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Immunogold localisation of ubiquitin-protein conjugates in primary (azurophilic) granules of polymorphonuclear neutrophils

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Ubiquitin-protein conjugates are found in the primary (azurophilic) lysome-related granules but not in the secondary (specific) granules in mature polymorphonuclear neutrophils prepared from bone marrow. This is the first reported demonstration of ubiquitin-protein conjugates in lysosome-related membrane-bound vesicles in granulocytes and complements our previous findings of ubiquitinated proteins in lysosomes of fibroblasts. The significance of the selective presence of conjugates in only one of the two main types of neutrophil granules remains to be elucidated but may relate to the presence of the complement of acid hydrolases, including proteases, in the azurophilic granules compared to the specific granules. Ubiquitin-protein conjugates may enter the primary granules during neutrophil maturation by an autophagic process or by a heterophagic process during the fusion of phagosomes with primary granules. Alternatively protein ubiquitination may be involved in granule biogenesis.

Ubiquitin; Azurophilic granule; Lysosome; Polymorphonuclear neutrophil

1. INTRODUCTION

Protein ubiquitination appears to take place for a variety of purposes but has been most studied as a post-translational protein modification required for rapid extralysosomal ATP-dependent protein degradation (reviewed in [1]). Recently there have been reports which show that free ubiquitin is present in lysosomes in hepatoma cells [2] and ubiquitin-protein conjugates are found in the lysosomes of fibroblasts [3,4]. Ubiquitin-protein conjugates are considerably enriched (12-fold) in the lysosomes of normal fibroblasts [4] implying that protein ubiquitination may have some role in lysosome function.

We are carrying out a study on the role of ubiquitin in lysosomal function in maturing bone marrow neutrophils. In the course of this work we have been able to show by immunogold electron microscopy that ubiquitin-protein conjugates are concentrated in the primary (azurophilic) granules but not in the secondary (specific) granules in polymorphonuclear neutrophils. The results show that ubiquitin-protein conjugates are specifically located in the lysosome-related azurophilic granules (i.e. containing acid hydrolases) of these phagocytic cells.

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2. MATERIALS AND METHODS

White blood cells were isolated from rabbit bone marrow by Percoll gradient fractionation [5]. Femoral marrow was scraped out from the bone and placed in disaggregation buffer (0.3 M lactose, containing 14 mM thio-ethanol, 2 mM EDTA, and 150 mM NaCl, pH 8). The cells were disaggregated by repeated pipetting, centrifuged at 150 × g for 5 min., washed by centrifugation with disaggregation buffer and resuspended in phosphate buffered saline (150 mM NaCl containing 20 mM sodium phosphate, pH 7.4). Cells were fixed for electron microscopy in 2% v/v paraformaldehyde/0.5% glutaraldehyde containing 1% w/v sucrose and 2 mM $CaCl_2$. After fixation the specimens were dehydrated in graded ethanol and embedded in araldite (TAAB Labs., Reading, UK) Immunogold labelling was carried out by a post-embedding biotin-antibiotin bridge method using affinity-purified rabbit antibody to ubiquitin-protein conjugates [6,7] at a dilution of 1:20 followed by biotinylated goat antibody to rabbit IgG (Vector Labs., Peterborough, UK; 1:100 dilution) and antibiotin-gold (10 nm, Bio-Rad, Hemel Hempstead, UK; 1:75 dilution). Immunostained sections were post-fixed with 1% w/v OsO_4 and counterstained with uranyl acetate and lead citrate.

3. RESULTS AND DISCUSSION

Fig. 1 shows a typical polymorphonuclear neutrophil from rabbit bone marrow with multilobate nucleus (N), primary granules (PG) and secondary granules (specific granules, SG). The secondary granules outnumber the primary granules by at least 2:1; this, together with the facts that the cytoplasm is virtually devoid of rough endoplasmic reticulum and that both the primary and secondary granules have a somewhat elongated appearance, indicates that the cell is a relatively mature neutrophil [8,9,12].

