BKV INFECTION AND HEMORRHAGIC CYSTITIS AFTER ALLOGENEIC BONE MARROW TRANSPLANT

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Hemorrhagic cystitis (HC) is a well-known complication after allogeneic bone marrow transplant (BMT) and can be related to adenovirus or human polyomavirus BK (BKV) infections. In this study a group of 20 patients after allogeneic BMT has been examined. BMT urine samples were analysed for the presence of Adenovirus and BKV DNA by means of polymerase chain reaction (PCR). 5/20 BMT patients developed HC after BMT. The presence of BKV DNA in urine samples was evident in 3/15 patients without HC and in 5/5 patients with HC. In 2/5 HC-patients the BKV DNA was not found after therapy with Cidofovir and Ribavirin. The search for adenovirus DNA in all samples was negative. The analysis of BKV non-coding control region (NCCR) isolated from urine samples revealed a structure very similar to the archetype in all samples. The RFLP (Restriction Fragment Length Polymorphism assay) showed the presence of BKV subtypes I and IV, with the prevalence of subtype I (4/5). This study supports the hypothesis that HC is mainly related to BKV rather than to adenovirus infection in BMT patients. Moreover, since BKV subtype I was predominant, it is reasonable to hypothesize that a specific BKV subtype could be associated with the development of HC.

Human Polyomavirus BK (BKV) usually infects young children and in adults the seroprevalence is 70-80% (1). After the primary infection, usually unapparent and without clinical signs, the virus disseminates and establishes a persistent infection in the urinary tract and in the lymphocytes (2-3). In allogeneic bone marrow transplanted (BMT) patients BKV reactivation has been suggested to be associated with hemorrhagic cystitis (HC) (4- 6). In fact since 1980 BKV has been frequently detected from urine of transplanted patients with HC, suggesting that BKV reactivation can be associated with this complication (4, 7-8). In particular little is known about the molecular aspects of how BKV contributes to hemorrhagic cystitis in the bladder, which can also be caused by adenovirus infection (2, 9). Whether the bladder is a reservoir of BKV that reactivates upon immunosuppression or whether the virus moves from kidney to bladder is still uncertain, although there is some evidence of BKV DNA presence in normal bladder (10-11).

Hemorrhagic cystitis is an important cause of morbidity and occasional mortality in patients undergoing bone marrow transplant (12). The manifestations vary from microscopic hematuria to severe bladder hemorrhage leading to clot retention and renal failure; HC incidence has varied from 7% to 68% of BMT cases (12-13). Some studies suggested the association between Polyomavirus BK and development of HC after BMT (4-5, 8). Other

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studies showed that BKV could be detected in urine of BMT patients with or without HC (7, 14). Moreover, after primary infection, BKV dormancy in uroepithelium was demonstrated (2). During BMT the intense immunosuppression leads to increased viral replication that results in viruria. Therefore the role of BKV in HC remains controversial. adenoviruses are increasingly recognized pathogens that affect bone marrow transplant recipients. Furthermore, the adenovirus infections are an important cause of morbidity and mortality in adult BMT recipients, particularly allogeneic transplant recipients with graft versus host disease (GVHD) under immunosuppressive therapy (15). In fact late-onset hemorrhagic cystitis is also attributed to Adenovirus infection (9).

In this study the aetiology of HC in BMT patients has been evaluated and sequence analysis of non-coding control region (NCCR) of BKV isolates was performed to verify the presence of viral variants and to subtype BKV by means of RFLP (Restriction Fragment Length Polymorphism) assay on viral protein-1 (VP1)- PCR products.

