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The Staining of Mast Cells: A Historical Overview

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Abstract
The specificity of several staining methods for mast cells provides the pathologist with a useful means for the differential diagnosis of mast cell tumors. Mast cells stain metachromatically with toluidine blue with greater intensity in cells containing smaller granules. Most stains for mast cells rely on the cell's content of heparin, other glycosaminoglycans, and esterase. As an alternative to histochemical stains, different antibodies have been used to identify mast cells in humans.

Introduction

Mast cells are present in mammalian as well as non-mammalian [1] vertebrates in virtually all vascularized tissues. The first phase of research on mast cells was by von Recklinghausen [2] in 1863, who noticed granular cells in the mesentery of frogs, almost certainly the first observation of mast cells in any species. Mast cells express, on their surface, the high-affinity receptor for IgE (FcεRI), and can be activated by IgE and specific antigens to release a diverse array of mediators, including histamines, leukotrienes, prostaglandins, serine proteases, and various cytokines, chemokines, and growth factors.

Mast cells are numerous. In fact, it has been estimated that if they were grouped together, they would make an organ equal to the size of the spleen [3]. The mast cells of rodents and humans may be subdivided into ≥2 subpopulations by various morphologic and functional criteria. The histochemical techniques for subdividing mast cells stemmed from the observation by Ehrlich in 1876 that their lysosomal granules have the capacity to take up and stain metachromatically with basic dyes, such as toluidine blue. Among the histochemical methods for staining mast cells worth mentioning are Sudan Black B, Luna stain, and Ziehl-Neelsen, which have been assessed historically [4]. In 1994, Simoes and Schoning [5] evaluated 18 methods for staining mast cells in dogs. Enerbäck [6–8] demonstrated in 1986 that rodent mast cell subpopulations could be differentially stained with Alcian blue and berberine sulfate. Enerbäck [6–8] also showed that mast cells from mucosal surfaces were sensitive to formaldehyde fixation, in that they failed to stain metachromatically upon additional exposure to dyes, whereas mast cells from connective tissues were insensitive to formaldehyde fixation. Before the introduction of monoclonal antibod-

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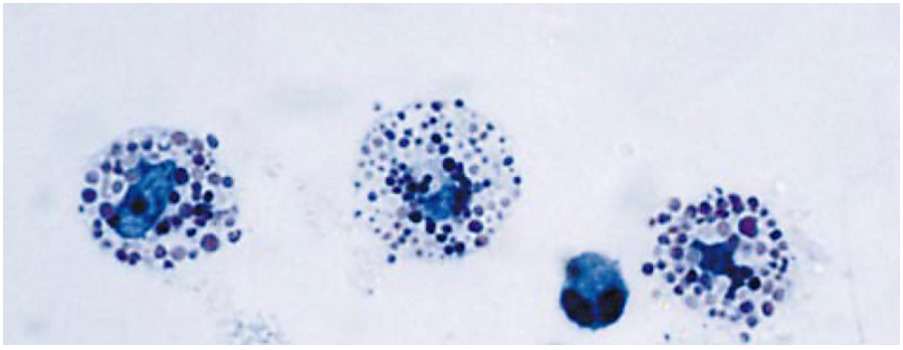

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Fig. 1. Semi-thin section of rat peritoneal mast cells stained with toluidine blue. Numerous cytoplasmic metachromatic granules are recognizable (reproduced from [45]).

ies against proteases, mast cells were stained by metachromatic stains, such as toluidine blue and Alcian blue.

This review article will analyze the most common techniques developed to stain different mast cell subpopulations.

Metachromasia

Mast cell granules (and some other tissue components such as cartilage matrix) can naturally induce metachromatic staining. Metachromatic stains include the Romanowsky combinations (Wright, Giemsa, May-Grünwald Giemsa, and Leishman), toluidine blue, and others.

Toluidine blue first emerged in 1856, courtesy of a British chemist called William Henry Perkin. Although he was working on the synthesis of quinine, Perkin instead produced a blue substance with good dyeing properties. Initially, it became known as aniline purple. Being mostly used in the dye industry, this was the first synthetic organic chemical dye. Later, it became known as toluidine blue, and began being used for medical purposes, in particular as a histological special stain to highlight certain components.

