α -1 antitrypsin gene exon use in stimulated lymphocytes

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Abstract

Aims: To investigate the expression of mRNA transcripts containing exon A or B in lymphocyte cultures.

Methods: An in situ hybridisation technique, using synthetic, biotinylated oligonucleotide probes was deployed to allow the demonstration of exon A, exon B, or the normal hepatocyte message containing exon C.

Results: Lymphocytes used the same alternative splicing technique as monocytes in the generation of their α -1 antitrypsin message. They also provided data on the frequency of exon A and B expression in cells from different subjects. Most circulating granulocytes failed to show the α -1 antitrypsin message, suggesting that this protein is synthesised in the marrow and represents a stored protein component in polymorph and circulating nuclear lymphocytes.

Conclusions: In situ hybridisation is a sensitive technique for the detection of individual gene exon use in cell populations. Lymphocytes show the same promoter use as that described for monocytes.

Immunoblastic lymphoma of T cell type and enteropathy associated T cell lymphoma express α -1 antitrypsin and show BER H2 positivity (CD30), an activation associated antigen, in the tumour cells.¹ These malignancies represent T cell tumours in which the malignant population has a blast-like morphological appearance.² Experiments on peripheral blood T lymphocytes stimulated with Concanavalin-A have shown the presence of α -1 antitrypsin using immunohistochemical techniques in the stimulated lymphocyte population after 48 hours in culture. Stimulated lymphocytes also express CD30.¹ Staining was cytoplasmic and granular, with apparent staining of the Golgi region favouring synthesis by the lymphocytes rather than uptake from the medium. The demonstration of α -1 antitrypsin messenger RNA (mRNA) in lymphocytes would provide conclusive evidence for synthesis by activated cells.

In 1983 Rogers *et al* showed the presence of a 1.4 kilobase α -1 antitrypsin mRNA in both liver and leucocytes.³ Subsequently, Perlmutter *et al* showed α -1 antitrypsin mRNA in monocytes by dot-blot hybridisa-

tion, but were unable to show α -1 antitrypsin message in lymphocytes.⁴ These authors showed that monocytes contained a 1.4 kilobase α -1 antitrypsin message which was similar to that in hepatocytes, which had a greater abundance of α -1 antitrypsin message.⁴ Mornex *et al* also showed the presence of α -1 antitrypsin mRNA in monocytes and hepatocytes.⁵ Quantitative studies have also shown that monocytes contained 2-100fold less α -1 antitrypsin message than hepatocytes.

Recently, Long *et al* reported the complete sequence of complementary DNA for the human α -1 antitrypsin gene.⁶ The gene has five exons and four introns. Of more interest is the observation by Perlino *et al*,⁷ who described an additional two exons in the α -1 antitrypsin gene. These authors described a macrophage specific promoter located 2000 base pairs upstream from the hepatocyte specific promoter. Transcription from the two promoters is mutually exclusive. In hepatocytes α -1 antitrypsin mRNA is 1.4 kilobases. In macrophages mRNAs are generated by alternative splicing, giving a 1.6 and 1.9 kilobase message. There are, therefore, a total of seven exons in the α -1 antitrypsin gene, the first two being transcribed in macrophages and not in hepatocytes. Alternative splicing leads to either the A or A and B exons being transcribed in macrophages.

In addition to the demonstration of mRNA for α -1 antitrypsin in lymphocytes, we decided to address the question of exon A and B expression in lymphocytes and other cells derived from the bone marrow. In situ hybridisation not only permits morphological identification of the cell expressing the gene but also its detection even when only a small subpopulation of cells in the preparation show gene expression.

Methods

Monocytes and lymphocytes were separated from peripheral blood by centrifugation over ficoll and percol.^{8,9} Lymphocytes were cultured in RPMI with 10% fetal calf serum at 37°C in an atmosphere of 5% carbon dioxide in air. Concanavalin A was added at a concentration of 100 μ l/10 ml of culture. Granulocytes were separated by dextran sedimentation. The cells were cytocentrifuged on to slides coated with amino-alkylsialin. The slides were dried in air for one minute and then fixed in 4% paraformaldehyde for 10 minutes. The paraformaldehyde was dissolved in phosphate buffered saline overnight at 60°C and filtered before use.