MATERIALS AND METHODS

Population

A group of 20 allogeneic BMT patients (11 males and 9 females) was analysed. The average age of the patients was 42,5 (19-64 years). Out of twenty bone marrow transplant recipients, 25% (5/20) of patients (2 males and 3 females) developed hemorrhagic cystitis at a median of 61,6 days after BMT. Urine samples were collected from patients, five with HC and fifteen without HC, the latter being used as controls, at zero, thirty, sixty and ninety days after allogeneic BMT. The patient characteristics are shown in Table I. All samples were analysed for the presence of BKV (NCCR and VP1 region) and Adenovirus (hexon region) DNA by polymerase chain reaction (PCR).

Processing of clinical specimens

Samples were incubated in lysis buffer (0.5 M TRIS, 0.02 M EDTA, 0.01 M NaCl, 1% SDS) with 200 mg/ml proteinase K at 55°C for 24 h. Digestion was followed by phenol-chloroform extraction and ethanol precipitation. One mg of total purified DNA was used for each PCR.

Condition for PCR

DNA suitability for analysis was checked by amplification of β-globin gene sequence (16). General precautions, conditions for PCR analysis and nested PCR procedures were as published (17-18). β-globin-positive samples were amplified in a GeneAmp PCR System 9600 (Perkin-Elmer Cetus, Emeryville, CA), and all assays included positive and negative controls to exclude false-positive and false-negative results (19). The PCR products were analysed on 2% agarose gel by ethidium bromide staining.

PCR for the non-coding control region (NCCR) of BKV

Nested PCR employed two pairs of primers that anneal to the invariant regions flanking the NCCR of BKV (17-18). Primers BKTT1 (+) and BKTT2 (-) generated a 748 bp DNA fragment (20). The second pair, BK1 (+) and BK2 (-), amplified a portion of the first round PCR product, generating a fragment of 354 bp (21).

PCR for the VP1 region of BKV

PCR primers were chosen to anneal to flanking region of BKV subtype-specific viral protein 1 (VP1) (22). Primers VP1-7 (+), 5' ATCAAAGAACTGCTCCTCAAT-3' (nucleotides 1480-1500) and VP1-2R (-), 5' GCACTCCCTGCATTTCCAAGGG-3' (nucleotides 2059-2038) (23) generated a 580 bp fragment after 35 amplification cycles of the first round PCR. Samples were subjected to 2 min. of denaturation at 94°C, followed by 35 rounds of an amplification cycle consisting of 1 min. at 91°C, 1 min. at 55°C and 1 min. at 72°C, followed by one extension cycle consisting of 4 min. at 74°C (17).

The second pair of primers [327-1 (sense) and 327-2 (antisense)] was designed to amplify a portion of the first PCR products. The samples were submitted to 35 amplification cycles and the expected BKV product was 327 bp length (22). BKV genomic DNA was used as positive control. BKV was grown in Vero cells and the tissue culture fluid was frozen at 20°C when the cytopathic effect was evident. DNA extraction was performed by DNeasy Tissue Kit (QIAGEN), according to the manufacturer's instructions.

PCR for adenovirus detection

According to Allard et al. (24) the PCR method was used for adenovirus detection. A specific target DNA sequence (308 bp) was amplified by means of PCR using the hexon region primers (hexAA1885 and hexAA1913). The specificity of the primers was tested on 18 different adenovirus types (24).

Sequencing of the control region of BKV

PCR products corresponding to BKV-NCCR were purified and sequenced (18). Briefly, amplified products were purified prior sequencing to remove the excess of primers with QIAquick PCR purification kit, according to

QIAGEN protocol. DNA sequencing was performed by automatic DNA sequencer (Applied Biosystem, mod. 370 A), according to manufacturer's specifications (Amplicycle Kit, Applied Biosystem). Sequences were organised and analysed using the Genetic Computer Group sequence analysis software package.

Restriction fragment length polymorphism (RFLP) assay

In order to subtype BKV, the VP1- PCR products were subjected to RFLP assay (18, 22). 10 ml of PCR product were digested, at 37° C for 2 h, with 1-2 U of an appropriate endonuclease enzyme in a total of 20 ml of supplied buffer. A two-step approach was performed: digestion with Alu I to distinguish BKV subtypes I and II from subtypes III and IV; subsequently, the digestion patterns produced by Xmn I and Ava II respectively were used to distinguish subtype I from II and subtype III from IV. The digested samples were electrophoresed in ethidium bromide-stained 2% agarose gel (Fig. 1).

