ISJ 1: 38-46, 2004 ISSN 1824-307X

Research Report

Role of cathepsin B in leech wound healing

A Grimaldi[1], G Tettamanti[1], L Rinaldi[1], G Perletti[2], R Valvassori[1], M de Eguileor[1]*

[1] *Department of Structural and Functional Biology, University of Insubria, Varese, Italy*
[2] *Department of Structural and Functional Biology, University of Insubria, Busto Arsizio, Italy*

Accepted June 30, 2004

Abstract

The wound healing process in leeches involves different types of cells like macrophages, NK-like cells and granulocytes. These cells that are involved in immune defence, can co-operate to attack and/or isolate the non self (de Eguileor *et al*., 1999; de Eguileor *et al*., 2000a; de Eguileor *et al*., 2000b). In addition other types of cells, like fibroblasts and endothelial cells, are involved in the formation of new vessels. To exert their functional role, all these cells must infiltrate and migrate through extracellular matrix (de Eguileor *et al*., 2001a; de Eguileor *et al*., 2003).

Here we show, by histochemical and biochemical methods, that the cathepsin B peptidase is present and active in all migrating cells, involved in immune responses of leeches subjected to different stimuli. Interstingly the cellular function of cathepsin B in invertebrates appear to be equivalent to that of vertebrates, where the secreted enzyme plays a role in basement membrane and matrix disruption operated by cells involved in angiogenesis, wound repair and immune defence.

Key words: leeches; cathepsin-B; immune cells; angiogenesis

Introduction

Both in invertebrates and vertebrates, inflammation is an acute reaction triggered by different types of lesions and aimed at preventing systemic infections. It is mediated by specific cells such as macrophages and neutrophiles that infiltrate the damaged tissue, removing tissue debris and controlling invading microorganisms. These cells synthesize different molecules such as growth factors and cytokines, inducing mesenchymal cell recruitment in the lesioned area (Moulin, 1995).

In addition, actively phagocytizing cells produce and secrete into the extracellular matrix a wide array of lysosomal enzymes, among which cathepsins play a pivotal role. The role of cathepsins as scavengers has been recently reconsidered due to the increasing identification and functional characterization of new lysosomal cysteine proteases (Bond and Butler, 1987; Kirschke *et al*., 1995; Turk *et al*. 2000; Turk *et al*. 2001). Cysteine proteases were believed to be mainly involved in intracellular protein degradation, since they are optimally active in the slightly acidic, reducing milieu found in lysosomes; these endopeptidases are synthesized as inactive precursors and they are regulated by several inhibitors or by pH, to regulate their lytic effects (Mach *et al*., 1994; Lah *et al*., 1995; Turk *et al*., 2000).

Recently, it has been demonstrated that besides their role within lysosomes, cysteine proteases can degrade proteins outside lysosomes, and can contribute to protein processing within cell cytoplasm (Kos and Lah, 1998; Lah and Kos, 1998, Pierre and Mellman, 1998, Saftig *et al*., 1998, Shibata *et al*., 1998). Cathepsins have been shown to be involved in cancer progression (Kos and Lah, 1998; Lah and Kos, 1998), apoptosis regulation (Shibata *et al*., 1998), MHC II antigen presentation (Pierre and Mellman, 1998) and tissue remodeling (Saftig *et al*., 1998). In particular, secreted cathepsins seem to have a role in degradation of basement membrane and extracellular matrix,

**Corresponding Author:*
Magda de Eguileor
Department of Structural and Functional Biology, University of Insubria, J.H. Dunant 3, 21100 Varese, Italy.
E-mail: magda.deeguileor@uninsubria.it

favouring migration of cancer (Kos and Lah, 1998) and immune (Diment *et al*., 1988; Young *et al*., 1991) cells.

Regarding invertebrates, cathepsin-like proteins have been characterized in several taxa like Protozoa (Judice *et al*., 2004), Platyhelminthes (Wong *et al*., 1997), Molluscs (Pipe, 1990), Arthropods (Deraison *et al*., 2004), while an involvement of cathepsin B in regulation of neo-angiogenesis has recently been hypothesized for the Annelid *Hirudo medicinalis* (Tettamanti *et al*., 2004, in press). In addition Lefebvre *et al*., (2004) have cloned a cystatin gene (Tt-cysb) in the leech *Theromyzon tessulatum*, suggesting that the proteinase inhibitor Tt-CYSB may regulate leech cathepsin B.

In leeches the wound healing process is characterized by the same sequence of events described in vertebrates. In particular, during the inflammation phase, immune cells numerically increase and move toward the lesioned area, while during the granulation tissue stage angiogenesis and fibroplasia take place (Tettamanti et al., 2003). During these events, different types of immune cells (macrophages, NK-like cells and granulocytes) that co-operate to attack and/or isolate the non self, as well as fibroblasts and endothelial cells, involved in the formation of new vessels, have to push their way through the extracellular matrix (de Eguileor *et al*., 1999; de Eguileor *et al*., 2000a; de Eguileor *et al*., 2000b; de Eguileor *et al*., 2001b; de Eguileor *et al*., 2003).

In the present study, utilizing histochemical and biochemical methods, it is shown that the peptidase cathepsin B is present in all migrating cells that are involved in a number of immune functions in leeches subjected to different stimuli.

Materials and methods

Leeches

Glossiphonia complanata (Annelida, Hirudinea, Glossiphoniidae) were collected from the Adda river, near Milan, Italy. They were kept in water at 18°C in aerated tanks and fed *ad libitum* with Planorbidae snails. Leeches were divided into two groups. Group 1 included 5 unstimulated leeches as control animals; group 2 included 15 leeches stimulated by lipopolysaccharide (LPS) injection. A solution of 10 mg/ml of LPS was used for immune stimulation (de Eguileor *et al*., 2000b).

