Localisation of lysozyme mRNA in rheumatoid synovial membrane by in situ hybridisation

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SUMMARY Type A synovial lining cells have been shown to contain lysozyme in their lysosomes. This might be phagocytosed because synovial fluid contains lysozyme originating from tissue macrophages and articular cartilage but in arthritides, in particular, from neutrophils. In situ hybridisation with ³⁵S labelled cDNA was used to detect mRNA for lysozyme over synovial lining in patients with rheumatoid arthritis. No hybridisation was found with lactoferrin cDNA, which was used as a negative control. Computer search against the EMBL gene bank (release 14) did not show any significant cross hybridisation to a known sequence. In cytological specimens ³⁵S-cDNA:mRNA hybrids were observed in positive but not in negative control cells. The presence of lysozyme and its mRNA suggests that type A synovial lining cells are of mononuclear phagocyte lineage.

Chronic synovitis is characterised by lining cell hyperplasia and villus formation often covered by a fibrin layer and containing areas of necrosis and extravasation.¹ Chronic inflammatory cell infiltrates, in particular, lymphocytes, have been a focus of research,^{2,3} although cells of the macrophage and fibroblast lineage are also present.⁴⁻⁷ In particular, the macrophage-like type A and fibroblast-like type B lining cells as well as intermediate type lining cells⁸⁻¹⁰ have been under scrutiny, also because of their role in pannus tissue formation.¹¹

From various animal and human studies it seems that type A synovial lining cells are peroxidase negative,¹² non-specific esterase positive¹³ Fc and C3b receptor carrying^{12,13} macrophages,¹⁴ which probably originate from the bone marrow.¹⁵

Recently, an interesting report giving further support to the mononuclear phagocyte origin of the type A lining cells was published. In an elegant immunoelectron microscopic study lysozyme (EC 3.2.1.17) was shown to be present in lysosomes of cells displaying macrophage-like ultramorphological

characteristics.¹⁶ This double identification seems quite convincing at first glance. Rheumatoid synovial fluid, however, contains high concentrations of lysozyme,¹⁷ which may originate from mononuclear phagocytes or articular cartilage,¹⁸ but probably is mainly derived from neutrophils. In rheumatoid effusions containing $25 \times 10^9/l$ neutrophils the breakdown in the synovial cavity may exceed one billion cells a day.¹⁹ Furthermore, synovial fluid neutrophils are very actively involved in the phagocytosis of, for example, immunoglobulin-rheumatoid factor complexes²⁰ and regurgitation into the extracellular environment during feeding will greatly contribute to high synovial fluid lysozyme concentrations. Therefore, it is quite possible that lysozyme, ultrastructurally localised in the lysosomes of macrophage-like type A synovial lining cells, has been phagocytosed from the synovial fluid. There are many examples of the way in which a phagocytosing cell may, in its lysosomes, contain immunoreactive substances phagocytosed but not synthesised by the cell. To take an example from the above, it has been shown that rheumatoid arthritis synovial fluid neutrophils with immunohistochemical staining often contain immunoglobulin, though they do not synthesise it.²⁰ Similarly

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macrophage-like type A lining cells might phagocytose synovial fluid lysozyme, though they would not synthesise it.

To further evaluate the question 'What are synoviocytes?', tentatively put forward by Revell and coworkers,²¹ we decided to extend their work and determine whether lysozyme in synovial lining is only phagocytosed or also synthesised in situ.

Patients and methods

PATIENTS

Synovial membrane biopsy samples obtained from three patients (mean age 54 years) fulfilling the 1987 revised American Rheumatism Association criteria for rheumatoid arthritis,²² and from two patients (mean age 41 years) with a traumatic joint lesion (meniscal cartilage rupture) were studied.

TISSUE PREPARATION AND FIXATION

The synovial membrane tissue samples were snap frozen in OCT compound (Lab-Tek, Naperville, IL) and stored at -20°C until sectioning on a cryostat. Sections were cut at $6\text{ }\mu\text{m}$ onto microscope slides coated with 0.5% gelatin, 0.05% $\text{CrK}(\text{SO}_4)_2$, and 0.02% diethylpyrocarbonate (RNase inhibitor; DEP; Sigma, St Louis, MO). To prevent RNase contamination gloves were worn when handling tissue and slides. Only RNase-free glassware and plasticware were used. They were treated with 0.02% DEP in water and then rinsed with distilled, deionised water before use. After air drying the cryostat sections were fixed in freshly prepared 3.5% neutral buffered paraformaldehyde containing 0.02% DEP for five minutes at room temperature. After fixation the slides were washed twice in $2\times\text{SSC}$ ($\text{SSC}=0.15\text{ M}$ sodium chloride, 0.015 M sodium citrate) for five minutes. To remove basic proteins, which may bind cDNA probes non-specifically, the sections were incubated in 0.2 M HCl for 20 minutes at room temperature after two five minute washes in $2\times\text{SSC}$. The sections were then dehydrated in 70%, 95%, and 100% ethanol, two minutes in each.

