Papers

Adenovirus pulmonary infections identified by PCR and in situ hybridisation in bone marrow transplant recipients

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Abstract

Aims—To investigate adenovirus pulmonary infections in bone marrow transplant (BMT) recipients.

Methods—Formalin fixed, paraffin wax embedded lung tissue was examined from 13 necropsy cases after BMT using PCR and in situ hybridisation to detect adenovirus DNA. The E1A region of the adenoviral genome was targeted for PCR. In situ hybridisation was performed only in the PCR positive cases.

Results—Of the 13 lung specimens analysed, nine cases were negative for adenoviral nucleic acid. Four (30%) PCR and two (15%) in situ hybridisation positive cases were found. In some of the patients there were clinical and pathological indications that some diseases might be associated with adenovirus infection—haemorrhagic cystitis (three cases); necrotising pneumonia (one case). In necrotising pneumonia in which no pathogenic agents had been shown by conventional histological study, the in situ hybridisation technique showed positive staining for adenovirus. In a patient who died of renal failure caused by adenovirus nephritis, both PCR and in situ hybridisation were positive in the lung as

well as in the kidney, although no histological change was found. Two PCR positive cases lacked positive sites for adenovirus by in situ hybridisation.

Conclusions—The combination of PCR and in situ hybridisation could be useful for diagnosing adenovirus infection of the lung in BMT recipients. These results provide a basis for exploring further the clinical use of PCR and in situ hybridisation to diagnose adenovirus infection.

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Adenovirus lytic infection in immunosuppressed patients can cause systemic infections and present a variety of clinical manifestations ranging from broncho-bronchiolitis to interstitial pneumonia, necrotising pneumonia, hepatitis, colitis, and haemorrhagic cystitis.¹⁻⁹ In 1985 Shields *et al* reported that after bone marrow transplantation (BMT) the incidence of adenovirus infection was 4.9% and invasive infection 1%.¹⁰ Diagnosis has been made mainly from isolation of the virus or an increase in antibody titre in addition to the characteristic adenovirus inclusion bodies seen on histological examination. However, it takes several days to isolate adenovirus from clinical specimens and the sensitivity is not very high. As latent adenovirus occurs in lymphocytes, lung, tonsils, and adenoids,¹¹⁻¹⁴ isolation from one site does not always mean systemic infection. Additionally, a low antibody titre does not rule out adenovirus infection in some immunosuppressed subjects¹⁵; and adenoviral inclusions could be mistaken for other viral bodies.

Like cytomegalovirus (CMV), latent adenovirus is thought to be reactivated in some conditions. It would therefore be of clinical use to differentiate latent from active infection in BMT recipients with a highly specific method. We tested the combination of the polymerase chain reaction (PCR) and in situ hybridisation (ISH) for this purpose. PCR can detect latent adenovirus infection with low copy numbers.¹¹ Only in the acute and active phase can adenovirus infection be demonstrated with ISH.^{7,16} Positive PCR results merely mean the presence of viral nucleic acid. Compatible pathological findings, positive

Table 1 Clinical and pathological findings in transplant recipients

Case No	Sex	Age	Basal haematological disorder	Pulmonary lesions	Survival period after BMT (days)
1	M	38	AA	Aspergillosis	74
2	M	18	MDS	Bacterial pneumonia	5
3	M	19	ALL	Fungus, CMV pneumonia	92
4	M	33	AMMoL	Aspergillosis	4
5	F	48	AMMoL	Pulmonary haemorrhage	76
6	F	33	MDS	Bacterial pneumonia	77
7	M	46	CML	Blast cell invasion	288
8	F	23	NHL	Lymphoma cell invasion	77
9	M	10	ALL	None	24
10	M	35	MDS	None	209
11	M	42	CML	Haemorrhagic necrotising pneumonia	308
12	M	36	ALL	Acute interstitial pneumonia	20
13	M	37	CML	CMV, <i>Candida</i> sp pneumonia	88

AA = aplastic anaemia; MDS = myelodysplastic syndrome; ALL = acute lymphocytic leukaemia; AMMoL = acute myelomonocytic leukaemia; CML = chronic myelocytic leukaemia; NHL = non-Hodgkin's lymphoma.