Leeches were kept in water in separate tanks and sacrificed 30 min, 2 hr and 4 hr intervals after LPS treatment.

*Hirudo medicinalis, (*Annelida, Hirudinea, Hirudidae), purchased from Ricarimpex, Eysines, France, were kept in water at 22-23°C in aerated tanks, and were fed monthly with calf liver. Prior to experiments, leeches had been kept fasting for a month. Leeches were subdivided into two groups. Group 1 included 5 unstimulated *H. medicinalis,* as control animals, used to document the normal body wall organization of this leech. Group 2 included 25 *H. medicinalis* subdivided into subgroups a and b. Subgroup a): 20 animals were subjected to lesions consisting of a tissue explant (about 2 mm $\times$ 2 mm x 2 mm) affecting the entire body wall. The tissue explant was surgically removed with microdissecting scissors, and the wounded leeches were observed at 3 and 5 hr intervals after surgical explantation. Subgroup b): 5 animals were injected with 30 ìl of the growth factor GM-CSF (10 ng/ìl) solution. Injected leeches were observed at 5 hr intervals after growth factor injection.

Prior to surgical procedures and fixation, leeches of both species were anesthetized with a saturated solution of mephenesin (3-*o*-toloxy-1,2-propanediol), a muscle relaxant.

Light and electron microscopy (TEM)

Leeches were dissected and fixed in 2% glutaraldehyde in 0.1 M Na-cacodylate buffer (pH 7.2) for 2 hr at room temperature, then washed in the same buffer and postfixed for 2 hr with 1% osmic acid in 0.1 M Na-cacodylate buffer (pH 7.2) at room temperature. After standard steps of serial ethanol dehydration, specimens were embedded in an Epon-Araldite 812 mixture. Semi-thin and thin sections were cut with a Reichert Ultracut E ultratome (Leica, Nussolch, Germany). Semi-thin sections (1 µm) were stained by conventional methods (crystal violet and basic fuchsin), and observed with a light microscope (Olympus, Tokyo, Japan).

Thin sections (80 nm) were collected on 300 mesh copper grids, stained with uranyl acetate and lead citrate, and observed with a Jeol 1010 EX electron microscope (Jeol, Tokyo, Japan).

Immunocytochemistry

Anaesthetised leeches were dissected in a cold Ringer solution (Miller and Aidley, 1973) into small blocks, which were immediately embedded in polyfreeze cryostat embedding medium (OCT) (Polyscience Europe, Eppelheim, Germany), and stored in liquid nitrogen according to Geiger and coworkers (1980). Cryosections (10 µm thick) of unfixed leeches were obtained with a Reichert-Jung Frigocut 2800; slides were immediately used or stored at -20°C. Sections were incubated for 15 min with Evans blue (De la Lande and Waterson, 1968) to reduce autofluorescence, washed with PBS, and incubated in a moist chamber for 1 h at 37 °C with a primary mouse anti-human cathepsin B monoclonal antibody (Clone CA10, Calbiochem, Cambridge, MA, USA), diluted 1:20. After incubation with the primary antibody, specimens were washed and incubated with appropriate secondary antibody conjugated with fluorescein isothiocyanate (FITC) (1:50 dilution, Jackson Immuno Research Laboratories, West Grove**,** PA, USA). Incubations were performed for 1 hr in a dark moist chamber at 37 °C. Double stainings were performed by firstly incubating samples with anti-human cathepsin B diluted 1:20 and FITC-conjugated secondary antibody. Subsequently the same specimens were incubated with a primary mouse monoclonal anti-human CD68 antibody (Clone PG-M1, diluted 1:20, DBA, Milan, Italy) and with a tetramethylrhodamine (TRITC)-conjugated secondary antibody. Antibodies were diluted with PBS containing 2% bovine serum albumin (BSA). In control samples, primary antibodies were omitted, and sections were treated with BSA-containing PBS.

Coverslips were mounted in Vectashield Mounting Medium for fluorescence (Vector Laboratories, Burlingame, CA, USA); slides were

examined at 40X (NA 1.30) magnification with a confocal laser microscope (laser 568 nm for rhodamine, laser 492 nm for fluoresceine; MRC 1024, Bio-Rad Laboratories, Hemel Hempstead, UK). Confocal images were superimposed using the Photoshop 5.0 program; fluorescent images were then overlayed onto transmission images showing the corresponding tissue sections.

Biochemical procedures

H. medicinalis tissues from the unstimulated body wall or from areas involved in surgical events were frozen in liquid nitrogen and then homogenized with a mortar.

For SDS-polyacrylamide gel electrophoresis (SDS-PAGE), leech homogenates were suspended in extraction buffer (2X Laemmli's Buffer in the presence of a protease inhibitor cocktail (Sigma, Milan, Italy)); the particulate material was removed by centrifugation at 13000 rpm for 10 min at 4°C in a refrigerated Eppendorf Minispin microcentrifuge. Supernatants were denatured at 100°C for 10 min.

SDS-PAGE

Proteins were separated in analytical SDS-PAGE using 15% acrylamide minigels (running conditions 200V for 1h). Molecular weights were determined by concurrently running broad range standards from Bio-Rad (Bio-Rad, Richmond, MA, USA). Gels were stained with 2.5% Coomassie blue (Bio-Rad) in methanol:acetic acid:water 4:1:5.

Western blot

Proteins separated by SDS-PAGE were transferred onto Bio-Rad nitrocellulose filters. Membranes were then saturated with 2% BSA in Tris-buffered saline (TBS, 20mM Tris-HCl buffer, 500mM NaCl, pH 7.5) at room temperature for 2 hr, and incubated overnight at 4°C with a mouse anti-human cathepsin B antibody (1:250 dilution in 2% TBS-BSA). After washing the membrane three times with TBS, the immunocomplexes were revealed with an appropriate alkaline phosphatase-conjugated secondary antibody (1:1000) (Sigma). Immunoreactivity was detected with a SIGMA FAST BCIP/NBT system (Sigma).

