Protease profile of normal and neoplastic mast cells in the human bone marrow

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Abstract

Mast cells (MC) are immune cells that produce a variety of mediators, such as proteases, that are important in the body's immune responses. MC proteases have pronounced multifunctionality and in many respects determine the biological characteristics of the organ-specific MC population. Although, increased numbers of MC are one of the objective mastocytosis signs, a detailed assessment of the proteases biogenesis and excretion mechanisms in the bone marrow has not yet been carried out. Here, we performed an analysis of the expression of proteases in patients with various forms of mastocytosis. We presented data on intracellular protease co-localization in the bone marrow MCs and discussed their implication in secretory pathways of MCs in the development of the disease. Characterization of MC proteases expression during mastocytosis can be used to refine the MC classification, make a prognosis, and increase the effectiveness of targeted therapy.

Introduction

Mastocytosis, a type of mast cells (MC) disease, caused by the accumulation of functionally defective MCs and MC precursors. When MCs undergo degranulation, the substances that are released can cause several symptoms that can vary over time and can range in intensity from mild to severe. The histochemical features of biosynthesis products of MCs made it possible for Paul Erlich to determine MC on micropreparations more than 130 years ago [1,2]. The arsenal of synthesized products in MCs is very diverse, including glycosaminoglycans, specific and nonspecific proteases, lysosomal enzymes, biogenic amines, mitogens, growth factors, cytokines, chemokines, etc. [3–5]. A special characteristic of the biology of MCs is the high presence of proteases in comparison with other immunocompetent cells [6,7].

Depending on the expression of specific proteases, human MCs are classified into tryptase-positive, chymase-positive MCs, and MCs expressing both proteases [3,8,9]. Specific proteases comprise up to 25% of the secretome proteins and form the characteristic phenotype of MC depending on their organization and accumulation in the cytoplasm. From exerting biological effects on the structures of connective tissue [10], tryptase and chymase are the most significant MC proteases, which are among the key factors influencing the phenotype formation of tissue microenvironment [11–13]. This allows us to consider specific MC proteases as relevant research objects in morphological practice, not only as diagnostic markers, but also as a promising pharmacological target for therapy [14–17]. The biological effects of tryptase and chymase depend on the secretion mechanism and have a selective effect on specific molecular microenvironment targets, modulating allergic and inflammatory reactions, angiogenesis, oncogenesis, causing remodeling of the extracellular matrix of connective tissue[10,18,19].

Due to the discovery of the hematopoietic cell carboxypeptidase gene, carboxypeptidase A3 has been shown to be an important marker for secretory granules, differentiation and MC phenotype [20–23]. At the same time, the detection of non-specific carboxypeptidases A1, A2, and B was carried out mainly to study pancreatic function, therefore the features of their distribution in MCs are still unexplored [24,25].

The cytomorphological examination of the bone marrow is an important diagnostic approach and an integral part of staging in mastocytosis. Cytomorphological aspects of MCs in smears of red bone marrow in systemic mastocytosis are very indicative of revealing a disease, have prognostic value and are important criteria for classifying a disease, not inferior to the effectiveness of molecular genetic studies, in particular, KITD816V mutation [26–31].

Immunohistochemical studies of MCs significantly expanded the informational content of ongoing studies to identify their functional state [28,30,32,33]. Immunohistochemical analysis of tryptase, chymase, and carboxypeptidase expression allows a better understanding of the development of mastocytosis and can be used both for the diagnosis of mastocytosis and as a promising pharmacological target. The aim of this study was a characterization of the protease profile of red bone marrow MCs in mastocytosis.

Results

We assessed protease expression in various types of MC: metachromatically granulated blast, atypical MC type II, atypical MC type I, and typical (mature) tissue MC [26,31]. Type I was differentiated into more mature MC (Ia type) and Ib type (more immature cells) [26].

Metachromatically granulated blast-like cells

Blast forms of MCs were small rounded cells of 5–7 μm in size with a predominant nucleus volume. The cytoplasm contained tryptase-positive and chymase-positive granules of type II and III and constituted a narrow rim around the nucleus (*Fig. 1).*. At the earliest stages of blast-like MC differentiation, protease-positive progranular formations or type I secretory granules could be seen in close contact with the nuclear membrane (*Figs. 1A, 1D, 1I)*.. At further stages of maturation, single protease-containing granules of type II or III were formed (*Fig 1F, 1G, 1I, 1J)*..

Multiple immunolabeling showed the existing identity of the synthesis of tryptase and chymase in blast forms of MC (*Fig. 1B, 1C)*.. In this case, the presence of specific proteases can be detected at the stage of type II secretory granules. From protease specificity, tryptase, and chymase co-localization was most often detected in blast-like MC granules. The formation of type III secretory granules is accompanied by a special folding of specific proteases along the periphery of the granule contents (*Fig. 1b)*.. As they mature, the blast forms of MC reveal individual characteristics of the protease phenotype, which can be expressed both in the spatial-quantitative ratio of tryptase and chymase in a single granule and the total number of secretory granules. Thus, in the early stages of development, the formation of MC is accompanied not only by phenotypic characteristics but also by the characteristic folding of specific proteases inside secretory granules. The specific tissue microenvironment of the red bone marrow is an important factor for the expression of MC proteases and determines the ratio of blast-like MC with a different protease phenotype (*Fig. 1C)*..

Interesting results were obtained by double immunolabeling of tryptase and carboxypeptidases A1, A2, and B. It turned out that the expression of carboxypeptidases was found in all blast forms, and most often they occupied a larger area in the cytoplasm compared to specific proteases (*Fig. 1D).*. During the formation of the secretory granules of type II and subsequently type III, not always was intragranular co-localization of tryptase and carboxypeptidases observed. *Fig. 1E* clearly shows that during the differentiation of a blast- like mast cell, the bulk of carboxypeptidases was located outside tryptase-positive granules. These facts indicates that at the stage of MC blast forms, the main stages of specific proteases processing are carried out extragranularly and reflect the MC activity level on post- translational proteins modification for various purposes. Less mature type II secretory granules do not have the level of intragranular tryptase and chymase processing characteristic of type III mature granules. Thus, the expression of specific proteases at the initial MC differentiation stages is quite variable and closely related to the specific tissue microenvironment state of the red bone marrow.

Atypical mast cell type Ia

Type Ia mast cells are the morphological equivalent of the most differentiated atypical MC variant of the red bone marrow in mastocytosis. They acquire an elongated shape and demonstrate pronounced cytoplasmic outgrowths (extensions), which can extend over considerable distances (*Fig. 2).*. In the perinuclear zone of the cytoplasm of such MCs, proteases can appear in mature type III secretory granules (*Figs. 2C, 2G, 2O).*

When the proteases intracellular organization is completed at the secretory granules level of type I or II, a hypogranulated MC phenotype is formed (*Figs. 2A, 2E, 2J, 2N, 2P, Fig. 6, Fig. 7)*.. Mature or immature secretory granules with specific proteases can be seen distally from the nucleus along the outgrowths of the cytoplasm while occupying the peripheral position, actually adjacent to the plasmalemma (*Figs. 2A, 2B, 2D, 2E, 2C, 2H, 2I)*.. Figure 2d demonstrates a transverse section of the cytoplasmic outgrowth characterizing the cytotopography of specific proteases in atypical type Ia MCs. Here, the central part free of secretory material is visible. The secretome may be tryptase-positive or contain both specific MC proteases (*Fig. 2D)*..

In atypical type Ia MCs, granules are formed in the perinuclear region, and then transported over fairly considerable distances through the cytoplasm via the corresponding intracellular transport systems. *Fig. 2N* can serve as the morphological evidence of this process, where the directed transport of tryptase into the peripheral region of the cytoplasmic outgrowth of MC is well visualized. Proteases contained in granules of various maturity stages and progranules formations can be transported along the periplasmalemma region (*Figs. 2G, 2H, 2I, 2O)*.. Apparently, the granules occupy certain loci throughout the cytoplasmic outgrowth and secrete proteases by the “kiss and run” mechanism. Interestingly, in some cases, the cytoplasmic outgrowths may have a considerable length (*Fig. 2F)*.. This might be a manifestation of the mechanism determining the MC influence on the endothelium functional activity, as well as on the entry of proteases into the bloodstream. The carboxypeptidases presence in the cytoplasm

of atypical type Ia MCs outgrowths indicates the universal mechanism of these proteases work, regardless of their disposition in the cell and the secretome proteins processing at a considerable distance from the nucleus. Localization of carboxypeptidases directly in the granules may indicate a partial cessation of post- translational modification of proteins in the cytoplasm after their entrance into granules.

Notably, the high content of carboxypeptidases in the cytoplasm indicates an active level of these enzymes expression in MC. However, in the case of hypogranulated MC, the high carboxypeptidases content may indicate an active modification of enzymes with the acquisition of biological activity and rapid excretion from the cell. Obviously, a decrease in the carboxypeptidases representation is evident of the secretome components expression level reduction, including specific proteases, and reflects the formation of a state of relative calm in biosynthetic processes.

It should be noted that the different cytotopographic localization of tryptase and carboxypeptidases in hypogranular forms of MC is a frequent morphological finding. Significant differences exist in the proteases content in the MC of hypogranulated and granular forms. Most likely, the hypogranular forms are a more unfavorable variant of the course of mastocytosis due to the rather high intensity of protease released into the extracellular matrix using piecemeal secretion. In this case, proteases are not formed into classical mature granules and are not stored in the cytoplasm of MC, but undergo rapid secretion. This fact is considered an unfavorable diagnostic sign of mastocytosis associated with the multifaceted biological effects of tryptase and chymase.

At the same time, by indolent forms of mastocytosis, atypical type Ia MCs of the spindle-shaped form with outgrowths of the cytoplasm contain mature type III secretory granules (*Figs. 2C, 2G, 2L)*.. This is an obvious morphological sign of a more favorable course of the disease and a slower proteases release into the extracellular matrix is evident with the wide palette provision of biological and undesirable specific proteases pathophysiological effects.

Atypical mast cell type Ib

This type of cell is less differentiated compared to type Ia MC. Two types of such cells can be distinguished: granular and hypogranular. In the first case, such MCs may resemble the structure of mature forms of MCs. However, the elongated shape of the nucleus and its eccentric position, as well as a possible pronounced polymorphism of the granules, indicate an atypical MC phenotype (*Fig. 3)*.. Protease-containing granules can be of various sizes, even if they are not numerous in the cytoplasm (*Figs. 3I, 3G, 3M)*.. Some granules have abnormally large sizes, which indicate a disturbance of the secretion mechanism and a distortion of the release of specific proteases into the extracellular matrix (*Figs. 3I, 3J)*.. At the same time, the presence of ordinary granules in such cells compensates for the MC regulatory potential failure by specific proteases. Depending on the course of mastocytosis, the MC granular and hypogranulated forms ratio changes with an increase in the MC and may indicate an unfavorable course of mastocytosis.

