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Evaluation of microvascular density in inflammatory lesions and carcinoma of palatine tonsil

CARMEN AURELIA MOGOANTĂ[1)], DANIELA ADRIANA ION[2)], V. BUDU[3)], GABRIELA MUŢIU[4)], D. ŞALPLAHTA[5)], EMILIA AFREM[6)]

[1)]*ENT Department, University of Medicine and Pharmacy of Craiova*
[2)]*Department of Pathophysiology*
[3)]*ENT Department "Carol Davila" University of Medicine and Pharmacy, Bucharest*
[4)]*Department of Histology, Faculty of Medicine and Pharmacy, University of Oradea*
[5)]*PhD student, Department of ENT*
[6)]*PhD student, Department of Histology University of Medicine and Pharmacy of Craiova*

Abstract

The tonsil carcinoma with squamous cells seems to be one of the neoplastic lesions with a growing incidence worldwide, even in those geographical areas where the smoking incidence has been reduced. In the disease etiopathogenesis, more factors are incriminated. Among these, the most frequently mentioned are smoking, alcohol consumption and the infection with the Human Papilloma Virus. Among the morphological modifications incriminated for the tumoral appearance and development, there is also included the angiogenesis process that involves the apparition of new blood vessels out from the pre-existent ones, vessels that bring a plus of oxygen and nutritive substances for the tumoral cells. Taking into consideration the fact that the tumoral process is most often accompanied by an inflammatory reaction, in our study we also determined the microvascular density in the carcinoma with squamous cells in the palatine tonsil and in chronic tonsillitis, compared to the vascular density in normal tonsil stroma. We quantified the reaction of the mast cells in the stroma of the two types of lesions, too. The microvascular density in the carcinoma with squamous cells in the palatine tonsil was a lot greater than the microvascular density in chronic tonsillitis. The maximum number of blood vessels in tumoral lesions as well as their area, quantified through the "hot spot" technique on the surface unit, was around two times greater than in chronic tonsillitis. The number of mast cells was significantly larger in chronic tonsillitis and in tonsil carcinoma, too, but the reaction of these cells in the inflammatory affections was more intense than in the neoplastic lesions.

***Keywords*:** microvascular density, angiogenesis, tonsil carcinoma, chronic tonsillitis, mast cells.

Introduction

The carcinoma with squamous cells of the head and neck represents a cause of significant mortality rate at global level [1] being, as incidence, the sixth type of cancer among the most common neoplasms [2]. The clinical-statistical data claim that this type of cancer represents 3–5% of the malignant tumors in Europe and the U.S., while in South-East Asia and India it tends to come up to 40–50% [3].

During the last years, there can be noticed a decreasing rate of the carcinoma incidence with squamous cells of head and neck, in the U.S. as well as in Europe. In spite of this decreasing tendency, generally because of a decreasing prevalence of smoking [4], the oropharyngeal incidence of the carcinoma with squamous cells is increasing, probably due to an epidemic with the *Human Papilloma Virus* (HPV) [5].

If 10–20 years ago numerous studies indicated the fact that around 80–90% of the cases of head and neck cancers were associated with common risk factors, such as smoking, alcohol abuse and chewing Bethel nuts [6], relatively recent genetic and molecular studies of these types of cancer indicated the fact that the infection with HPV is ever more implicated in the appearance of these lesions [7, 8]. According to some studies, the most powerful association between the head and neck cancers and the HPV was found in the carcinoma with oro-pharyngeal squamous cells at tonsils level, just when the DNA with the HPV was identified in 45–70% of the cases [9, 10] and in the cancer at the tongue base where the DNA with HPV was identified in 40% of cases [11]. Other data indicate the fact that in the West-European countries, most cases of the oropharyngeal cancers are HPV-positive; the association between the cases of tonsils cancer and tongue base cancer and the HPV infection is over 80% in Stockholm [5, 12, 13]. At

present, it is unanimously accepted that the tumoral development depends on the blood and lymphatic vascularization. That is why it is considered that the forming of new blood vessels represents an important mechanism in the tumoral pathobiology [14]. Taking into consideration that important vascular modifications take place within the tumoral processes as well as in the inflammatory ones at the level of the palatine tonsils, in the present study, we proposed to quantify the micro vascular density in the chronic tonsillitis and the carcinoma with squamous cells developed at the level of the palatine tonsils, as well as the density of the mast cells as cells involved into the development of the vascular system.

