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A simple and rapid method for the detection of RNA in formalin-fixed, paraffin-embedded tissues by PCR amplification

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Summary: A simple and rapid method for the extraction, reverse transcription and PCR amplification of RNA from formalin-fixed, paraffin-embedded tissue is described. The procedure can be completed within 24 hours. In a first application of this method we detect human albumin mRNA in liver tissue, demonstrating the feasibility to retrospectively analyze gene expression and RNA viruses in fixed tissue.

The polymerase chain reaction (PCR) has been successfully applied for the amplification and subsequent analysis of DNA from formalin-fixed, paraffin-embedded (FFPE) tissues (1) allowing both histological and molecular analysis on small amounts of tissue. However, the reverse transcription of RNA followed by PCR amplification (RNA-PCR) in FFPE tissues has proven more difficult, involving a five day RNA extraction procedure (2).

Here, we report a rapid and sensitive method to reproducibly amplify RNA from FFPE tissues. As a first application of this method, we demonstrate mRNA from the human albumin gene in 10/10 FFPE liver biopsies. The amplified cDNA could easily be distinguished from chromosomal DNA on the basis of intron splicing during mRNA processing. Possible biological and clinical applications of our method are discussed.

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Abbreviations: PCR, polymerase chain reaction; FFPE, formalin-fixed, paraffin-embedded.

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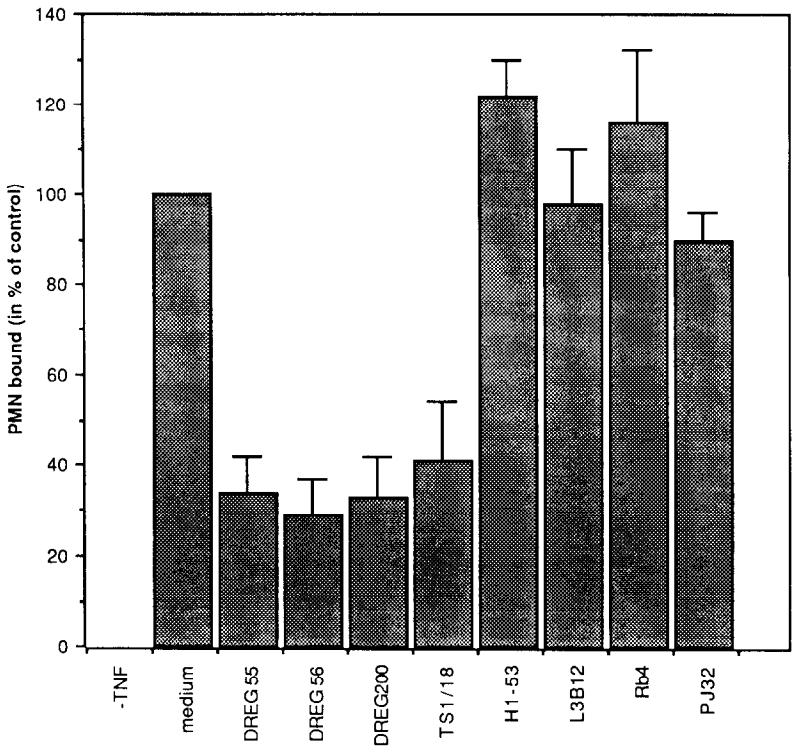

Fig.3. Adhesion of unactivated PMN to TNF-activated HUVEC at 7°C under shear (assay II) is inhibited by DREG antibodies and TS1/18 (antiCD18), while four isotype matched controls showed no inhibitory effect. Results are mean values ± SEM from 12 assays, derived from four independent experiments.

neutrophils (18 ± 2% of contact cells), even in the presence of DREG-56 and TS1/18, raises the possibility that additional cell adhesion molecules participate in neutrophil binding.

The results presented here are consistent with a important role for LECAM-1 in CD18-independent adhesion, and with the loss of this LECAM-1-based adhesion mechanism in association with shedding of LECAM-1 during neutrophil activation. Recent experiments suggest that the activated endothelium per se could signal the attached PMN (27,28), triggering the shedding of LECAM-1(29) and arresting the PMN through activation of the leukocyte integrins. The leukocyte integrins probably play an important role in subsequent diapedesis as well, since in vitro studies of neutrophil interactions with cytokine-activated endothelium demonstrate the importance of CD18-dependent mechanisms both in adhesion and subsequent transmural migration (6,21).

Furthermore, the findings demonstrate a common molecular basis underlying two previously uncompared models for studying leukocyte - endothelial cell interactions, the Stamper-Woodruff frozen section assay (24) of binding to venules in frozen sections, and the cultured HUVEC model. This common basis strongly supports the physiologic relevance of both systems.

Visualization of PCR products

After PCR amplification the samples were precipitated with isopropanol (see above), resuspended in 20 µl TAE-buffer and fractionated over a 1% or 1.5% agarose gel containing 1 µg/ml ethidium bromide. DNA was transferred to a nylon-membrane (Genescreen, New England Nuclear) and hybridized as described (5). A probe was prepared by cloning of the PCR product generated by primer pair 230/231 in the M13 vector (5) using Hind III and Bam HI as cloning sites. Probes were [^{32}P]-labeled by random priming (Amersham).

Results

We first determined the incubation time required to yield maximum amounts of RNA for analysis. Fig. 1 shows that the maximum extraction of RNA, as detected on agarose gel, is completed after only five hours of incubation at 60 °C. This makes it possible to complete the outlined procedure for RNA-PCR within 24 hours. There was no need to add RNA inhibitors to the lysis buffer under the conditions described (data not shown). By comparison, for maximum DNA yields an incubation time of 24 hours was required. This may reflect the sequential lysis of the cytoplasmic and nuclear cell compartments.