Unlike type Ia, type Ib MCs do not form significant outgrowths of the cytoplasm, but they can form wide protrusions (*Figs. 3B, 3L)*.. At the same time, the perinuclear cytoplasmic region presence in many hypogranulated cells which is almost immunonegative for specific TK proteases, suggests very active processes of post-translational specific proteases modification in the Golgi complex (*Figs. 3B, 3D, 3K)*.. Such cells can represent the majority of MC in the red bone marrow population with aggressive forms of mastocytosis.

At the same time, it is possible to suggest the secretion of proteases in granular atypical type Ib MCs using the “kiss and run” mechanism, in which mature granules line up along the plasma membrane, come into contact with it and form selective transport of the necessary secretory components (*Fig. 3A, Fig. 7)*.. Granules of atypical type Ib MCs most often contain both tryptase and chymase. In the non-aggressive course of mastocytosis, extensive protease- negative zones are formed in the cytoplasm of type 1b MC, which may indicate low activity of protease secretion into the extracellular matrix.

In hypogranulated atypical type Ib MCs, proteases are diffusely distributed over the cytoplasm, which implies active piecemeal degranulation of proteases into the extracellular matrix. Unfortunately, immunohistochemical confirmation of microvesicular transport is very difficult due to the small size of secretory formations released from the cell (*Fig. 3E)*..

However, it should be noted that the hypogranulated cells are quite clearly differentiated into tryptase-positive, and MC, which express both tryptase and chymase (*Figs. 3C, 3D, 3E)*.. In some cases, individual mature granules are observed in hypogranulated cells, which may contain both tryptase and chymase.

Noteworthy, the protease profile of atypical type Ib MCs is characterized by a high content of carboxypeptidases A1, A2, and B (*Figs. 3F, 3H)*.. This indicates a high level of post-translational protein modification processes for constitutive exocytosis or induced secretion. Three variants of cytotopography of carboxypeptidases can be noted: mainly outside the granules, in granules, and combined localization. Most likely, this is associated with the stage of the intracellular process in the biogenesis of specific proteases. The presence of small progranular formations that do not contain carboxypeptidases with their high concentration in the cytoplasm indicates the actively proceeding extragranular stage of the processing of specific proteases (*Fig. 3F)*.. Localization of carboxypeptidases exclusively in mature MC granules may indicate proteases modification suspension in the cytoplasm and the intragranular proteases local modification (*Fig. 3G)*.. Finally, the carboxypeptidases detection both in the cytoplasm and inside mature granules indicates an actively ongoing process of granule biogenesis at all stages of proteases processing (*Fig. 3H)*..

Atypical mast cell type II

Atypical type II MCs are a morphological sign of the progression of mastocytosis. Their striking feature is the segmentation of the nucleus (nuclei bi- or polylobed) (*Fig. 4)*..

Interestingly, a rather atypical shape of the nuclei or their segments, which forms the “dentation” of the contours, was observed (*Fig. 4I).*. In some cases, protease-positive mast cells had atypical segmentation in the form of disproportionately small outgrowths or had nuclear segments of different sizes (*Figs. 4L, 4M, 4N).*. The sizes of such MCs can differ significantly from each other in the range from 10 to 20 μm. Also, the nuclear-cytoplasmic ratio can be different, from high to low. Having in some cases a small volume of cytoplasm, atypical type II MCs contain very small secretory granules (*Fig. 4A).*. Small granules are evenly distributed in the cytoplasm, without the formation of local clusters. The cytoplasm of MCs can be immunopositive for tryptase and chymase not only within the granular formations or granules but also between them. Often different in size, type II MCs are closely adjacent to each other (*Figs. 4A, 4B).*. Moreover, neighboring MCs may differ in the expression of specific proteases from each other. For example, type II MC with simultaneous expression of tryptase and chymase can be contacted with a smaller tryptase-positive MC (*Figs. 4A, 4C).*.

Also, large MCs of type II may also affect other types of MCs, obviously affecting further differentiation, protease expression, and secretory activity (*Fig. 4B).*.

The intracytoplasmic distribution of proteases can be almost uniform. However, immature granules of type II may be of three variants: tryptase-positive, chymase-positive, and also with the simultaneous content of both proteases (*Fig. 4D).*. Binuclear MCs are sometimes granular and have mature secretory granules with characteristic edge localization of tryptase and chymase in the form of a ring, however, their number may depend on the specificity of the disease (*Figs. 4E, 4J, 4K).*. Large mature granules can be represented in various numbers, from a single (*Fig. 4K)* to the almost complete filling of the cytoplasm (*Fig. 4J).*. Despite the generally high expression of specific proteases in hypogranulated type II MC, it can vary from moderate to high (*Fig. 4H).*.

Binuclear cells were typically characterized by high levels of carboxypeptidases (*Fig. 4F, 4G).* This is the indirect evidence of a high degree of secretogen biogenesis in MC. However, the intracytoplasmic distribution of carboxypeptidases was characterized by significant variation.

Typical tissue MCs

Typical mature MCs of the red bone marrow were well-granulated, large cells, reaching a size of 15–20 μm. Their nuclei, as a rule, had a rounded shape and were located in the center of the cell. Specific proteases were formed into well-distinguishable granules about 1 μm in size; their number was significantly high and they could fill almost the entire cytoplasm (*Fig. 5).*.

In mature cells, the predominant intragranular localization of specific proteases became quite obvious. As a rule, both tryptase and chymase were located on the periphery of the granule, which was earlier shown by us in MCs of other organs (*Figs. 5A’, 5B’)* [18,34]. At the same time, mature MCs separate phenotypes existence of three variants in the red bone marrow was noted: with the chymase predominance, the prevalence of tryptase, or the specific proteases simultaneous maintenance in relatively equal amounts

(*Figs. 5A, 5B, 5C)..* The mode of the intragranular distribution of proteases is noteworthy. In particular, epifluorescence microscopy gives the impression of a larger area occupied by the chymase compared with tryptase-positive material (*Figs. 5A, 5B)..*

In contrast to blast forms, it is apparent that in mature granular MCs the process of modifying the protein components of the secretome occurs at the intragranular level. *Fig. 5E* demonstrates the localization of carboxypeptidases within the granules. When it is necessary to synthesize an additional amount of proteins, including specific proteases, activation of intracytoplasmic processing is realized in the appearance of carboxypeptidases outside the granules (*Fig. 5D)..*

Sometimes protease-positive granules were visualized autonomously in a specific tissue microenvironment of the red bone marrow. This indicates the possibility of their autonomous existence in the extracellular matrix sometime after secretion, which allows the delayed regulatory functions of tryptase and chymase to be realized.

Discussion

This study showed that each type of atypical MC in mastocytosis has its protease profile, which is characterized by the ratio of proteases content within a cell. The total set of MCs determines the integral functional potential of tryptase and chymase proteases in the bone marrow. We have also revealed the expression of nonspecific carboxypeptidases A1, A2, and B in all MCs. Three MC states can be distinguished, depending on the cytotopographic features of the distribution of carboxypeptidases. The presence of carboxypeptidases A1, A2, and B in the cytoplasm of MC indicates actively occurring protein processing processes.

Localization of carboxypeptidases mainly in mature type III granules or immature type II granules indicates the absence of post-translational proteases modification necessary for incorporation into granules.

At the same time, intragranular protease processing continues even when the transcription of tryptase and chymase genes ceases, indicating the possibility of certain rearrangements in the enzyme's structure and biopolymers localized in secretory granules. The identified feature is of great importance for the realization of the specific proteases functional potential after secretory granules exocytosis into the extracellular matrix, implying the possibility of prolonging the MC secretome regulatory role, including tryptase and chymase. The formation of atypical MC hypogranular forms may be due to the specificity of the mechanisms responsible for secretion into the extracellular matrix.

Additional criteria for mastocytosis: cytotopography of processing and secretion of specific proteases

The pathogenesis of mastocytosis is closely associated with the biogenesis of specific proteases. About 5% of the genome encodes information on human MC proteases, and the protease mRNA content is comparable to the content of housekeeping genes transcripts. This reflects the high content of proteases in the cytoplasm of MC [7,15,35–39].

Tryptase and chymase processing ends with the formation of monomeric or tetrameric forms of enzymes. In particular, the tryptase tetramer with a molecular weight of 140–142 kDa has maximum biological activity and consists of four identical monomers stabilized in the presence of heparin or other glycosaminoglycans at pH values below 6.5 [40]. The stability of tryptase in MC granules also depends on the histamine content [41]. Chymase, in contrast to tryptase, is active in monomeric form and is localized in granules also in combination with heparin. Once in the extracellular environment, the chymase remains in complex with heparin, which protects it from neutralization by endogenous inhibitors [42]. At the same time, the chymase activity is known in the absence of heparin, which makes it an understudy to a certain extent of tryptase, ensuring the development of similar physiological and pathological effects in the formation of conditions for a local decrease in glycosaminoglycans in the tissue microenvironment.

MC proteases during the accumulation of granules in the cytoplasm do not show enzymatic activity due to the characteristics of the pH level and interaction with serglycin and glycosaminoglycans [9,12,43]. Therefore, an indirect judgment on the content of proteases in MC granules can be obtained after using various metachromatic staining options [26,44–46].

Packaging specific proteases in granules

The classical model of the formation of MC secretory granules suggests that after protease synthesis in rough endoplasmic reticulum they enter the Golgi complex, where they undergo post-translational modification and are packaged in small-sized granular formations surrounded by a membrane [47,48]. These granules containing specific and non-specific proteases complex with proteoglycans in the cytoplasm can merge, and after combining with acid hydrolases from type early endosomes, type I secretory granules are formed (*Fig. 7)*..

Morphologically, they are indistinguishable, small in size, and are further combined with the formation of secretory granules of type II, reaching visualized sizes from 0.2 to 0.4 μm (*Fig. 7)*.. At this stage of maturation, type II secretory granules have a certain secretome composition, including glycosaminoglycans, proteases, lysosomal enzymes, etc. Type II secretory granules are enlarged in size by combining with similar or secretory type I granules (*Fig. 6, 7)*.. Their further maturation leads to a further increase in volume and is accompanied by the formation of type III secretory granules with sizes of 0.4–1 μm with the accumulation of a unique composition of specialized secretome components. At the same time, a broad individuality in the accumulation of certain mediators in the formed granules is possible, the amount of which in each MC can be hundreds, with a significant individuality in the content

of proteases (*Fig. 7).*. Such granules can be tryptase-positive, chymase-positive, as well as with the simultaneous presence of tryptase and chymase (*Fig. 7).*.