Results

General characteristics of leech body wall in unstimulated and stimulated animals

The body wall of leeches is virtually avascular (Figs 1, 2) and is made of tightly-packed muscle fibers, grouped in fields and separated only by extracellular matrix or by dorsoventral fibers (Figs 1, 2).

After stimulation there is always a proliferation and movement of immune cells and macrophages, NK-like cells and granulocytes migrate towards the injured area (Figs 3, 4, 5) forming a plug (Fig. 6). While in Glossiphoniidae proliferation and migration of immune cells are basic events, in Hirudidae proliferation and migration are associated to important effects as angiogenesis and fibroplasia.

Thus in these leeches the formation of a network of blood vessels, spanning the entire body wall of the animal, occupies the space among the fields of muscle fibers (Fig. 7) within the whole thickness of the body wall.

Cathepsin B detection in Glossiphonia complanata

Cathepsin B immunolocalization was performed, both in control and stimulated leeches. In untreated animals anti-cathepsin-B antibody stained the cytoplasmic core of circomyarian helical muscle fibers (Figs 8, 9). After 30' from LPS administration a marked signal was detectable not only in the basal lamina surrounding the muscle fibers of the treated area, but also in the cytoplasm of migrating cells localized among the muscle fields (Fig. 10). Double immunofluorescence localization with CD68 (a specific marker for vertebrate macrophages) and cathepsin B antibodies, showed that migrating cells, were macrophage-like cells since CD68 and cathepsin B signal co-localized in the same cell types (Fig. 11). Two and 4 hr after LPS injection, cathepsin B signal was detectable in migrating cells: in particular the signal was mostly visible in cell basal lamina (Fig. 12). Double staining with anti-CD68 and anti-cathepsin B antibodies showed that 4 hr interval from LPS treatment the cathepsin B signal was mainly localized in the basal lamina surrounding $CD68^+$ macrophage-like cells (Fig.13).

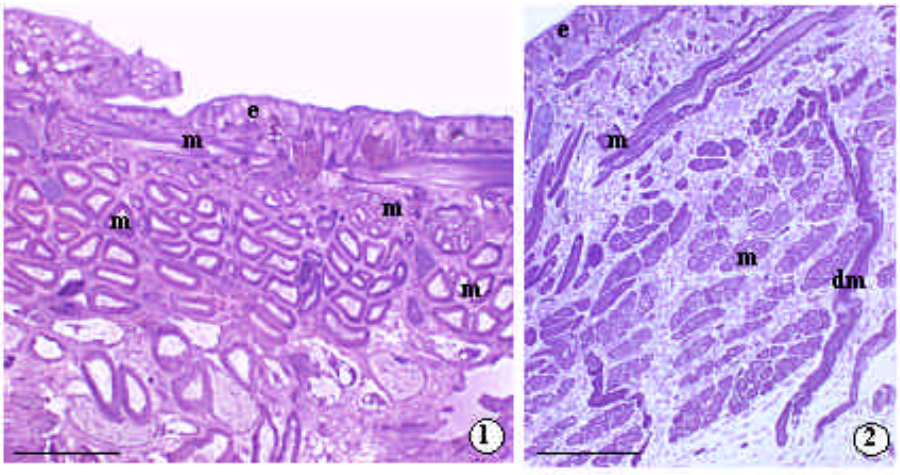
Cathepsin B detection in Hirudo medicinalis

In unstimulated animals cathepsin B was mainly expressed in the cytoplasm of muscle fibers (Figs 14, 15).

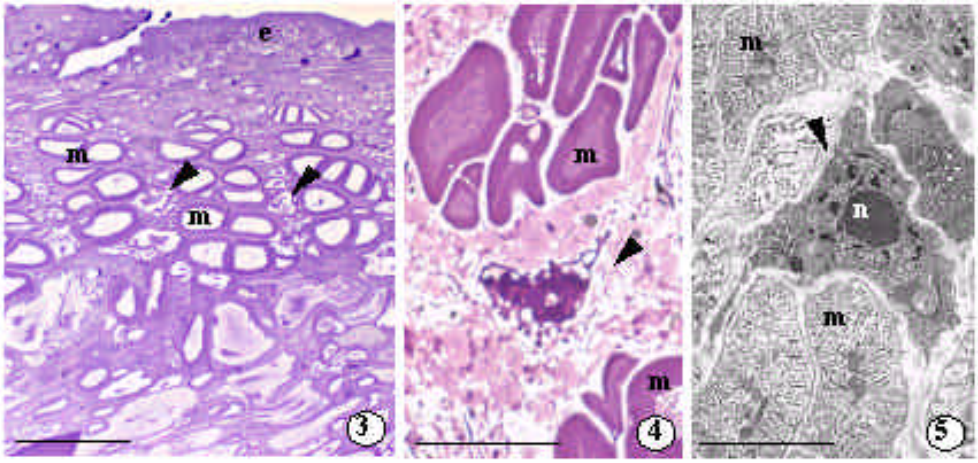
About 3 hr after a surgical lesion, cathepsin B immunoreactivity was detectable, as in *G. complanata,* in migrating cells localized in the connective tissue among muscle fibers (Fig. 16). Most migrating cells were macrophages, as demonstrated by co-localization of cathepsin B and CD68 (Fig. 17). The two antibodies recognized both migrating cells in the injured area and cells filling the explanted area and forming the "plug" (Figs. 6, 18). Five hours after tissue explantation, cathepsin B and CD68 signals did not co-localize. In the plug area, CD68 stained macrophage-like cells, while cathepsin B was mainly found in the surrounding connective tissue (Figs 19, 20). After 5 hr from injection of GM-CSF growth factor, cathepsin B was detected in migrating cells as well as in endothelial cells lining the new vessels of stimulated animal (Figs 21, 22).

