Biol. Chem., Vol. 383, pp. 1277 – 1283, July / August 2002 ·

Short Communication

Modulation of the Endosomal and Lysosomal Distribution of Cathepsins B, L and S in Human Monocytes/Macrophages*

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* Dedicated to Professor Burkhard Bültmann on the occasion of his 60th birthday
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Endosomal and lysosomal fractions of human monocytes/macrophages prepared from buffy coats were analyzed for activities of cathepsins B, L and S, and expression of cathepsin proteins along with major histocompatibility complex class I and class II molecules under control and immunomodulatory conditions. While the total activity of cathepsins B, L, and S together remained unchanged in lysates of control cells during culture for 72 h, the subcellular distribution of cathepsin activities underwent a shift from a predominantly endosomal localization in freshly isolated cells to a lysosomal pattern after 72 h of culture. Interferon-γ treatment for 72 h resulted in an upregulation of both major histocompatibility complex proteins and cathepsins with differential changes in cathepsin B, L and S activities in endosomes *versus* lysosomes. These changes suggest a remodeling of the endocytic machinery and imply different functions of cathepsins B, L and S during monocyte differentiation.

Key words: Antigen presentation / Enzyme secretion / Limited proteolysis / Major histocompatibility complex class II / Maturation / Phagocytosis.

Monocytes/macrophages represent a primary line in the host defense against infectious agents. They display a variety of functions that are a prerequisite for the initiation of specific immune responses, such as phagocytosis and antigen presentation.

Macromolecules internalized by phagocytosis or endocytosis are degraded by hydrolases within the large endosomal/lysosomal apparatus of phagocytes. The bulk of such proteolytic activities is catalyzed by various cathepsins (Bohley and Seglen, 1992). In contrast to lysosomal proteolysis, prelysosomal proteolysis is considered to be more controlled according to the cellular requirements (Berg *et al.*, 1995; Authier *et al.*, 1996), such as limited proteolysis of complex antigens into antigenic peptides (Fineschi and Miller, 1997). In endosomal compartments of antigen-presenting cells major histocompatibility complex (MHC) class II molecules acquire antigenic peptides (Neefjes, 1999) after removal of the invariant chain through the catalysis of cathepsin S (Riese *et al.*, 1996) and HLA DM (Miyazaki *et al.*, 1996). This implies that at least cathepsin S and MHC class II molecules are colocalized at this first stage of antigen presentation (Villadangos *et al.*, 1999). Considerably less is known about processing of antigens by endosomal/lysosomal proteinases which appears to depend on the type of antigen-presenting cell as well as on the proteins to be cleaved (Nakagawa and Rudensky, 1999; Beck *et al.*, 2001). The exact intracellular localization of processing enzymes, such as cathepsins (cat) B, L and S, is also a matter of debate. Their expression varies in different types of antigen-presenting cells. All three enzymes are present in mouse macrophages (Nakagawa and Rudensky, 1999). Different endosomal/lysosomal localization of various hydrolases, including cat B and L, has been found in murine J774 macrophages (Claus *et al.*, 1998). These enzymes serve different functions along the endosomal/lysosomal pathway.

The functional state of monocytes varies during their maturation to macrophages and under the influence of cytokines. Interferon-γ (IFN-γ) has been reported to increase the activities of cat B and L in cell lines derived from human primary monocytes/macrophages (Lah *et al.*, 1995; Li *et al.*, 1997). However, the distribution of the activities of cat B, L and S between endosomal and lysosomal compartments of human primary monocytes/macrophages is not known. Here the subcellular distribution of cat B, L and S was analyzed in freshly isolated hu-

man monocytes as well as in cultured monocytes under the influence of IFN-γ. Enzymatically active cathepsins were assessed by active site-directed labeling as well as by determination of specific activities.