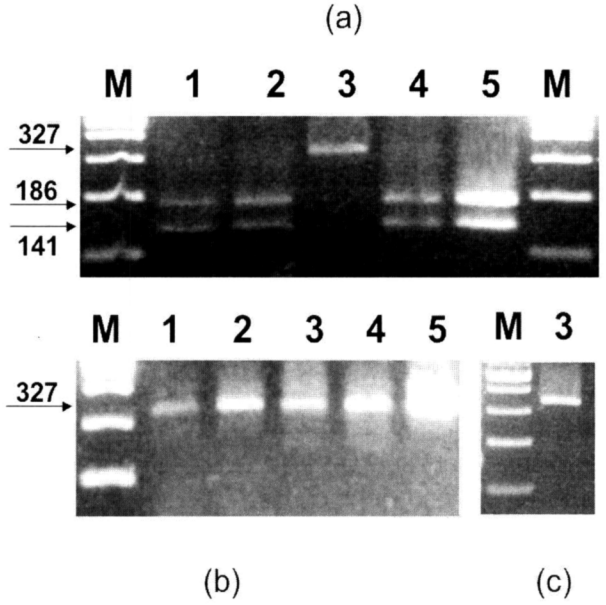

Fig. 1. *Electrophoresis on an ethidium bromide-stained 2% agarose gel of VP-1 PCR products digested with different enzymes.*
M= Marker 100 bp DNA ladder; 1, 2, 3, 4, 5= VP-1 PCR products obtained from different urine samples.
a) The digestion with Alu I enabled to distinguish BKV subtypes I and II (186 bp and 141 bp fragments) from subtypes III and IV (327 bp).
b) Patterns produced by Xmn I digestion distinguish subtype I (327 bp) from II (244 bp and 83 bp).
c) Patterns produced by Ava II digestion distinguish subtype III (237 bp and 90 bp) from IV (327 bp).

Diagnosis and treatment of HC

The urine samples were collected from patients at zero, thirty, sixty and ninety days after allogeneic BMT. All patients were analysed for the presence of BKV and AdV as previously reported. At the time of HC, the treatment of BKV-associated HC included: Cidofovir (5 mg/Kg once a week for 2 weeks, followed by drug administrations on alternate weeks) associated with Ribavirin (1gr every 12 hours), in alternative, Ribavirin (1gr every 12 hours) or only Ganciclovir (5 mg/Kg every 12 hours for 14 days, followed by drug administrations every 24 hours for 5 days/week).

Statistical analysis

Statistical analysis was performed utilizing the Mann-Whitney U test. *P* values of less than 0.05 were considered statistically significant.

RESULTS

Twenty recipients of allogeneic bone marrow transplants were monitored prospectively after BMT, for urinary excretion of Adenovirus and Human Polyomavirus BK by polymerase chain reaction. All suitable DNA samples (analysed by PCR for β-globin gene) were utilized for subsequent analysis. Our results showed that 5/20 (25%) of BMT patients developed hemorrhagic cystitis with a median onset of 61,6 days (range 28-90) after BMT.

All urine samples resulted negative for adenovirus DNA. BKV DNA was present in 5/5 of urine samples of patients with HC and in 3/15 urine samples without HC ($p<0.05$). Moreover the patients with HC were positive for both BKV NCCR and VP-1 genomic DNA, whereas the patients without HC were positive for NCCR only (Table II). In 2/5 HC patients after antiviral therapy with Cidofovir/Ribavirin and Ribavirin only, BKV DNA was not found; these patients died within a few months after HC complete remission, for recidivate myeloid acute leukaemia and for metabolic coma respectively. Moreover, one patient was treated with Ganciclovir and died for non-Hodgkin lymphoma and HC progression. Finally two patients, both with HC, were untreated because they died within a few days. These data are shown in Table III.