Formation of a hypogranulated TC phenotype

The MC's hypogranular forms formation reflects the active, unfavorable course of the disease, which is accompanied by a high level of protease synthesis, coupled with active liberation into the extracellular matrix. The formation of immature and mature granules activity can be explained by the stochastic model for MC granule growth and elimination with the direct participation of nano-machines [48]). In this case, the homotypic fusion of small granules with each other is possible, leading to the unification of their contents and the outer coating with a common plasma membrane (*Fig. 7).*. At the same time as shown with confocal microscopy, it is possible to suggest the possibility of unequal granules further merging, which might be a common granule maturation and modification mechanism of the secretome intragranular composition (*Fig. 6).*.

Ultimately, the content and composition of the granules reflect the cell physiological state, adequate to the conditions of local homeostasis, which can be significantly distorted in pathology. The dependence of the various sizes in MC granules formation is closely related to the needs of a specific tissue microenvironment in one or another secretome component and an integral secretory activity. As a rule, the size of the granules correlates with the duration of their stay in the mast cell cytoplasm. Small granules have a higher rate of post- translational modification of the proteases enclosed in them, are practically not stored, and are characterized by rapid exchange of secretome components in the course of active secretion into the extracellular matrix. Obviously, in the case of hypogranulated MC in mastocytosis, the specific proteases maturation can be completed at the level of type I and II secretory granules with further active secretion into the extracellular matrix, leading to various clinical or organ-specific manifestations (*Fig. 6, Fig. 7).*. Moreover, in atypical MCs, a certain level of mature secretory granules in the cytoplasm may not be maintained, which is a kind of buffer for an adequate response to the need of the extracellular matrix in a particular secretory component. Further on, when discussing the content of proteases in atypical hypogranulated forms of MC during mastocytosis, one should take into account the fact that young granules may be preferred in secretion over mature ones (Hammel I, Meilijson I, 2015). This fact can serve as an additional explanation for the formation of the hypogranulated MC phenotype in mastocytosis.

Secretory granule as a structural entity of functional activity of MC

The granules structure depends on the degree of maturity, the proteases processing stage and the activity of secretory pathways adequately to the tissue microenvironment challenges.

Therefore, in the morphological aspect, it is important to evaluate the topographic features of the arrangement of enzymes in granules. The study of the localization of secretome components in MC granules in mastocytosis was carried out using electron microscopy [49]. In the granules, various contrast-rich objects were identified in the form of twisted plates - “scrolls”, as well as grating and/or lattice-like structures, etc. However, these results cannot provide information on the qualitative composition of the visualized structures [50]. In several works with the improved technique of immune-electronic histochemistry, the granules ultrastructure dependence on the presence of proteases was shown; for example, granules with tryptase contained “scrolls” that could overlap each other, and granules with chymase were characterized by the presence of a crystalloid lattice [49,51,52]. Tryptase could be co-localized with chymase, carboxypeptidase A and cathepsin G in the same granules [49]. However, it should be noted that the solution to the problem of the protease co-localization in granules during electron microscopic examination is very difficult due to the effect of fixing the biomaterial, the histological section plane under study and first, the methodological issues of double immunolabeling.

Our data suggest that contrast-reach formations along the periphery of the granules observed by electron microscopy, as well as possibly scrolls, are a morphological reflection of specific proteases location at these loci (*Figs. 5, 6)*.. An immunomorphological study is a very informative addition to electron microscopy, allowing the detection of fluorochrome-labeled proteases in MC, including inside the granules. Modern advances in confocal microscopy with an ultra-high-resolution option provide unique molecular morphological information about the topography of intragranular co-localization of specific MC proteases (*Fig. 6)*..

Protease profile of TC in the diagnosis of mastocytosis

The ratio of tryptase-positive and chymase-positive MC in the red bone marrow will be of great importance for the specific tissue microenvironment formation, indicating the vector of functional changes presence at a given time. At the same time, it should be noted that the high variability of the expression of the specific protease within each MC, regardless of its type. The co-localization of tryptase and chymase in the same granules (*Fig. 6, Fig.7)* emphasized the variability of this criterion depending not only on organ affiliation but also on the development of pathology. Thus, the cytological characteristic of the expression and localization of tryptase and chymase in MC is a separate diagnostic value for assessing the progression of mastocytosis.

Secretory pathways of proteases

Evaluation of the secretory pathways, which exert the physiological effects of tryptase and chymase in the intercellular matrix, has a significant information potential. In detail, these processes are described in our previous works [18,34]. In parallel with the secretome maturation, the molecular nano-machines associated with the granules allow precise regulation of the release of the necessary mediators from the

granules with further transportation to the cytoplasm and extracellular matrix [Blank U et al, 2014} (*Fig. 6, Fig. 7).* Unfortunately, microscopy does not allow visualization of some events associated with the hypogranulated forms of atypical TC in mastocytosis, such as transgranulation, microvesicular transport, and exosome formation for the secretion of mediators, etc.

Gradual degranulation or a microvesicular transport provide background (constitutive) secretion of tryptase and chymase into the intercellular space, the intensity, despite its weak severity, is determined by the scale of proteases local participation in the local homeostasis regulation [53,54] (*Fig.7).*. However, it is obvious that with the development of mastocytosis, this tryptase and chymase secretion mechanism can acquire significant activity, despite the absence of morphological evidence. MCs are known to have the ability, under certain conditions, to accelerate secretion hundreds of times per unit time [48]. Vesicles of 30–150 nm in size that are peeling from mature granules undergo after intracellular transport a separate secretion into the extracellular matrix. Gradual degranulation is an important signaling system for the interaction of MCs with each other. Considering the tight MC neighboring to each other in the red bone marrow, as a pathognomonic sign of the disease, we can assume the active piecemeal degranulation participation in the MC intercellular signaling using specific proteases.

Proteases can be secreted into the tissue microenvironment via the “transgranulation” mechanism, during which micro bulging of MC cytolemma is formed at specific loci in contact with other cells (*Fig. 7).*. For example, this can be observed during the contact of MC with each other, as well as in contact with cells of the fibroblastic differon, endothelium, etc. 10,18,34. Finally, a visually indetectable mechanism of MC proteases secretion into the extracellular matrix is possible through the exosomes formation [55] (*Fig. 7).*.

Along with the above degranulation mechanisms, there are other options for proteases release into the extracellular matrix, which was observed in granular forms of atypical MC in mastocytosis, as well as in typical MC of red bone marrow. With the kiss-and-run secretion mechanism [53], MC granules came into contact with the plasma membrane to form a temporary pore releasing the proteases into the extracellular matrix in corresponding amount with slightly higher intensity compared to the microvesicular secretion mechanism [53] (*Figs. 2C, 2G, 2H-K, 3A, Fig. 7).*. The peripheral arrangement of granules in type I MC indicates the active use of this mechanism in specific proteases secretion. The further fate of tryptase and chymase in the extracellular matrix depends on many parameters that determine the rate of protease cleavage from serglycin and subsequent diffusion in the intercellular matrix.

Proteases can enter the extracellular matrix using the mechanism of “macrovesicles” formation, which are fragments of the TC cytoplasm containing mediators that are gradually able to be secreted by other mechanisms and diffuse to the target substrates [34]. MC granules or individual fragments of their cytoplasm in the stroma of the organ have autonomy in decision-making and can participate in achieving the required chymase and tryptase concentration within the tissue microenvironment necessary limits without a “maternal” MC participation [34]. However, in mastocytosis, this mechanism is quite rare and is a characteristic of predominantly mature MCs.

In the case of allergy, anaphylactic degranulation of MC can be observed. It is accompanied by a massive excretion of granules into the extracellular matrix with a generalization of the process. Perhaps, in some cases, this can significantly aggravate the course of mastocytosis.

Specific MC proteases as multifunctional mediators

Specific proteases bio-effects development in the red bone marrow begins from the moment they enter the extracellular matrix and is characterized by a number of specific features. In particular, specific proteases are involved in collagen fibrillogenesis (Atiakshin D, Buchwalow I, Tiemann M., 2020). This explains the frequent detection of sclerosis and collagen fibrosis in the red bone marrow associated with the prevailing presence of atypical MCs in mastocytosis [56].

Tryptase

Tryptase has a high biological activity, affecting the state of many cellular and non-cellular components of the tissue microenvironment [8,38–40,57]. At the same time, secreted MC proteases can lead to further intensification of degranulation using the autocrine mechanism, as well as to increase the liberalization of biogenesis products in eosinophilic granulocytes [38].

Tryptase has its molecular targets on the cells or components of the extracellular matrix, causing any pro- or anti-inflammatory effects [38,42,58–62]. Most often, tryptase initiates the development of inflammation, causing an increase in the permeability of the capillary wall, increasing the migration of neutrophils, eosinophils, basophils and monocytes beyond the microvasculature [63]. These effects of tryptase can be mediated by the induction of the formation of kinins, IL–1, and IL–8 in the endothelium, which is combined with a change in the synthesis of ICAM–1 intercellular adhesion protein. Several studies have shown the close involvement of tryptase in the processes of angiogenesis. Moreover, the formation of new vessels is combined with the pronounced connective tissue remodeling, associated primarily with the degradation of the extracellular matrix amorphous and fibrous components, the growth factors secretion, cytokines and chemokines, matrix metalloproteinases (MMPs).

The synchronous secretion of MMPs and tryptase may have a reasonable explanation since the latter has the properties to activate various MMPs synthesized not only by MC but also by other cells of the connective tissue in an inactive form within the tissue microenvironment. This list includes MMP–1, MMP–2, MMP–3, MMP–9, MMP–13, etc.

Thus, tryptase, by activating MMP, is capable of exerting far-reaching extracellular matrix rearrangements associated with degradation of both the fibrous component and ground substance components, including laminin, fibronectin, several proteoglycans, etc. [10,18,40,57]. Finally, the effects of tryptase on the fibroblastic differone cells are shown, causing their active movement, mitotic division, and stimulation of

the collagen proteins synthesis. As a result, the effects of tryptase may promote wound healing and can lead to fibrotic effects[10,57,64].