The monocyte preparation obtained by a combination of centrifugations with density and continuous gradients consisted of 60–80% monocytes. The majority of the remaining cells are T lymphocytes as inferred from FACS analysis (not shown). Subcellular fractionation of freshly isolated monocytes by differential centrifugation followed by hypotonic lysis of the mitochondrial/endosomal/lysosomal fraction (Schröter *et al.*, 1999) resulted in a particular endosomal/mitochondrial fraction and a soluble lysosomal fraction. The endosomal fraction was highly enriched in the activities of cat B, L and S (factors 11.5; 13.2 and 96.9, respectively) compared to whole cell lysates, while in the lysosomal fraction the enzymes were less concentrated by factors of 2.2, 7.1 and 3.7, respectively (Figure 1). In both fractions the ratio between cat B, L and S activities differed considerably from that in whole cell lysates. In the endosomal fraction, cat S activity contributed to 4% of total cat B+L+S activity, while cat L activity showed a relative amount of 8%, similar to whole cell lysates. In the lysosomal fraction the contribution of

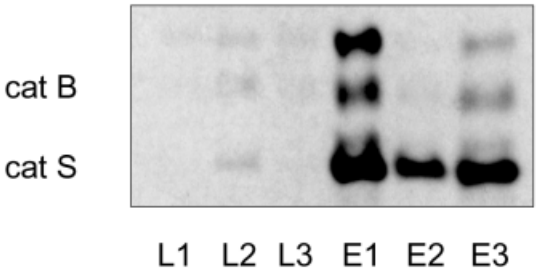

Fig. 2 Cat B and cat S in Endosomal (E) and Lysosomal (L) Fractions from Three Individual Preparations of Freshly Isolated Monocytes Visualized by Active Site-Directed Labeling.
Nine µg of each protein fraction (see Figure 1) were subjected to active site-directed labeling of cysteine proteinases with biotinylated JPM-565 (kindly provided by A.-M. Lennon-Duménil, Boston, MA, USA) (Bogyo *et al.*, 2000) followed by SDS-PAGE (12.5%) and visualization using the horseradish peroxidase/ECL system (Amersham-Pharmacia, Uppsala, Sweden). Cat B and cat S were identified immunochemically (not shown) by reprobing the blot membrane with polyclonal rabbit antisera against cat B and cat S (generated by E. Weber, Halle, Germany) after removal of the streptavidin horseradish peroxidase conjugate with 20 mM Tris pH 6.7, 2% SDS and 100 mM mercaptoethanol. The cat anti-cat complexes were also visualized by luminescence detection.