Materials and Methods

The biological material was represented by 34 tonsils carcinomas with squamous cells (15 well-differentiated and 19 moderately-differentiated), by 47 chronic tonsillitis sampled after surgical interventions in the Emergency County Hospital of Craiova, Romania, between 2009 and 2011, from a group of patients aged between 47 to 75-year-old. For the comparative study of the tonsils microvascularization, we used seven fragments of normal tonsils kept in paraffin, from the collection of the Laboratory of Histology, University of Medicine and Pharmacy of Craiova.

Immediately after being taken out, the biological material was introduced into 10% neutral formalin solution for 72 hours and included into paraffin. The sectioning of the biological material was made at a Microm HM350 rotative microtome equipped with a transferring system of the sections on water bath (STS, Microm). The histological study was performed in the classical staining with Hematoxylin–Eosin and the trichromic Goldner–Szekely.

For the immunohistochemical study, the histological sections were collected on slides covered with poly-L-Lysine that were kept in a thermostat at 37^0C for 24 hours. After deparaffining and hydrating the sections, the biological material was incubated for 30 minutes in 1% hydrogen peroxide solution and washed in flowing water. For antigene demasking, the sections were boiled in a Na^+ pH 6 solution of citrate for 20 minutes at the microwave oven, and then cooled for 15 minutes. Then, they were washed in a bisaline buffer phosphate solution (PBS), followed by the blocking of the endogenous peroxidase in 2% skimmed milk for 30 minutes. After this preparation, the sections were incubated overnight with primary antibodies, at 4^0C, and next day, the signal was amplified for 30 minutes by using the secondary antibody with peroxidase on polymer support (EnVision, Dako). The signal was detected with 3,3'-diamino-benzidine (DAB, Dako). After that, there was made the contrasting with Hematoxylin, dehydration, clarifying and coupling with DPX (Fluka).

In order to emphasize the blood microvascularization within the inflammatory and tumoral processes at the level of the palatine tonsils, we used the CD31 antibody (JC70A clone, Dako) to mark the vascular endothelial cells and the mast cell tryptase antibody (AA1 clone, Dako) for emphasizing the mast cells involved in the appearing and extending of the angiogenesis vessels. After performing the histological preparations, these were examined at the microscope. For the study of the microvascular density, we selected the areas with the greatest vascular density and, respectively, the greatest density of mast cells (the „hot spot" technique). From every case to be studied, there were made four microscopic images with the 20× objective.

All slides were photographed by using a Nikon 55i microscope (Nikon, Apidrag, Romania) equipped with a 5-megapixel Nikon DS-Fi1 CCD color camera, a frame grabber and the Image ProPlus AMS image analysis software (Media Cybernetics, Bethesda, MD, US). Based on the immunostainings, vascular silhouettes were delimitated by hand as the regions of interest (ROIs) in order to prevent any errors of an automatic thresholding method that might have been induced by the endothelial staining discontinuities. In order to reduce biases, all vessels touching the left and lower edges of the images were not considered in the study, while those touching the right and the upper edges were. Next, the vessels were automatically measured in Image ProPlus for their total areas and respective total numbers and these were reported as means for a 20× objective area. An inter-group comparative analysis for normal tonsils, tonsillitis and tonsil cancer was performed by using ANOVA testing, the bi-group analysis was performed by using Student's *t*-test, and the correlations were reported according to Pearson's correlation index. The entire statistical analysis was performed in Excel and SPSS14.

Results

In our study, we evaluated the microvascular density in chronic tonsillitis and in tumoral stroma of the carcinoma with squamous cells developed at the level of the palatine tonsils, compared to the microvascular density in normal tonsil stroma. In order to obtain relevant data regarding the blood vascular system developed in the tonsil pathological processes, we proposed to quantify the maximum number of blood vessels on surface unit, as well as the area of these vessels. We also proposed to evaluate the reaction of the mast cells within the two types of tonsil lesions.