Tryptase has a high tropism for PAR–2 receptors, potentiating the development of inflammation. Localization of these receptors on various cells of a specific tissue microenvironment can lead to pro-inflammatory signaling, including afferent neurons. An important tryptase regulatory mechanism in potentiation of inflammation is the induction of a persistent increase in PAR–2 receptor expression in various connective tissue cells. In particular, in the airways, this leads to the formation of functional prerequisites for exacerbation of bronchoconstriction, mucus secretion by mucocytes. PAR2 promotes M1 macrophage polarization and inflammation via FOXO1 pathway [65]. In cells of certain areas of cartilage tissue, PAR–2 receptors increased expression leads to arthritis progression, degradation in osteoarthritic cartilage, inflammation, chondrocyte apoptosis, and cartilage breakdown [66]. After surgical interventions, an increase in the presence of PAR–2 on soft tissue cells significantly complicates the course of the postoperative period. It was shown that PAR2 enhanced the expression of MYO10 through the repression of miR–204. PAR2 mediated tryptase-induced cell migration and might contribute to the invasion of cancer cells at the edge of tumor [67]. In addition, an important effect of tryptase is the activation of secretion by cells of a specific tissue microenvironment of pro-inflammatory mediators into the intercellular matrix, creating an increased background content of cytokines chemokines[68,69.]

In light of the previously described effects of tryptase, the progression of allergic reactions is also important [70]. Tryptase leads to the stimulation of histamine liberalization from intracellular depots, which, in turn, causes a new increase in tryptase secretion. This contributes to the involvement of new MCs in the degranulation process, creating conditions for the realization of the biological effects of histamine over a larger area [71].

In the literature, various mechanisms of the influence of tryptase are considered that contribute to the growth and differentiation of new blood vessels, including oncogenesis [72,73]. The stimulating role of tryptase in neoangiogenesis in B-cell non-Hodgkin lymphoma, multiple myeloma, chronic lymphocytic leukemia, melanoma, etc. has also been shown [Ribatti D. 2016]. Recently, interesting information has appeared about the possible anti- oncogenic mechanisms of tryptase [55].

Chymase

The substrates of another specific TK protease, chymase, are various components of the extracellular matrix, receptors, proteins, as well as chemokines and cytokines [Pejler G et al., 2007]. Human chymase actively hydrolyzes angiotensin I to its active form of angiotensin II, participating in both local and systemic mechanisms of blood pressure regulation in physiological and pathological conditions, in the pathogenesis of hypertension. In addition to the function of angiotensin II as an effector peptide of the renin-angiotensin system, it also has effects on the regulation of cell growth, angiogenesis, regeneration, and tissue remodeling[10,74].

Chymase is capable of causing MC migration to the place of destination in tissues and thus acts as an inducer of directional movement [75]. Chymase is involved in the development of pulmonary hypertension and fibrosis. Chymase inhibitors reduced pulmonary hypertension, improved hemodynamics, decreased right ventricular hypertrophy, remodeled blood vessels, and reduced connective tissue in the lungs [76].

Chymase is directly capable of changing the state of many extracellular matrix components and, in comparison with tryptase, has a more pronounced destructive potential [8]. On the other hand, chymase is less resistant to inhibition and neutralization by extravascular antipeptidases, including serpins and α2-macroglobulin, and has a shorter interval of enzymatic activity in the tissue microenvironment. Chymase can lead to direct effects of the fibronectin degradation and its fragments accumulation in the intercellular substance of connective tissue, as well as vitronectin, laminin, and other components. The chymase effects can be mediated, in particular, by activation of collagenase, MMP–2, MMP–9, inhibition of TIMP–1, etc. Chymase can induce an increase in mitotic and biosynthetic activity of fibroblasts. An important fact is the participation of chymase in the procollagen molecules enzymatic rearrangement, which makes possible the tropocollagen polymerization with the collagen fibrils and fiber growth formation both in length and thickness [10].

An increase in chymase level correlated with the development of fibrosis in experimental diabetes and autoimmune liver fibrosis, while the use of chymase inhibitors reduced its progression [8]. The role of chymase in the formation of keloid skin scars has also been shown [13]. Our previous studies convincingly indicated MCs direct participation in fibrillogenesis by using a pronounced inductive effect on the ratio of the tissue microenvironment components, including areas around the fibroblastic differone cell [10]. Also, the ability of MC to actively secrete fibrillogenesis inducers is often expressed in the formation of its initiation points close to the MC cytoplasm.

Chymase has a different effect on bioactive peptides. On the one hand, it can activate IL–1β, IL–8, IL–18, neutrophil-activating peptide 2, transforming growth factor-beta, endothelin–1, etc. In this regard, the ability of a chymase, like a tryptase, to provide pro- inflammatory effects causes the recruitment of neutrophils, eosinophils, basophils, monocytes, and lymphocytes into the tissue microenvironment [77]. Like tryptase, chymase is actively involved in angiogenesis, which contributes to the progression of cancer [78]. On the other hand, it can correct the area of the ischemic lesion, the number, and histoarchitectonics of intraorgan vessels, including the myocardium. Chymase can cause allergic reactions in the skin with the degradation of the structures responsible for attaching the epidermis to the basement membrane in dermatitis, atopic dermatitis and eczema, increase the permeability of the mucous membranes, reduce the epithelium barrier function, increase the vascular wall permeability, the synthesis of IgE and IgG1, inhibit smooth muscle cell proliferation and cause their apoptosis development.

In particular, chymase is involved in the pathogenesis of increased vascular permeability in preeclampsia and Crohn’s disease. Chymase is a powerful MC degranulation inducer and a stimulator of histamine

excretion into the extracellular matrix, which leads, to one degree or another, to the generalization of peritonitis, the aortic aneurysm formation, the myocardial region's expansion with metabolic disorders in heart attack, the secretory activity activation of glandular cells in the respiratory tract, etc. On the other hand, chymase causes degradation of tumor necrosis factor, substance P, VIP, kallikrein, bradykinin, complement component C3a, IL–1β, IL–5, IL–6, IL–13, IL–18, IL–33, preproendothelin –1, tumor necrosis factor-α and eotaxin [77]. Reducing the transition processes intensity of cholesterol into macrophage lipoprotein bodies, chymase leads to the formation of "foamy cells" and, accordingly, to the atherosclerosis progression. A close association of the chymase effects with an aortic aneurysm, chronic trophic leg ulcers, lung, liver and kidney diseases, diabetic nephropathy and retinopathy, conjunctival epithelium apoptosis, the development of systemic scleroderma, arthritis, etc. has also been shown [8,79].

Methods

Patients. 8 mastocytosis patients were included in this study. Informed consent was obtained from all subjects. The samples were retrieved from the files of the Institute of Pathology, Ludwig-Maximilians University, Munich, Germany. These samples were redundant clinical specimens that had been de-identified and unlinked from patient information. Histological diagnoses were established according to the WHO classification [80–82]. This study was conducted in accordance with the principles of the World Medical Association Declaration of Helsinki "Ethical Principles for Medical Research Involving Human Subjects" and approved by the Institutional Review Board of the Institute for Hematopathology, Hamburg, Germany.

Tissue probe stainings. Tissue probes were fixed in buffered 4% formaldehyde and routinely embedded in paraffin. Deparaffinised and rehydrated sections (2 µm thick) were subjected to antigen retrieval by heating in a steamer with sodium citrate buffer, pH 6.0, at 95°C x 30 min. We reported earlier that endogenous Fc receptors in routinely fixed cells and tissue probes do not retain their ability to bind Fc fragments of antibodies [83]; therefore, blocking the endogenous Fc receptors prior to incubation with primary antibodies was omitted. After antigen retrieval, sections were immunoreacted with primary antibodies to tryptase, chymase, and carboxypeptidases (*Table 1).* Principally, immunohistochemical staining was performed according to the standard protocols described earlier [84–86].

For brightfield microscopy, bound primary antibodies were detected with AmpliStain™ Horseradish Peroxidase (HRP) conjugates (SDT GmbH, Baesweiler, Germany) according to the manufacturers' instructions [87]. The HRP label was visualized using a 3,3'- diaminobenzidine (DAB) substrate kit (Vector Laboratories, Burlingame, CA, USA). The nuclei were counterstained with hematoxylin.

For fluorescence detection, bound primary antibodies were visualized using secondary antibodies (purchased from AbCam, United Kingdom) conjugated with Cy3 or Alexa Fluor-

488. Nuclei were counterstained with 4',6-diamidino–2-phenylindole (DAPI, 5 µg/ml in PBS) for 15 s, and the sections were then mounted using VectaShield (Vector Laboratories, Burlingame, USA). The list of

secondary antibodies and other reagents used in this study is presented in *Tables 2.*

Table 1. Primary antibodies used in this study

Antibodies	Host	Catalogue Nr.	Dilution	Sourse
Tryptase	mouse monoclonal Ab	#ab2378	1:3000	AbCam, United Kingdom
Tryptase	rabbit monoclonal Ab	#ab151757	1:2000	AbCam, United Kingdom
Chymase	mouse monoclonal Ab	#ab2377	1:2000	AbCam, United Kingdom
Carboxypeptidases A1, A2 and B	rabbit monoclonal Ab	#ab 181146	1:500	AbCam, United Kingdom

Table 2. Secondary antibodies and other reagents

Antibodies and other reagents	Source	Dilution	Label
Goat anti-mouse IgG Ab (#ab97035)	AbCam, United Kingdom	1/500	Cy3
Goat anti-rabbit IgG Ab (#ab150077):	AbCam, United Kingdom	1/500	Alexa Fluor 488
AmpliStain™ anti-Mouse 1-Step HRP (#AS-M1-HRP)	SDT GmbH, Baesweiler, Germany	ready-to-use	HRP
AmpliStain™ anti-Rabbit 1-Step HRP (#AS-R1-HRP)	SDT GmbH, Baesweiler, Germany	ready-to-use	HRP
4',6-diamidino-2-phenylindole (DAPI, #D9542-5MG)	Sigma, Hamburg, Germany	5 µg/ml	w/o
VECTASHIELD® Mounting Medium (#H-1000)	Vector Laboratories, Burlingame, CA, USA	ready-to-use	w/o
DAB Peroxidase Substrat Kit (#SK-4100)	Vector Laboratories, Burlingame, CA, USA	ready-to-use	DAB
Mayer's hematoxylin (#MHS128)	Sigma-Aldrich	ready-to-use	w/o

Image acquisition. Immunostained tissue sections were observed on a ZEISS Axio Imager.A2 equipped with a Zeiss alpha Plan-Apochromat objective 100x/1.46 Oil DIC (UV)

VIS-IR and AxioCam digital microscope cameras (Axiocam 506 color and Axiocam 503 monochrome CCD). Captured images were processed with the software program ZEN 2.3 (Carl Zeiss Vision, Germany) and submitted with the final revision of the manuscript at 300 DPI (*Figs. 1–5).*. Two- and three-dimensional images with a higher optical resolution were obtained using a confocal scanning microscope ZEISS LSM 880/Airyscan equipped with a Zeiss Plan-apochromat objective 63x/1.40 oil (*Fig. 6).*.

