ORIGINAL PAPER

Simultaneous demonstration of mast cells and blood vessels by the combined method CD34 – alcian blue–safranin in lip tumors

PUŞA GAJE[1)], VIORICA BOCAN[1)], ANCA MARIA CÎMPEAN[1)], D. A. IZVERNARIU[1)], FELICIA STREIAN[2)], M. RAICA[1)]

[1)]*Histology and Cytology Department*
[2)]*Surgery Department, Faculty of Dental Medicine*
"Victor Babeş" University of Medicine and Pharmacy, Timisoara

Abstract

The aim of the study was to evaluate the mast cell–blood vessel relationship using double staining CD34/AAS. Sections from 14 cases with lip tumors have been stained with Hematoxylin–Eosin. On additional sections from each case, we highlighted blood vessels by immunohistochemistry for CD34 antigen using the method LSAB2–HRP/DAB, followed by alcian blue–safranin stain for mast cells. We quantified the density, distribution and the mast cell types as well as the correlation with the number of blood vessels. All cases have been positive for both staining. We observed a significant correlation between the number of vessels and the mast cells ($p = 0.003$). In one case, we observed the mast cells stained with safranin (red), the vascular density being less than the mast cells density. Our results confirmed the data from the literature with respect to the large number of mast cells observed in the malignant tumors. The increased vascular density together with the mast cell density suggests a correlation between these two elements in the tumor angiogenesis, possibly though the VEGF secretion. The CD34/AAS stain is a quick and simple method and it allows an optimal correlation between the number of mast cells and blood vessels on the same section. The type of mast cells correlated with microvessel density is a powerful argument towards the involvement of the mast cells in the tumor angiogenesis of the malignances of the lips.

***Keywords*:** CD34, mast cells, lip, carcinoma.

Introduction

The origin of the mast cells is not well known. The enzymes of the mast cells are numerous. Some of these enzymes are specific and considered markers for them: mast cell tryptase, mast cell chymase and naphtol-AS-D-chloroacetate esterase.

In normal conditions, mast cells are predominantly situated in the perivascular space but the meaning of their disposition is controversial. A mast cells reactive hyperplasia has been noted in malignancies, accompanied by the increase of the number of granules per cell, which activates metachromasia through accumulation of GAG sulfates.

In the basocellular carcinoma has been noted a 15–20-fold increase as opposed to standard values and they are situated around the tumor cells at the proliferation limit.

The prognostic value of mast cells in the malignant tumors has not been yet demonstrated even though numerous authors [1, 2] have reported the hyperplasia. Currently, there is no plausible explanation for the mast cells hyperplasia of the tumors in the early evolution phase and the numeric decrease up to complete disappearance in the advanced malignances. Mast cells inside the epithelial tissues represent a particular aspect of histology.

Their existence has been proven among keratinocytes from the spinous layer of the lip epithelium, surrounding by chromophobe halo. In addition, they have been noted in the dermis, in the neighborhood of the basal membrane.

The function of the intraepithelial mast cells is not known, but it supposed that the glycosaminoglycans are involved in accelerated keratin production in the precancerous conditions or malignant transformation. The tumor angiogenesis is essential for the progression and metastasis.

The identification of blood vessels is based on pan-endothelial markers (CD34, CD31, von Willebrand factor).

The CD34 antibody is frequently used in the study of the tumor angiogenesis and microvessel density.

The QBEnd10 clone is most reliable on paraffin sections.

The aim of the study was to evaluate the relationship between mast cells and blood vessels applying the double staining CD34/alcian blue–safranin.

Material and methods

The study included 14 cases of lip tumors. Morphologic staining of the 5 μm sections used the standard Hematoxylin–Eosin method.

By immunohistochemistry, the blood vessels have been stained using anti CD34 clone QBEnd 10, at 1 : 25 dilution after an enzymatic pretreatment. The incubation with primary antibody was followed by LSAB+ working system and the final product was visualized with 3,3-diaminobenzidine.