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Probe 1 (50–1)

5' amino-link-TTGGTTCAGCTCAGGACTCTGAGGGTT
GCTGCGTGGAGGCAGTGAATTC 3'

Probe 2 (200–151)

5' amino-link-GCTCTCTCAAGCTCTCTCAAGCTCTG
TCTCTTCTGGAGGCACAGGAG 3'

Probe 3 (263–214)

5' amino-link-ATCTACCATTACTGAGTCACCCCAAAA
TGCCTGATGCTGAAGACTTACT 3'

Probe 4 (450–401)

5' amino-link-TTAAGCAGTGGATCCAGAGGGGCAACG
GGGGAGGCGAAAGAGTCATTGT 3'

Figure 1 Probe sequences.

Four oligonucleotide probes were synthesised from the complementary DNA sequence of exons A and B of the α -1 antitrypsin gene using an Applied Biosystems automated synthesis apparatus. Each probe was 50 base pairs in length. The probe sequences are given in fig 1. The four probes were used as a cocktail for in situ hybridisation. For the demonstration of alternative splicing the exon A probes—that is, number 1 and number 2—and the exon B probe—that is, number 3—were used separately (fig 2). Each probe was biotinylated at the 5' end by a biochemical reaction with biotinamido-caproate N-hydroxysuccinamide ester. The 3' end was enzymatically biotinylated with bio-11dUTP using terminal deoxynucleotidyl transferase (TdT). The length of the linker arm was optimised to ensure that reaction of the detection reagents with biotin was unaffected by steric hindrance. An 11 atom linker arm has been shown to be suitable for this purpose¹⁰ and was used in our system. Both sense, which have the same sequence as the α -1 antitrypsin mRNA, and anti-sense probes, which have a sequence complementary to α -1 antitrypsin mRNA (and thus hybridise to it), were

Figure 2 Structure of α -1 antitrypsin gene showing position of probes used. Hatched area represents exons expressed in monocytes.

prepared. The former acted as a control for the oligonucleotide hybridisation and the poly-d(T) probes were each 25–30 nucleotides in length and were obtained from Pharmacia (27-7986, 27-839-01). They were enzymatically labelled at the 3' end.

The specificity of the hybridisation reaction was confirmed with a number of parallel controls. The control without probe and the poly-d(A) control both show non-specific interactions with the detection reagents on cultured cells. The poly-d(T) control was a positive control to confirm the adequate preservation of RNA in the cell preparation via hybridisation to mRNA poly-d(A) tails. RNase digestion allowed the class of nucleic acid to which the probe had hybridised to be determined. Finally, the sense α -1 antitrypsin probe was used as a control for the specificity of the binding of the anti-sense probe to α -1 antitrypsin mRNA.

Before treatment with RNase slides were washed with $2 \times$ sodium citrate and sodium chloride (SSC) and distilled water treated with DEPC. The slides were hybridised for one hour at 37°C in a mixture containing 50% formamide, 600 mM sodium chloride, 50 mM TRIS-hydrochloric acid, 0.1% w/v sodium pyrophosphate, 0.2% w/v polyvinylpyrrolidone, 0.2% w/v ficoll, 5 mM EDTA, 150 μ g/ml sheared salmon sperm DNA and 10% polyethylene glycol. The hybridisation solution contained the probe at a concentration of 2 ng/ μ l. Hybridisation was carried out at 37°C overnight. Hybridisation was followed by one wash in $2 \times$ SSC and then three changes of $2 \times$ SSC with 50% formamide at 37°C for 30 minutes. This was followed by a $2 \times$ SSC wash for 10 minutes and then a brief wash in 0.1% Triton-X in TBS. The sections were then developed with a multi-step streptavidin/biotinylated alkaline phosphatase system. The use of a multi-step avidin/biotin complex method as a reporter system for in situ hybridisation gives results of very high sensitivity.¹¹ Streptavidin has a slightly acidic isoelectric point which precludes binding to charge groups seen with avidin. The alkaline phosphatase system offers a high degree of sensitivity.^{12,13}