Controls. Control incubations were: omission of primary antibodies or substitution of primary antibodies by the same IgG species (Dianova, Hamburg, Germany) at the same final concentration as the primary antibodies. The exclusion of either the primary or the secondary antibody from the immunohistochemical reaction, the substitution of primary antibodies with the corresponding IgG at the same final concentration resulted in a lack of immunostaining.

Data availability

The authors declare that all the data supporting the findings of this work are available within the article and its Supplementary Information files or from the corresponding author upon reasonable request.

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Declarations

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Author contributions

Contribution: D.A and I.B. designed the study, did immunohistochemistry, conceived, and prepared the manuscript. P.H. collected the clinical data. P.H. and M.T. performed the analysis and supervised the study. All authors read and approved the final manuscript.

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Competing interests

The authors declare no competing interests.

Additional information

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Figures

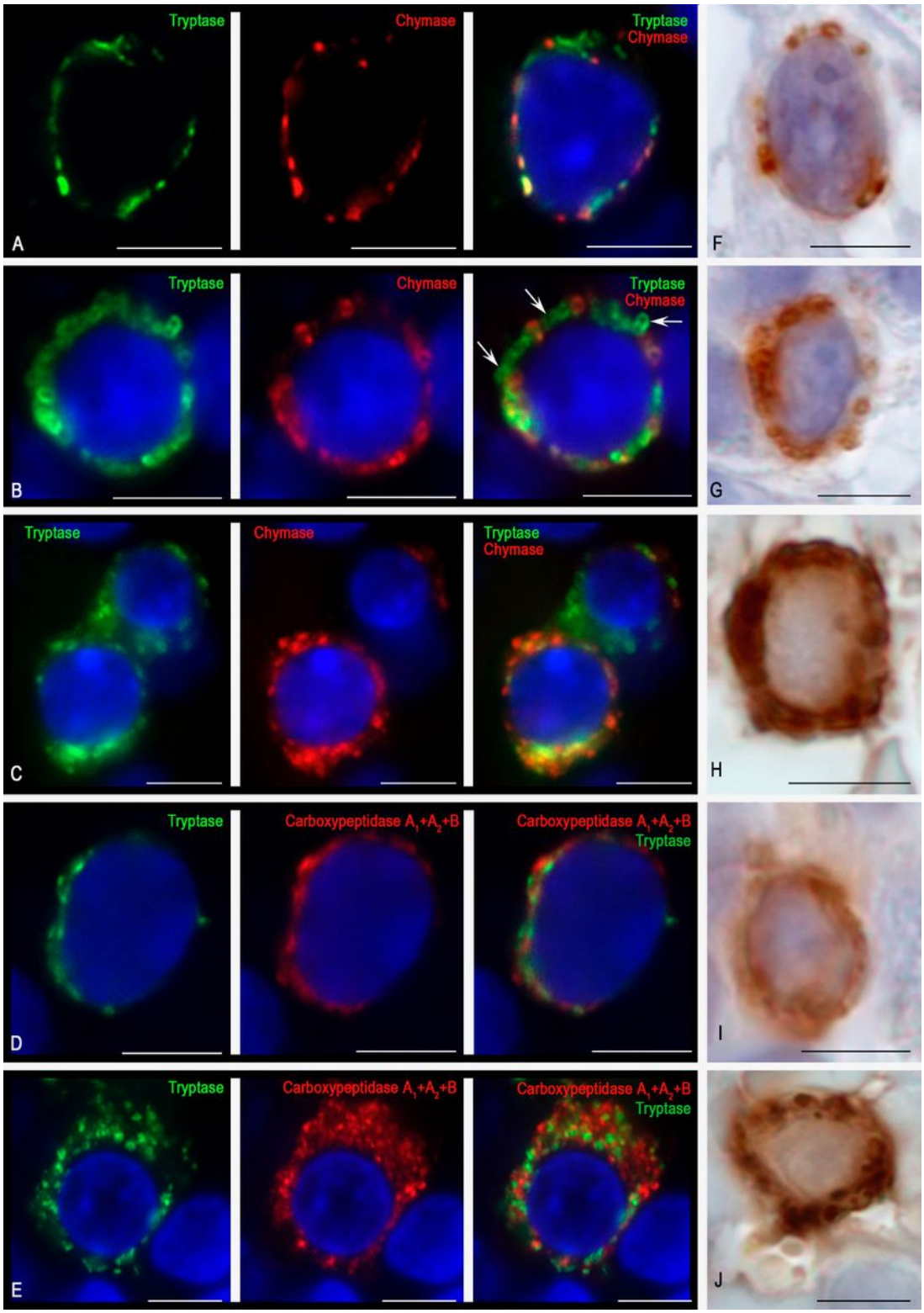

Figure 1

Cytological characterization of proteases in blast forms of MCs of red bone marrow during mastocytosis. Tryptase was detected using immunolabeling with mouse monoclonal anti-tryptase AB (D, E, H, I) or rabbit monoclonal anti-tryptase AB (A, C). Chymase was detected using immunolabeling with mouse monoclonal anti-chymase AB (A, C, F, G). Carboxypeptidases was detected using immunolabeling with rabbit anti-carboxypeptidase A1 + A2 + B AB (D, E). Visualization of bound primary AB was performed using fluorochromes Alexa Fluor 488 and Cy3 (A-E) or DAB Chromogen (F-J). A - Initial level of synthesis of specific proteases in MC. Typical secretory granules are absent. Chymase and tryptase are localized in a narrow zone of the perinuclear region. B - Stage of specific proteases formation in granules of types II and III. Granules containing both tryptase and chymase are prevailing; granules containing the only tryptase are in a lower amount (marked with an arrow). C - Accumulation of secretory granules in blast forms of MCs with the formation of MCs with different chymase contents. Granules containing exclusively chymase are marked with an arrow. D - Mast cell in the initial stages of the formation of secretory material in the form of immature granules and granules of type I, in which tryptase is partially co-localized with carboxypeptidases. E - Blast form of a mast cell at the stage of differentiation into a more mature state. The cytoplasm is predominantly filled with carboxypeptidases localized extragranularly; tryptase takes up a smaller volume and is partially visualized in granules. F-G - Single (F) and more numerous (G) chymase-positive granules in MC with clearly visible nucleoli. H - Mast cell cytoplasm is filled with large tryptase-positive granules of type III. I - Chromogenic detection of the initial stages of tryptase synthesis in the cytoplasm of the MC blast form. J - Uneven accumulation of tryptase-containing granules in the cytoplasm of the MC blast form, while the perinuclear zone remains free of proteases. Bar 5 μm for the entire layout.

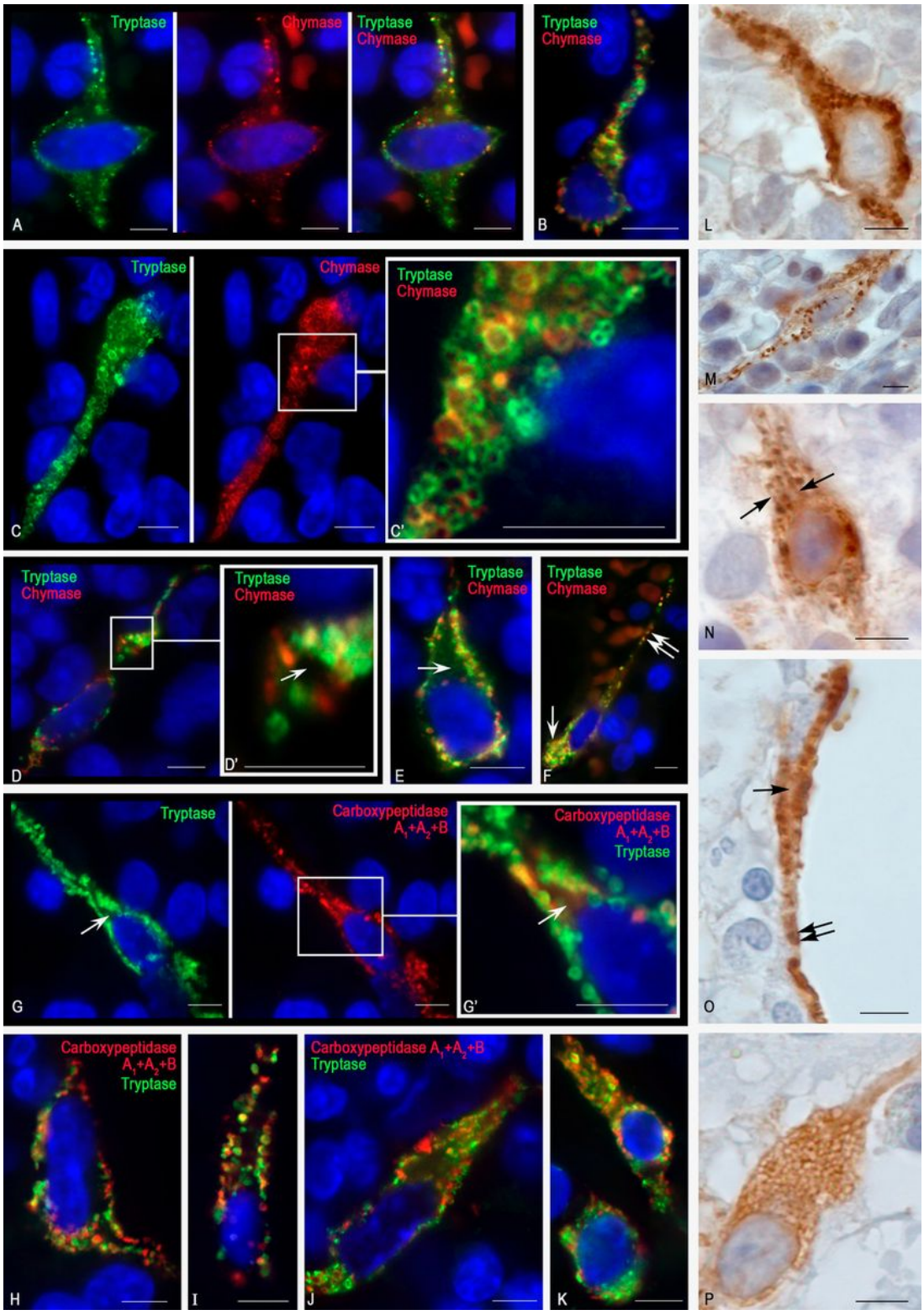

Figure 2

Specific proteases in MCs of type 1a of the red bone marrow in mastocytosis. Tryptase was detected using immunolabeling with mouse monoclonal anti-tryptase AB (G-K, L-N) or rabbit monoclonal anti-tryptase AB (A-F). Chymase was detected using immunolabeling with mouse monoclonal anti-chymase antibodies AB (A-F, O-P). Carboxypeptidases were detected using immunolabeling with rabbit anti-carboxypeptidase A1+A2+B AB (G-K). Visualization of bound primary AB was performed using