On the same sections, we applied a histochemical method with alcian blue–safranin for specific identification of the mast cells (Figures 1 and 2). The slides were mounting with permanent medium.

The microscopic examination was performed with the Nikon Eclipse E600 microscope. Photos were taken in JPEG format.

We were interested in the vascular density correlated with the mast cell density as well as the distribution and the type of mast cells.

The correlation between blood vessels and the mast cells was done using SPSS system.

Results

On Hematoxylin–Eosin stain, we established the histopathologic diagnosis.

We found 12 cases of squamous cell carcinoma and two cases of basal cell type carcinoma. The basal cell carcinoma showed low density and staining for blood vessels and mast cells too. The microvessel density was higher for squamous cell carcinomas. We identified all types of tumor blood vessels (single endothelial cells, immature, and mature types) but the most blood vessels had lumen and thin wall probably without perivascular cells around them.

Many branches on the blood vessels around the tumor areas represented another particular aspect of the blood vessels. This pattern was correlated with a high number of degranulated mast cells around them. Inside the tumor the blood vessels positive for CD34 were small and most of them without lumen. The vascular branching was rare phenomenon inside the tumor areas. In 13 of the 14 cases we identified blue (alcianophil) and violet (mixed) mast cells situated around the blood vessels at a distance from tumor sites (Figure 3).

We have not identified mast cells within the tumor. At the epidermal level, we observed isolated mast cell in the spinous layer. We found a significant correlation between mast cells density and the number of blood vessels ($p = 0.003$) (Figure 4).

In one case of basal cell carcinoma, we found safraninophil (red) mast cells. For this case, the vascular density was lower than the mast cell density. Blood vessels showed a different morphology and they increased in number and color intensity in the areas where mast cell degranulations appear (Figure 5).

Alcianophil and mixed mast cells was observed at the edge of the tumor area, adjacent to blood vessels (Figure 6).

Discussions

In 1993, Norrby K and Woolley D [3] found a correlation between the number of mast cells and angiogenesis and established a relationship between the normal processes, inflammatory diseases and carcinogenesis.

Starting with the second half of the 1990's, a strong correlation between mast cells and angiogenesis [4, 5] was observed on lymphomas, angiomas and multiple myeloma. Histochemistry using AAS method revealed the mast cells at the periphery of the tumor areas, for most studied cases.

In the case of mammary neoplasm [6], close to the tumor, the number of mast cells was increased compared to benign tumors. An increased number of mast cells were sparsely observed in the case of gastrointestinal tumors (colon, stomach). The mast cells contain a series of active substances at the granules level, having direct implications upon the different tumor angiogenesis stage [7–9].

Our results obtained with combined imunohistochemical/histochemical stains CD34/AAS showed the existence of degranulated mast cells close to a high number of blood vessels intensely stained and with a different morphology than normal ones.

In the case of non-degranulated alcianophil and mixed mast cells, the number of blood vessels was significantly lower, with morphology.

Several studies of various researchers [3, 10] showed the following:

- Tryptase activates metalloproteinase and the plasminogen activators, fact that causes the degradation of the extracellular matrix, which is the critical moment in the beginning of angiogenesis. Tryptase and the heparin stimulate the migration and the division of the vascular endothelial cells.
- Interleukin 8 [11] increases the expression of the ICAM1 protein, which expresses itself at the surface of the endothelium and is responsible with cellular adhesion during the formation of the capillary vessels.
- VEGF and b-FGF stimulates the proliferation and migration of endothelial cells (as shown by Bikfalvi A [12]). The heparin secretion at the mast cells level increases the receptor sensitivity for the release of b-FGF on the endothelial cell.

In the first angiogenesis stage, enzymes are released at the granule level: cathepsin G, elastase, collagenase [13, 14]. This degrades the extracellular matrix components.