In patients with hemorrhagic cystitis BKV DNA of NCCR and VP1 regions was found during the disease but it was not detectable after HC remission. In patients without HC, BKV DNA (NCCR only)

Table I. *Patients characteristics.*

Total number	20
Median age in years (range)	42.5 (19-64)
Sex (female/male)	9/11
Disease	MAL (10); LAL (5); NHL (3); HD (2)
Disease status at BMT	REL (12= 60%); CR (6= 30%); PR (2= 10%)
Stem cell donor	HLA identical sibling (16= 85%); HLA identical unrelated donor (4= 15%)
Conditioning regimen	Bu Cy (10= 50%); Cy TBI (4= 20%); Flu Mel (4= 20%); Other (2= 10%)
ATG with conditioning regimen	6= 30 %
GvHD prophylaxils	MTX and Csa (17); Other (3)

MAL= myeloid acute leukaemia; LAL= lymphoblastic acute leukaemia; NHL= non Hodgkin lymphoma; HD= Hodgkin lymphoma; REL= relapse; CR=complete remission; PR= partial remission; Bu= busulphan; Cy= endoxan; TBI= total body irradiation; Flu= fludarabine phosphate; Mel= melphalan; ATG= anti- thymocite globulin; GvHD= graft versus host disease; MTX= methotrexate; Csa= Cyclosporine A.

Table II. *Presence of BKV-DNA in analysed urine samples.*

Number of positive samples for	**Days after BMT**				
	0	**30**	**60**	**90**	**>90**
NCCR	0	1/5 (HC) 3/15 (no HC)	3/5 (HC) 3/15 (no HC)	5/5 (HC) 3/15 (no HC)	3/5 (HC) 3/15 (no HC)
VP-1	0	1/5 (HC)	3/5 (HC)	5/5 (HC)	3/5 (HC)

Presence of BKV-DNA in urine samples. HC = haemorrhagic cystitis, NCCR = non coding control region, VP-1 = Viral Protein-1, BKV = BK virus, BMT =. Bone marrow transplant.

Table III. *Characteristics of patients who developed hemorrhagic cystitis.*

Patient	Sex/age	Diagnosis	Onset HC (days)	BKV NCCR	BKV VP1	AdV	Therapy	HC resolution	Cause of death	BKV Subtype
1	M/30	MAL	60°	+	+	-	Cidofovir[1] + Ribavirin[2]	Yes	Recidive MAL	I
2	F/56	MAL	80°	+	+	-	Ribavirin[2]	Yes	Metabolic coma	I
3	F/19	MAL	28°	+	+	-	Ganciclovir[3] (no resolution)	No	NHL	IV
4	M/19	LAL	90°	+	+	-	No, for premature death	No	Recidive LAL, HC	I
5	F/53	LAL	50°	+	+	-	No, for premature death	No	Chronic GvHD HC	I

MAL = Myeloid acute leukaemia; LAL = Lymphatic acute leukaemia; NHL = Non Hodgkin lymphoma; GVHD = Graft Versus Host Disease. [1]= 5 mg/Kg once a week for 2 weeks, followed from drug administrations on alternate weeks; [2]= 1gr every 12 hours; [3]= 5 mg/Kg every 12 hours for 14 days, followed from drug administrations every 24 hours for 5 days/week.

was found in 3/15 samples (Tab. II). Moreover the NCCR structure of BKV isolates from HC-patients was analysed to verify the presence of different viral variants. The obtained sequences were compared with the archetype strain (WW) by means of homology analysis and multiple alignment. Our results indicated the presence of a NCCR structure very similar to that of the archetypal strain in all urine samples (Lines 1, 2, 3, 4, 5) with the exception of some point mutations (Fig. 2).