fluorochromes Alexa Fluor 488 and Cy3 (A-K) or DAB Chromogen (L-P). A, B - Various variants of hypogranulated MC with cytoplasmic outgrowths in which tryptase and chymase-positive secretory material are visualized. C - Elongated MC with an eccentric position of the nucleus and well-defined secretory granules of type II and III; the peripheral annular part of granules is tryptase and/or chymase positive. D - Spindle-shaped MC with the formation of cytoplasmic outgrowths at considerable distances from the nucleated region of the cell. D' – Cross-section of the cytoplasmic outgrowth. Granule-containing peripheral region and protease-free central part are (marked with an arrow). E - Hypogranulated MC with a cytoplasmic outgrowth in which proteases are localized in the peripheral region, whereas the central region is free of proteases (indicated by an arrow). F - Hypogranular mast cell with the formation of two morphologically different poles. The wide pole (indicated by the arrow) is filled with granules with the equal expression of tryptase and chymase. A narrow cytoplasmic outgrowth (indicated by a double arrow) contains fewer specific proteases and accompanies a vessel of the microvasculature at a considerable distance. G - Elongated mast cell with a cytoplasmic outgrowth of a considerable length. The peripheral region of the appendix contains proteases, while the central region is free of granular secretory material (indicated by the arrow). H, I, J - Various forms of localization of tryptase and carboxypeptidases in MC with elongated nuclei, cytoplasmic outgrowths, and the peripheral arrangement of secretory granules in them. Both tryptase- or carboxypeptidase-positive granules are visible, as well as granules with simultaneous expression of both proteases. K - MC with different ratios of tryptase and carboxypeptidases expression. L - Tryptase-positive MC with a cytoplasmic outgrowth containing tryptase-positive granules. M - Elongated MC with a small content of secretory granules both in the perinuclear region and in the cytoplasmic outgrowths. N - Hypogranular MC. In the cytoplasm, single secretory granules and longitudinally directed small structural formations that are immunopositive to tryptase are obvious (indicated by an arrow). O - Spindle-shaped MC neighbouring an adipocyte, filled with a large number of chymase-positive granules. In the perinuclear cytoplasm, the central region is free of granules (indicated by an arrow), while the distance from the nucleus, the size of granules is comparable with the volume of the cytoplasm (indicated by a double arrow). P - Hypogranulated chymase-positive MC with the formation of a narrow cytoplasmic outgrowth on the periphery. Bar 5μm for the entire layout.

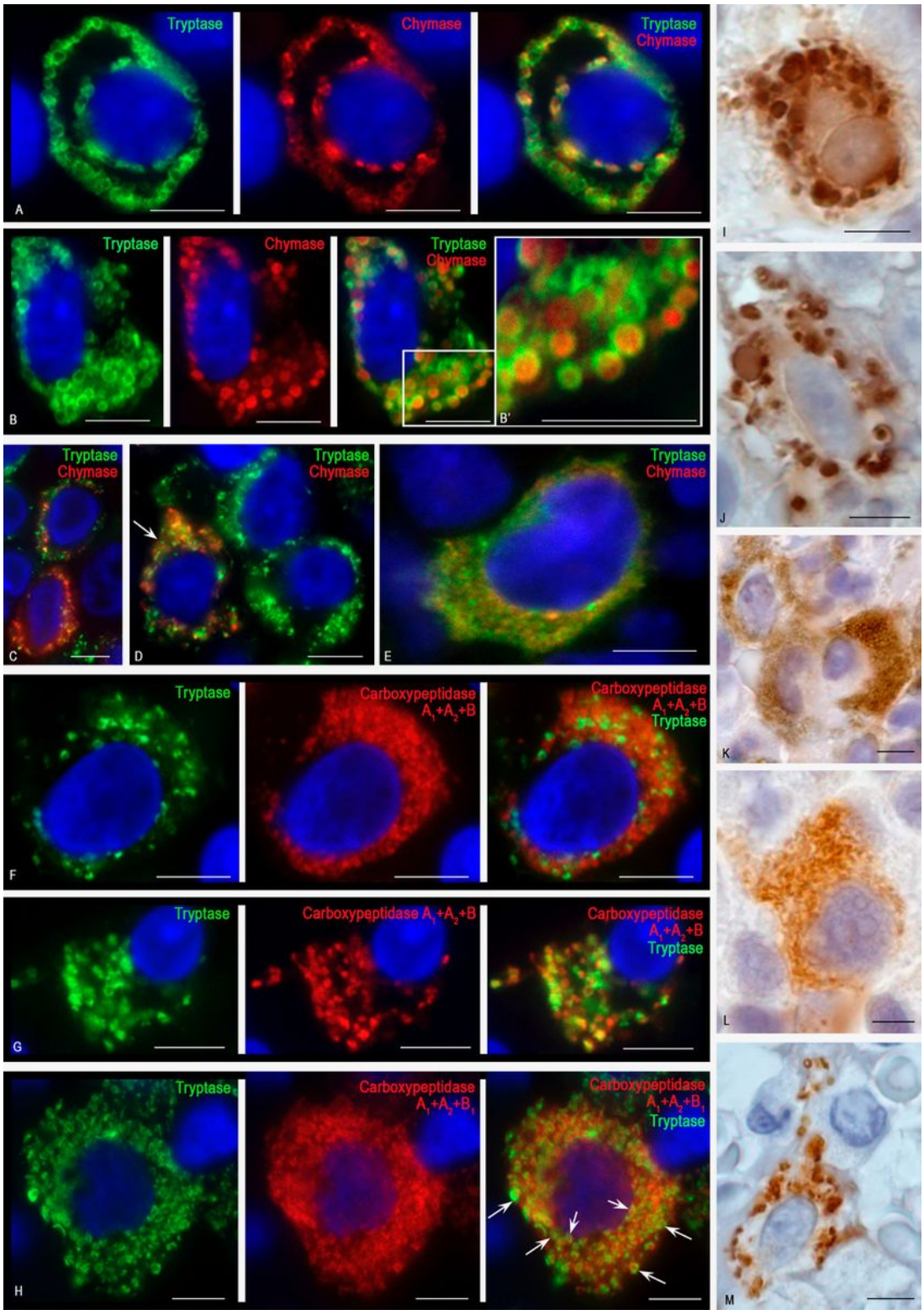

Figure 3

Cytological features of the distribution of proteases in MCs of type 1b in the red bone marrow in mastocytosis. Tryptase was detected using immunolabeling with mouse monoclonal anti-tryptase AB (A-E, I-K) or rabbit monoclonal anti-tryptase AB (F-H). Chymase was detected using immunolabeling with mouse monoclonal anti-chymase AB (A-E, L-M). Carboxypeptidases were detected using immunolabeling with rabbit anti-carboxypeptidase A1+A2+B AB (F-H). Bound primary AB was visualization using

fluorochromes Alexa Fluor 488 and Cy3 (A-H) or DAB Chromogen (I–M). A - Localization of protease granules in the peri-plasmalemmal and perinuclear regions of the MC. Tryptase and chymase are located on the periphery of granules of type III. B - A large number of mature secretory granules in the MC cytoplasm with uneven accumulation. High accumulation of chymase in the MC secretory granules with filling the central region. C - Variable content of chymase in hypogranular MCs. D - MC with high chymase expression (indicated by an arrow) neigbouring two tryptase-positive MCs. E - Hypogranulated MC with an eccentrically positioned nucleus, with the simultaneous content of specific proteases in the cytoplasm. F - Hypogranulated MC with predominant localization of carboxypeptidases outside of tryptase-positive granules and an eccentric arrangement of the nucleus. G - Localization of tryptase and carboxypeptidases in MC granules with an eccentric position of the nucleus. H - Tryptase-positive MC with a high content of carboxypeptidases, co-localized intragranularly with tryptase (indicated by an arrow) or extragranularly in the cytoplasm. Neighbouring to another tryptase-positive MC is observed. I - Localization of tryptase in various granules, which have a pronounced polymorphism. Near the nucleus with a clearly visible nucleolus, a cytoplasm region, not filled with granules (presumably occupied by the Golgi complex) is identified. J - Pronounced polymorphism of tryptase-positive granules in MC with an elongated nucleus. K – A group of tryptase-positive hypogranular MC with a low level of protease in the perinuclear region. L - Absence of mature type III protease granules in a hypogranulated chymase-positive MC with an eccentrically located nucleus. M - Elongated chymase-positive MC whose cytoplasm is unevenly filled with mature type III secretory granules. Bar 5μm for the entire layout.