Mast cells were first identified by Paul Ehrlich [9] in 1878 when he was still a medical student; in his doctoral dissertation, he described a class of aniline-positive cells of the connective tissues endowed with cytoplasmic metachromatic granules, for which he coined the name "Mastzellen." The first use of the term "metachromatic" was by Ackroyd [10] in 1876, to indicate that the structure being dyed assumed a color different from that of the dye itself. In 1879, Ehrlich [11] used the word for the first time in a biological context, to describe the staining reaction of blood leukocytes on the basis of their specific affinities for various dyes [11, 12]. He encountered cells with basophilic, metachromatic granules, and thus came to recognize two types of mast cells. The first type, which could be identified and differentiated by its repertoire of coarse basophil granules (gamma granulation), was to be found in the connective tissues and apparently derived from them (tissue mast cells). The second, the counterpart of the neutrophil polymorph and eosinophil leukocyte, contained basophilic granulation of the fine type (delta granulation); its origin was in the bone marrow and it was to be found in the peripheral blood (blood mast cells and basophils).

At the end of the 1930s, Scandinavian researchers provided fundamental new insights into mast cell structural profile. The mast cell component prophesized by Ehrlich as the responsible agent for granule metachromasia was revealed [12, 13]. Holmgren and Wilander [14], following the discovery by Jorpes [15] that the anticoagulant heparin was subject to stain metachromatically with toluidine blue, reconsidered Ehrlich's observation that mast cell granules stained metachromatically with toluidine blue. These authors were able to set a correlation between the number of toluidine blue-positive mast cell in various tissues and their heparin content.

Metachromatic staining is important in the detection of mast cells and is strongly recommended as a routine stain for this purpose. One of the most frequently metachromatic stains is toluidine blue which stains the mast cell granules purple-to-red (Fig. 1). Dilute staining solutions should be used in order to demonstrate strongly metachromatic elements [16]. The pH of the dye solution used is important. Lennert [17] showed that the use of a series of toluidine blue solutions at pH levels varying from 2.62 to 7.00 might help differentiate benign from malignant cases of human mastocytosis. This issue was further developed when it was shown that benign lesions stained with toluidine blue at a lower pH whereas malignant lesions were poorly stained at a pH <3.5 [18].

Metachromasia is due to the presence of tissue polyanions that induce a polymerization of dye molecules. It has

been shown that a distance of around 0.5 nm between negatively charged groups is needed to induce such polymerization [19]. The process involves a shift in the absorption spectrum of the dye towards shorter wavelengths, and is accompanied by a hypochrome color changes of the dye from blue towards violet, red, or orange. Metachromasia of anionic tissue can be demonstrated with many cationic dyes such as thiazine, oxamine, azine, and xanthene, and with fluorescent dyes such as Acridine orange. The thiazine dyes toluidine blue and Azure A are by far the most widely used. Metachromasia is best observed in a water solution of low ionic strength. A large number of anionic tissue sites are metachromatic under such conditions [20].

Mast cells are round in the proximity of blood vessels, but display an elongated shape in the interstitial regions. In addition, mast cells often have a reduced number of granules and a disorganized granule content, suggesting an ongoing degranulation process. Analysis at higher magnifications allows the identification of degranulating mast cells, characterized by numerous extracellular metachromatic granules and/or by a poor intracytoplasmic granule content, and nondegranulating mast cells without any granule in the proximal extracellular space.

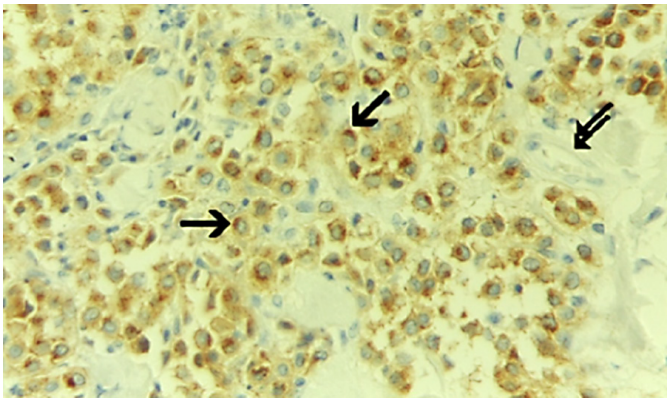

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Fig. 2. Predominantly focal paranuclear c-Kit protein expression in mast cells of a canine mast cell tumor. Immunohistochemistry is performed with primary anti-c-Kit receptor antibody. Single arrows indicate focal brown paranuclear immunostaining, while double arrows indicate a vessel (reproduced from [46]).

