Distribution of cathepsin D in human polymorphonuclear and mononuclear blood cells

K. BARABASI and L. NÄSSBERGER

Department of Clinical Immunology and Medical Microbiology, Lund University, Lund, Sweden


We have investigated the distribution of cathepsin D in mononuclear and polymorphonuclear cells using an anti-rabbit human cathepsin D antibody and indirect immunofluorescence. Lymphocytes showed the same fluorescence intensity as control cells incubated with normal serum. Fluorescence staining patterns of polymorphonuclear cells differed according to the fixative used. Monocytes and granulocytes fixed in ethanol, methanol, glutaraldehyde, and Bouin's fixative caused a homogeneous cytoplasmic staining with a distinct nuclear sparing. A perinuclear staining was seen in acetone-fixed cells in both monocytes and granulocytes. Another interesting difference was observed between monocytes and granulocytes when formalin was used as fixative. Monocytes appeared with a cytoplasmic staining, but in formalin-fixed granulocytes redistribution to the nuclear membrane was seen.

Key words: Cathepsin D; polymorphonuclear cells; mononuclear cells; blood.

L. Nässberger, Department of Medical Microbiology and Clinical Immunology, Sölvegatan 23, S-223 62 Lund, Sweden.

Antibodies directed against neutrophil granulocyte components have become increasingly important in the diagnostic field of systemic vasculitis. For instance, Wegener's granulomatosis has been associated with a high frequency of classical ANCA (6, 12), nowadays designated c-ANCA according to the new nomenclature (14). On immunofluorescence, this antibody causes a clear cytoplasmic fluorescence pattern with nuclear sparing. However, antibodies directed to other granule proteins such as myeloperoxidase, lactoferrin (9), and elastase (5) cause a peripheral nuclear fluorescence staining, due to a redistribution of these proteins during ethanol fixation of granulocytes. The neutrophil granulocytes play an important role in inflammatory conditions, and release of lysosomal enzymes causes tissue damage (3). It has been shown that cathepsins are released during pathological conditions. High levels of cathepsin D have been measured in synovial fluid especially in rheumatoid arthritis and it has been found that cathepsin D may digest IgG (7). Furthermore, increased levels of cathepsin D have been measured in patients with systemic lupus erythematosus, progressive systemic sclerosis, and glomerulonephritis accompanied by nephrotic syndrome. The highest values were found in patients with systemic lupus erythematosus (8). Granulocytes harbour a large number of components, including several cathepsins. The aim of this study was to undertake an investigation of intracellular cathepsin D in mononuclear cells and redistribution of the enzyme during different fixation procedures in polymorphonuclear and mononuclear cells.

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MATERIALS AND METHODS

Chemicals
All chemicals used were of analytical purity.

Cell preparation
Polymorphonuclear and mononuclear blood cells from healthy subjects were isolated on polymorph gradient (Nycoderm A/S, Oslo, Norway). Briefly, blood was drawn into tubes containing heparin (12.5 IE/ml). Whole blood was layered onto the gradient and centrifuged at 450 g for 30 min. Polymorphonuclear and mononuclear cells are separated at different bands. The bands are pipetted off and the cells washed twice. Cells were prepared using ethanol-washed slides on a cytocentrifuge (Shandon, England) at 1000 rpm for 5 min, followed by immediate fixation.

Anti-cathepsin D antibody
A polyclonal antibody against human cathepsin D, a kind gift from Dr. B. Johansson, Dako A/S, Glostrup, Denmark, was raised in rabbit. The second antibody was an anti-rabbit IgG raised in swine and FITC-conjugated.

Experimental design
After pilot studies we decided to use the following dilutions: 1:100 for anti-cathepsin D and 1:200 for the second antibody.

Mononuclear cells
Lymphocytes were only fixed in ethanol for 5 min at 4°C. Monocytes were fixed in ethanol, methanol, acetone, formalin 1% and 5%, glutaraldehyde 1.5%, and Bouin's fixative for 5–10 min at 4°C. After washing (×3) with phosphate-buffered saline (PBS), pH 7.2, cells were incubated with rabbit anti-human cathepsin D 1/100 for 30 min. Washing was performed three times in PBS. Cells were thereafter incubated with swine anti-rabbit IgG conjugated with FITC (Dakopatts A/S, Glostrup, Denmark) diluted 1:200 for 30 min. After washing (× 3) in PBS the slides were mounted.

Neutrophil granulocytes
Neutrophil granulocytes were fixed in the same fixatives as mononuclear cells. Incubations with the primary and secondary antibodies were carried out as described above.

HL-60 cells
In order to investigate the presence of cathepsin D in the promyelocyte cell line, unfixed as well as HL-60 cells fixed in ethanol and formalin 5% were examined.

Normal serum
In control experiments, normal sera diluted 1–100 were incubated instead of the primary antibody.

RESULTS

Lymphocytes
The fluorescence intensity observed resembled that seen in control cells.

Monocytes
Ethanol- and methanol-fixed cells showed a homogeneous cytoplasmic fluorescence with a clear nuclear sparing (Fig. 1). The same staining pattern was seen for monocytes fixed with formalin, glutaraldehyde, and Bouin's fixative. Cells fixed in acetone, however, showed an accentuated perinuclear fluorescence staining.

Neutrophil granulocytes
Granulocytes fixed in ethanol or methanol showed a homogeneous cytoplasmic pattern with nuclear sparing (Fig. 2). The same pattern was seen in neutrophils fixed in Bouin's fixative or glutaraldehyde. Acetone-fixed cells appeared with an accentuated perinuclear fluorescence staining.