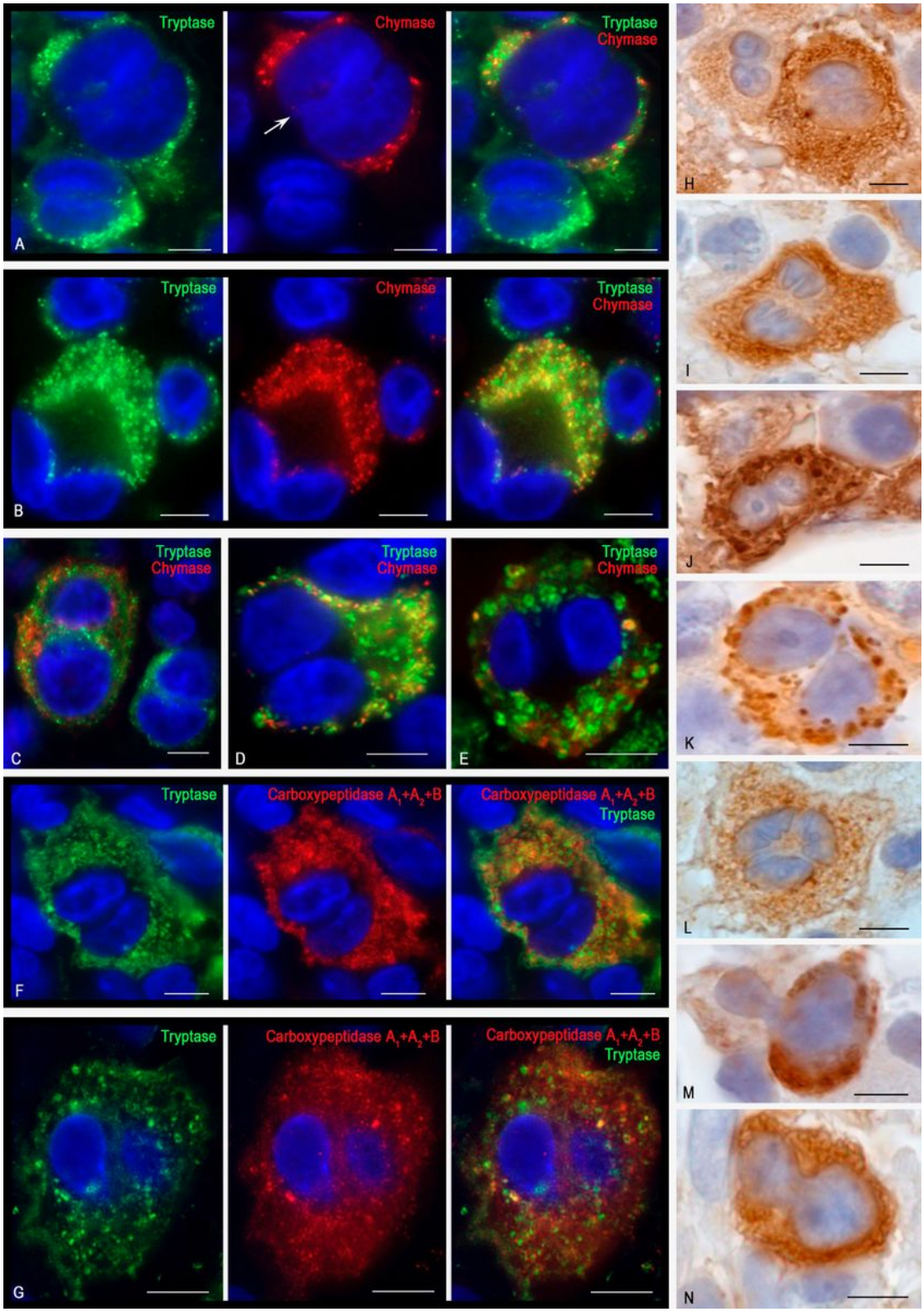

Figure 4

Cytotopography of proteases in MCs of type II of the red bone marrow in mastocytosis. Tryptase was detected using immunolabeling with mouse monoclonal anti-tryptase AB (F-G, H-J, N) or rabbit monoclonal anti-tryptase AB (A-C). Chymase was detected using immunolabeling with mouse monoclonal anti-chymase AB (A-C, K-M). Carboxypeptidases were detected using immunolabeling with rabbit anti-carboxypeptidase A1+A2+B AB (F-G). Bound primary ABs were visualized using fluorochrome

Alexa Fluor 488 and Cy3 (A-G) or DAB Chromogen (H-N). A, B, C – Variants of the localization of hypogranular mast cells with unequal expression of tryptase and chymase. A – Large MC containing tryptase and chymase with a segmented nucleus (indicated by an arrow) in a contact with a binuclear tryptase-positive mast cell that does not express chymase. B - Large binucleated MC with simultaneous expression of tryptase and chymase contacting with blast MCs, one of which contains only tryptase (top), and the other both proteases (right). C - A large hypogranulated binucleated MC containing both specific proteases is localized near a smaller binucleated MC cell that does not express chymase. D, E - Variants of co-expression of tryptase and chymase in hypogranulated (D) and granulated (E) binuclear MC. F, G - MCs with a high (F) and low (G) expression of tryptase and carboxypeptidase. H - Contact of hypogranulated binuclear MCs with high (right) and moderate (left) tryptase expression. The pronounced nucleoli in the nuclei of an MC with lower protease content are noteworthy. I - Hypogranulated tryptase-positive TK with abnormal shape of the nuclei. J – A large MC with large nucleoli in the nuclei and with well-defined mature tryptase-positive granules of type III. K - Binuclear MC with the expression of chymase, formed into large secretory granules. A large nucleolus is visible in one of the nuclei. L - Hypogranulated chymase-positive MC with well-formed nucleoli in the nuclei. M - Abnormal form of the nucleus in a chymase-positive MC with a small content of large secretory granules. N - Binuclear hypogranulated tryptase-positive MC. Bar 5μm for the entire layout.

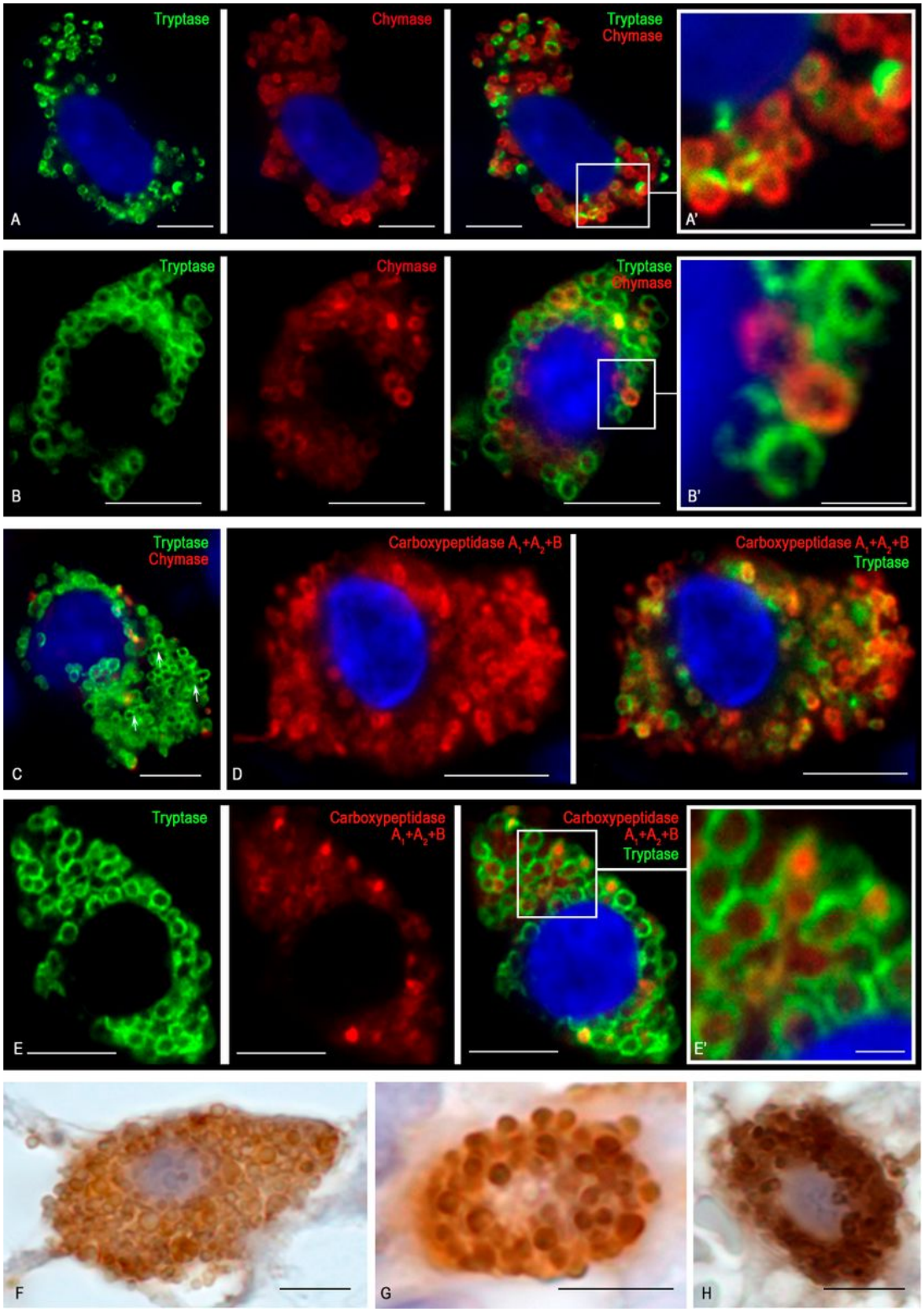

Figure 5

Proteases in mature mast cells of red bone marrow in patients with mastocytosis. Tryptase was detected using immunolabeling with mouse monoclonal anti-tryptase AB (D-E, H) or rabbit monoclonal anti-tryptase antibodies AB (A-C). Chymase was detected using immunolabeling with mouse monoclonal anti-chymase antibodies AB (A-C, F-G). Carboxypeptidases were detected using immunolabeling with rabbit anti-carboxypeptidase A1+A2+B AB (D-E). Visualization of bound primary AB was performed using

fluorochromes Alexa Fluor 488 and Cy3 (A-E) or DAB Chromogen (F-H). A - A mast cell with a high level of chymase expression. Chymase prevails both in the number of granules in the MC cytoplasm and the intragranular content. In granules, chymase is concentrated on the periphery (A'). B - A MC with approximately equal contents of tryptase and chymase. Granules containing exclusively chymase or tryptase are detected, as well as with simultaneous protease content (B'). Proteases are localized on the periphery of mature granules of type III. C - A MC with a predominance of tryptase-positive granules, some of which tend to fusion with each other (marked by an arrow). D – A tryptase-positive MC with an intra- and extra-granular carboxypeptidase content. Tryptase is localized exclusively on the periphery of the granules. E – A tryptase-positive mast cell with predominant intragranular localization of carboxypeptidases in the central region of the granules (e'). F, G - The mast cell cytoplasm is filled with large chymase-positive granules, some of which are secreted by the exocytosis mechanism. One can see single freely lying secretory granules in the extracellular matrix (F). H – A tryptase-positive mast cell with low secretory activity. Bar 5 μm for the entire layout and 1 μm for the A', B', E'.