Regarding VP1 region, the same was detectable only from the urine of HC patients during the disease, and it was analysed by RFLP to identify BKV subtype. Results showed the presence of subtypes I and IV. In particular the subtype I was found in 4 cases and only one case of infection was related to subtype IV. Results are shown in figure 1 and in table III.

DISCUSSION

Hemorrhagic cystitis is an important cause of morbidity and occasional mortality in patients undergoing bone marrow transplant (12). This pathology is mainly attributed to infection with BKV or adenovirus. Adenovirus infections are an important cause of morbidity and mortality in adult BMT recipients, particularly allogeneic transplant recipients with graft versus host diseases (GVHD) who are receiving immunosuppressive therapy (15). Polyomavirus BK, after BMT, the intense immunosuppression increases viral replication that results in viruria. The role of BKV in HC remained contentious. Therefore in this study the aetiology of HC in BMT patients has been evaluated and the structure of NCCR of BKV isolates has been analysed in order to verify the presence of viral variants and to identify BKV subtypes associated to hemorrhagic cystitis after BMT.

In this study twenty recipients of allogeneic bone marrow transplant were monitored for urinary excretion of Adenovirus and Human Polyomavirus BK by polymerase chain reaction. The excretion of Adenovirus was absent in all samples, whereas excretion of BK virus was established in 40 % of transplant recipients (5 with HC and 3 without HC) and could be associated with viral reactivation from latency. Therefore this study showed that after allogeneic BMT, the hemorrhagic cystitis was predominantly related to BKV infection. In fact in the

examined population the association hemorrhagic cystitis- adenovirus infections, found by others, has not been confirmed, whereas BKV DNA was evident in 5/5 of urine samples of patients with HC and in 3/15 of urine samples of patients without HC ($p<0.05$). In patients with hemorrhagic cystitis BKV DNA of NCCR and VP1 regions was found during the disease but not after HC remission. In patients without HC, only NCCR of BKV DNA was found in 20% of samples. The finding of a BKV NCCR structure very similar to the archetypal strain and the presence of VP1 region in all urine samples of HC-patients showed that the virus is in active replication. In fact, the presence of VP1 sequences has been proposed to be related to recent infection or reactivation (25-28). Finally the finding of BKV subtypes I and IV, with prevalence of subtype I (4/5), demonstrated that there is a probable correlation between the HC and a specific BKV subtype. Moreover, this study supports the hypothesis that after BMT, HC was mainly related to BKV rather than to Adenovirus infection.

In the follow-up of the patients after BMT we observed that all HC patients died with an average survival of four months after BMT (Tab. III), so we hypothesize that the hemorrhagic cystitis could represent a predictive factor for a shorter survival after BMT.

In conclusion, since after a specific antiviral therapy (cidofovir and/or ribavirin) in HC subjects the BKV viruria appeared negative, a rapid diagnosis of BKV productive infection could be useful to begin a specific and immediate antiviral therapy for a complete resolution of viral infection.

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Archetype  TTGAGAGAAAGGGTGGAGGCAGAGGCGGCCTCGGCCTCTTATATATTATAAAAAAAAAGG  60
1          TTGAGAGAAAGGGTGGAGGCAGAGGCGGCCTCGGCCTTTTATATATTATAAAAAAAAAGG  60
2          TTGAGAGAAAGGGTGGAGGCAGAGGCGGCCTCGGCCTCTTATATATTATAAAAAAAAAGG  60
3          TTGAGAGAAAGGGTGGAGGCAGAGGCGGCCTCGGCCTCTTATATATTATAAAAAAAAAGG  60
4          TTGAGAGAAAGGGTGGAGGCAGAGGCGGCCTCGGCCTCTTATATATTATAAAAAAAAAGG  60
5          TTGAGAGAAAGGGTGGAGGCAGAGGCGGCCTCGGCCTTTTATATATTATAAAAAAAAAGG  60