On the other hand, b-TGF action upon plasminogen activators. Proteolysis activity of mast cells favors tumor cells infiltration in tissues. Some researchers believe that the high number of mast cells in tumor metastases favors tumor progression in the lymphatic vessels. Mast cells have been observed at the edge of the tumor in case of the skin neoplasm [15–17]. Mast cell density is independent of inflammatory infiltrate.

Other authors [1, 18] have shown that the inflammation at the peritumoral level does not associate with the mast cells.

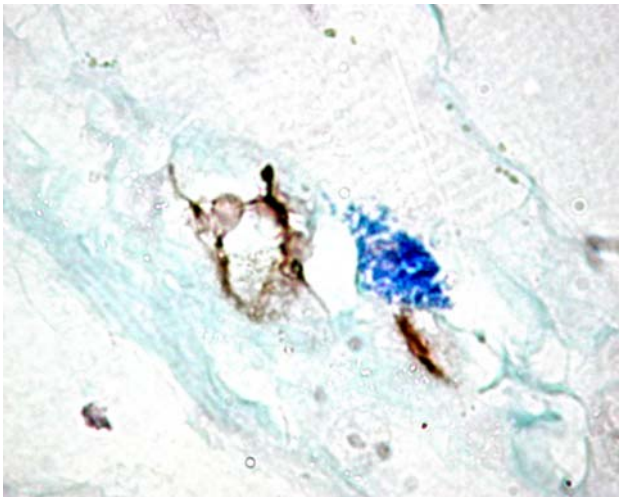

Figure 1 – ***Combined CD34/AAS method. Note the specific stain of blood vessel (brown, CD34) and mast cells (blue, AAS), ×100***

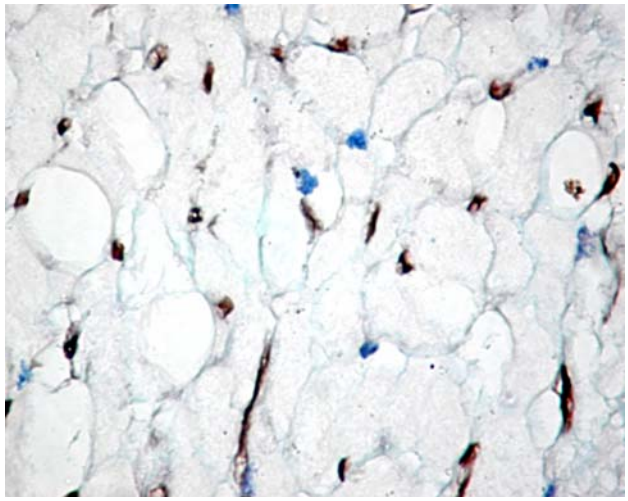

Figure 2 – ***CD34/AAS stain, ob. ×10***

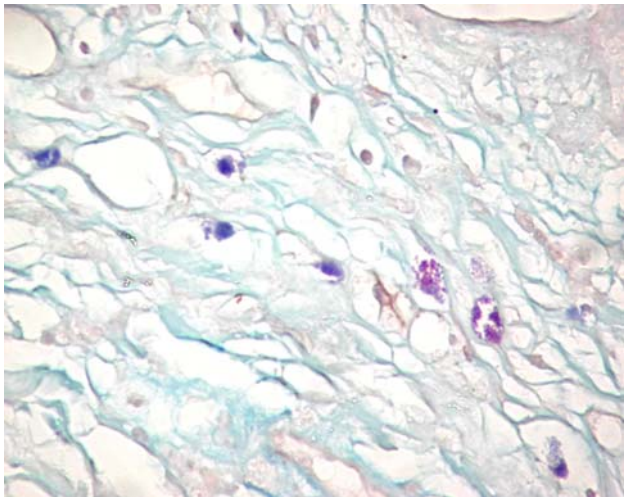

Figure 3 – ***Non-degranulated alcianophil and mixed mast cells, few blood vessels (CD34/AAS stain, ob. ×10)***