Western blot analysis of cathepsin B in stimulated Hirudo medicinalis

The presence of cathepsin B in leeches was assayed in protein extracts from stimulated *H. medicinalis* body wall. Tissues from area close to explant were taken 3 and 5 hr intervals after surgical lesion (Fig. 23). Western blot showed that the anti-cathepsin B antibody detected a single band of about 34 kDa in protein extracts of leeches sacrificed 3hr after surgery, while in proteins extracts of leeches sacrificed 5hr after surgery two immunoreactive bands of about 34 kDa and 31 kDa were detected.



Figs. 1, 2 Semi-thin cross sections of unstimulated leeches *G. complanata* (Fig. 1) and *Hirudo medicinalis* (Fig. 2). Under the epithelium (e) muscle layers, circularly, obliquely and longitudinally oriented, are visible (m). Helical muscle fibers are tightly packed and organized into groups sometimes separated by dorsoventral muscles (dm). Bars = 80 μm and 100 μm respectively.



Figs 3,4,5. Semi-thin cross section of stimulated *G. complanata* (Fig 3). Migrating cells (arrowheads) are visible in the extracellular matrix among muscle fields. e (epithelium), m (muscle fibers). Bar = 80 μm. Semi-thin (Fig. 4) and thin (Fig. 5) sections of surgically lesioned *Hirudo medicinalis*. Macrophage-like cells (arrowhead) are detected among muscle fibers (m) in the newly synthesized extracellular matrix. n (nucleus). Bars = 20 μm, 4 μm.

Discussion

As we reported previously, characterization and expression studies have stressed the existence of several similarities between leech and vertebrate immune responses. In leeches, processes in response to surgical wounds, grafts or injections of stimulating factors (LPS, growth factors) are similar to equivalent responses in vertebrates and involve a wide range of sequential events triggered by inflammatory reactions (de Eguileor *et al*., 2003, Tettamanti *et al*., 2003, Tettamanti *et al*., 2004). Macrophage-like cells, NK-like cells and granulocytes migrate through the extracellular matrix from the inner regions of the body towards stimulated areas. Contextually, as demonstrated for *H. medicinalis*, beside the migration of activated fibroblasts involved in the production of the scaffold, supporting the network of new capillaries (Tettamanti *et al*., 2004), the formation of new vessels begins from the botryoidal tissue. In fact, within a short time interval after an angiogenic stimulus, botryoidal tissue cells forming a solid cord, proliferate and reshape into tubes, the new capillaries, that stretch towards the lesioned area by moving between muscle fiber fields through the ECM (de Eguileor *et al*., 2001b). It is important to underline that all steps of these complex responses have the migration of cells as a common denominator. In fact either immune cells or endothelial cells or fibroblasts (involved directly or indirectly in neovascularization) need the degradation of extracellular matrix in order to move and change their spatial position in the leech body.

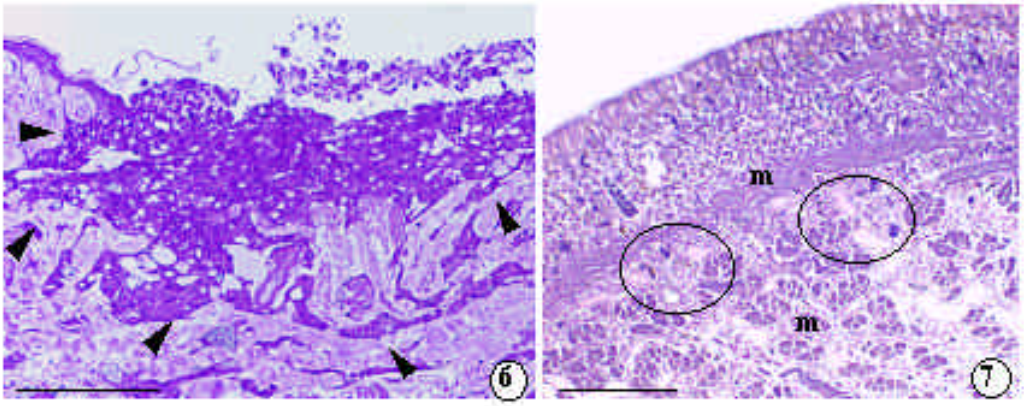


Fig. 6 Semi-thin cross section of surgically lesioned *H. medicinalis.* The area of lesion is infiltrated by a high number of migrating cells forming a “plug” (arrowheads). Bar = 200 μm

Fig. 7 Semi-thin cross section of surgically lesioned *H. medicinalis.* Numerous new vessels (encircled areas) are visible among muscle fibers (m). e, (epithelium). Bar = 100 μm.