Results

Granular, cytoplasmic staining with the α -1 antitrypsin anti-sense probe was seen in lymphocytes. Granular signal was seen in medium sized lymphocytes (fig 3). Monocytes present in the cytocentrifuge preparations acted as an internal positive control and showed granular cytoplasmic hybridisation reactions with the α -1 antitrypsin probes. The monocytes and lymphocytes did not react with the α -1 antitrypsin sense probe. The positive staining was abolished by RNase before hybridisation. The control without added probe and the poly-d(A) control were always negative; the poly-d(T) probe gave granular cytoplasmic signal in all lymphocytes and monocytes.

A small percentage of granulocytes showed cytoplasmic positivity with the α -1 antitrypsin

Figure 3 Peripheral blood lymphocytes stimulated with Con-A hybridised with the α -1 antitrypsin-antisense probe. Lymphocytes show a granular cytoplasmic staining (arrow).

probe (fig 4). The positive granulocytes were cells with trilobed nuclei. The α -1 antitrypsin sense (fig 5) and the poly-d(A) controls were unreactive. The poly-d(T) control stained all the cells in the preparation.

Figure 4 Peripheral blood granulocytes hybridised with α -1 antitrypsin-antisense probe. The granulocytes give a cytoplasmic reaction (arrow).

Figure 5 Peripheral blood granulocytes hybridised to α -1 antitrypsin sense probe. No reaction is seen in the granulocytes (arrow).

Monocytes were hybridised with probes for exon A and B. Eighty seven per cent showed exon B expression and 99% exon A expression. Twenty eight per cent of the lymphocytes expressed exon A and 14% exon B. In both cases the percentage of cells positive with the cocktail of four probes was the same as that with the exon A probe alone.

Discussion

The use of in situ hybridisation offers several advantages over other methods of nucleic acid detection. It has a greater relative sensitivity than methods that homogenise and extract nucleic acids. The latter may cause dilution of message in complex cell mixtures. Another advantage is that analysis of single cells requires minimal sample size and reagents.¹⁴ In situ hybridisation also permits morphological identification of the cell expressing the gene of interest.

The use of oligonucleotides allows probes unique to mRNA regions to be constructed. Synthetic oligonucleotides more easily penetrate cells but, as they hybridise only to small portions of the target sequence, they can lack sensitivity.¹⁴ This was, however, compensated for by simultaneous hybridisation with several oligonucleotides complementary to different sequences within the α -1 antitrypsin mRNA. The advantage of using a single-stranded probe is that there is no reannealing in the hybridisation solution to compete with hybridisation to

cellular RNA. Hybridisation therefore occurs with greater efficiency than with double-stranded probes and the hybrid formed is more stable.¹⁴ Biotinylated probes are stable for many months when stored at -20°C and provide excellent resolution in tissues and cells.

Using synthetic oligonucleotide probes against exons A and B, we have shown the presence of α -1 antitrypsin mRNA in stimulated lymphocytes. Monocytes in the same preparation acted as an internal control and also contained α -1 antitrypsin mRNA. It is clear, from the probe sequences used for hybridisation, that lymphocytes and granulocytes employ exons A and B as do monocytes and, thus, use the same promotor as the macrophage. The failure of Perlmutter *et al*⁴ and Mornex *et al*⁵ to show α -1 antitrypsin mRNA in lymphocytes may reflect the use of preparations of total cellular RNA in which α -1 antitrypsin mRNA may form only a small part and fall below the level of sensitivity of their detection system. The specificity of our hybridisation was confirmed by the results obtained with the parallel controls. Attempted hybridisation in the absence of specific probe and in the presence of biotinylated poly-d(A) probe always gave a negative result, excluding non-specific interaction of the detection system with the sample. RNase digestion abolished staining, showing that the probe hybridises to RNA in the sample. The poly-d(T) probe served as a positive control for the system. Negative staining with the sense α -1 antitrypsin probes confirmed that we were, in fact, demonstrating specific hybridisation to α -1 antitrypsin coding mRNA sequences.