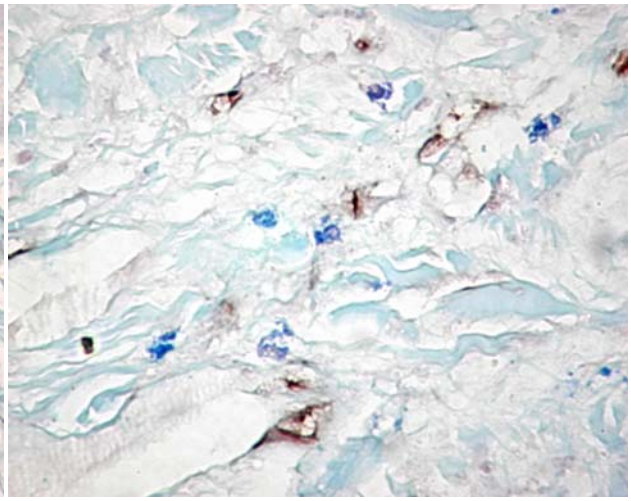

Figure 4 – ***Significant correlation between the number of vessels and mast cells (p = 0.003) (CD34/AAS stain, ob. ×20)***

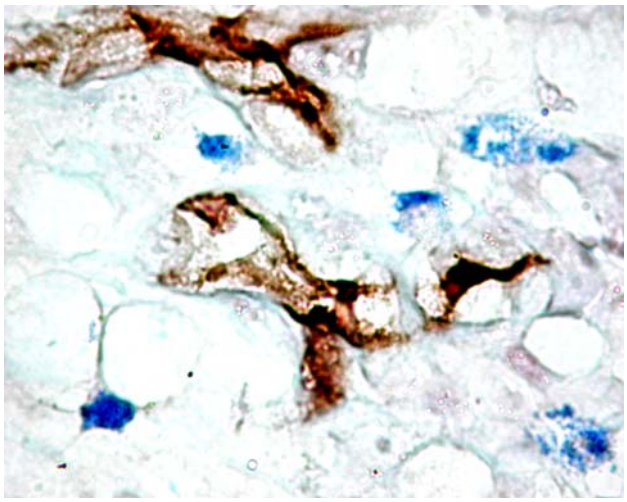

Figure 5 – ***Degranulated alcianophil mast cells, numerous blood vessels with different morphology (CD34/AAS stain, ob. ×40)***

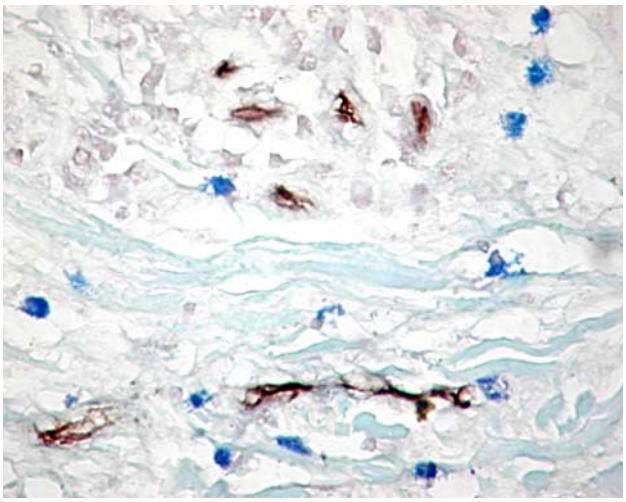

Figure 6 – ***Mast cells (alcianophil), adjacent to the blood vessels concentrated at the edge of the tumor area. Sparse intraepithelial mast cells (CD34/AAS stain, ob. ×10)***

In an experiment on mice, Riley JF [19] proved the massive progression of mast cells in the case of squamous cell tumors migrations from the dense connective tissue to the lax connective tissue are done by formation of some multi-chamber sachets within mast cells due to the fusion of granules. These ultrastructural modifications take place through mast cells stimulation. Observations suggest that the mast cells are attracted by the carcinoma microenvironment and, at the same time induced their degranulation. Histopathological studies on the human basal cell and squamous cell carcinomas have shown that the mast cell density is increased in the aggressive forms. Our results have shown a tight relation between the number of vessels and mast cells, proven with the correlation index of $p = 0.003$.