Table 2 Results of adenovirus PCR and in situ hybridisation with associated clinical and pathological findings

Case No	Necrotising pneumonia	Colitis	Hepatitis	Haemorrhagic cystitis	Adenovirus isolation (organ)	PCR	In situ hybridisation
1	-	-	+	+	+(urine)	+	-
2	-	-	-	-	-	-	ND
3	-	+	-	+	-	-	ND
4	-	-	-	-	-	-	ND
5	-	-	-	-	-	-	ND
6	-	-	-	-	-	-	ND
7	-	-	-	-	-	-	ND
8	-	-	-	-	-	-	ND
9	-	-	-	-	-	+	-
10	-	-	-	+	+(urine)	+	+
11	+	-	-	-	-	+	+
12	-	-	-	-	-	-	ND
13	-	-	+	-	-	-	ND

PCR, and in situ hybridisation results indicate active adenovirus infection.

Methods

Lung tissue obtained from 13 consecutive necropsy cases, who died within one year of BMT between 1986 and 1991 at the Institute of Medical Science, University of Tokyo, were studied. The clinical histories of these patients are shown in table 1. Adenovirus was isolated from the urine of cases 1 and 10 (table 2). Some of the cases had the pathological manifestations compatible with adenovirus infection, including haemorrhagic cystitis, hepatitis, necrotising pneumonia, and colitis (table 2). However, adenovirus infection was not demonstrated histologically except in case 10 who had a renal infection with adenovirus inclusion bodies. This patient had died of renal failure caused by adenovirus nephritis. The kidney sample from this case served as a positive control for PCR and in situ hybridisation.

DNA extraction was performed according to a method described before.¹⁷ The DNA extracted from paraffin wax embedded tissues was analysed by PCR targeted at the E1A region of adenovirus. As a positive control, DNA was extracted from formalin fixed, paraffin wax embedded Graham 293 cells, transformed with four to five copies of E1A region of the adenovirus type 5 genome.¹⁸

PCR was performed using our previously described method.¹¹ Thirteen lung and one renal sample were examined simultaneously along with the positive and negative controls. For PCR, adenovirus type 2 DNA (5 ng) served as a positive control in addition to the DNA extracted from Graham 293 cells. "No template DNA," which consisted of a sample containing all the components for PCR except template DNA, served as a negative control. The oligonucleotide primer was chosen from the E1A region of adenovirus types 2 and 5 genomes, according to the sequences in the Genebank DNA database.¹¹ The primer sequences were: 5'-CTG CCA CGG AGG TGT TAT TACC-3' and 3'-TCC AGG ACA CAG ACT TGG ACTC-5'. After PCR, 20 µl of the PCR mixture was subjected to 1% agarose gel electrophoresis. The amplification products were transferred to a nylon mem-

brane (Hybond-N, Amersham, Illinois, USA) by diffusion blotting in 20 × SSC (3M sodium chloride, 0.3 M sodium citrate, pH 7.0). The filter was prehybridised at 65°C for five hours in 6 × SSC, 5 × Denhardt's solution, 0.5% sodium dodecyl sulphate (SDS), and 20 µg/ml of salmon sperm DNA. The radio-labelled probe was added to the filter and hybridised at 65°C. The filter was washed twice at room temperature for 10 minutes in 2 × SSC, 0.1% SDS; then at 65°C for 30 minutes in 0.1 × SSC, 0.1% SDS. The filter was then subjected to autoradiography. The probe DNA for the E1A region was a 756 base pair product of a double digest with *Pst* I and *Bam*HI of a 742 base pair *Alu* I fragment from pXC-15 containing the E1A region which had been subcloned into the *Hinc* II site of pUC13 (gift of Dr JC Hogg, Vancouver, Canada). This 756 base pair fragment was purified by low melting agarose gel electrophoresis and labelled with ³²P-dCTP using a random priming kit (Amersham).