Alcian Blue-Safranin

Strongly metachromatic polyanions such as the sulfated glycosaminoglycans (GAGs) of mast cell granules retain red or violet metachromasia after dehydration in ethanol and mounting in synthetic resins. Two copper phthalocyanin dyes are of special interest for the staining of mast cells, Alcian blue 8GX and Astrablau 6GLL. Alcian blue, formulated by Scott et al. [21] is more easily available and better specified, and should therefore be preferred. Alcian blue interacts with polyanions such as heparin in aqueous solutions, to give insoluble precipitates in which the two components are bound by ionic linkages. The connective tissue mast cells of rats and mice have a low affinity for Alcian blue. Mast cells in the cervical lymph nodes and uterus stain strongly with Alcian blue at a pH of 2.5 [22].

In the 1960s, Enerbäck [6–8] described two morphologically distinct subpopulations of rodent mast cells, based on their specific staining characteristics and preferential tissue homing, i.e., connective tissue mast cells (CTMCs) present in the connective tissues, and serosae and mucosal mast cells (MMCs) located on the mucosae of the respiratory and gastrointestinal tracts. CTMCs could be distinguished from MMCs by red staining with safranin due to the presence of large amounts of heparin in their secretory granules. In the mouse, indeed, the proteoglycan content of mast cell granules varies in the different mast cell subtypes. CTMCs contain heparin that is lacking in MMCs. Conversely, MMCs express chondroitin sulfates A and B, which are not found in CTMCs, but both subtypes store chondroitin sulfate E in their granules. Thus, in contrast to CTMCs, MMCs are sensitive to routine formalin fixation and cannot be identified in standard histological sections. CTMCs can be detected after fixation with 10% neutral-buffered formalin, while MMCs require fixation in nonaldehyde solutions such as Carnoy solution [23]. After appropriate fixation and sequential staining with Alcian blue and safranin, MMCs stain blue, being thus differentiated from CTMCs which stain with safranin and are red. Differential affinity for Alcian blue can be visualized with sequential staining, consisting of Alcian blue followed by safranin [24]. Immature embryonic mast cells contain granules which stain with Alcian blue rather than safranin. With the Alcian blue-safranin sequence, the maturation of mast cells is accompanied by a change in the staining properties from blue to red [25].

The Tyrosine Kinase Kit Receptor

Mast cells, but not basophils, express the Kit receptor for the stem cell factor (Fig. 2), which not only drives the terminal differentiation of mast cells but plays other im-