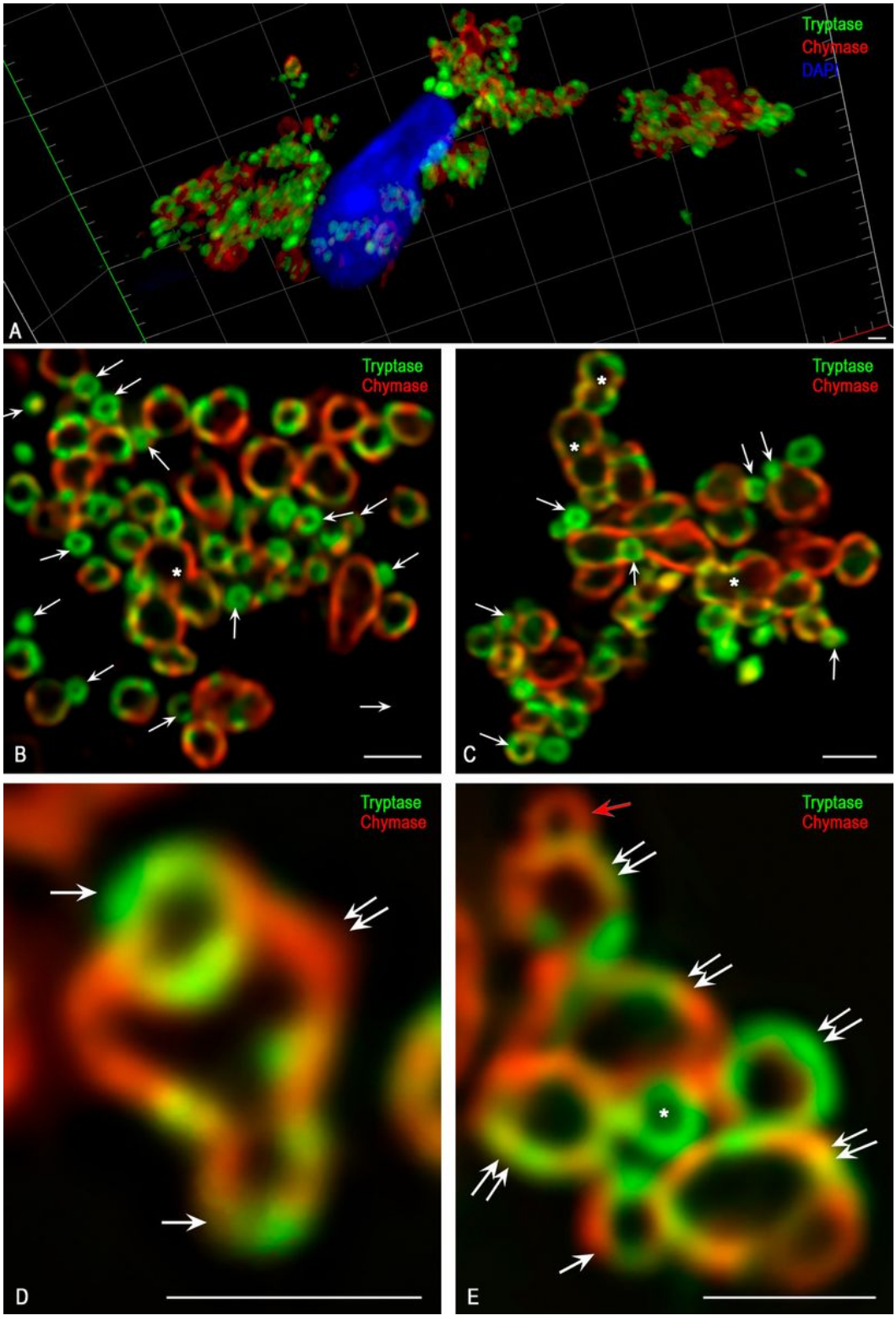

Figure 6

Cytotopography of specific proteases in the secretion of an atypical mast cell of type Ia with mastocytosis. Tryptase was detected using immunolabeling with rabbit monoclonal anti-tryptase AB. Chymase was detected using immunolabeling with mouse monoclonal anti-chymase AB. Visualization was performed using fluorochrome Alexa Fluor 488 and Cy3. Images were obtained using a confocal scanning microscope ZEISS LSM 880/Airyscan equipped with a Zeiss Plan-apochromat objective

63x/1.40 oil. A - A general plan for the localization of tryptase and chymase in the secretion of MC. B, C - various options for the localization of chymase and tryptase in secretory granules. The cytoplasm contains a large number of small granules of type II (indicated by an arrow) contacting with each other and adjacent to large mature secretory granules of type III. In secretory granules, proteases are localized on the periphery. Well-pronounced wide variability of intragranular co-localization of tryptase and chymase. Some mature granules have visually distinguishable anastomoses with each other (indicated by an asterisk). D, E - Morphological equivalents of different variants of fusion of immature secretory granules of type II with large mature granules (indicated by a double arrow) during post-translational modification of specific proteases. At the stage of an immature granule, granules can contain mainly chymase (red arrow), tryptase (asterisk), or both specific proteases (white arrow). Bar 1 μm for the entire layout.

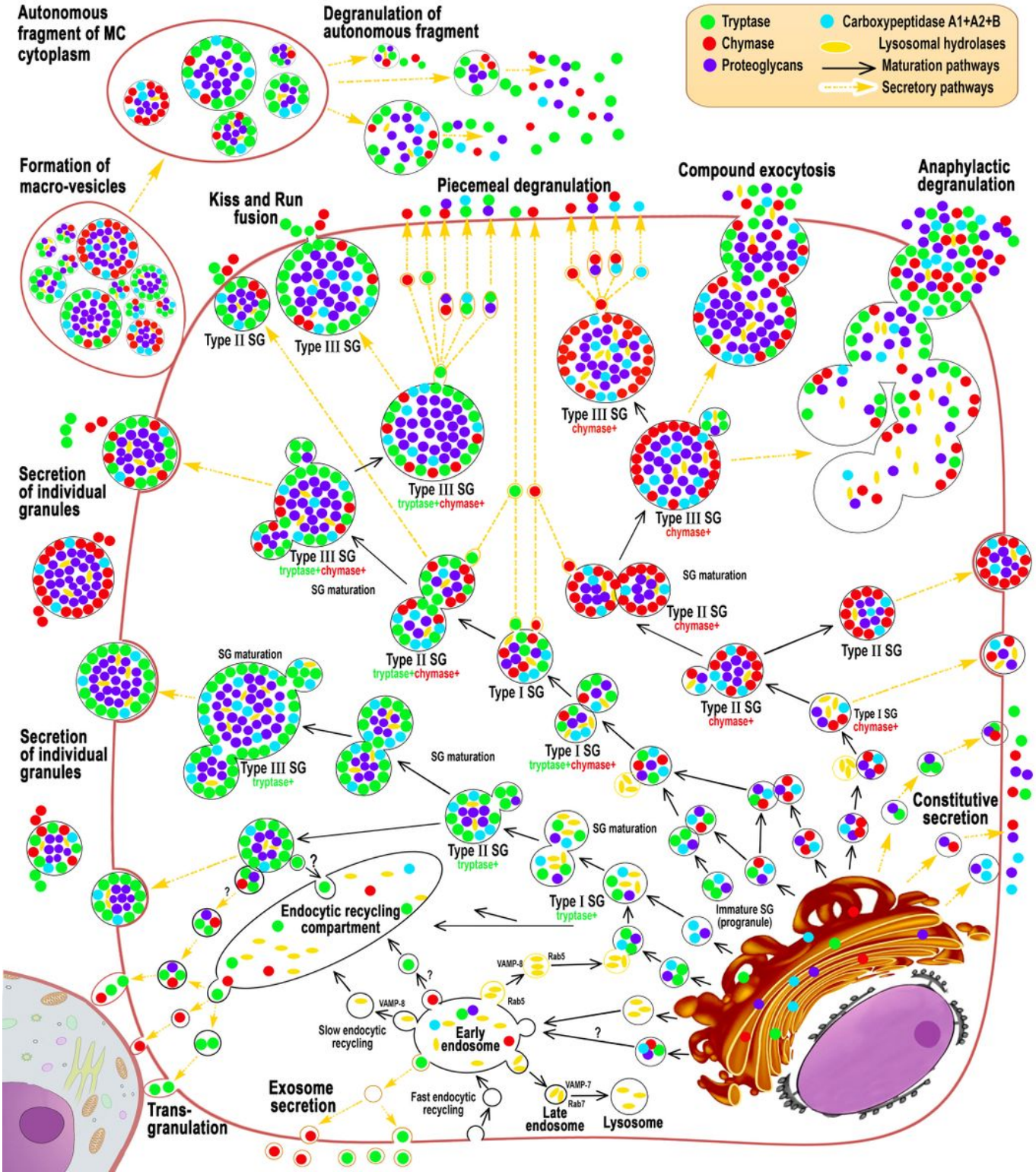

Figure 7

Cytotopography and secretory pathways of mast cell proteases in red bone marrow in mastocytosis. The scheme (Fig. 7) shows the main stages of post-translational modification of proteases, taking into account their cytotopography, intragranular localization, and secretory mechanisms. Protease biosynthesis starts in the granular endoplasmic reticulum of mast cells (MCs) and continues in the Golgi apparatus (GA), where immature granules are formed. According to morphometric characteristics and the

specificity of the intragranular folding of proteases, secretory granules can be divided into 3 types. Secretory granules of type I are formed after the fusion of the lysosome with the granules coming from the Golgi complex having the smallest size and the lowest protease content. As granules are enlarged through homotypic fusion, granules of type II are formed with a size of 0.2-0.4 μm; a characteristic feature is the laying of proteases on the periphery of the secretory granule, while proteoglycans in the central region. Secretory granules of type II are chymase +, tryptase +, as well as with the simultaneous content of specific proteases. As a result of the completion of maturation stages, type III secretory granules with sizes of 0.5 μm or more are formed, which are characterized by the largest volume of secretome while maintaining the peripheral localization of tryptase and chymase in the form of a ring. These MC granules contain also carboxypeptidases A1, A2, and B; their content and cytotopography are bound with tryptase and chymase biogenesis. Hypogranulated atypical MCs red bone marrow formed during mastocytosis is characterized by the prevalence of type I and II secretory granules in the cytoplasm that are morphologically indistinguishable and lead to diffuse immunohistochemical staining of the cytoplasm. The main mechanisms for the removal of specific proteases from hypogranulated MCs into the extracellular matrix are pacemaker secretion, transgranulation, and exosome formation. In the secretory granules of type II or III in atypical MCs, protease secretion is possible using the “Kiss and run” mechanism, exocytosis of individual granules, or lacing of macrovesicles, which for a long time retain autonomous secretory activity in a specific tissue microenvironment of the red bone marrow.