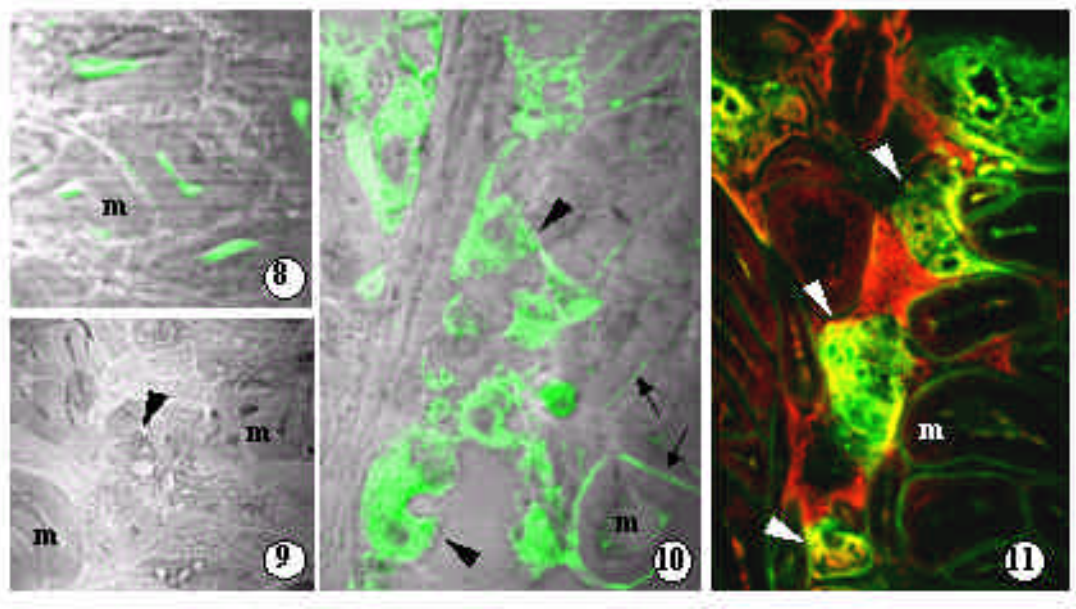


Fig. 8 Cryosection of unstimulated leech *G. complanata.* An anti-cathepsin B antibody stains the cytoplasmic core of helical muscle fibers (m).

Fig. 9 Cryosection of stimulated *G. complanata.* Negative control for cathepsin B staining. Positivity is detected neither in muscle cells (m) nor in migrating cells (arrowheads).

Fig. 10 Cryosection of stimulated *G. complanata* sacrificed 30 min after LPS administration. Cathepsin B is markedly expressed in the cytoplasm of migrating cells (arrowheads). The signal is detected also in the cytoplasm of a few helical fibers (m) and, widely, in their basal membrane (arrows).

Fig. 11 Cryosection of stimulated *G. complanata* analyzed 30 min after LPS administration. Co-localization of cathepsin B (green) and CD68 (red) is indicated by yellow fluorescence in migrating cells (arrowheads) localised between muscle fibers (m). A signal for cathepsin B is also detected in basal lamina of muscle fibers (m).

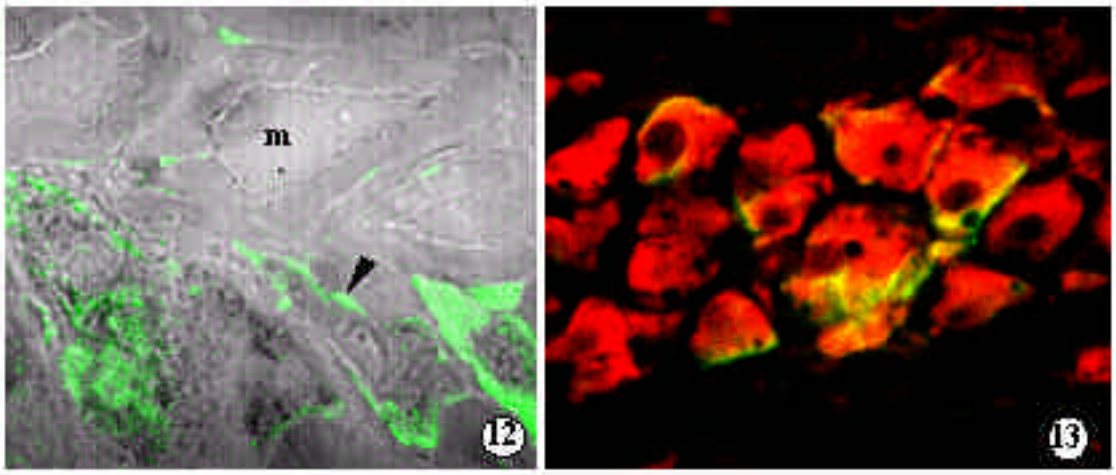


Fig. 12 Cryosection of stimulated *G. complanata,* analyzed 2-4 hr after LPS injection. Cathepsin B signal is detected in the cytoplasm and in the basal lamina of macrophages (arrowheads) and muscle fibers (m).

Fig. 13 Cryosection of stimulated *G. complanata,* analyzed after 2-4 hr from LPS administration. Double immunofluorescence shows that while anti-CD68 antibody (red) stains the cytoplasm of all macrophages, only some of them are positive also for cathepsin B (green) as demonstrated by co-localization (yellow fluorescence).