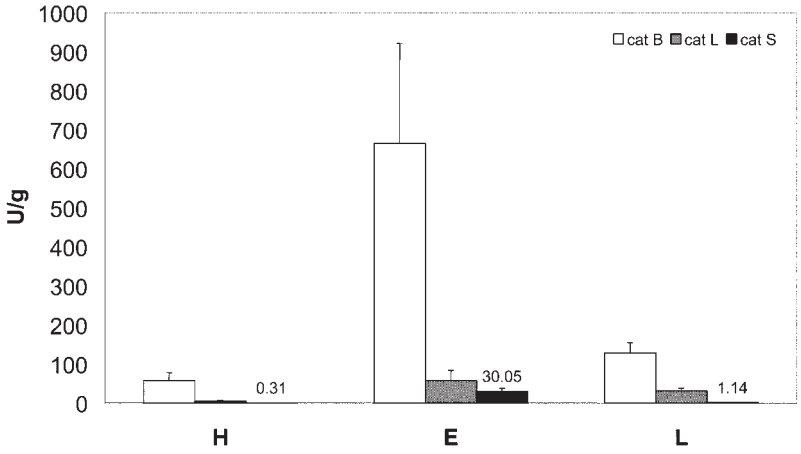

Fig. 1 Specific Activities of cat B, L and S in Whole Cell Lysates (H), Endosomal (E) and Lysosomal (L) Fractions of Freshly Isolated Monocytes.
Data are given in U/g protein; mean ± SD, n=4. Human monocytes were enriched from two buffy coats using density gradient centrifugation with ficoll-hypaque (Boyum, 1968) and subsequent purification on a continuous percoll gradient (Gmelig-Meyling and Waldmann, 1980). The preparation consisted of 60–80% monocytes as detected by FACS analysis with anti-CD 14 and anti-HLA DR (Becton-Dickinson, Mountain View, CA, USA).
Freshly isolated monocytes (~4 × 10^8 cells) were immediately homogenized for subsequent subcellular fractionation according to Schröter *et al.* (1999). The mitochondrial/endosomal/lysosomal fraction obtained by differential centrifugation of cell homogenates was separated into mitochondrial/endosomal and lysosomal fractions by hypotonic lysis of the lysosomes with water (15-fold volume of the pellet volume). Lysates of whole cell homogenates (H) and mitochondria/endosomes (E) were prepared by addition of water at 4 °C in addition to freezing and thawing.
Activities of cat B, L and S (µmol/min·g protein=U/g) were determined fluorometrically (Schmid *et al.*, 1997) using 0.5 mM Z-Phe-Arg-AMC as the common substrate. Total cat B+L+S activity was inhibited by 10 µM E-64. Cat B activity was obtained as the fraction of total cat B+L+S activity at pH 5.0 that is inhibited by 10 µM CA-074 (Bachem, Heidelberg, Germany; Towatari *et al.*, 1991). The residual activity inhibited by 0.5 µM Z-Phe-Phe-CHN_2 was attributed to cat L activity. Cat S activity, which was determined at pH 7.5 after inactivation of cat B and L activities at pH 7.5 and 37 °C for 1 h, was inhibited by 5 nM LHVS (kindly provided by H. Ploegh, Boston, MA, USA) (Riese *et al.*, 1996).

cat L activity amounted to 22% of total cat B+L+S activity, while that of cat S activity remained nearly as low as in whole cell lysates with 0.8%. The high endosomal enrichment of cat B and S was confirmed by active site-directed labeling of the enzymes with JPM-565 (Bogyo *et al.*, 2000; Figure 2). This method is considered to detect several active cysteine proteinases. The identity of only cat B and cat S was confirmed immunochemically on the same blotting membrane (not shown). The amount of active cat S relative to that of active cat B visualized by this method optically exceeds that obtained by measuring specific catalytic activities.

In order to follow cat B, L and S activities in correlation to the expression of MHC class I and MHC class II molecules under immunomodulatory conditions, monocytes were cultured for 0–4 days in serum-free medium with IFN-γ (100 U/ml). Crude organellar fractions containing mitochondria, endosomes and lysosomes were prepared daily for active site-directed labeling of cat with JPM-565 followed by Western blotting for the detection of cat and MHC molecules (Figure 3). Under control conditions, the levels of active cat B and S (Figure 3A) as well as cat S protein (Figure 3B) remained largely unchanged during culture compared to freshly isolated monocytes (day 0). In parallel, the amount of MHC class I molecules also remained unchanged in control cells, while class II molecules were slightly upregulated upon culture (Figure 3C). Under the influence of IFN-γ the amount of active enzymes was substantially increased at days 2, 3 and 4 (Figure 3A). This was corroborated by the immunochemical detection of an increased amount of cat S protein (Figure 3B) in parallel to an increased amount of MHC class I molecules and MHC class II molecules (Figure 3C). Note that the upregulation of MHC molecules starts earlier than upregulation of cat B and cat S, as seen at day 1. Upregulation of cat B, cat S and MHC molecules reached a maximum at day 3.

