Research Article

Comparing Effects of BK Virus Agnoprotein and Herpes Simplex-1 ICP47 on MHC-I and MHC-II Expression

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Background. Among human polyomaviruses, only BK virus (BKV) and JC virus (JCV) encode an agnoprotein upstream of VP1 on the viral late transcript. BKV agnoprotein is abundantly expressed late in the viral life cycle, but specific cellular and humoral immune responses are low or absent. We hypothesized that agnoprotein might contribute to BKV immune evasion by downregulating HLA expression, similar to Herpes simplex virus-1 ICP47. Methods UTA-6 or primary human renal proximal tubular epithelial cells (RPTEC) were co-transfected with plasmids constitutively expressing agnoprotein, or ICP47, and enhanced green-fluorescent protein (EGFP). EGFP-gated cells were analyzed for HLA-ABC and HLA-DR expression by flow cytometry. HLA-ABC and HLA-DR expression was also analyzed on UTA-6 cells bearing tetracycline-regulated agnoprotein or ICP47. Effects of agnoprotein on viral peptide-dependent T-cell killing were investigated using 

51Cr release.

Results. ICP47 downregulated HLA-ABC without affecting HLA-DR, whereas agnoprotein did not affect HLA-ABC or HLA-DR expression. Interferon-γ treatment increased HLA-ABC in a dose-dependent manner, which was antagonized by ICP47, but not by agnoprotein. In UTA-6 cells, agnoprotein expression did neither impair HLA-ABC or -DR expression nor peptide-specific killing impaired by HLA-matched T-cells. Conclusion. Unlike the HSV-1 ICP47, BKV agnoprotein does not contribute to viral immune evasion by down-regulating HLA-ABC, or interfere with HLA-DR expression or peptide-dependent T-cell cytotoxicity.

1. Introduction

Human polyomavirus (HPyV) species comprise currently at least 9 members that infect 30 to 90 percent of the general population without severe clinical manifestations [1–6]. In immunocompromised individuals, however, significant pathologies have been linked to HPyVs, for example, nephropathy and haemorrhagic cystitis to BKV, progressive multifocal leukoencephalopathy to JCV, trichodysplasia spinulosa to TSPyV and Merkel cell carcinoma to MCPyV [7, 8]. The general architecture of the approximately 5.1 kb circular dsDNA genome is shared among all HPyVs consisting of the noncoding control region separating the early nonstructural genes encoding small and large T antigen and late capsid VP1, -2, and -3 genes [6]. So far, however, only BKV and JCV contain an additional small conserved open reading frame (ORF) in the late gene transcript upstream of the major capsid protein VP1 [9, 10], a feature shared by the simian virus SV40, but not by other HPyVs [11]. The encoded protein of 60–70 amino acids (aa) has been termed agnoprotein, and, despite multiple studies, its major function in the biology of BKV or JCV has not been identified [12–14]. Agnoprotein is abundantly expressed late in the viral life cycle, and localizes predominantly to the cytoplasm both in vitro [9, 15] and also in vivo as shown in biopsies of BKV-associated nephropathy [16]. In the cytoplasm, BKV agnoprotein shows a reticular distribution but also colocalizes with lipid droplets, the latter being mediated by an amphipathic helix of 20 aa in the central part of the 66 aa BKV agnoprotein [15].

Despite its abundant expression of BKV agnoprotein in vivo, agnoprotein-specific humoral and cellular immune responses were low or undetectable in individuals exposed
to BKV [16]. This observation was contrasted by the strong immune responses found for the BKV capsid protein VP1 located on the same transcript or for the amino-terminal domain shared between small and large T antigen [16–18]. Since BKV and JCV persist lifelong after primary infection and are asymptomatically shed in immunocompetent healthy individuals [3], we speculated that agnoprotein might have a role in immune evasion. Interestingly, histopathology studies reported decreased MHC-II expression of BKV-infected renal tubular epithelial cells as a potential marker distinguishing BKV-associated nephropathy from acute cellular rejection [19].

Multiple mechanisms of viral immune evasion have been described. The immediate early protein ICP47 of Herpes simplex virus-1 (HSV-1) has been reported to downregulate surface expression in transiently and stably transfected cells. BKV agnoprotein might have a similar immunomodulatory role and investigated its effects on HLA-ABC and HLA-DR surface expression in transiently and stably transfected cells.