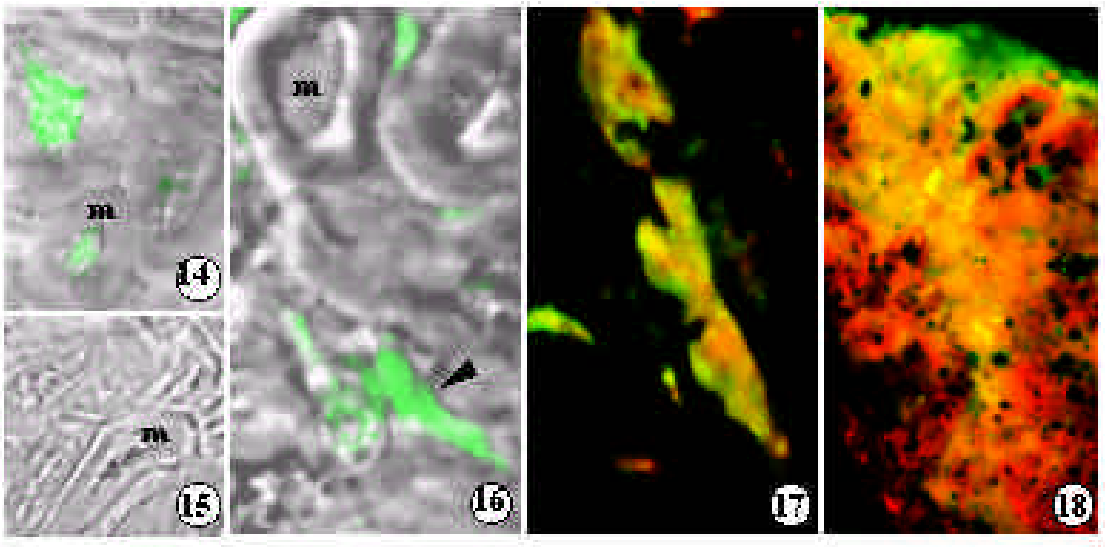
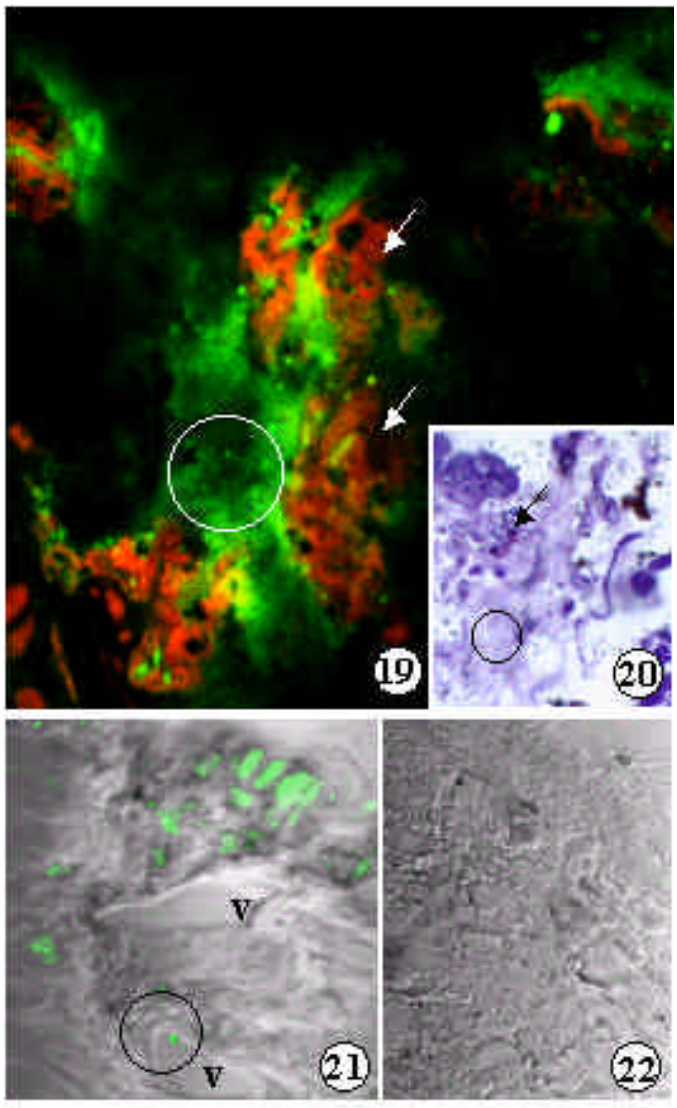


Fig. 14 Cryosection of unstimulated *H. medicinalis*. Cathepsin B is expressed in the cytoplasm of muscle fibers (m).

Fig. 15 Cryosection of *H. medicinalis*. Negative control for cathepsin B staining. No positivity is detected. Muscle fibers (m).

Fig. 16 Cryosection of stimulated *H. medicinalis* analyzed after 3 hr from surgical stimulation. Cathepsin B is mainly located in the cytoplasm of migrating cells (arrowheads) localized among muscle fibers (m).

Figs. 17, 18 Cryosections of *H. medicinalis* analyzed 3 hr after surgical stimulation. Double staining using anti-cathepsin B (green) and anti-CD68 (red) antibodies shows that migrating cells (Fig. 17) and those forming the plug are macrophages (Fig. 18).



Figs. 19, 20 Cryosections of *H. medicinalis* analyzed 5 hr after surgical stimulation. Double immunofluorescence evidences that in plug area (Figs. 19, 20) macrophages (arrowheads) are CD68 positive (red stain) while extracellular matrix (encircled areas) is cathepsin B positive (green stain).

Figs. 21, 22 Cryosections of stimulated *H. medicinalis* analyzed 5 hr after GM-CSF injection. Cathepsin B is detectable in cells migrating towards the injected area and in endothelial cells (encircled area) of neo-vessels (v) (Fig. 21). In negative control for cathepsin B staining (Fig. 22) no positivity is detected.

In vertebrates cell migration involves the degradation of extracellular matrix through enzymatic digestion. Cathepsin B, is a protease that can degrade components of ECM outside the cells. It is active against large substrate components such as laminin, fibronectin and collagen IV. In the animal model used in the present study, in addition to data previously described regarding immune responses, it is important to underline that the extracellular matrix is formed of the same components typical of vertebrates (de Eguileor *et al*., 1999); for this reason we have hypothesized the involvement of cathepsin B in stimulated and lesioned *G. complanata* and *H. medicinalis*. In unstimulated leeches (both species), cathepsin B is detected in the cytoplasmic core of muscle fibers and in the very few migrating cells present, while there is a considerable change in the intensity and in the localization of the immunoreactivity against the enzyme in the period of time following any stimulating events. Following surgical stimulation, the enzyme is not only detectable in muscle fiber cytoplasm, but also in the basement membrane of muscle fibers. In addition, a strong immunoreactive signal is detected predominantly in the cytoplasm of migrating cells, mostly CD68-positive macrophages, and, as previously demonstrated, in activated fibroblasts (Tettamanti *et al*., 2004). Local degradation of basement membrane followed by ECM digestion, is a crucial step in cellular migration. In *H. medicinalis*, 5 hr after surgical stimulation, cathepsin B is mainly localised in the connective tissue surrounding