Coussens L [18] showed that the mast cells contribute to neovascularization in squamous cell carcinomas. The tryptase has a role in the reorganization of the matrix in correlation with neovascularization.

Blair H *et al.* [13] demonstrated their role in capillary vessel formation through endothelial cell cultures. The heparin is the dominant proteoglycan from the mast cells and has several features, such as the mitogen for the endothelial cell. It was proven that mast cells around basocellular carcinoma and melanoma are a major source of VEGF [20–22].

This is one of the most important angiogenetic factors, which contributes to neovascularization through endothelial cell mitosis, induction of hyper-permeability in capillary vessels and surfacing of other proangiogenetic factors in the extracellular matrix. Our results were correlated to the data from the literature with respect to the high number of mast cells (7–13 mast cells on site, small lenses) observed in malignant tumors.

Mast cell degranulation is associated with an increased number and staining of the blood vessels. High vascular density associated with the mast cell density ($p = 0.003$) suggests the cooperation of both elements in the tumor angiogenesis, possibly through the secretion of VEGF.

Information obtained by us is correlated with the results of the literature, with respect to peritumor mast cell localization and mast cell degranulation correlated with the blood vessel morphology.

Conclusions

The CD34/AAS method is simple yet rapid, allowing an optimal correlation between the number of mastocytes on the same section and their type.

The vascular tumor density (10–20 vessels on site) is an added argument regarding the involvement of mast cells in tumoral angiogenesis of lip malignant injuries.

References

[1] HERMES B., WELKER P., FELDMANN-BÖDDEKER I., KRÜGER-KRASAGAKIS S., HARTMANN K., ZUBERBIER T., HENZ B. M., *Expression of mast cell growth modulating and chemotactic factors and their receptors in human cutaneous scars*, J Invest Dermatol, 2001, 116(3):387–393.

[2] HERMES B., FELDMANN-BÖDDEKER I., WELKER P., ALGERMISSEN B., STECKELINGS M. U., GRABBE J., HENZ B. M., *Altered expression of mast cell chymase and tryptase and of c-Kit in human cutaneous scar tissue,* J Invest Dermatol, 2000, 114(1):51–55.

[3] NORRBY K., WOOLLEY D., *Role of mast cells in mitogenesis and angiogenesis in normal tissue and tumour tissue*, Advanc Biosci, 1993, 89:71–115.

[4] RIBATTI D., VACCA A., MARZULLO A., NICO B., RIA R., RONCALI L., DAMMACCO F., *Angiogenesis and mast cell density with tryptase activity increase simultaneously with pathological progression in B-cell non-Hodgkin's lymphomas*, Int J Cancer, 2000, 85(2):171–75.

[5] AZIZKHAN R. G., AZIZKHAN J. C., ZETTER B. R., FOLKMAN J., *Mast cell heparin stimulates migration of capillary endothelial cells in vitro*, J Exp Med, 1980, 152(4):931–944.

[6] KANKKUNEN J.-P., HARVIMA I. T., NAUKKARINEN A., *Quantitative analysis of tryptase and chymase containing mast cells in benign and malignant breast lesions*, Int J Cancer, 1997, 72(3):385–388.

[7] BEIL W. J., FÜREDER W., WIENER H., GROSSSCHMIDT K., MAIER U., SCHEDLE A., BANKL H. C., LECHNER K., VALENT P., *Phenotypic and functional characterization of mast cell derived from renal tumor tissues*, Exp Hematol, 1998, 26(2):158–169.