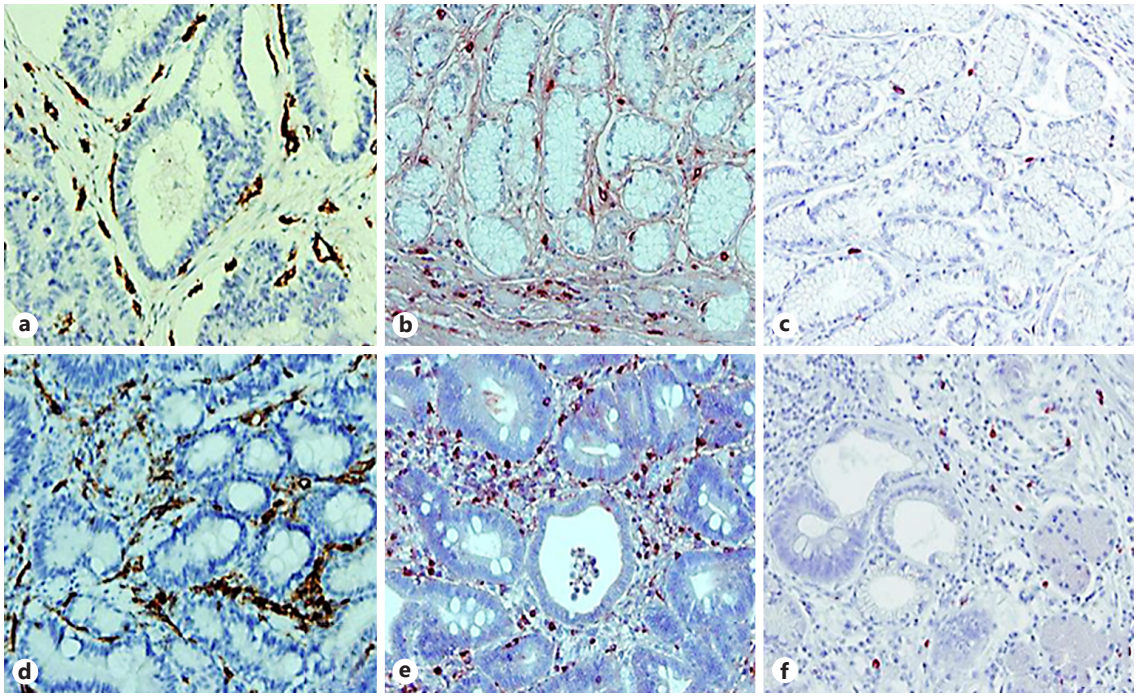

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Fig. 3. Immunohistochemical staining for CD31, tryptase, and chymase in stage II (**a–c**) and stage IV (**d–f**) human gastric cancer. **a**, **d** In endothelial cells immunoreactive for CD31. **b**, **e** In tryptase-positive mast cells. **c**, **f** In chymase-positive mast cells. Blood vessels and mast cells are distributed around the gastric glands. The number of blood vessels and mast cells is higher in stage IV than in stage II biopsy specimens, and the number of chymase-positive cells is lower than the number of tryptase-positive cells (reproduced from [47]).

portant roles in regulating mast cell biology, such as the survival, activation, and degranulation of mature cells. The importance of the stem cell factor as a mast cell growth factor is underlined by the fact that mice with certain loss-of-function mutations affecting either the stem cell factor or its receptor Kit are devoid of mast cells. Indeed, the lack of expression of a functional Kit receptor due to spontaneous mutation in both copies of Kit, as it occurs in genetically mast cell-deficient WBB6F1-Kit^{W}-$Kit^{W\text{-}v}$ mice (W/W^{v} mice), results in the absence of tissue mast cells [26].

On immunohistochemical staining, the c-Kit proto-oncogene encodes a transmembrane tyrosine kinase receptor, c-Kit (CD117), which is closely related to the platelet-derived growth factor family. This antibody recognizes the extracellular domain, and is expressed by a variety of normal and abnormal cell types. In normal cells, the CD117 antibody has been shown to label breast epithelium, germ cells, melanocytes, stem cells, mast cells, salivary glands, and the esophagus, cerebellum, hippocampus, and spinal cord [27]. In mast cells, the marking is at the level of the plasma membrane (in normal cells); in other cells, it is at the level of the cytoplasm. Furthermore, a large proportion of mast cells, including those in the colon, stomach, lung, uterus, and bladder, do not stain with c-Kit [28]. Because CD117 is also expressed on immature hematopoietic progenitor cells, additional markers should be applied to define the mast cell component by flow cytometry in bone marrow samples. To distinguish CD117-positive mast cells from CD117-nonhematopoietic cells, the staining of mast cells with a monoclonal antibody against CD45 is recommended [29].

Tryptase and Chymase

Mast cell proteases represent major protein components of secretory granules, but the role of each individual protease in the mast cells remains poorly understood. The proteases are classified into carboxypeptidase, chymase, and tryptase. Mast cells differ in their protease expression pattern, depending on the tissue where they are to be found.