In order to examine the endosomal/lysosomal distribution of cat B, L and S activities under conditions of maximal upregulation of MHC class II molecules, endosomal and lysosomal fractions of the monocytes were prepared for assessment of cat activities after 72 h of culture under control conditions (Figure 4) and in the presence of IFN-γ, respectively. Whole cell lysates of control monocytes displayed activities of cat B, L and S which were very similar to those found in freshly isolated monocytes (Figure 1). However, the intracellular distribution of the enzymes differed considerably. Highest activities were found in the lysosomal fraction, while endosomal activities were as low as those found in whole cell lysates. This finding and the variability of activities might be explained by lower amounts of cytosolic cysteine proteinase inhibitors

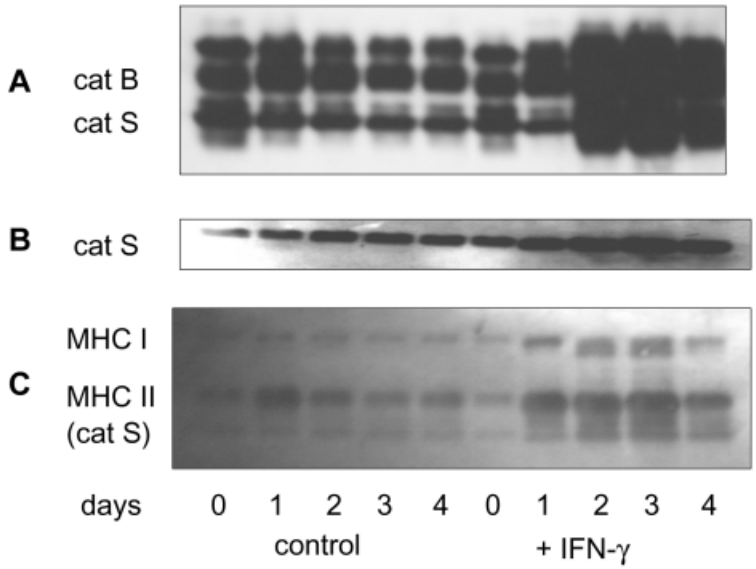

Fig. 3 Kinetics of the Upregulation of cat B and cat S Levels in Correlation to That of MHC Class I and II Molecules Visualized by Active Site-Directed Labeling (A) and Western Blotting (B,C).
Protein levels were determined in crude organelle fractions of monocytes upon treatment with IFN-γ (100 U/ml) for 0–4 days. Monocytes freshly isolated from two buffy coats (~4×10^8 cells) were cultured for 0–4 days suspended in 5 ml serum-free medium (X-Vivo 15, BioWhittacker, Walkersville, MD, USA) (control) or in medium supplemented with IFN-γ (100 U/ml, Strathmann Biotech, Hamburg, Germany). The cells were harvested daily by centrifugation, homogenized and fractionated by differential centrifugation to obtain crude organelle fractions (mitochondria, endosomes and lysosomes; pellet at 100 000 *g*, 2 min). These fractions (45 µg protein each) were analyzed for active cat B and cat S using biotinylated JPM-565 (A) and subsequently by Western blotting for cat S protein (B) (for procedure see legend to Figure 2). After a second stripping procedure the same blot membrane was treated simultaneously with polyclonal antisera against human MHC class I α heavy chain (46 kDa) and human MHC class II β chain (33 kDa) kindly provided by H. Ploegh, Boston, MA, USA (C).