In situ hybridisation analysis was performed only for the PCR positive cases. Four PCR positive lung and one kidney (from case 10) samples were examined. For each sample, two identical sections were put on a slide coated with poly-L-lysine. One side was hybridised with adenovirus DNA probe using the ENZO PathoGene DNA probe assay for the identification of adenovirus (ENZO Diagnostics, New York, USA), the other side served as a control and was hybridised with biotinylated pBR 322 DNA. In situ hybridisation was performed according to the manufacturer's instructions. The presence of hybridised probe was determined by the reaction of hydrogen peroxide and 3-amino-9-ethylcarbazole.

Cultured Hep-II cells infected with adenovirus types 2 and 5 served as the positive control. They were fixed with formalin and embedded in paraffin wax.

Results

The PCR product was subjected to Southern blotting after electrophoresis, and probed with radiolabelled DNA from the corresponding region of the adenovirus genome. The expected size of the amplified products was 675 base pairs. The autoradiograph result of PCR for the E1A region is shown in fig 1. The DNA extracted from cases 1, 9, 10, and 11 (30%) was positive for the E1A region by PCR, as were the positive controls (table 2). The positive controls consisted of 5 ng of adenovirus type 2 genomic DNA and the extracted DNA from paraffin wax sections of Graham 293 cells, and the kidney sample with adenovirus inclusion bodies. The negative control "no template DNA" did not show bands either in the gel or the autoradiograph.

Figure 2A shows that the cultured cells infected with adenovirus type 5 positively stained for adenovirus with in situ hybridisation as well as the cultured cells infected with adenovirus type 2. Among the four PCR positive cases, two (cases 10 and 11) (15%)

Figure 1 Autoradiograph of Southern hybridisation of PCR products probed with adenovirus E1A region. Thirteen samples from necropsy cases and two positive (Adeno virus, Kidney 10) and one negative (No template) control samples are hybridised with ^{32}P labelled probe. Lanes from top to bottom are PCR products from "No template" DNA, 5 ng of adenovirus type 2 DNA, DNA extracted from lungs (BMT cases 1 to 13) and one kidney (BMT case 10). Cases 1, 9, 10, and 11 were hybridised with the probe for the E1A region as well as two positive controls. "No template" = no template DNA; Adeno virus = 5 ng of adenovirus DNA, Kidney 10 = kidney sample from case 10.

stained positively on situ hybridisation for adenovirus (fig 2B); the others did not. All four PCR positive samples were not hybridised with non-specific DNA (biotinylated pBR322) probe, a step included for negative controls (fig 2C). Both the lung and kidney specimens from case 10 showed positive results with PCR and in situ hybridisation. Case 11 was diagnosed as having haemorrhagic necrotising pneumonia. However, the causal agent was not known because a specific feature had not been histologically demonstrated and no pathogen had been clinically isolated. We found sites positively stained with adenovirus DNA probe by in situ hybridisation in the lesions showing necrotising haemorrhagic pneumonia (case 11), but the positive cells were scattered throughout.

Discussion

Patients immunocompromised as a result of

immunosuppressive treatment, organ transplantation, or chemotherapy are increasing in number, as are those with AIDS. In such patients viral pulmonary infections have become one of the most important causes of death. However, viral infection in the lung is difficult to distinguish from other interstitial pulmonary disorders, such as drug toxicity, neoplasia, pulmonary haemorrhage, allergic reactions, and pulmonary oedema.¹⁹ Besides, it is difficult to determine which species of virus is responsible for the lesion with or without viral inclusion bodies.

The incidence of adenovirus infections after BMT has been based on the clinical isolation of the virus, the prevalent serotypes being adenovirus types 1, 2, 5, 7, 11, 34, and 35.¹⁰ However, the isolation rate is not an indicator of the morbidity. To diagnose invasive infections, samples of the diseased organs for culture and histological examination are needed; and these should not be highly specific and sensitive.