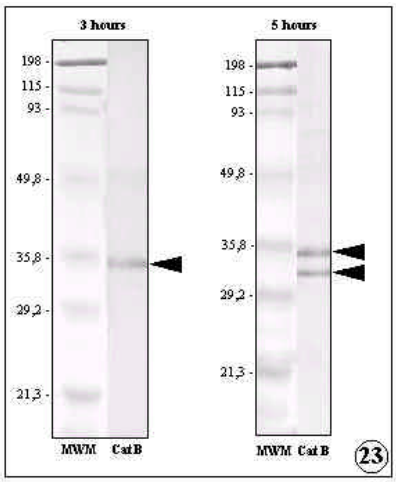


Fig. 23 Western blot analysis of surgically stimulated *H. medicinalis.* The antibody anti-cathepsin B detects a single band of about 34 kDa and 2 bands of 31 and 34 kDa 3 and 5 hr after surgery, respectively. MWM (molecular weight marker), Cat B (cathepsin B).

numerous migrating cells as immune cells, fibroblasts and endothelial cells. These cells push their way among groups of muscle fibers. Some of these cells are involved in the obstruction of the wound and in tissue repair, whereas cellular elements, involved in agiogenesis, create a "pipe system" to transfer nutrients, oxigen and immune cells in stimulated area (Tettamanti *et al*. 2003, de Eguileor *et al*., 2003). It is interesting to underline that the increased immunoreactivity towards cathepsin B that follows the surgical stimulation, has been also detected after activating the immune system by injection of LPS (de Eguileor *et al*., 2000b). In addition, in vertebrate it is well known that the growth factor administration may upregulate the expression and activity of cathepsin B (Koblinski *et al*., 2000); thus it is suggested here that in leeches the same regulation might be present since VEGF, EGF, bFGF and GM-CSF figure among the most potent angiogenic factors and since the formation of new vessels requires massive movements of cells (de Eguileor *et al*., 2004, Tettamanti *et al*. 2003), Our hypothesis is supported by the markedly increased immunopositivity for cathepsin B in the stimulated areas by GM-CSF administration. This growth factor released in the ECM, is generally produced by a variety of different cells such as macrophages, endothelial cells and fibroblasts.

It must be emphasized that, to validate the immunocytochemical localization of cathepsin B, western blot analysis of this protease confirms a degree of similarity between hirudinean and vertebrate cathepsin B signal. Immunostaining with antibody raised against cathepsin B resulted, in control animals (Tettamanti *et al*., 2004, in press) and in stimulated leeches after short time elapsed from stimulation, in one band, while two bands that probably could correspond to the mature form and proform (Takahiro *et al*., 1993) resulted after 5 hr from stimulation.

In conclusion, the results reported here on the differentiated release of cathepsin B from unstimulated and variously stimulated leeches confirm, also in these invertebrates, the role that this enzyme plays in wound healing processes.

References

Bond JS, Butler PE. Intracellular proteases. Ann. Rev. Biochem. 56: 333-364, 1987.

De la Lande IS, Waterson JG. Modification of autofluorescence in the formaldehyde-treated rabbit ear artery by Evans blue. J. Histochem. Cytochem. 16: 281-282, 1968.

Deraison C, Darboux I, Duportets L, Gorojankina T, Rahbe Y, Jouanin L. Cloning and characterization of a gut-specific cathepsin L from the aphid *Aphis gossypii*. Insect Mol. Biol. 13: 165-177, 2004.

Diment S, Leech MS, Stahl PD. Cathepsin D is membrane-associated in macrophage endosomes. J. Biol. Chem. 263: 6901-6907, 1988.

de Eguileor M, Tettamanti G, Grimaldi A, Boselli A, Scari G, Valvassori R, Cooper EL, Lanzavecchia G. Histopathological changes after induced injury in leeches. J. Invertebr. Pathol. 74: 14-28, 1999.

de Eguileor M, Grimaldi A, Tettamanti G, Valvassori R, Cooper EL, Lanzavecchia G. Different types of response to foreign antigens by leech leukocytes. Tissue Cell. 32: 40-48, 2000a.

de Eguileor M, Grimaldi A, Tettamanti G, Valvassori R, Cooper EL, Lanzavecchia G. Lipopolysaccharide-dependent induction of leech leukocytes that cross-react with vertebrate cellular differentiation markers. Tissue Cell. 32: 437-445, 2000b.

de Eguileor M, Grimaldi A, Tettamanti G, Ferrarese R, Congiu T, Protasoni M, Perletti G, Valvassori R, Lanzavecchia G. *Hirudo medicinalis*: a new model for testing activators and inhibitors of angiogenesis. Angiogenesis 4: 299-312, 2001a.

de Eguileor M., Grimaldi A., Tettamanti G., Congiu T., Protasoni M., Reguzzoni M., Valvassori R., Lanzavecchia G. Ultrastructure and functional versatility of hyrudinean botryoidal tissue. Tissue Cell. 33: 332-341, 2001b.

de Eguileor M, Tettamanti G, Grimaldi A, Congiu T, Ferrarese R, Perletti G, Valvassori R, Cooper EL, Lanzavecchia G. Leeches: immune response, angiogenesis and biomedical applications. Curr. Pharm. Design 9: 133-147, 2003.

de Eguileor M., Tettamanti G., Grimaldi A., Perletti G., Congiu T., Rinaldi L., Valvassori R., *Hirudo medicinalis*: avascular tissues for clear-cut angiogenesis studies? Curr. Pharm. Design 2004 (in press).

Geiger B, Tokuyasu KT, Dutton A, Singer SJ. Vinculin an intracellular protein localized at specialized sites where microfilament bundles terminate at cell membranes. Proc. Natl. Acad. Sci. USA 77: 4127-4131, 1980.

Judice WA, Puzer L, Cotrin SS, Carmona AK, Coombs GH, Juliano L, Juliano MA. Carboxydipeptidase activities of recombinant cysteine peptidases. Cruzain of *Trypanosoma cruzi* and CPB of *Leishmania mexicana*. Eur. J. Biochem. 271: 1046-1053, 2004.