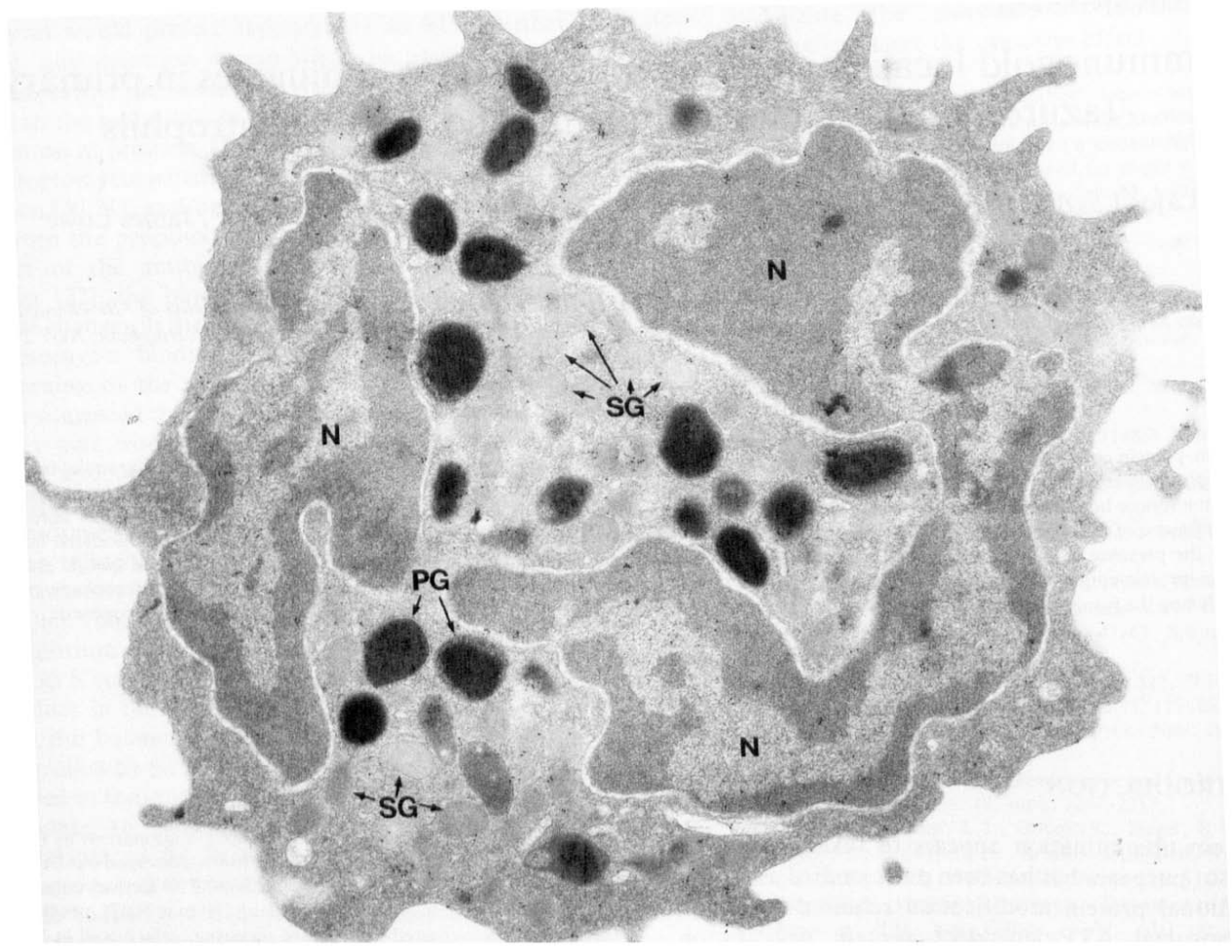

Fig. 1. Ubiquitin-protein conjugates detected by immunogold electron microscopy of polymorphonuclear neutrophils. Gold particles (10 nm), indicating the presence of ubiquitin-protein conjugates, are detected in primary (azurophilic) granules (PG). The nucleus is indicated by (N) and the secondary granules by (SG). Fig. 1 illustrates a typical polymorphonuclear neutrophil at 34 500 magnification.

