Technical report

Use of proteinase K for RT-PCR of cytokine mRNA in formalin fixed tissue

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Abstract

Fresh tissue from cases of sudden infant death syndrome is becoming increasingly scarce and therefore researchers interested in studying the aetiology of this syndrome have had to resort to archival tissue, usually in the form of paraffin wax sections. A simple method for isolating mRNA from formalin fixed, paraffin wax embedded material of sufficient purity for reverse transcription (RT)-PCR is described. Proteinase K treatment of formalin fixed, wax embedded tissue followed by RNA STAT-60 extraction was successful in isolating mRNA suitable for RT-PCR. Interleukin (IL)-1 α , IL-6 and tumour necrosis factor (TNF) transcripts were amplified successfully from heart, but not thyroid, kidney or liver tissue, of a patient who died following rejection of a transplanted heart, and IL-1 α , but not IL-6 or TNF, transcripts from lung tissue of a six month old baby who died of viral pneumonia. Transcripts of a housekeeping gene were detected in all tissues. This method should be useful for examining gene expression in archival material.

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sensitivity we wished to use reverse transcription (RT)-PCR to study cytokine gene expression in such tissues. While amplification of DNA isolated from archival material is well established,² few studies have examined gene expression in such tissue using PCR; and, to our knowledge, no such studies on the detection of cytokine mRNA have been published. In the present paper we describe a method which has been used to identify successfully cytokine expression in lung tissue obtained from a six month old baby who died of viral pneumonia and in heart tissue from a patient who died following rejection of the transplanted heart but not in the thyroid, kidney or liver tissue of the same patient. Messenger RNA from the constitutively expressed porphobilinogen deaminase (PBGD) gene was detected in all tissues tested.

Methods

Lung tissue from a six month old baby with viral pneumonia was collected within 36 hours of death in 1988 in Denmark. Thyroid, heart, liver, and kidney tissue was collected from a patient who had died following rejection of a heart transplant in Birmingham in 1994. The tissues in each case were fixed in formal saline and embedded in paraffin wax following standard histological procedures.

Primers were designed from sequence data published previously to amplify specifically sequences from the human IL-1 α ,³ IL-6,⁴ TNF α ,⁵ and PBGD⁶ genes. The sequences of the oligonucleotide primers (synthesised by Alta Biosciences, Birmingham University, Birmingham, UK) were as follows: IL-1 α forward 5'-GCCAAGTTCCAGACATG-3' and reverse 5'-GATGATCAATGGAGGAACTG-3'; IL-6 forward 5'-CTGAACCTTCCAAAGATGGC-3' and reverse 5'-TGTACTCATCTGCACAGC TC-3'; TNF α forward 5'-GCCTGTAGCC CATGTTGTAG-3' and reverse 5'-AGAGGAC-CTGGGAGTAGATG-3'; PBGD forward 5'-TGTCTGGTAACGGCAATGCGGGTGC AAC-3' and reverse 5'-TCAATGTTGCCA CCACACTGTCCGTCT-3'. The amplified DNA products were 1037, 880 and 458 base pairs (bp) for IL-1 α , IL-6 and TNF α , respectively. The same primers were used to amplify RNA by RT-PCR but gave different amplification products

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Figure 1 (A) RT-PCR amplification of IL-1 α , IL-6 and TNF α mRNA extracted from unfixed U937 cells. Lane 1, 1 kilobase DNA size markers; lanes 3–5, products amplified from mRNA obtained using primers for IL-1 α , IL-6 and TNF α , respectively; lane 7, products amplified from DNA using all three primer sets. (B) RT-PCR amplification of IL-1 α and PBGD mRNA extracted from formalin fixed, paraffin wax embedded lung tissue of a six month old baby who died of viral pneumonia. Lane 1, 1 kilobase DNA size markers; lanes 2 and 4, and 3 and 5, products amplified from two separate mRNA extractions using primers specific for IL-1 α and the constitutively expressed housekeeping gene PBGD, respectively; lane 6, product of PCR amplification of DNA isolated from the same sample using IL-1 α primers. The images have been generated from scanned original photographs using Adobe Photoshop.