2. Materials and Methods

2.1. Plasmids. The pCMV-agn plasmid was a kind gift from Dr. Christine Rinaldo, Tromso, Norway [9]. The hygromycin selectable expression plasmid pH TRE-agn was generated by subcloning the agnoprotein coding sequence from pH TRE-agn into pH TRE2hyg using BamH I and NotI restriction sites. The pCMV-ICP47 and pH TRE-ICP47 were generated by replacing the agnoprotein coding sequence with the corresponding sequence encoding ICP47 (a kind gift from Dr. Paul Zajac, University Hospital of Basel, Switzerland). pEGFP-N1 was purchased from BD Biosciences.

2.2. Transfection. Cells were transfected with GeneExpresso 8000 (IV-1047; Lab Supply Mall, Inno-Vita Inc., Gaithersburg, MD, USA), according to manufacturers instructions. Briefly, for a well of a 6-well plate, cells were seeded in 1.5 mL of culture medium; 3 μL of transfection reagent were diluted in 250 μL of OptiMEM (Gibco) and kept for 5 minutes at RT; 4 μg of DNA were diluted in 250 μL of OptiMEM, and then mixed with the diluted transfection reagent for 20 minutes at room temperature before dropwise adding to the cells. After 4 hours of incubation at 37°C and 5% CO₂, medium was replaced. UTA-6 cells were cultured in presence of Tet and G418, unless indicated otherwise.

2.3. Cells. UTA-6 cells [23] were maintained in high glucose Dulbecco’s Modified Eagle’s Medium (DMEM, Sigma) with 10% Fetal Bovine Serum (FBS, Biochrome AG, Berlin, Germany), 2 mM L-glutamine, supplemented with 500 μg/mL G418 (Geneticin, Sigma) and 1 μg/mL tetracycline (Tet) (Sigma). For inducible protein expression experiments, medium containing 1 μg/mL Tet was replaced with medium without Tet for 48 hours.

Primary human renal proximal tubular epithelial cells (RPTEC; ATCC PCS-400-010, lot 5321) were maintained in epithelial cell medium (EpiCM, ScienCell, Carlsbad, CA, USA) and passaged with Passage Kit 2 (2040002, Provitro GmbH, Berlin, Germany).

UTA-6 agno clones stably expressing the BKV agnoprotein in a Tet-dependent manner were obtained by cotransfecting the hygromycin-selectable BKV agnoprotein-encoding construct pH TRE-Agno and the EGFP encoding construct pEGFP-N1 (Clontech, Mountain View, CA, USA) in a 10:1 ratio. One day after transfection, cells expressing EGFP were sorted using a cell sorter (FACSARIA, BD) and seeded in 96 multwell plates at a limiting dilution of 0.3 cells per 100 μL of DMEM containing 10% FCS supplemented with 500 μg/mL G418 (Geneticin, Sigma), 1 μg/mL Tet (Sigma) and 800 μg/mL hygromycin-B (Calbiochem). Medium was changed every 48 hours for 14 days to select clones. Subsequently the clones were expanded in medium containing 500 μg/mL G418, 1 μg/mL Tet and 200 μg/mL hygromycin-B, tested by immunofluorescence for BKV agnoprotein expression after Tet removal. Clones with a Tet-dependent BKV agnoprotein expression were cryopreserved.

2.4. Interferon-γ Treatment. Recombinant human interferon-γ (IFNγ) was purchased from Peprotech (Rocky Hill, NJ, USA) and diluted in the cell medium at 100 U/mL, 10 U/mL and 1 U/mL. UTA-6 cells (4 × 10⁴) or RPTECs (1 × 10⁵) were seeded in a 24 multiwell plate. Serial dilutions of IFNγ were added to the culture medium and left at 37°C, 5% CO₂ for 48 hours.

2.5. Antiagnoprotein Rabbit Serum. To detect expression of BKV agnoprotein or HSV ICP47, the following antibodies were used: antiagnoprotein rabbit serum (a kind gift from Dr. Klaus Frueh, Portland, ON, USA) diluted 1:750 in 3% milk/PBS. Cells were seeded onto glass coverslips and, after 2 days, washed with phosphate-buffered saline w/o Ca²⁺ and Mg²⁺ (PBS) and fixed using 4% paraformaldehyde at room temperature for 20 minutes. After two rounds of washing with PBS, the cell membranes were permeabilized with 0.2% Triton at room temperature for 10 minutes, washed again with PBS, and blocked for 15 minutes at 37°C using 3% milk/PBS. Coverslips were then stained with primary antibodies for 60 minutes at 37°C and washed three times in PBS. Then, Hoechst 33342 (0.5 μg/mL; H21492, Invitrogen) and fluorescently labeled secondary antibody (anti-rabbit-Cy3 1:2000, 111-165-144, Jackson Immunoresearch, West Grove, PA, USA) were added and incubated for 60 minutes at 37°C. After 3 washes in PBS, the slides were mounted in 90% Glycerol (1.04095, Merck, Darmstadt, Germany) in PBS containing 1% N-propyl gallate (P-3130, Sigma) as an antifading agent.
2.6. HLA Quantitation by FACS. Cells were harvested and 5 \times 10^4 were resuspended in 50 \mu L of washing buffer (0.5% BSA/PBS) and stained with 10 \mu L of antibody anti-HLA-ABC PE conjugate and 5 \mu L of anti-HLA-DR APC conjugate (Becton Dickinson). Samples were incubated for 30 minutes at 4\degree C in the dark, then washed twice with washing buffer, and centrifuged at 400 x g for 5 minutes at 4\degree C. Supernatant was discarded, and cells were resuspended in 400 \mu L of washing buffer and analyzed using the FACS Canto (Becton Dickinson Franklin Lakes, NJ, USA) and FACS Diva or Flow Jo software.