When fixation was carried out with formalin 1% or 5%, a very marked redistribution to the nuclear membrane was observed (Fig. 3).

C-ANCA-pattern
Neutrophil granulocytes fixed in ethanol were incubated with a positive control c-ANCA serum, titre 1/1200. The serum was obtained from a patient with classical Wegener's granulomatosis. The cells were incubated in a 1:100 dilution for 30 min and afterwards washed (× 3) in phosphate-buffered saline, pH 7.2. A rabbit anti-human IgG F(ab')2, conjugated with FITC was then incubated for 30 min. The slides were mounted after washing with phosphate-buffered saline (× 3).

Microscopy
Fluorescence microscopy was performed using a Zeiss Axioskop. Photography was carried out with Ektachrome 800 (Kodak).
Fig. 1. Shows a monocyte fixed in ethanol for 5 min at 4°C. A homogeneous cytoplasmic staining with clear nuclear sparing is seen. Magnification x 760, oil immersion.

Fig. 2. Neutrophil granulocytes fixed in ethanol for 5 min at 4°C. A homogeneous cytoplasmic staining with sparing of the nuclear segments is seen. Magnification x 760, oil immersion.

Fig. 3. Neutrophil granulocytes fixed in formalin 1% for 5 min at 4°C. A marked fluorescence of the nuclear membrane and a weak cytoplasmic staining are demonstrated, indicating a redistribution. The same pattern was seen with formalin 5%. Magnification x 760, oil immersion.

Fig. 4. Neutrophil granulocytes fixed in glutaraldehyde cause a distinct homogeneous cytoplasmic staining. Magnification x 760, oil immersion.

Fig. 5. Antibodies against myeloperoxidase cause an intensive nuclear membrane staining on ethanol-fixed neutrophil granulocytes.

Fig. 6. The c-ANCA serum causes a homogeneous cytoplasmic staining pattern with nuclear sparing on ethanol-fixed neutrophil granulocytes.
Glutaraldehyde-fixed neutrophils, on the other hand, presented with distinct homogeneous cytoplasmic staining (Fig. 4).

**HL-60 cells.**

Ethanol-fixed cells showed a strong cytoplasmic fluorescence. Unfixed as well as formalin-fixed cells showed a weaker cytoplasmic staining compared to the ethanol-fixed cells.

**p-ANCA (anti-myeloperoxidase antibody)**

As can be seen in Fig. 5, antibodies against myeloperoxidase cause an intensive peripheral nuclear staining pattern.

**c-ANCA**

The c-ANCA serum from the patient with Wegener's granulomatosis causes a homogeneous cytoplasmic staining with nuclear sparing (Fig. 6).

**DISCUSSION**

Cathepsin D, an acidic lysosomal protease with a molecular weight around 46 kDa, has been demonstrated in several cells. It is of major interest for the diagnostic field of breast cancer (10). Breast cancer cells, unlike normal cells, contain high levels of cathepsin D (2).

Cathepsin D, like many other lysosomal enzymes, may play a role in immunologically mediated disease. It has been found to be responsible for the degradation of interleukin-2. Blocking of cathepsin D activity causes prolongation of serum IL-2 T1/2 and augmentation of lymphokine-activated killer cell activity (7).

It has also been suggested the cathepsin D plays an important role in the processing of antigens, showing a strong preference for peptide bonds, which is indeed an essential step in their recognition by T cells (11).

Further supporting evidence came from another study where high cathepsin D activity in macrophages was demonstrated (4). The macrophage is one of the most important antigen-presenting cells. These authors also found a high activity in monocytes, which is in accordance with our findings from an immunofluorescence viewpoint. We were, however, not able to detect cathepsin D in lymphocytes. Even if lymphocytes are lacking in lysosomes, we cannot rule out the presence of cathepsin D, but then the levels are below those required for detection with our immunofluorescence technique. On the other hand, high levels are seen in circulating neutrophils, as well as in monocytes. Alcohol-fixed neutrophils caused a cytoplasmic staining pattern, which means that cathepsin D does not undergo redistribution due to alcohol fixation. These findings can be of interest also from the point of view of laboratory diagnosis. During recent years human granulocytes have been used as a substrate for detecting antineutrophil cytoplasmic antibodies (ANCA) in systemic vasculitic disorders (6, 12).

Autoantibodies occurring in patients with vasculitis usually cause two major staining patterns, named c-ANCA and p-ANCA according to the new nomenclature (14). The c-ANCA pattern corresponds to a cytoplasmic staining with nuclear sparing, similar to that found for cathepsin D in alcohol-fixed granulocytes. The corresponding autoantigen to c-ANCA is proteinase-3 (PR-3). The reason why these proteins are not redistributed during alcohol fixation may be an intense membrane binding. One remarkable finding was that formalin caused a redistribution of cathepsin D to the nuclear membrane. This is not the case for proteinase-3. Formalin may in some way destabilize cathepsin D without changing its antigenicity. Furthermore, it was puzzling to find that no such redistribution was seen in monocytes. This may suggest that there are differences between synthesized cathepsin D in granulocytes and monocytes. A study by Levy and co-workers (4) provides support for such an assumption. They found that cathepsin D derived from monocytes was completely inhibited by pepstatin, whilst cathepsin D from neutrophils was only partially inhibited by pepstatin A.

At present there is no evidence of circulating autoantibodies directed against cathepsin D. If this proves to be the case and furthermore has any clinical significance, it will be difficult to use neutrophil granulocytes as a substrate for screening of c-ANCA.

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