HYBRIDISATION PROBES

A recombinant plasmid, pLZM, containing a cDNA insert covering the coding sequence for the chicken egg white lysozyme was grown in *Escherichia coli* RRI cells and purified by standard procedures.²³ The cDNA_{LZM} insert was isolated from the vector DNA (pBR322) by digestion with Pst I restriction endonuclease (Boehringer Mannheim, West Germany) followed by preparative 4% polyacrylamide gel electrophoresis. The cDNA_{LZM} fragment (593 bp) was labelled with $\alpha\text{-}^{35}\text{S}$ -dCTP

and $\alpha\text{-}^{35}\text{S}$ -dTTP (Amersham International, UK) to a specific activity of $5\text{--}9\times 10^7\text{ cpm}/\mu\text{g}$ of DNA by a nick translation method.²⁴

A recombinant plasmid, pHLF1 containing a partial cDNA (990 bp) for the human lactoferrin²⁵ was prepared and labelled as described above. The specific activity of the ^{35}S labelled insert was $0.5\text{--}1\times 10^8\text{ cpm}/\mu\text{g}$ DNA.

PREHYBRIDISATION AND HYBRIDISATION BUFFERS

Prehybridisation was carried out in $100\text{ }\mu\text{m}$ TRIS-HCl (pH 7.5), 144 mM NaCl, 50% deionized formamide, Denhart's solution (0.02% Ficoll 400, 0.02% polyvinylpyrrolidone, and 0.02% bovine serum albumin), $7\text{ }\mu\text{M}$ EDTA, 0.05% yeast total RNA (type III), 0.005% t-RNA (type X), 0.05% herring sperm DNA, and 0.05% inorganic sodium pyrophosphate. Hybridisation was performed in the prehybridisation buffer, which was supplemented with 10% dextran sulphate (mol.wt 8000) and 0.005% polyadenylic acid.²⁶ The probe was heat denaturated at 95°C for 10 minutes and quickly cooled in ice.

PREHYBRIDISATION

The slides were incubated in prehybridisation buffer at room temperature for two hours, gently rinsed in $2\times\text{SSC}$, and air dried.

HYBRIDISATION

The hybridisation buffer was prepared so that each section received $3\text{--}5\times 10^4\text{ cpm}$ in $50\text{ }\mu\text{l}$ of buffer. Hybridisation was allowed to proceed in a sealed moist chamber at 37°C for 48 hours. After hybridisation the unhybridised probe was removed by rinsing two times, 10 minutes each, in $2\times\text{SSC}$ with 0.05% inorganic sodium pyrophosphate (NaPPi) at room temperature, followed by a wash in $0.5\times\text{SSC}$ with 0.05% NaPPi for 24–48 hours (two changes).

AUTORADIOGRAPHY

The dried slides were dipped in NTB2 nuclear track emulsion gel (Eastman Kodak Co, Rochester, NY) exposed in darkness for 12 days, developed for two minutes (Rodinal; Agfa-Gevaert, Leverkusen, FRG), fixed (Rapid Fix, Agfa-Gevaert), counterstained with haematoxylin, and mounted.

CONTROL EXPERIMENTS

The cDNA probe was from the nucleotide sequence encoding for chicken egg white lysozyme. The reactivity of the probe with mRNA for human LZM had therefore to be tested. Human peripheral blood leucocytes were cytocentrifuged onto microscope slides and hybridisation was performed as in the

Fig. 1 *In situ* hybridisation with the lysozyme ^{35}S -cDNA probe of normal human peripheral blood leucocytes. Autoradiography positive monocytes containing mRNA for lysozyme can be seen (thick arrows). Lymphocytes (small arrows) are not labelled. Note, that polymorphonuclear leucocytes, known to contain lysozyme at protein level, do not synthesise it and are thus not autoradiographically labelled (large arrows). This experiment indicates homology between hen egg lysozyme and human lysozyme to the extent that the cDNA probe against the former also hybridises with the mRNA of the latter. Haematoxylin counterstaining. Bar=20 μm .

tissue sections. In these experiments the chicken cDNA_{LZM} hybridised only to human peripheral blood monocytes, which are known to synthesise lysozyme. No hybridisation was seen in mature polymorphonuclear leucocytes or lymphocytes, which do not synthesise lysozyme (Fig. 1).

The ^{35}S -pBR322 fragment was used as a negative probe control in each hybridisation experiment.