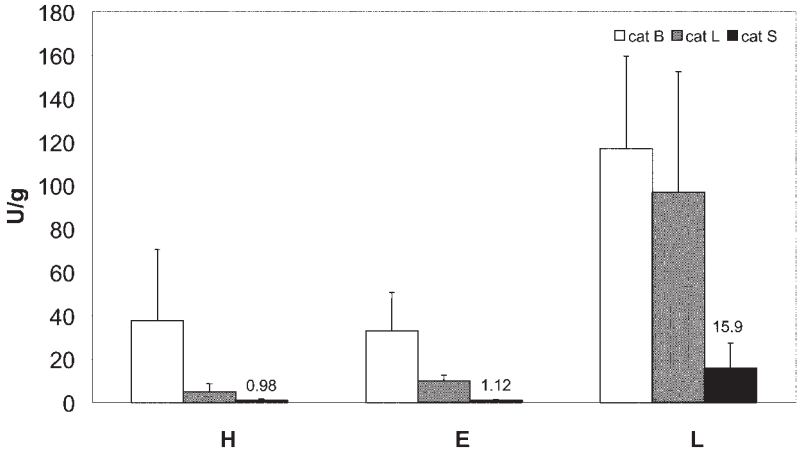

Fig. 4 Specific Activities of cat B, L and S in Whole Cell Lysates (H), Endosomal (E) and Lysosomal (L) Fractions of Monocytes after 72 h of Culture (Controls).
Data are given as U/g protein; mean ± SD, n=3. After 72 h of culture of freshly isolated monocytes in serum-free medium (see Figure 3) the cells were harvested and homogenized for subcellular fractionation (see Figure 1). The determination of enzyme activities was performed as described in the legend to Figure 1.

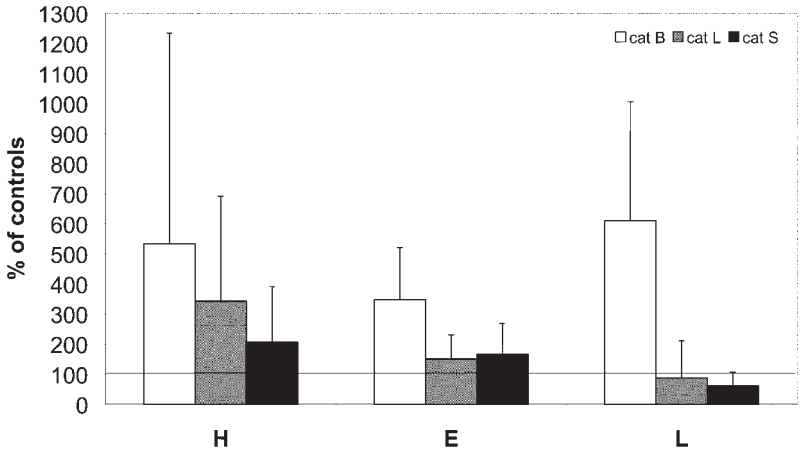

Fig. 5 Effect of IFN-γ (100 U/ml) for 72 h on the Specific Activities of cat B, L and S in Whole Cell Lysates (H), Endosomal (E) and Lysosomal (L) Fractions of Monocytes.
Data are given as per cent of the controls; mean ± SD, n=3. After 72 h of culture of freshly isolated monocytes in serum-free medium supplemented with IFN-γ (100 U/ml) (Figure 3) the cells were processed as described in the legend to Figure 4.

(Abrahamson, 1994) in lysates from cultured cells compared to lysates from freshly isolated cells. Again, cat B, L and S activities were distributed differently between the endosomal and lysosomal fractions. The relative portions of cat L and S activities were increased in both fractions, but they were 2- to 3-fold higher in the lysosomal compartment. In turn, relative cat B activity decreased from 80–90% of total cat B+L+S activity found in freshly isolated monocytes to 74% and 46%, respectively, in the endosomal and lysosomal fractions after 72 h of culture.

Seventy-two hours of treatment with IFN-γ resulted in a significant upregulation of cat B and S in crude organelle fractions accompanied by upregulation of MHC molecules (Figure 3). After IFN-γ treatment (Figure 5) the predominant lysosomal localization of cat B, L and S in control cells at this time point (Figure 4) was maintained, but again the individual enzyme activities varied. A 3- to 6-fold increase in cat B activity in whole cell lysates and subcellular fractions in contrast to smaller changes of cat L and cat S activities led to 80–90% relative contribution of cat B activity to total cat B+L+S activity similar to freshly isolated monocytes. In whole cell lysates cat L

and cat S activities were also increased, however with great individual variability, which was attributed to variations in the presence of cytosolic cysteine proteinase inhibitors. In endosomal and lysosomal fractions cat L and S activities were found to be moderately changed. The high increase in cat B activity in endosomal and lysosomal fractions relative to small changes in cat L and cat S activities might be caused by parallel induction of unknown proteins upon IFN-γ treatment in these fractions which occurs in a similar order of magnitude as induction of cat L and cat S.