(79, 174 and 158 bp, respectively). This was because the primers spanned an intronic sequence between exons 2 and 3 for IL-1 α and TNF α , and between exons 3 and 4 for IL-6. The product

amplified from PBGD mRNA was 127 bp. The sequences of the probes used to confirm the identity of the amplified products were as follows: IL-1 α 5'-TTCCAGACATGTTTGAAGACCTGAAGAACT-3'; IL-6 5'-AATCATCACTGGTCTTTTGGAGTTTGAGGT-3'; TNF α 5'-AGCTGAGGGGCAGCTCCAGTGGCTGAACCG-3'.

A lymphoblastoid cell line (U937) known to express these cytokines on stimulation was used to establish that the primers were suitable for amplification of DNA and RNA of the required specificity. U937 cells at a concentration of 1×10^6 cells/ml were incubated with 50 ng/ml PMA (phorbol myristate acetate) at 37°C for 24 hours. Total DNA was extracted from the cells using the single tube method (Biotecx Laboratories, Oxford, UK) while total RNA was extracted using RNA STAT-60 (Biogenesis Ltd, Poole, Dorset, UK) based upon a conventional guanidinium hydrochloride/phenol/CHCl₃ single-step extraction method.

Amplification from DNA followed a standard procedure. Briefly, 1 μ g DNA in 100 μ l reaction volume (20 mM Tris-HCl, pH 8.4, 50 mM KCl, 2.5 mM MgCl₂, 1 mM each primer, 200 mM each dNTP, and 2.5 units of Taq DNA polymerase (Gibco-BRL, Paisley, UK) was amplified in a Techne PHC-2 temperature cycler under the following conditions: denaturation for three minutes at 94°C followed by 30 cycles of incubation at 94°C for 30 seconds, 60°C for 30 seconds for primer annealing and at 72°C for 60 seconds for strand elongation. This was followed by incubation at 72°C for nine minutes before amplified products were electrophoresed through agarose or acrylamide for analysis. Amplification of RNA was similar except that 500 ng of total RNA was reverse transcribed in 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.7 mM MgCl₂, 0.005% gelatin, 1 mM each primer, 200 μ M each dNTP, and 5 units of avian myeloblastosis virus reverse transcriptase (Pharmacia Biotech, St Albans, UK) for one hour at 42°C in the presence of 0.5 units RNAGuard (Pharmacia Biotech). The resultant cDNA was then denatured, 2.5 units Taq polymerase added and amplified as described earlier.

Oligonucleotide probes were 5'-end labelled with γ -[³²P] ATP using the T4 bacteriophage enzyme polynucleotide kinase.⁷ PCR products were electrophoresed through an 8% polyacrylamide gel and electroblotted onto a nylon membrane (Amersham, Aylesbury, UK) following the protocol supplied by the manufacturer. Membranes were prehybridised for two hours at 68°C in a solution containing 6 $\times$ SSC buffer (1 $\times$ SSC = 0.15 M sodium chloride, 0.015 M sodium citrate, pH 7.0), 0.5% (w/v) sodium dodecyl sulphate (SDS), 5 $\times$ Denhardt's solution (1 $\times$ Denhardt's solution = 0.02% (w/v) Ficoll, 0.02% (w/v) polyvinylpyrrolidone, 0.02% (w/v) bovine serum albumin) and 100 μ g/ml denatured calf thymus DNA. Hybridisation was carried out overnight at 5–10°C below the T_m of each individual probe in prehybridisation solution containing 2–5 $\times$ 10⁷ cpm/ml of radioactive probe (see earlier). The membrane was then progressively washed