Polymerase chain reaction (PCR) and in situ hybridisation for detecting viral nucleic acid have been established for the diagnosis of several viral infections. However, the sensitivity of PCR is extremely high, and positive PCR results merely indicate the presence of the viral genome, and not necessarily that the virus is related to the present disease. In this study the E1A region of the adenoviral genome was targeted by the PCR method. The E1A gene can sensitise cells to destruction by cytokines²⁰ and it could have an essential role in adenovirus infection through activation of other adenoviral genomic DNA.²¹ The sensitivity of in situ hybridisation for the detection of adenoviral genome has been reported before.¹⁶ A detection limit of five to 10 copies of adenoviral DNA using in situ hybridisation has been estimated, and adenovirus was demonstrated by in situ hybridisation only in acute cases of adenovirus pneumonia.¹⁶

We examined adenovirus infections in necropsy lung specimens from 13 necropsy patients after BMT using PCR and in situ hybridisation techniques, because adenovirus pneumonia is one of the most common causes of mortality in transplant recipients. Some of our cases had some pathological evidence suggestive of adenovirus infection: necrotising pneumonia (n = 1); colitis (n = 1); hepatitis (n = 1); and haemorrhagic cystitis (n = 3) (table 2). In this study organs other than the lungs were not examined.

In our cases the clinical isolation rate of adenovirus was 15% and the incidence of adenovirus detection by PCR was 30% in the patients who died after BMT (table 2). Both were higher than previously reported rates of adenovirus infection.¹⁰ This finding should be interpreted with caution, because the number of subjects we studied was small. However, this difference might be explained by the fact that we examined only necropsy cases and that PCR was more sensitive than isolation of the virus. PCR analysis was positive in two of the three patients with haemorrhagic cystitis

Figure 2 In situ hybridisation with biotinylated probe to the sections of formalin fixed, paraffin wax embedded lung tissue and cultured cells. **A** Hybridisation of adenovirus probe to cultured Hep-II cells infected with adenovirus type 5. **B** Hybridisation with the biotinylated adenovirus probe to lung tissue from case 10. Epithelial cells stained positively for adenovirus (arrowheads). **C** Hybridisation with the pBR322 probe to the same tissue as in **B** showing no positive signal. **D** Pulmonary lesion from case 11, showing necrotising pneumonia with haematoxylin and eosin staining. The arrowhead indicates a nucleus with an homogeneously basophilic appearance, so-called ground glass appearance, which was thought to be an adenovirus inclusion body (haematoxylin and eosin stain).

(cases 1 and 10), one patient with necrotising pneumonia (case 11), and one without any clinical and pathological findings related to adenovirus (case 9). Because PCR is highly sensitive for the detection of a viral genome, the positive PCR results do not necessarily indicate active viral infection. Therefore, the in situ hybridisation study was performed in these PCR positive cases.

Of the four positive PCR cases, two were also positive on in situ hybridisation. The lung section from the patient with necrotising pneumonia stained positively on in situ hybridisation. The PCR and in situ hybridisation findings, taken together with the clinical and histological data, indicate that the necrotising pneumonia was caused by adenovirus. However, the in situ hybridisation positive cells were scattered throughout the lesions. This is probably because the lesion was necrotic and the normal lung structure had been destroyed. As a result, the number of infected but viable and well preserved epithelial cells had decreased.

After the in situ hybridisation method had disclosed adenovirus in the lung tissue, we re-examined haematoxylin and eosin stained sections and were able to point out cells with viral inclusion bodies in the lung tissue of the

case with necrotising pneumonia (case 11) (fig 2D). Definite diagnosis of active adenovirus infection can be established, if histological findings correlate with positive staining on in situ hybridisation.

In conclusion, our results raise the possibility that in addition to conventional histological and virological investigations, the combination of PCR and in situ hybridisation might be useful for detecting and diagnosing adenovirus infections in patients undergoing BMT. Further study with larger numbers of subjects is needed to evaluate the sensitivity and clinical value of these techniques.

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