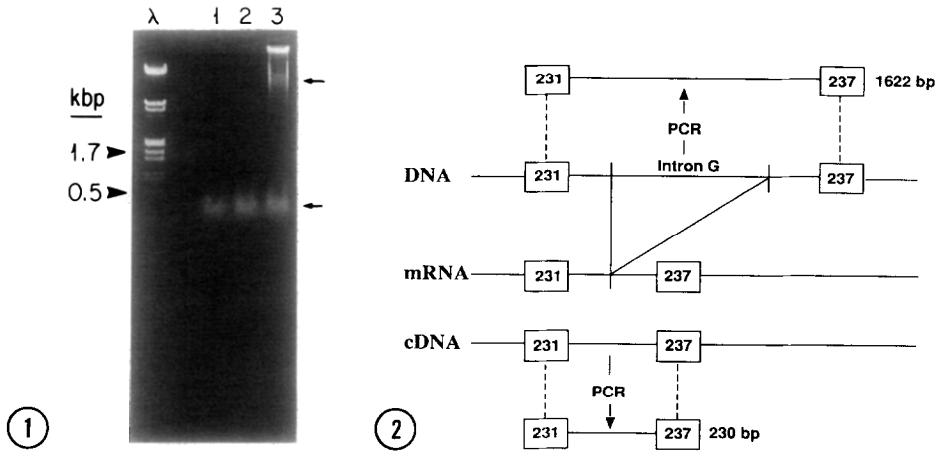

Fig. 1. Time dependent release of nucleic acids from FFPE human liver tissue. Lane **1**: 6 hrs extraction, lane **2**: 12 hrs extraction, lane **3**: 24 hrs extraction. Analysis by 1.2 % agarose gel elecrophoresis. Arrows on the right side indicate chromosomal DNA (upper arrow) and RNA (lower arrow) The identity of RNA and DNA were established by RNase or DNase treatment, respectively. λ: λ–DNA cut with Hind III and Eco RI.

Fig. 2. Positions of primers 231 and 237 (see Materials and Methods) relative to intron "G" of the human albumin gene. Upper part: amplification of chromosomal DNA. Lower part: amplification of cDNA.

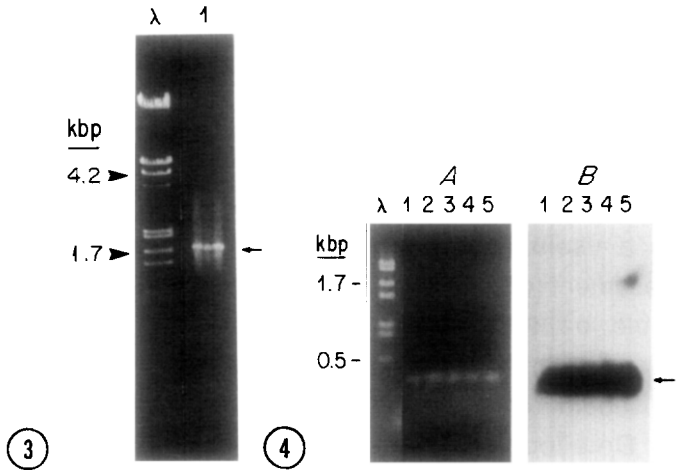

Fig. 3. PCR amplification product of chromosomal DNA using primer pair 231/237. Analysis by 1.2 % agarose gel electrophoresis. arrow on the right side indicates the position of the PCR product (lane **1**). λ: see Fig. 1.

Fig. 4. PCR amplification product of cDNA using primer pair 231/237. Lanes **1-5**: analysis of five different samples by 1.5 % agarose gel electrophoresis (***A***) and Southern blot hybridization (***B***). Arrow on the right side indicates the position of the PCR product. λ: see Fig. 1.

We then designed a set of primers that would allow the distinction between amplified cDNA and chromosomal DNA. As a model target sequence we chose mRNA encoded by the human albumin gene. As outlined in Fig. 2, the primer pair 231/237 was predicted to yield a 230 bp PCR product when cDNA is amplified and a 1622 bp product if chromosomal DNA is amplified. This difference in size is based on the expected splicing of intron "G" (4) during mRNA processing. Indeed, we found a 1622 bp product when no reverse transcription was performed (Fig. 3) and a 230 bp product when reverse transcription was performed prior to PCR amplification (Fig. 4A). To achieve a preferential amplification of cDNA sequences we chose a short extension time at 72°C for RNA-PCR-cycles. Under these conditions there was no 1622 bp band detectable in these samples. The specificity of the RNA-PCR products was confirmed by Southern blot analysis and hybridization to a human albumin specific DNA probe (Fig. 4B).

Discussion

Our data show that RNA can be reproducibly amplified from small amounts (1-2 mg) of FFPE human tissue within 24 hours. The ease of the described technique make it suitable not only for research studies but also for immediate clinical applications. Since FFPE human tissues are

routinely stored for many years, without affecting RNA-PCR (2), the method should allow systematic retrospective studies.

Numerous applications of our method are conceivable. For instance, the presence of RNA viruses, including HCV and HAV in liver tissue could be studied. For such studies the human albumin model presented here should provide a useful RNA-PCR control. Furthermore, the method should allow the assessment of gene expression using FFPE tissues. Particularly interesting would be the analysis of oncogene/ anti-oncogene mRNA to supplement histological tumor diagnosis. Structural changes in some of these mRNAs should render them readily distinguishable from their normal counterparts. Detailed structural information could be obtained by applying established methods such as restriction enzyme analysis (6) and direct sequencing (5) of the RNA-PCR product.

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