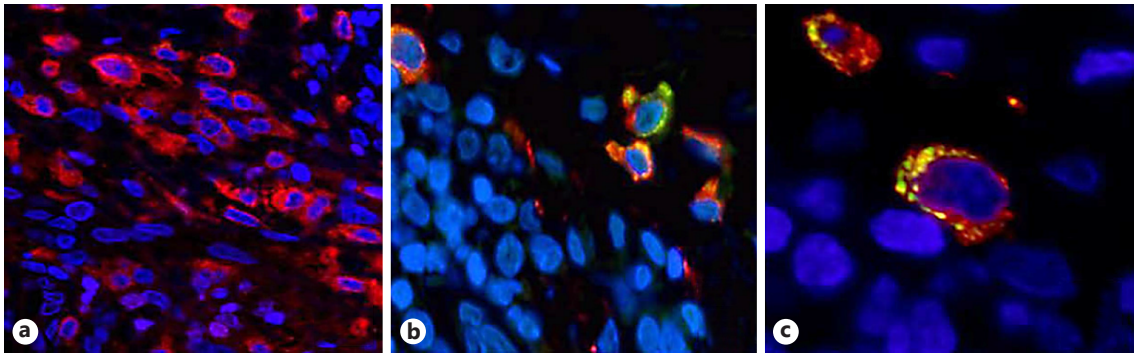

Fig. 4. Dual immunofluorescence for tryptase (red, **a**), cathepsin-G (green, **b**), and both proteins (orange, **c**) in a biopsy specimen of human cutaneous mastocytosis (reproduced from [48]).

By 1960, two proteases, with chymotrypsin- and trypsin-like activity were identified in mast cells [30–32], and enzyme activity was recognized to localize within intracellular granules. The enzymes were purified in the 1980s and renamed tryptase and chymase [33, 34]. Mast cells from different anatomical sites contain different profiles of these enzymes as well as of other proteases (Fig. 3). Human mast cells were divided into two subtypes, depending on the expression of different proteases in their granules [35]. The first, containing tryptase only, was designated MC_T or "immune cell-associated" mast cells, predominantly located in the respiratory and intestinal mucosa, where they colocalize with T lymphocytes. A type of mast cell that contained both tryptase and chymase, along with other proteases such as carboxypeptidase A and cathepsin G (Fig. 4), was referred to as MC_{TC}, and these cells are predominantly found in connective tissue areas, such as the skin, submucosa of the stomach and intestine, breast parenchyma, myocardium, lymph nodes, conjunctiva, and synovium. A third type of mast cell, called MC_C, expresses chymase without tryptase and is to be found mainly in the submucosa and mucosa of the stomach, small intestinal submucosa, and colonic mucosa [36]. Interestingly, the human MC_T type was seen to correspond most closely to rodent MMCs, whereas the MC_{TC} type resembled rodent CTMCs.

Other Stains

Chloroacetate-esterase is found in mast cells. Specimens are incubated with naphthol AS-D chloroacetate in the presence of freshly formed diazonium salt. Enzymatic hydrolysis of ester linkages liberates free naphthol compounds which couple with diazonium salt, forming highly colored deposits at the site of enzyme activity [37]. Unlike most enzyme stains, chloroacetate-esterase can be used on fixed, paraffin-embedded tissue. The slides are incubated in a solution containing the substrate naphthol AS-D chloroacetate, and the esterase contained in the neutrophils and mast cells then binds with the chloroacetate. This releases the naphthol group, which binds to the diazonium dye pararosanalin (basic fuchsin), another component of the incubating solution. Pararosanilin or basic fuchsin gives a deep pink-red color to the granules, while hematoxylin counterstains the nuclei blue.

Berberine forms a strongly fluorescent complex with heparin, proportional to the heparin content in the mast cell granules [38], enabling the quantitation of heparin by both microscope fluorometry [38] and flow cytometry [39]. Berberine staining may also be used for the visualization of mast cells in tissue sections [40].

Mast cells stain intensely at a pH of 8.00–10.5 with the anionic bis-azo dye, Biebrich scarlet [41], and stain strongly with naphtol AS-D chloroacetate as a substrate and diazo-coupling fixation in acetone or neutral formalin [42]. Histamine can be detected histochemically by means of a fluorescent reaction with o-phthalaldeyde [43, 44].

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Disclosure Statement

There are no conflicts of interest.