Qualitative evaluation of the blood micro-circulation

The qualitative evaluation of the blood micro-circulation within the normal tonsil stroma allowed us to notice the fact that the blood vessels had a relatively uniform disposition regarding orientation, dimensions and number. The blood vessels were identified in the lymphoid tissue in a greater number within the inter-lobular conjunctive septa and at the edges of the lymphoid follicles. In the superficial chorion, the blood micro-vessels had a reduced caliber, most often blood capillaries being identified with an ascendant development towards the limit between the surface epithelium and the subjacent chorion (Figure 1).

In chronic tonsilitis, the qualitative analysis showed a greater number of blood vessels on surface unit, most of them being congested, with a slightly irregular lumen and with moderate perivascular edema (Figure 2). Also, there was noticed the presence of some angiogenesis capillaries, formed of prominent endothelial cells, with big hypochrome nucleus and nucleoli. In the moderately-differentiated tonsils carcinoma with squamous cells, blood vessels appeared more numerous than in the chronic tonsillitis, with an irregular development and ununiform caliber (Figure 3). In the well-differentiated carcinoma, the blood microvessels appeared more numerous in the area of proliferation of the carcinoma, in the area where the stroma was strongly infiltrated with cells of inflammatory type and around the carcinoma islands (Figure 4).

In the areas of tumor invasion, the number of the blood vessels appeared much more numerous compared to other areas of the carcinoma stroma, and the angiogenesis capillaries were preponderant.

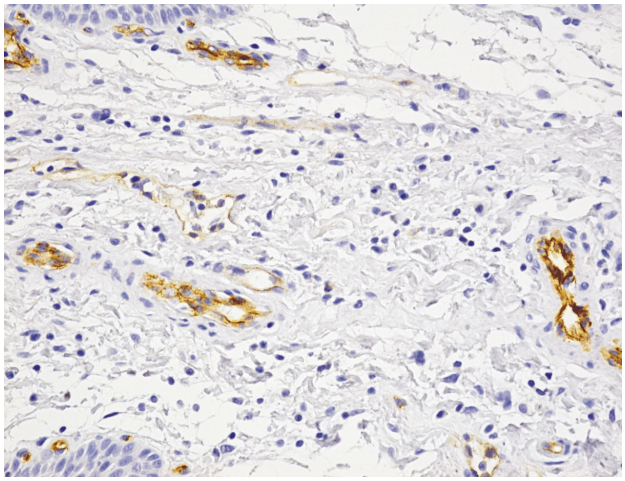

Figure 1 – ***Blood vessels in normal tonsil stroma. Anti-CD31 immunostaining, ×200.***

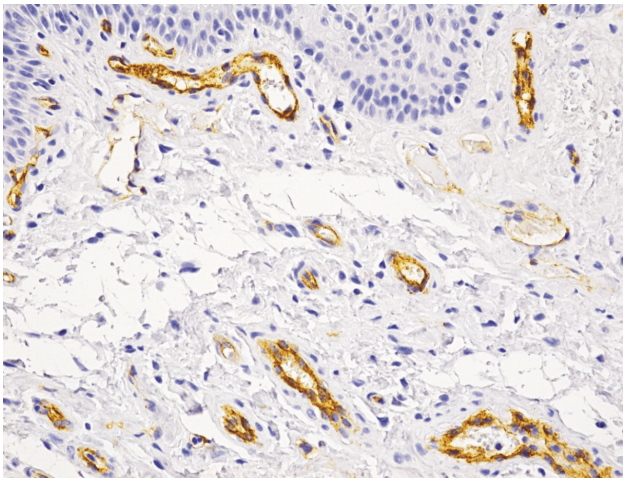

Figure 2 – ***Microscopic aspect of the blood vessels in chronic tonsillitis. Anti-CD31 immunostaining, ×200.***