2.7. Peripheral Blood Mononuclear Cells Recovery and In Vitro Stimulation. Peripheral blood mononuclear cells (PBMCs) were obtained from two healthy donors, who gave written informed consent to the protocol (IRB 267/06) approved by the local institutional review board. Blood samples were diluted 1:2 in PBS, overlaid on Ficoll (Lymphoprep, Axis-Shield PoC AS, Oslo, Norway), and centrifuged at 800 x g for 25 minutes at RT. The PBMCs band was recovered and washed twice at 300 x g for 10 minutes at RT in PBS, and cells were then counted and resuspended in RPMI-1640 (Sigma-Aldrich Chemie GmbH Buchs SG, Switzerland) supplemented with 5% Human Serum AB (Sigma) and 2mM L-Ala-Glutamine termed R5. PBMCs were seeded in a concentration of 6 x 10^6 cells per well in 3 mL of R5 medium in 6-well plates and incubated overnight at 37\degree C and 5% CO_2. Floating cells were resuspended at a concentration of 2 x 10^6 in 500 \mu L of R5 in wells of 24 multiwell plate. Adherent cells were scraped, and the viable ones counted with Trypan Blue solution and resuspended at the concentration of 4 x 10^6 per 1 mL in R5. After incubation with 5 \mu g/mL of peptide for antigenic stimulation for 2 h at 37\degree C 5% CO_2, the adherent cells were added to the floating cells in a 1:10 ratio. Incubation was continued for 5 to 12 days prior to testing. The peptides were derived from cytomegalovirus (CMV)-pp65 consisting of a 15-mer peptide (AGILARNLVPMVATV), a 9-mer peptide (NLVPVMATV) and a pool of peptides of 15-mer length overlapping for II aa length spanning the entire open reading frame of CMV-pp65 (Eurogentec Deutschland GmbH, Köln, Germany).

2.8. Interferon-γ EliSpot Assay (ESA). PDVF multiscreen 96-well filter plates (MSIPS4W10, Millipore Bedford, MA, USA) were coated with 100 \mu L of anti-IFNγ mAb I-D1 K (Mabtech, Nacka, Sweden) at 10 \mu g/mL and incubated overnight at 4\degree C. In vitro stimulated T cells were seeded at 1 x 10^7/well in presence of 2 \mu g/mL of peptide. Cells without any peptide were used as negative control, whereas cells stimulated with phytohemagglutinin (PHA; Sigma-Aldrich Chemie GmbH Buchs SG, Switzerland) were used as positive control. After incubation for 24 h at 37\degree C, anti-IFNγ mAb 7-B6-1-Biotin (Mabtech, Nacka, Sweden) at 1 \mu g/mL was added for 2 h at RT, then Streptavidin ALP (Mabtech, Nacka, Sweden) at 1 \mu g/mL for 1 h at RT, and SigmaFast BCIP/NBT (Sigma-Aldrich Chemie GmbH Buchs SG, Switzerland) for 20 minutes at RT in the dark. Plates were rinsed with water and dried, and spots were counted with an EliSpot reader (Cellular Technology Ltd Europe, Bonn, Germany).