References

1 Baccari GC, Pinelli C, Santillo A, Minucci S, Rastogi RK: Mast cells in nonmammalian vertebrates. Int Rev Cytol 2011;290:1–53.
2 von Recklinghausen F: Über Eiter- und Bindegewebskörperchen. Virchows Arch Patholog Anat Physiol Klin Med 1863;83:247–287.
3 Bradding P, Holgate ST: Immunopathology and human mast cell cytokines. Crit Rev Oncol Hematol 1999;31:119–133.
4 Rest JR, Lee RL: Staining of mast cell granules by the Ziehl-Neelsen method and differential diagnosis of malignant dermal tumours in the dog. Vet Rec 1979;104:79.
5 Simoes JP, Schoning P: Canine mast cell tumors: a comparison of staining techniques. J Vet Diagn Invest 1994;6:458-465.
6 Enerbäck L: Mast cells in rat gastrointestinal mucosa. I. Effects of fixation. Acta Pathol Microbiol Scand 1966;66:289–302.
7 Enerbäck L: Mast cells in rat gastrointestinal mucosa. 2. Dye-binding and metachromatic properties. Acta Pathol Microbiol Scand 1966;66:303–312.
8 Enerbäck L: The evolution of the concept of a specific mucosal mast cell; in Befus AD, Bienenstock J, Denburg JA (eds): Mast Cell Differentiation and Heterogeneity. New York, Raven Press, 1986, pp 1–26.
9 Ehrlich P: Beiträge zur Theorie und Praxis der histologischen Färbung (thesis, 1878); in: The Collected Papers of Paul Ehrlich. Elsevier, 2013, pp 29–64.
10 Ackroyd W: Metachromism or colour change. Chem News 1876;33:60.
11 Ehrlich P: Beiträge zur Kenntnis der granulierten Bindegewebszellen und der eosinophilen Leukozyten. Arch Anat Physiol 1879; 36:166–169.
12 Ehrlich P: Farbenanalytische Untersuchungen zur Histologie und Klinik des Blutes. Dtsch Med Wochenschr 1894;20:135–136.
13 Ehrlich P: Die Anemie. 1. Normale und Pathologische Histologie des Blutes. Wien, Holder, 1898.
14 Holmgren H, Wilander O: Beitrag zur Kenntnis der Chemie und Funktion der Ehrlichschen Mastzellen. Z Mikr Anat Forsch 1937; 42:242–278.
15 Jorpes E HH, Wilander O: Über das Vorkommen von Heparin in den Gefässwänden und in den Augen. Ein Beitrag zur Physiologie der Ehrlichschen Mastzellen. Z Mikr Anat Forsch 1937;42:279–301.
16 Kramer H, Windrum GM: The metachromatic staining reaction. J Histochem Cytochem 1955;3:227–237.
17 Lennert K: Zur pathologischen Anatomie von Urticaria pigmentosa und Mastzellenreticulose. Klin Wochenschr 1962;40:61–67.
18 Klatt EC, Lujes RJ, Meyer PR: Benign and malignant mast cell proliferations. Diagnosis and separation using a pH-dependent toluidine blue staining in tissue section. Cancer 1983; 51:1119–1124.
19 D'mello AXP, Sylvester TV, Ramya V, Britto FP, Shetty PK, Jasphin S: Metachromasia and metachromatic dyes: a review. Int J Adv Health Sci 2016;2:12–17.
20 Spicer SS HR, Leppy TJ (eds): Histochemistry of connective tissue mucopolysaccharides; in: The Connective Tissue. Baltimore, Williams and Wilkins, 1967.
21 Scott JE, Quintarelli G, Dellovo MC: The chemical and histochemical properties of Alcian blue. Histochemie 1964;4:73–85.
22 Spicer SS: Histochemical properties of mucopolysaccharixe and basic protein in mast cells. Ann NY Acad Sci 1963;103:322–333.
23 Irani AM, Schwartz LB: Mast cell heterogeneity. Clin Exp Allergy 1989;19:143–155.
24 Spicer SS: A correlative study of the histochemical properties of rodent acid mucopolysaccharides. J Histochem Cytochem 1960;8: 18–35.
25 Combs JW: Differentiation and proliferation of embryonic mast cells of the rat. J Cell Biol 1965;25:577–592.
26 Kitamura Y, Go S, Hatanaka K: Decrease of mast cells in W/Wv mice and their increase by bone marrow transplantation. Blood 1978;52: 447–452.
27 Lammie A, Dobnojak M, Gerald W, Saad A, Cote R, Cordon-Cardo C: Expression of c-Kit and Kit ligand proteins in normal human tissues. J Histochem Cytochem 1994;42:1417–1425.