Results

The ^{35}S -cDNA_{LZM}:mRNA hybrids were detected in

the positive but not in the negative control cells (Fig. 1). In rheumatoid synovial tissue the ^{35}S -cDNA_{LZM} probe localised in the lining cell layer (Figs 2 and 3) whereas the negative control ^{35}S -pBR322 and ^{35}S -cDNA_{LF} probe did not label any lining cell area (Fig. 4). A computer search²⁷ of the ^{35}S -cDNA_{LZM} nucleotide sequence against the known sequences in the EMBL gene bank (release 14) did not show any cross hybridisation which would favour false positive labelling. In between the areas containing lysozyme mRNA, however, long stretches of negative areas were seen. It is not known at present

Fig. 2 *In situ* hybridisation of rheumatoid synovial tissue. The ^{35}S -cDNA_{LZM}:mRNA hybrids are localised in some lining cells and in some mononuclear cells in synovial tissue stroma. From lysozyme expression at transcriptional level it seems that these cells represent macrophage-like type A lining cells (thick arrows) and tissue macrophages (thin arrows) respectively. Haematoxylin counterstaining. Bar=20 μm .

Fig. 3 A high power view of the sample shown in Fig. 2. Specific localisation of ^{35}S -cDNA_{LZM} probe over some but not all cells, indicating active local transcription of lysozyme gene at least three times that over the background (grain counts over positive cells). Bar=10 μm .

if this is caused by RNase mediated degradation of the target lysozyme mRNA or if there is local patchy—for example, cell cycle dependent—down regulation of lysozyme synthesis at transcriptional level. The last interpretation is lent some support by the ultrastructural observations of Mapp and Revell,¹⁶ which showed in some specimens, many empty cytoplasmic vacuoles not expressing lysozyme.

Discussion

Macrophage-like type A lining cells have many ultrastructural characteristics which distinguish them from fibroblast-like type B lining cells.^{8,9} In type A cells chromatin is condensed near the nuclear membrane in a horseshoe-shaped nucleus.²⁸ Thin but short pseudopodia are many.²⁸ Rough endoplasmic reticulum and Golgi complex are sparse or

Fig. 4 In situ hybridisation of rheumatoid synovial tissue. Lactoferrin ^{35}S -cDNA was used as a hybridisation probe. No rheumatoid synovial lining cells contained lactoferrin at the mRNA level. Bar=10 μm .

poorly developed, whereas vacuoles and lysosomes are numerous.²⁸ Recently, lysozyme has been identified at the ultrastructural level in the lysosomes of type A lining cells.^{16, 21} This lysosomal localisation may be due to phagocytosis of synovial fluid lysozyme.¹⁷ It has been shown clearly that phagocytosed material retains its immunoreactivity for a while until, probably, the intralysosomal degradation eventually causes a loss of immunoreactivity.²⁰ This seems to be particularly relevant in a continuous process such as lysozyme phagocytosis by macrophage-like type A lining cells. Lysozyme is present in synovial lining cells as shown by light^{29, 30} and electron microscopic^{16, 21} studies. Its mRNA was also detected by in situ hybridisation (Figs 2 and 3), which indicates that lysozyme in macrophage-like type A lining cells is synthesised locally at least partially. This is also supported by the observations of Mapp and Revell,¹⁶ because although preservation of the fine detail was somewhat variable, the vacuoles containing immunogold labelled lysosome appeared to be primary lysosomes.¹⁶ Unfortunately, the degree of preservation did not allow clear visualisation of components such as Golgi apparatus or rough endoplasmic reticulum, which would have given more direct evidence on synthesis and intracellular location of lysozyme.¹⁶ Lysozyme is a marker for mononuclear phagocytes and the presence of the protein and its mRNA clearly indicates that type A cells are of mononuclear phagocytic lineage.

Lysozyme (EC 3.2.1.17) is a highly conserved molecule, which is also shown by the homology and cross reaction of ³⁵S labelled hen lysozyme cDNA with the human mRNA counterpart. This has already been shown by the localisation of ³⁵S-cDNA:mRNA hybrids in human peripheral blood monocytes.³¹ As a negative control in the white LZM cell smears polymorphonuclear cells were not labelled because mature neutrophils do not actively synthesise lysozyme,^{32, 33} though lysozyme is located both in the primary or azurophil and secondary or specific granules of these cells.^{32, 34} False positive hybrid formation seems unlikely because an unrelated probe, human ³⁵S-cDNA_{LF}, did not localise in the rheumatoid synovial lining. Furthermore, the results of a computer search comparing the probe sequence with the nucleotide sequences available in the EMBL (release 14) gene bank indicate that cross hybridisation²⁷ is also unlikely. In situ hybridisation is a highly specific method of localising nucleotide markers in tissue sections because it is based on precision of the nucleotide base pair formation (A/T, A/U, and C/G) between the probe and the target nucleic acids.³⁵ Therefore, our study indicates that

lysozyme is at least partially synthesised locally in rheumatoid synovial lining. Together with the findings by Revell, Mapp, and coworkers, these results suggest that type A synovial lining cells belong to a mononuclear phagocytic lineage.

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