[8] JOŚKO J., MAZUREK M., *Transcription factors having impact on vascular endothelial growth factor (VEGF) gene expression in angiogenesis*, Med Sci Monit, 2004, 10(4):RA89–98.

[9] FOLKMAN J., INGBER D., Inhibition of angiogenesis. In: PICKNETT T. (ed), *Seminars in Cancer Biology*, Academic Press, London, 1992, 3:89–96.

[10] NIENARTOWICZ A., SOBANIEC-ŁOTOWSKA M. E., JAROCKA-CYRTA E., LEMANCEWICZ D., *Mast cells in neoangiogenesis*, Med Sci Monit, 2006, 12(3):RA53–56.

[11] GRÜTZKAU A., KRÜGER-KRASAGAKES S., KÖGEL H., MÖLLER A., LIPPERT U., HENZ B. M., *Detection of intracellular interleukin-8 in human mast cells: flow cytometry as a guide for immunoelectron microscopy*, J Histochem Cytochem, 1997, 45(7):935–945.

[12] BIKFALVI A., KLEIN S., PINTUCCI G., RIFKIN D. B., *Biological roles of fibroblast growth factor-2*, Endocr Rev, 1997, 18(1):26–45.

[13] BLAIR R. J., MENG H., MARCHESE M. J., REN S., SCHWARTZ L. B., TONNESEN M. G., GRUBER B. L., *Human mast cells stimulate vascular tube formation. Tryptase is a novel, potent angiogenic factor*, J Clin Invest, 1997, 99(11):2691–2700.

[14] AOKI M., PAWANKAR R., NIIMI Y., KAWANA S., *Mast cells in basal cell carcinoma express VEGF, IL-8 and RANTES*, Int Archives Allergy Immunol, 2003, 130(3):216–223.

[15] CH'NG S., WALLIS R., YUAN L., DAVIS P. F., TAN S. T., *Mast cells and cutaneous malignancies*, Modern Pathol, 2006, 19(1):149–159.

[16] SIMU G., CSABA G., *Mast cells in tumor-bearing patients*, Acta Morphol Acad Sci Hung, 1972, 20:327–338.

[17] CSABA G., ACS T., HORVATH C., MOLD K., *Genesis and function of mast cells. Mast cell and plasmacyte reaction to induced, homologous and heterologous tumours*, Br J Cancer, 1961, 15:327–335.

[18] COUSSENS L. M., RAYMOND W. W., BERGERS G., LAIG-WEBSTER M., BEHRENDTSEN O., WERB Z., CAUGHEY G. H., HANAHAN D., *Inflammatory mast cells up-regulate angiogenesis during squamous epithelial carcinogenesis*, Genes Devel, 1999, 13(11):1382–1397.

[19] RILEY J. F., *Mast cells and cancer in the skin of mice*, Lancet, 1966, 2(7479):1457–1459.

[20] ROCHE W. R., *Mast cells and tumor angiogenesis: the tumor-mediated release of an endothelial growth factor mast cell*, Int J Cancer, 1985, 36(6):721–728.

[21] DUNCAN J. I., BROWN F. I., MCKINNON A., LONG W. F., WILLIAMSON F. B., THOMPSON W. D., *Patterns of angiogenic response to mast cell granule constituents*, Int J Microcirc Clin Exp, 1992, 11(1):21–33.

[22] DVORAK H. F., BROWN L. F., DETMAR M., DVORAK A. M., *Vascular permeability factor/vascular endothelial growth factor, microvascular hyperpermeability, and angiogenesis*, Am J Pathol, 1995, 146(5):1029–1039.

Corresponding author
Marius Raica, Professor, MD, PhD, Department of Histology & Cytology, "Victor Babeş" University of Medicine and Pharmacy, 2 Eftimie Murgu Square, 300 041 Timişoara, Romania; Phone/Fax +40256–200 256, E-mail: raica@umft.ro

Received: January 20th, 2007

Accepted: June 20th, 2007