The results in Fig. 1 show that the dense primary (azurophilic) granules in a polymorphonuclear neutrophil contain large numbers of gold particles detected by the affinity-purified antibodies to ubiquitin-protein conjugates. The antiserum has a marked preference for ubiquitinated proteins rather than free ubiquitin, immunohistochemically and on Western blots [6,7]. The antibodies have previously been used to show ubiquitinated proteins in the lysosomes of fibroblasts [4,10] as well as ubiquitin-protein conjugates in intracellular inclusions in a family of ubiquitin-filament chronic degenerative diseases, particularly neurodegenerative diseases [11]. In contrast to the primary granules, there is little or no immunostaining of ubiquitinated proteins in the secondary (specific) granules.

The higher power magnification (Fig. 2) again clearly shows that the gold particles corresponding to the ubiquitin-protein conjugates are concentrated over the dense azurophilic granules. A background immunostaining is seen over the nucleus (N) and in the cytoplasm including the secondary granules (SG). The primnary azurophilic granule marked (X) close to the cell surface seems to be either extending a process or has recently undergone a fusion process.

The primary (azurophilic) granules are synthesised first in granulocytopoiesis. They contain a battery of cathepsins (B, D and G), together with other enzymes which include β-glucuronidase, β-glucosaminidase, β-galactosidase, β-glycerophosphatase, aryl sulphatase, a myeloperoxidase and 'defensins' [12]. The combined complement of enzymes and proteins is involved in the destruction of microorganisms which enter the cells by phagocytosis. The granules share many molecular components in common with the classical lysosomes seen in other cell types. Therefore the finding of gold particles corresponding to ubiquitinated proteins in the primary granules supports and extends our previous findings of ubiquitinated proteins in the lysosomes of fibroblasts [3,4]. Ubiquitinated proteins appear to be common if not essential components of lysosomes in eukaryotic