Kirschke H, Barrett AJ, Rawlings ND. Proteinases 1: lysosomal cysteine proteinases. Protein Profile 2: 1581-1643, 1995.

Koblinski J., Ahram M., Sloane BF. Unraveling the role of proteases in cancer. Clin. Chim. Acta 291: 113-135, 2000.

Kos J, Lah TT. Cysteine proteinases and their endogenous inhibitors: target proteins for prognosis, diagnosis and therapy in cancer (review). Oncol. Rep. 5: 1349-1361, 1998.

Lah TT., Hawley M., Rock K., Goldberg A. Gamma-interferon causes a selective induction of the lysosomal proteases, cathepsin B and L in macrophages. FEBS Lett. 363: 85-89, 1995.

Lah TT, Kos J. Cysteine proteinases in cancer progression and their clinical relevance for prognosis. Biol. Chem. 379: 125-130, 1998.

Lefebvre C., Cocquerelle C., Vandelbulcke, Hot D., Huot L., Lemoine Y., Salzet M. Transcriptomic analysis in the leech *Theromyzon tessulatum*: involvement of cystatin B in innate immunity. Biochem. J. 380: 617-626, 2004.

Mach L., Mort JS., Gloss J. Maturation of human procathepsin B. J. Biol. Chem. 17: 13030-13035, 1994.

Mann KH. Leeches (Hirudinea): their structure, physiology, ecology and embriology. Pergamon Press, Oxford, 1962.

Miller JB, Aidley DJ. Two rates of relaxation in the dorsal longitudinal muscle of leech. J. Exp. Biol. 58: 91-103, 1973

Moulin V. Growth factors in skin wound healing. Eur. J. Cell Biol. 68: 1-7, 1995.

Pierre P, Mellman I. Developmental regulation of invariant chain proteolysis controls MHC class II trafficking in mouse dendritic cells. Cell 93: 1135-1145, 1998.

Pipe RK. Hydrolytic enzymes associated with the granular haemocytes of the marine mussel *Mytilus edulis*. Histochem. J. 22: 595-603, 1990.

Saftig P, Hunziker E, Wehmeyer O, Jones S, Boyde A, Rommerskirch W, Moritz JD, Schu P, von Figura K. Impaired osteoclastic bone resorption leads to osteopetrosis in cathepsin-K-deficient mice. Proc. Natl. Acad. Sci. USA 95: 13453-13458, 1998.

Salzet M. Vertebrate innate immunity resembles a mosaic of invertebrate immune responses. Trends Immunol. 22: 285-288, 2001.

Shibata M, Kanamori S, Isahara K, Ohsawa Y, Konishi A, Kametaka S, Watanabe T, Ebisu S, Ishido K, Kominami E, Uchiyama Y. Participation of cathepsins B and D in apoptosis of PC12 cells following serum deprivation. Biochem. Biophys. Res. Commun. 251: 199-203, 1998.

Takahiro K, Yukio N, Masahide H, Keitaro K. Purification and processing of rat liver Procathepsin B. J. Biochem. 113: 389-394, 1993

Tettamanti G, Grimaldi A, Ferrarese R, Palazzi M, Perletti G, Valvassori R, Cooper EL, Lanzavecchia G, de Eguileor M. Leech responses to tissue transplantation. Tissue Cell. 35: 199-212, 2003.

Tettamanti G, Grimaldi A, Rinaldi L, Arnaboldi F, Congiu T, Valvassori R, de Eguileor M. The multifunctional role of fibroblasts during wound healing in *Hirudo medicinalis* (Annelida, Hirudinea). Biol. Cell 2004 (in press).

Turk B, Turk D, Turk V. Lysosomal cysteine proteases: more than scavengers. Biochim. Biophys. Acta 1477: 98-111, 2000.

Turk V, Turk B, Turk D. Lysosomal cysteine proteases: facts and opportunities. EMBO J. 20: 4629-4633, 2001.

Wong JY, Harrop SA, Day SR, Brindley PJ. Schistosomes express two forms of cathepsin D. Biochim Biophys Acta 1338: 156-160, 1997.

Young PR, Karanutilake C, Zygas AP. Binding of cathepsin D to the mannose receptor on rat peritoneal macrophages. Biochim. Biophys. Acta 1095: 1-4, 1991.

Appendix

Leeches are invertebrates characterized by a relatively simple anatomy. The body wall of unstimulated, control leeches (*Glossiphonia complanata* and *Hirudo medicinalis*), contains several organs embedded in a loose connective tissue. It is composed of a monolayered epithelium surrounding three different layers of circular, oblique and longitudinal helical muscle fibers.

In spite of the basic plan, several differences can be noted in the body organization of leeches belonging to different families. The differences that may be remarked vary from the degree of longitudinal muscle layer thickness to a reduction of blood system, to the presence of peculiar tissues, embedded in the connective tissue and localized between the gut and the body wall. The more primitive leeches like *G. complanata* have a reduced longitudinal muscle layer, distinct blood vessels lying within coelomic sinuses, and no evidence of peculiar tissues. Conversely *H. medicinalis* shows a very thick longitudinal muscle layer, it has completely lost all traces of blood vessels, and the blood circulates in coelomic sinuses. In addition, between the gut and the body wall muscle fibers, *Hirudo* shows tissues characteristic of jawed leeches, called botryoidal and vaso-fibrous tissues (Mann, 1962).

The combination of the varied complexity of body organization and the different types of physical, biochemical or immune stimuli leads to modulated selected responses including production of antibacterial peptides, proliferation and migration of immune cells, new synthesis of extracellular matrix, phagocytosis, encapsulation of cumbersome foreign bodies and angiogenesis (characterised by a proliferation and migration of endothelial cells) (de Eguileor *et al*., 1999, 2000a, 2000b, 2001a, 2003; Lefebvre *et al*., 2004, Salzet 2001, Tettamanti *et al*, 2003).