2.9. Killing Assay. Specific cytotoxic activity of in vitro stimulated T cells on UTA-6 cells was assessed by ⁵¹Cr release assay. Target cells were cultured for 24 hours prior to testing in presence or absence of Tet, labeled for 1h at 37\degree C with 250 \mu Ci per 1 x 10^6 cells with ⁵¹Cr (Sodium Chromate Hartmann Analytic, Braunschweig, Germany) and then pulsed for 1h at 37\degree C with 2 \mu g/mL with CMV-pp65 peptides or without as negative control. The stimulated T cells were incubated with 5000 target cells at an effector:target (E:T) ratios of 80:1, 40:1, 20:1, 10:1, 5:1, and 2.5:1 at 37\degree C and 5% CO_2 for 4 h, and then 50 \mu L of the supernatants were transferred to a lumaplate (Perkin Elmer, Waltham, MA, USA), dried and counts per minutes (cpm) were counted in a β-counter (TopCount, Perking Elmer). Killing assay data are reported as average of duplicate wells and as percentage of lysis, which is calculated according to the following formula: (Sample cpm-Spontaneous Release cpm) / (Maximum Release cpm / Spontaneous Release cpm) x 100, where the spontaneous release corresponds to the ⁵¹Cr released by the target cells alone and the maximum release corresponds to the ⁵¹Cr released by the target cells lysed with 2.5% SDS. Percentage of specific lysis was obtained subtracting percentage of lysis of the negative control. Data were considered reliable when the minimum release was less than 50% of the maximum release.

3. Results

To investigate the effect of the BKV agnoprotein on MHC class-I and class-II expression, we transfected UTA-6 cells with plasmids containing BKV agno or HSV-1 ICP47 under the control of a Tetracycline-(Tet-) off regulated promoter. Both expression constructs were cotransfected in 10-fold excess to an EGFP expression vector to permit enriching for transfected cells. In fixed cells, prominent expression of BKV agno or ICP47 could be seen after 24 and 48 hours following Tet removal, whereas expression was suppressed in the presence of Tet (not shown). To enumerate HLA class-I and class-II expression in transfected cells, the cells were stained using anti-HLA-ABC and anti-HLA-DR antibodies and the corresponding signals on EGFP-gated cells were quantified by flow cytometry. As shown, the removal of Tet was associated with a significant downregulation of HLA-ABC molecules in the Tet-regulated ICP47 transfectants, whereas no effect on HLA-ABC expression was seen for the Tet-regulated BKV agno transfectants (Figure 1).

To investigate the possibility that BKV agno elicited its function more prominently under circumstances of inflammation and immune activation such as interferon-γ (IFNγ) exposure, we treated transiently transfected UTA-6 cells with increasing concentrations of IFNγ known to increase MHC-expression. Indeed, significant upregulation of HLA-ABC expression was seen in response to 1U/mL IFNγ with little further increases in response to 10 or 100 U/mL IFNγ (Figure 2). This IFNγ -induced HLA-ABC upregulation was clearly reduced in HSV-1 ICP47-expressing cells (Figure 2(A)) but not affected by BKV agno expression (Figure 2(B)). For HLA-DR expression, a significant increase
Figure 1: HLA-ABC cell surface expression in transfected UTA-6. Cells were cotransfected with pEGFP-N1 and the Tet-off regulated phTRE-agn or pTRE-ICP47 at a 1:10 ratio. HLA-ABC expression was analyzed by flow cytometry using labeled antibodies on EGFP-gated UTA-6 cells 48 h after transfection and 24 h after Tet-off induction.
in response to IFNγ became also apparent but was not affected by BKV agno- or by ICP47-expression (Figures 2(A) and 2(B)). Together, the results indicated that dynamic changes in HLA-expression could be monitored by flow cytometry as indicated by the responses to IFNγ. HLA-ABC or HLA-DR expression was not significantly affected by BKV agnoprotein, with or without IFNγ-mediated upregulation. By contrast, HSV-1 ICP47 selectively interfered with HLA-ABC expression but not with HLA-DR.

To examine the effects of BKV agno and HSV ICP47 on HLA class-I and class-II expression of RPTECs (Figure 3), the corresponding constructs under the control of the constitutive CMV promoter were cotransfected with the EGFP plasmid in a 10:1 ratio. HLA-ABC and HLA-DR expression was evaluated in EGFP-gated cells by flow cytometry at 24 h with or without IFNγ stimulation. As shown (Figure 3(a)), IFNγ treatment significantly increased HLA-ABC expression but had only minor effects on HLA-DR expression. HLA-ABC expression was reduced in ICP47-transfected RPTECs, whereas HLA-DR expression levels were not significantly affected. By contrast, agnoprotein-transfected cells had no discernible effect on HLA-ABC or HLA-DR expression. Similar results were obtained when UTA-6 cells were cotransfected with either the constitutive.
CMV-driven BKV agnoprotein or HSV-1 ICP47 together with EGFP (Figure 3(b)).