Archetype  CCACAGGGAGGAGCTGCTTACCCATGGAATGCAGCCAAACCATGACCTCAGGAAGGAAAG  120
1          CCACAGGGAGGAGCTGCTTACCCATGGAATGCAGCCAAACCATGACCTCAGGAAGGAAAG  120
2          CCACAGGGAGGAGCTGCTTACCCATGGAATGCAGCCAAACCATGACCTCAGGAAGGAAAG  120
3          CCACAGGGAGGAGCTGCTTTCCCATGGAATGCAGCCAAACCATGACCTCAGGAAGGAAAG  120
4          CCACAGGGAGGAGCTGCTTACCCATGGAATGCAGCCAAACCATGACCTCAGGAAGCAA-G  119
5          CCACAGGGAGGAGCTGCTTACCCATGGAATGCAGCCAAACCATGACCTCAGGAAGGAAAG  120

Archetype  TGCATGACTGGGCAGCCAGCCAGTGGCAGTTAATAGTGAAACCCCGCCCCTGAAATTCTC  180
1          TGCATGACTGGGCAGCCAGCCAGTGGCAGTTAA-AGTGAAACCCCGCCCCTGAAATTCTC  179
2          TGCATGACTGGGCAGCCAGCCAGTGGCAGTTAATAGTGAAACCCCGCCCCTAAAATTCTC  180
3          TGCATGACTGGGCAGCCAGCCAGTGGCAGTTAATAGTGAAACCCCGCCCCTAGAATTCTC  180
4          TGCATGACTGGGCAGCCAGCCAGTGGCAGTTAATAGTGAAACCCCGCCCCT-AAATTCTC  178
5          TGCATGACTGGGCAGCCAGCCAGTGGCAGTTAATAGTGAAACCCCGCCCCTGAAATTCTC  180

Archetype  AAATAAACACAAGAGGAAGTGGAAACTGGCCAAAGGAGTGGAAAGCAGCCAGACAGACAT  240
1          AAATAAACACAAGAGGAAGTGGAAACTGGCCAAAGGAGTGGAAAGCAGCCAGACAGACAT  239
2          AAATAAACACAAGAGGAAGTGGAAACTGGCCAAAGGAGTGGAAAGCAGCCAGACAGACAT  240
3          AAATAAACACAAGAGGAAGTGGAAAGTAGCCAAAGGAGTGGAAAGCAGCCAGACAGACAT  240
4          AAATAAACACAAGAGGAAGTGGAAACTGTCCAAAGGAGTGGAAAGCAGCCAGACAGACAT  238
5          AAATAAACACAAGAGGAAGTGGAAACTGGCCAAAGGAGTGGAAAGCAGCCAGACAGACAT  240

Archetype  GTTTTGCGGGCCTAGGAATCTTGGCCTTGTCCCCAGTTAAACTGGACAAAGGCCA  293
1          GTTTTGCGGGCCTAGGAATCTTGGCCTTGTCCCCAGTTAAACTGGACAAAG-CCA  291
2          GTTTTGCGAGCCTAGGAATCTTGGCCTTGTCCCCAGTTAAACTGGACAAAGGCCA  293
3          GTTTTGCGAGCCGAGGAATCTTGGCCTTGTCCCCAGTTAAACTGGACAAAGGCCA  293
4          GTTTTGCGAGCCTAAGAATCTTGGCCTTGTCCCCAGTTAAACTGGACAAAGGCCA  290
5          GTTTTGCGAGCCTAGGAATTTTGGCCTTGTCCCCAGTTAAACTGGACAAAG-CCA  292
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Fig. 2. *Multiple alignment of BKV isolates NCCR-sequences compared with BKV archetype strain. Lines 1, 2, 3, 4, 5 = sequences obtained from urine samples of HC patients. Point mutations are designed in bold.*

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