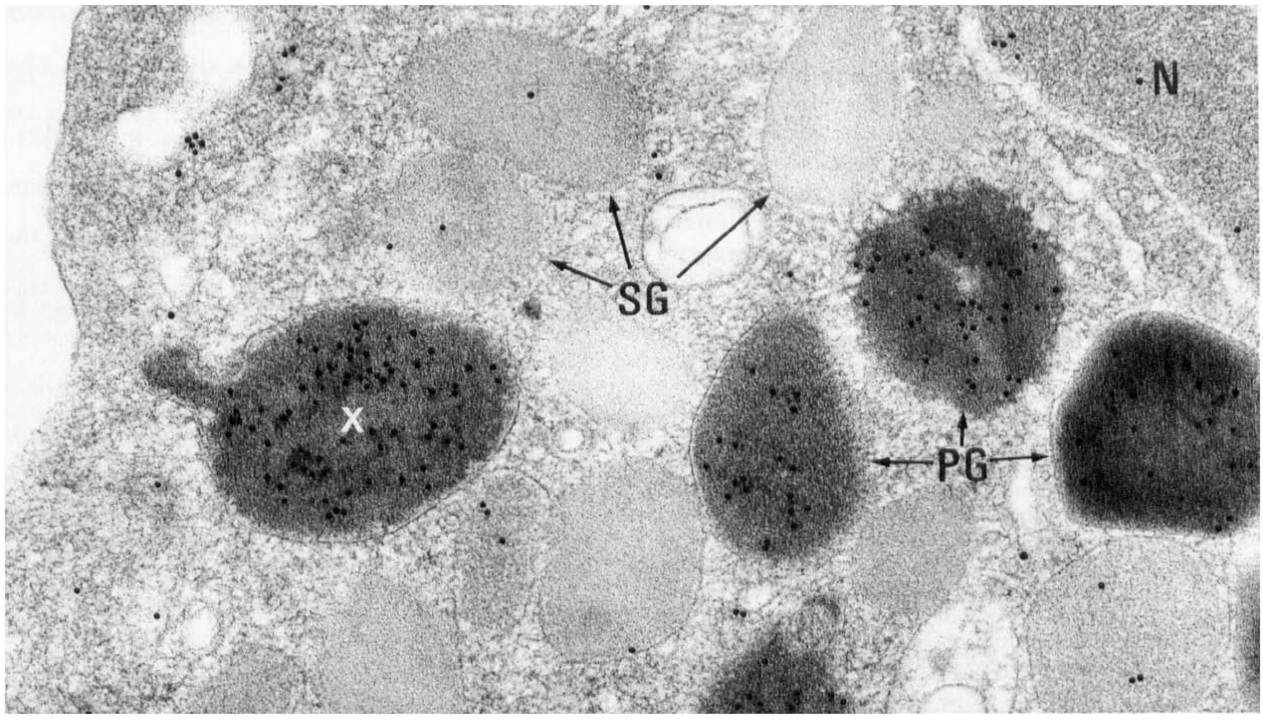

Fig. 2. Electron micrograph showing a higher power magnification (108 800) to demonstrate the specific concentration of gold particles in the azurophilic dense granules. The symbols are the same as in Fig. 1. X marks a primary (azurophilic) granule close to the cell surface.

cells. This conclusion is further supported by our demonstration of ubiquitinated proteins in lysosomes which also contain the latent membrane protein of Epstein-Barr virus (EBV) in EBV transformed lymphoblastoid cells (unpublished observations).

The secondary (specific) granules do not contain gold particles corresponding to ubiquitin-protein conjugates in excess of the general background level. These granules are synthesised later in granulocytopoiesis and come to exceed the primary granules by approximately 2:1 in mature neutrophils (e.g. as in Fig. 1). The secondary granules contain many proteins including lysozyme, lactoferrin, collagenase, plasminogen activator and a complement activating enzyme [12]. The contents of the secondary granules are secreted following neutrophil emigration in inflammation.

Although the acid hydrolases of the azurophilic granules are delivered by fusion into the phagosomes containing the engulfed microorganisms they may be secreted and cause tissue destruction in inflammatory reactions. Such a process may deliver uniquitinated proteins or protein fragments into the circulation which may result in immune responses to the ubiquitinated peptides. This certainly occurs in systemic lupus erythematosus although antibody production is probably through a different mechanism [13,14].

The specific finding of ubiquitin-protein conjugates in the granule type related to lysosomes in mature neutrophils begs the question of how the ubiquitinated proteins enter this intracellular compartment. Mature neutrophils are a non-dividing cell type ostensibly concerned with the phagocytosis and killing of microorganisms or mediating tissue repair in the inflammatory response. We have previously proposed that ubiquitinated proteins may enter the lysosomes of fibroblasts by microautophagy: protein ubiquitination may be one of the signals for protein uptake into the lysososmal system [3,4]. Recently the importance of the ubiquitin-mediated metabolic pathway in the lysosomal degradation of long-lived proteins was emphasised by the demonstration that the ubiquitin-activating enzyme E_1 is essential for the degradation of proteins by the lysosomal system after cell stress and during cell starvation [15].

Neutrophil primary granules may serve an autophagic function during cell development, i.e. from the promyelocyte to the metamyelocyte stages, which is subsequently lost, since there is evidence for microautophagy or macroautophagy in mature neutrophils. Such an explanation suggests that intralysosomal ubiquitinated proteins may be very stable, perhaps surviving the lifetime of the cell which is approximately 15–16 days [12]. A second explanation is that protein ubiquitination is involved in granule biogenesis. Such an explanation must account for the fact that both primary and secondary granules seem to be formed by budding from the Golgi apparatus [12] and would imply a selective partition of ubiquitinated proteins in granule biogenesis. A third explanation is that primary granules may take up extracellular ubi-

quitin-protein conjugates when acid hydrolase containing vesicles from the Golgi fuse with heterophagosomes to form secondary lysomes. This notion implies that there should be a cell surface receptor for ubiquitin-protein conjugates since the extracellular concentration of such conjugates may be very low. Finally the ubiquitin-protein conjugates in the primary granules may be derived from surface receptors internalised during phagocytosis since some surface receptors are known to be ubiquitinated [16].

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