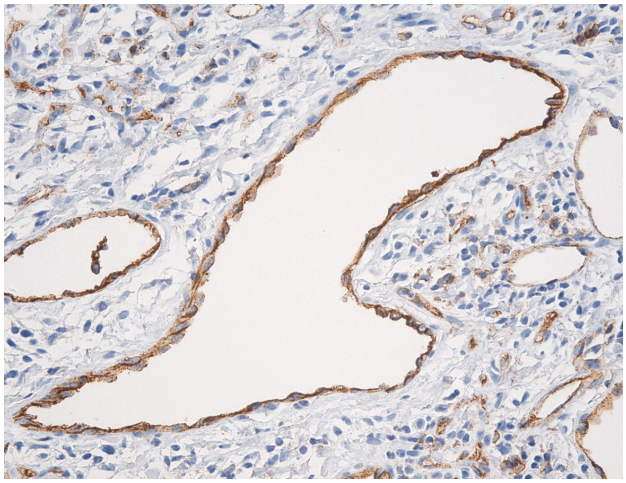

Figure 3 – ***Moderately-differentiated tonsil carcinoma with numerous blood vessels of various caliber. Anti-CD31 immunostaining, ×200.***

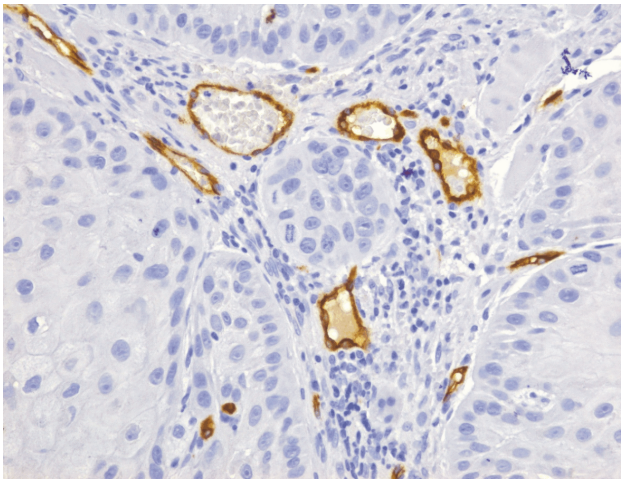

Figure 4 – ***Strongly vascularized tumoral stroma in a case of well-differentiated tonsil carcinoma. Anti-CD31 immunostaining, ×200.***

Qualitative evaluation of the microvascular density

The qualitative evaluation of the microvascular density using the „hot spot" technique and the Image ProPlus analysis software proved the fact that the maximum number of microvessels was 110–120/mm^2 in normal tonsils stroma, 125–140/mm^2 in chronic tonsillitis, and 220–230/mm^2 (Figure 5) in carcinomas. In other words, regarding the density of the blood vessels on the surface unit, this number was significantly greater in the stroma of the tumoral tissue compared to the stroma in chronic tonsillitis or normal tonsils stroma (Student *t*-test, $p<0.05$). On the other hand, there is no difference between the vascular densities in chronic tonsillitis and in normal tonsils.

As far as the areas of the vascular microvessels are regarded, the quantitative evaluation showed the fact that, overall, the means of the three groups were significantly different (ANOVA testing, $p<0.05$). The medium vascular area (on 20× objective) was minimum in the normal tonsils stroma, compared to the vessels in the inflamed tonsil tissue or to the tonsil cancer (Student *t*-test, $p<0.05$) (Figure 6). Overall, there existed a good correlation between the medium vascular area and the medium number of vessels (the Pearson coefficient of correlation, $r=0.746$, $p<0.01$), indicating the fact that the vessels increase simultaneously in number and in total

area. Also, there could be noticed the fact that, in the tonsil cancer, the area of the small vessels was extremely variable from a vessel to another and from a lesion to another. Thus, on the same histological section, we identified blood vessels with areas from 5 to 8200 μm^2.

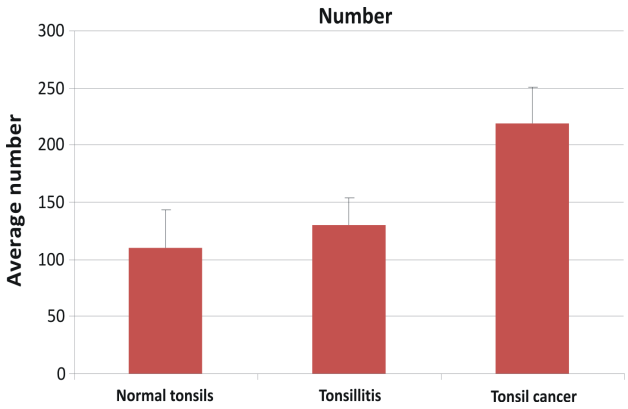

Figure 5 – ***Evaluation of the microvessels number in normal tonsil stroma, in chronic tonsillitis and in tonsil cancer.***