In spite of the low class II expression of freshly isolated monocytes at day 0 (Figure 3C), the predominant endosomal localization of cat B, cat L and cat S (Figures 1 and 2) may be indicative of higher cellular capacities for limited proteolysis of antigenic peptides. The endosomal cat S activity is also required for maturation of MHC class II molecules (Riese *et al.*, 1996). Thus, the cells are well equipped for efficient processing of the invariant chain once class II upregulation upon stimulatory signals occurs.

Monocytes cultured for 72 h in serum-free medium, but not adhering to a matrix, may repesent an early state of differentiation of monocytes into resident tissue macrophages. This state is characterized by an upregulation of MHC class II molecules and a shift of cat B, L and S activities from endosomal to lysosomal compartments, associated with a relative increase in cat L and S activities as compared to total cat B+L+S activity. The increased relative portion of endosomal cat L and cat S activities may indicate an improved capacity of the cells for antigen presentation. The high lysosomal cat activities may merely reflect an essentially improved capacity of the cells for complete proteolysis of endocytosed or phagocytosed proteins with the involvement of cat L and cat S in addition to cat B.

The differential regulation of cat activities under the influence of IFN-γ may reflect an increase in the capacity to degrade internalized proteins during macrophage maturation. In addition, secretion of active cat B, L and S might occur for extracellular degradation of proteins as demonstrated for human long-term cultured macrophages (Reddy *et al.*, 1995).

The quantitative findings (Figures 1, 4 and 5) show some differences to the qualitative findings obtained by active site-directed labeling of cysteine proteinases (Figures 2 and 3). The different methods may well account for this discrepancy. Since JPM-565 binds covalently to the active site of different cysteine proteinases presumably to different degrees (Bogyo *et al.*, 2000), it allows a comparison of the relative amount of a given active cysteine proteinase between different samples, but provides no information regarding the absolute amount of activity. Cat L may not be detectable by this method. However, immunochemical analysis has not yet been performed to verify this finding of Bogyo *et al.* (2000). Cat B could be clearly identified at 30 kDa, while cat S appears not always well-resolved. It is visualized in close proximity to cat H and an additional cysteine proteinase running slightly lower than cat S on 12.5% SDS-PAGE (Driessen *et al.*, unpublished results). Thus, cat B might be underestimated when compared to cat S. By contrast, quantitative determination of cat activities allows the discrimination of cat B, cat L and cat S on the basis of their different kinetic characteristics to catalyze the cleavage of the same artificial substrate (Schmid *et al.*, 1997). This catalytic method shows higher sensitivity than the labeling method, but does not discriminate between various species of an active enzyme. In spite of different chemical characteristics of the substances (inhibitor, substrate) used to assess active cat B and S, the two methods lead to qualitatively comparable findings.

The variations in the specific catalytic activities measured may be caused by impurities of the monocyte preparations due to the presence of lymphocytes, as lymphoid cells display considerably lower specific catalytic activities of cat B+L+S in whole cell lysates, endosomal and lysosomal fractions (Schwarz *et al.*, 2002). The unknown secretory characteristics of the different types of cells with respect to active cat may further account for the variations observed.

The present results demonstrate that human monocytes/macrophages adapt cellular levels of cat B, L and S to their functional state. This adaptation occurs at the subcellular level, *i.e*. in the distribution of these enzyme activities between endosomal and lysosomal compartments, which may be linked to different functions of these enzymes. Interestingly, recent morphological evidence demonstrates substantial reorganization of the endocytic compartments in dendritic cells upon stimulation (Kleijmeer *et al.*, 2001).