28 Qi JC, Li L, Li Y, Moore K, Madigan MC, Katsoulotos G, Krilis SA: An antibody raised against in vitro-derived human mast cells identifies mature mast cells and a population of cells that are FcεRI(+), tryptase (–), and chymase (–) in a variety of human tissues. J Histochem Cytochem 2003;51:643–653.
29 Escribano L, Díaz-Agustín B, Bellas C, Navalón R, Nuñez R, Sperr WR, Schernthaner G-H, Valent P, Orfao A: Utility of flow cytometric analysis of mast cells in the diagnosis and classification of adult mastocytosis. Leuk Res 2001;25:563–570.
30 Benditt EP, Arase M: An enzyme in mast cells with properties like chymotrypsin. J Exp Med 1959;110:451–460.
31 Benditt EP: An enzyme in mast cells with some properties resembling chymotrypsin. Fed Proc 1956;15:507.
32 Glenner GG, Cohen LA: Histochemical demonstration of a species-specific trypsin-like enzyme in mast cells. Nature 1960;185:846–847.
33 Schechter NM, Choi JK, Slavin DA, Deresienski DT, Sayama S, Dong G, Lavker RM, Proud D, Lazarus GS: Identification of a chymotrypsin-like proteinase in human mast cells. J Immunol 1986;137:962–970.
34 Schwartz LB, Lewis RA, Austen KF: Tryptase from human pulmonary mast cells. Purification and characterization. J Biol Chem 1981; 256:11939–11943.
35 Irani AA, Schechter NM, Craig SS, DeBlois G, Schwartz LB: Two types of human mast cells that have distinct neutral protease compositions. Proc Natl Acad Sci USA 1986;83:4464–4468.
36 Irani A-MA, Schwartz LB: Human mast cell heterogeneity. Allergy Asthma Proc 1994;15: 303–308.
37 Moloney WC, McPherson K, Fliegelman L: Esterase activity in leukocytes demonstrated by the use of naphthol AS-D chloroacetate substrate. J Histochem Cytochem 1960;8: 200–207.
38 Enerbäck L: Berberine sulphate binding to mast cell polyanions: a cytofluorometric method for the quantitation of heparin. Histochemistry 1974;42:301–313.
39 Enerbäck L, Berlin G, Svensson I, Rundquist I: Quantitation of mast cell heparin by flow cytofluorometry. J Histochem Cytochem 1976;24:1231–1238.
40 Dimlich RVW, Meineke HA, Reilly FD, McCuskey RS: The fluorescent staining of heparin in mast cells using berberine sulfate: compatibility with paraformaldehyde orophthalaldehyde induced fluorescence and metachromasia. Stain Technol 1980;55:217–223.
41 Spicer SS: Histochemical properties of mucopolysaccharide and basic protein in mast cell. Ann NY Acad Sci 1963;103:322–333.
42 Gomori G: Chloroacyl esters as histochemical substrates. J Histochem Cytochem 1953;1: 469–470.
43 Shelley WB, Öhman S, Parnes HM: Mast cell stain for histamine in freeze-dried embedded tissue. J Histochem Cytochem 1968;16:433–439.
44 Juhlin L, Shelley WB: Detection of histamine by a new fluorescent o-phthalaldehyde stain. J Histochem Cytochem 1966;14:525–528.
45 Ribatti D, Vacca A, Nico B, Crivellato E, Roncali L, Dammacco F: The role of mast cells in tumour angiogenesis. Br J Haematol 2001; 115:514–521.
46 Patruno R, Marech I, Zizzo N, Ammendola M, Nardulli P, Gadaleta C, Introna M, Capriuolo G, Rubini RA, Ribatti D, Gadaleta CD, Ranieri G: c-Kit expression, angiogenesis, and grading in canine mast cell tumour: a unique model to study c-Kit driven human malignancies. BioMed Res Int 2014;2014:1–8.
47 Ribatti D, Guidolin D, Marzullo A, Nico B, Annese T, Benagiano V, Crivellato E: Mast cells and angiogenesis in gastric carcinoma. Int J Exp Pathol 2010;91:350–356.
48 Ribatti D, Nico B, Finato N, Crivellato E, Beltrami CA: Co-localization of tryptase and cathepsin-G in mast cells in cutaneous mastocytosis. Cancer Lett 2009;279:209–212.