establishment of hu-SCID chimeras

Following the injection of an appropriate amount of PBMC ($3\text{-}6x10^7$) chimerism may be established in SCID mice. Interspecies immunological chimerism becomes functional after approx. 4 weeks.

Exogenous EBV strain

The EBV containing supernatant of the B95-8 marmoset cell-line came from the Regional Laboratory of Virology, Institute of State Public Health Service at Pécs. The supernatant contained infective virions introduced by i.p. injection 10 days following the PBL injection.

Examination of hu-SCID chimera system

Peritoneal cells and the lymphoid organs were removed from moribund mice due to human B-cell lymphoproliferation. Following surface and intracellular staining the cells were assayed by **flow-cytometry**. From the lymphoid organs (spleen, lymphatic glands) sections were

made, that were assayed by **immuno-histochemistry** following fixation and staining as above. Following RNA isolation from the spleen of certain animals active EBV strains were identified using known primers and commercially available reagents by **RT-PCR**. DNA from known EBV strains was used as control.

B-cell monoculture system

Highly pure (see above) human primary B-cells were activated by CpG oligo, ant-Ig, human recombinant IL-2 and IL-10. Cells were efficiently transducible by lentiviral cultures three days later. Cultures last most max. 10 days during which proliferation and Ig secretion occur.

Preparation of lentiviral vetors

The used mono- (GFP) and bicistronic vectors (vFLIP-GFP or IL-4-GFP) were prepared in collaboration with the Medical University Centre (CMU) and the Geneva University Hospital (HUG).

Examination of transduced B-cells

Transgenic proteins are active in the cultures 2 days after infection. Commercially available reagents and antibodies were used for all the applied methods. Cells were examined for transgene expression by **flow-cytometry** following surface and intracellular staining. The presence of the introduced transgenic vFLIP protein was shown by **Western-blot**, its protective effect against FasL was assayed in native cultures and on cells enriched by **flow-cytometric sorting**. The quantity of IL-4 secreted by transgenic cells was measured by **ELISA**. The functionality of transgenic IL-4 was examined in secondary cultures (increased proliferation in the CD40L system, IgE secretion in the EL4-B5/B co-cultures.

RESULTS

1. As a result of endogenous EBV reactivation fatal human B-cell lymphoproliferation develops in the hu-SCID chimera system. Several aspects of the disease show similarity to the human PTLD. The survival of experimental animals shows significant variance (46-67 days).

2. The PTLD model may be significantly standardised in terms of survival (30-32 days) following *in vivo* superinfection with a known EBV strain. The dominance of the exogenous EBV strain (B95-8) is shown chimera organs.

3. The utilisation of CpG oligo, α-Ig, IL-2 and IL-10 allows for the establishment of a human primary B-cell monoculture system in which B-cells are efficiently transduced by monocistronic lentiviral vectors (approx. 25-30% GFP+ population).

4. Bicistronic lentiviral vectors allow for the efficient transduction of functionally active transgenes along with GFP (approx. 15% GFP+ population). The vFLIP gene introduced by lentiviral vector efficiently protected GFP+ cells from FasL-mediated apoptosis. If the IL-4 gene was introduced into B-cells by lentiviral vectors, then transgenic IL-4 secreted by GFP+ cells was also functional in secondary B-cell culture systems (increased proliferation, IgE isotype switch).

DISCUSSION

During the establishment of the hu-SCID chimera system we repopulate immune-deficient mice (SCID) with leukocytes (PBMC) isolated from healthy human donors. Immunological chimerism is functional in the system, lymphocyte homing is normal, immunological memory may be recalled and developed. Still, as a result of EBV reactivation resembling PTLD, polyclonal, fatal human B-cell lymphoproliferation occurs. During reactivation EBV gene expression pattern is known to undergo significant changes. Starting from latency programme I the group of active EBV genes expands to latency programme III. Human PTLD develops at higher chances and rate in patients and in the model system in cases of exogenous EBV superinfection, compared to endogenous reactivation, as the former immediately initiates latency programme III.

Previously described human primary B-cell culture systems used for transduction either gave low efficiencies (i.e. the CD40L system) or were co-culture systems (i.e. EL4-B5/B system). It is a milestone in the field to combine the efficient lentiviral transduction method with a human primary B-cell monoculture system (CpG system). Along with a marker gene further genes, encoding ‘proof-of-principle’ intracellular and secreted transgenic proteins were introduced into the target cells. The transgenic proteins were functionally intact.

The adaptation of the lentiviral technique in a human primary B-cell monoculture system offers new perspectives to the research of individual EBV genes and complete EBV gene clusters. This allows researchers to perform experiments on human primary B-cells instead of human B-cell lines that are often genetically instable (i.e. due to endogenous EBV) in a manner, so that the employed (HIV-1 based) lentiviral vector is functionally inert in terms of the assayed (i.e. EBV) genes or gene-clusters.