Intracellular levels of active cat B, L and S are balanced by enzyme induction, activation of proenzymes, intracellular inhibitors, enzyme degradation and, in the case of macrophages, secretion into the extracellular space, any of which might account for the effects observed. Upregulation *via* induction of cat B, L and S has been described in different types of monocytes/macrophages under the stimulatory effect of IFN-γ (Lah *et al.*, 1995; Li *et al.*, 1997; Nakagawa and Rudensky, 1999). Recently, regulation of cat L expression has been suggested on the basis of splice variants (Abudula *et al.*, 2001). Activation of the proenzymes, which occurs autocatalytically in an acidic environment *in vitro* (Nissler *et al.*, 1998), might be processed not only autocatalytically, but also through the action of cat B *in vivo* (Driessen *et al.*, 2001). However, activation of cat from their zymogens has not yet been demonstrated *in vivo*, presumably due to the short half-life of proenzymes, as described for cat S (Nissler *et al.*, 1999). Intracellular inhibitors, such as endosomically localized cystatin C, have been suggested to regulate cat S activity in mouse dendritic cells (Pierre and Mellman, 1998). Recently, p41, a fragment of the invariant chain, has been found to regulate the level of active cat L *in vivo*, not by inhibiting the enzyme (Bevec *et al.*, 1996), but by stabilizing its mature form as a chaperone (Lennon-

Duménil *et al.*, 2001). In addition, cat B appears to regulate the turnover of cysteine proteinases (Driessen *et al.*, 2001). On the other hand, regulation of cat L expression by cat S has been found in lymphocytes, but not in macrophages (Honey *et al.*, 2001). Thus, different types of antigen-presenting cells show variations in regulation of the expression of cat B, L and S. The unique feature of macrophages to secrete active cat reduces the cellular levels of these enzymes (Reddy *et al.*, 1995; Petanceska *et al.*, 1996; Claus *et al.*, 1998; Liuzzo *et al.*, 1999; Punturieri *et al.*, 2000). Interestingly, there is evidence that macrophages are capable to secrete endosomal/lysosomal hydrolases specifically and selectively under distinct conditions (Claus *et al.*, 1998).

Altogether, cat B, L and S of monocytes/macrophages are involved in general lysosomal proteolysis leading to complete protein degradation, limited and specific endosomal proteolysis resulting in activated enzymes or antigenic peptides as well as pericellular proteolysis, which is considered to be destructive and involved in tissue remodeling. Cat B as a housekeeping enzyme (Qian *et al.*, 1991) has been assigned a role in general lysosomal protein turnover. Although cat B is dispensable for presentation of a number of antigens (Deussing *et al.*, 1998), the present finding of considerable endosomal cat B activity points also to specific functions. Entirely consistent with this notion, it was recently demonstrated that cat B is involved in the degradation of exogenous protein taken up *via* Fcγ receptors by macrophages (Driessen *et al.*, 2001). This might be due to a regulatory role of cat B in controlling cysteine proteinase activities. Cat S is also involved in antigen processing (Beck *et al.*, 2001; Driessen *et al.*, 2001) in addition to its well-known function in cleaving the invariant chain (Riese *et al.*, 1996). Both cat B and cat S are suggested to degrade mature cat L, which is prevented by p41 (Lennon-Duménil *et al.*, 2001). Cat L is also suggested to participate in degradation of invariant chain in macrophages (Nakagawa and Rudensky, 1999). In contrast to the regulated secretion of endosomal/lysosomal enzymes during phagocytosis (Claus *et al.*, 1998), secretion of active cat B, L and S allows elastinolysis of extracellular matrix for tissue remodeling in long-term differentiated macrophages of the tissue-destructive type (Punturieri *et al.*, 2000).

In conclusion, monocytes freshly isolated from human blood or cultured for 0–4 days in defined medium and in the presence of IFN-γ may represent an *in vitro* model for early states of differentiation into resident tissue macrophages under normal or immunomodulatory conditions. Differences in the activities of cat B, L and S and their localization to endosomal and lysosomal fractions may serve as markers for differentiation. Monocytes modulate the subcellular distribution of endosomal/lysosomal proteinases and, presumably, thereby different functional characteristics of the enzymes according to the cellular requirements.

Acknowledgements

This work was supported by the Deutsche Forschungsgemeinschaft (Dr 378/2–1, Ka 767/4–2 and Sonderforschungsbereich 510; H.K.)

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Received January 15, 2002; accepted March 6, 2002