The staining reaction seen in lymphocytes stimulated with Concanavalin A using in situ hybridisation for mRNA and antibody staining for α -1 antitrypsin protein was different.¹ With immunohistochemical techniques, large blast cells showed abundant α -1 antitrypsin protein. Following in situ hybridisation, the medium sized lymphocytes gave the most intense reaction for α -1 antitrypsin mRNA. These results indicate that the production of α -1 antitrypsin mRNA precedes the cytoplasmic demonstration of α -1 antitrypsin protein on lymphocyte activation. α -1 antitrypsin is stored in the cytoplasm while the α -1 antitrypsin mRNA undergoes rapid degradation, accounting for the difference in the reactions in the two preparations.

Only a small percentage of peripheral blood granulocytes had α -1 antitrypsin mRNA in the cytoplasm. The granulocytes with trilobed nuclei were those which showed a positive reaction. Immunohistochemistry shows abundant α -1 antitrypsin protein in the cytoplasm of granulocytes. We presume that this also reflects a difference in the timescale of α -1 antitrypsin mRNA and protein expression, an early stage in granulocyte maturation giving peak mRNA expression that is rapidly degraded. Granulocytes stain strongly with the poly-d(T) probe showing abundant total mRNA.

Monocytes have been shown to have alternative splicing of exons A and B of the α -1 antitrypsin gene.⁷ Exon B was only expressed in

a minority of monocytes in most individuals. In some subjects, however, exons A and B were expressed in equal proportions. Our data show that 87% of the monocytes expressing α -1 antitrypsin mRNA expressed exon B. In lymphocytes 50% of the cells expressing α -1 antitrypsin mRNA expressed exon B. Our data cannot be directly compared with those of Perlino *et al*⁷ as they identify α -1 antitrypsin mRNA by northern blot analysis using a full length 1400 base pair human liver cDNA α -1 antitrypsin probe. We used separate oligonucleotide probes for exons A and B to identify expression in individual cells.

The phenomenon of alternative splicing is well known. In the calcitonin/calcitonin gene related peptide (CGRP) gene alternative splicing results in the production of CGRP mRNA in neurones and calcitonin mRNA in thyroid C cells.¹⁵ Alternative splicing of the third exon of the T3 γ gene accounts for the production of two species of mRNA.¹⁶ A protein produced by translation of the shorter mRNA could lack a transmembrane region and might be secreted or associated with the outer surface of the cell.¹⁶ In B lymphocytes the shift from membrane bound IgM to secreted IgM following activation is encoded by separate mRNAs, generated by alternative splicing.¹⁷

In the α -1 antitrypsin gene the entire structural information for the protein is coded by the last four exons,¹⁸ which do not participate in alternative splicing. The changes in the 5' non-coding region may affect α -1 antitrypsin synthesis directly.¹⁹ On the basis of observations of other examples of alternative splicing, we propose that the two α -1 antitrypsin mRNAs have different coding capacity.⁷ They code for an identical molecule, but the larger transcript shows two short open reading frames, one contained in exon B and the other beginning towards the end of exon B and terminating in the third macrophage specific exon. This observation is reminiscent of the situation observed in the 5' flanking region of the genes coding for the human transferrin receptor²⁰ or yeast GCN4 regulatory protein.²¹ In the latter convincing evidence shows that short open reading frames are essential for translational repression. Our data seem to indicate that lymphocytes and granulocytes synthesise α -1 antitrypsin and use the same promotor as monocytes, leading to the expression of exons A and B. Two different, mutually exclusive promotors are, therefore, involved in the synthesis of α -1 antitrypsin in unrelated cell types—that is, hepatocytes, monocytes, and lymphocytes. The functional importance of alternative splicing in the α -1 antitrypsin gene is not yet known.

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