To verify that any potential effect of BKV agno expression could be compared in the same cell population, we analysed selected UTA-6 cell clones that were stably transfected with a Tet-regulated construct and expressed agno in a tightly regulated manner. BKV agno was expressed at different levels depending on the Tet concentrations (Figure 4(a)). After 48 hours of incubation with or without IFNγ, HLA-ABC expression was not significantly altered in the presence or absence of agno expression, and if at all, a slight increase in HLA-DR expression was seen after induction in UTA-6 cells clones (Figure 4(b)).

We next investigated the potential effect of BKV agnoprotein on HLA-peptide-dependent killing by antigen-specific cytotoxic T lymphocytes (CTL). The HLA-0201 positive UTA-6 cells expressing agnoprotein under Tet-off regulation were used as targets and HLA-A0201 specific T cells were used as effectors in a 51Cr release assay. Peripheral blood mononuclear cells (PBMCs) from two HLA-A0201 healthy donors (HD1 and HD2) were stimulated with a 15-mer peptide derived from CMV-pp65 (AGILARNLVPVATV) containing the well-characterized HLA-A0201 restricted immunodominant nonameric sequence NLVPVATV. The EliSpot assay of the HD1 and HD2 cell preparations showed a peptide-specific IFN-γ response with the 15-mer and with the
Figure 4: Continued.
Figure 4: (a) Regulation of BKV agnoprotein expression in the stably transfected UTA-6 clone 2C9. The panel shows a time course experiment using different concentrations of tetracycline. Blue: DNA stained with Hoechst 3342. Red: agnoprotein detected by indirect immunofluorescence. (b) HLA-ABC and -DR surface expression in UTA-6 clonal cells with Tet-off inducible agnoprotein expression. HLA-ABC and -DR surface expression was detected by flow cytometry in the UTA-6 clone 1C5 (first row) and 2C9 (second row) and cultured for 48 hours with different concentration of IFNγ and for 24 h in the presence (◻) or absence (✧) of Tet. MFI: mean fluorescence intensity.

CD8-specific CMV 9-mer NLVPMVATV (Figure 5(a)). The cells were added to $^{51}$Cr-labeled UTA-6 cells pulsed with CMV 9-mer NLVPMVATV 24 hours after inducing or not inducing BKV agnoprotein (Figure 5(b)). The results of $^{51}$Cr release assay showed that CTL of HD1 could efficiently kill UTA-6 cells in an E:T- dependent ratio, which was not affected by agnoprotein expression (Figure 5(b)). Similar results were obtained with HLA A-0201 matched T cells obtained from HD2 (not shown). The data demonstrated that agnoprotein had no influence on HLA-0201—peptide-dependent killing by cytotoxic T cells.

4. Discussion

Immune evasion has been recognized as an important feature of viruses to subvert innate and adaptive immune responses [24]. Frequently, different steps of the MHC-I peptide loading and surface presentation are targeted in line with the notion
Figure 5: (a) IFN$\gamma$ production by CMVpp65-specific T cells: IFN$\gamma$ SFUs are shown for $1 \times 10^5$ T cells from healthy donor 1 (HD1) and 2 (HD2) challenged with medium (neg), phytohemagglutinin (PHA), CMV-pp65-15 m123, and CMV-pp65-9 m495, CMV-pp65-15 Mp. (b) Effect of Tet-off inducible BKV agnoprotein expression on CTL activity. UTA-6 clone 2C9 cells were cultured for 24 h in the presence of Tet (continuous line) or absence of Tet expressing agnoprotein (dotted line, agno), labeled with $^{51}$Cr and then pulsed without (△, □) or with (▲, ○) CMV 9-mer peptide NLVPVMVATV. CMV antigen-specific T cells from a healthy donor was added in the indicated effector:target ratios and percent release of $^{51}$Cr measured.

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on HLA-ABC rendering a severe limitation of our approach unlikely.

As the HLA-ABC and -DR molecules were not numerically reduced on the cell surface, we investigated the possibility of a functional impairment mediated by BKV agnoprotein. To test this hypothesis, we used a well-characterized, immunodominant 9-mer peptide from the CMV-pp65 antigen, which is an immunodominant target of cytotoxic T cell response in vitro and in vivo in HLA-A0201-positive individuals. As shown, BKV agnoprotein expression failed to interfere with CMV 9-mer NLVPVMATV-dependent killing arguing against a major role of BKV agnoprotein in modulating a functional immune evasion of this kind. In conclusion, we demonstrate that BKV agnoprotein does not modulate surface expression of HLA-ABC and -DR or interfere with HLA-A0201-mediated CTL activity. Thus, other functions and mechanisms of BKV agnoprotein need to be considered which may or may not include a role in immune evasion.

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