Figure 2 (A) RT-PCR amplification of IL-1 α , IL-6 and TNF α mRNA extracted from formalin fixed, paraffin wax embedded thyroid, heart, kidney, and liver tissue from an adult who died following rejection of a heart transplant. Lane 1, 1 kilobase DNA size markers; lanes 2–5, 6–9 and 10–13, amplification products using IL-1 α , IL-6 and TNF α specific primers amplifying RNA isolated from thyroid (lanes 2, 6 and 10), heart (lanes 3, 7 and 11), kidney (lanes 4, 8 and 12) and liver (lanes 5, 9 and 13), respectively. (B) RT-PCR amplification of IL-1 α , IL-6 and TNF α mRNA extracted from formalin fixed, paraffin wax embedded heart and liver tissue from an adult who died following rejection of a heart transplant. Lane 1, 1 kilobase DNA size markers; lanes 2 and 5, 3 and 6, and 4 and 7 contain amplification products from IL-1 α , IL-6 and TNF α specific primers, respectively, used with RNA from heart (lanes 2–4) and liver (lanes 5–7) tissue. (C) Southern transfer of (B) probed with specific oligonucleotides for (i) IL-1 α , (ii) IL-6 and (iii) TNF α amplification products.

in SSC, varying the concentration of SSC and SDS and the temperature depending upon the probe. Membranes were then dried and exposed against Hyperfilm MP (Amersham) overnight at -70°C .

Results and Discussion

IL-1 α , IL-6 and TNF α DNA and mRNA were detected in PMA stimulated U937 cells (fig 1A). Both the size of the amplified products and Southern blotting (data not shown) confirmed their identity. Other bands in lanes 5 and 7 represent non-specific priming as they did not hybridise with the oligonucleotide probes.

IL-1 α DNA could be detected readily in deparaffinised, formalin fixed tissue (fig 1B) but extraction of RNA from such tissue proved more problematical. The RNA STAT-60 method alone failed to yield suitable RNA as did disruption of tissue by one to three 30 second bursts of sonication in a water bath or three rapid freeze-thaw cycles in liquid nitrogen and thawing at 65°C followed by RNA STAT-60 extraction. However, RNA was extracted successfully by incubating four 10 μm sections of deparaffinised lung tissue from a six month old baby in 100 μl of deionised water containing 20 mg/ml proteinase K, either alone or with 1% SDS, followed by incubation at 37°C for 48 hours and extraction with RNA STAT-60. This RNA was satisfactory for amplification of PBGD and IL-1 α gene transcripts (fig 1B).

This procedure was limited neither to lung nor infant tissue. The PBGD gene transcript was amplified successfully from liver, kidney, heart, and thyroid tissue of an adult (data not shown) and transcripts of the three cytokine genes were evident in heart tissue but not thyroid, kidney or liver tissue of this patient

(fig 2A). The identity of the amplified products was confirmed by Southern blotting (fig 2B).

As in our studies, others have failed to detect mRNA (from the house keeping gene hypoxanthine phosphoribosyltransferase) in formalin fixed tissue although it was detected in tissue fixed in Carnoy's fixative or Omnifix II.⁸ Using proteinase K digestion followed by RNA STAT-60 extraction, we have identified transcripts from the house keeping gene PBGD in several tissues (heart, lung, liver, kidney, and thyroid) and cytokine gene expression in tissues undergoing an inflammatory response: in lung as a result of pneumonia and in heart because of immune rejection, but not in other organs which were pathologically normal. Proteinase K digestion is also suitable for extraction of viral RNA.⁹

DNA contamination of RNA samples is a potential problem in RT-PCR⁸ but this was overcome by the use of RNA STAT-60 and primer pairs spanning intronic sequences which gave unambiguous evidence of RNA amplification as RNA and DNA amplified products were of different sizes.

This method is robust, easy to use, has been repeated by two different workers in several tissues and, in a blind trial, identified tissue undergoing inflammatory changes. We now intend to use the method to examine cytokine gene expression in tissues from cases of SIDS.

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