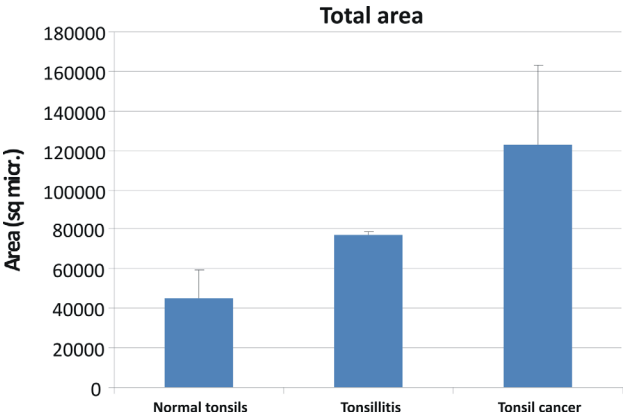

Figure 6 – ***Evaluation of the mean area of the microvessels in normal tonsil stroma, in chronic tonsillitis and in tonsil cancer.***

Qualitative evaluation of the mast cells reaction

The study of the mast cells reaction in the inflammatory and tumoral processes was suggested by the fact that, lately, more and more researchers have shown an involvement of these cells in the stimulation of the angiogenesis and in the tumoral progression.

In our study, the distribution of the tryptase-positive mast cells was not a homogenous one in the normal palatine tonsil. They appeared in a greater number in the superficial chorion and around the blood vessels, rarely being present in the structure of the lymphatic follicles structure (Figure 7).

In chronic tonsillitis, the mast cells appeared in an even greater number, being not homogenously distributed, preponderantly around the blood vessels and in the conjunctive septa between the tonsils lobules and even in the edge area of the lymphatic follicles (Figures 8 and 9).

In the tonsil cancers, the number of the mast cells appeared, on the whole, much more reduced than in chronic tonsillitis, being more abundant in the area of tumoral invasion where there was a more abundant inflammatory infiltrate, too (Figure 10).

Between the two forms of tonsil cancers, we noticed the fact that, in the more moderately differentiated squamous cancers, the reaction of the mast cells had a lower intensity than in the well-differentiated squamous cancers (Figure 11).

Quantitative evaluation of the mast cells

The quantitative evaluation of the mast cells was achieved by using the „hot spot" technique and the Image ProPlus analysis software.

The results of the statistical study showed the fact that the maximum number of the mast cells was around 60 cells/mm^2 in the normal tonsil stroma, 263 cells/mm^2 in the stroma of chronic tonsillitis, and 221 cells/mm^2 in tonsil tumoral stroma (Figure 12).

The tissue of control illustrated a superior density of the tryptase-positive cells in the inflammatory lesions and in the carcinoma with squamous cells compared to the normal tonsil tissue (ANOVA testing, p<0.01).

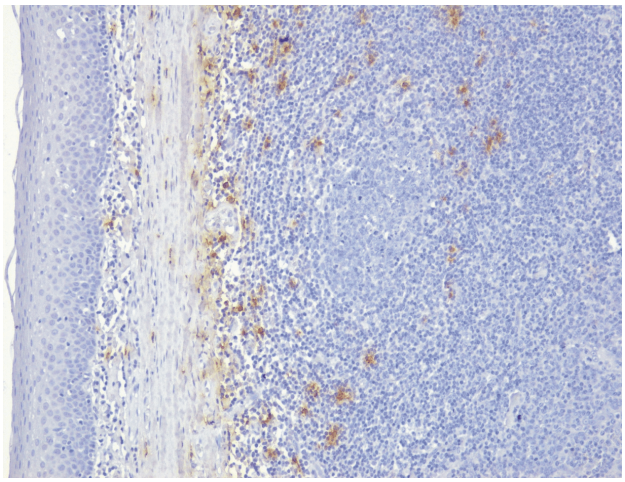

Figure 7 – ***Image of the reaction of the mast cells in normal tonsil stroma. Anti-tryptase immunostaining, ×100.***

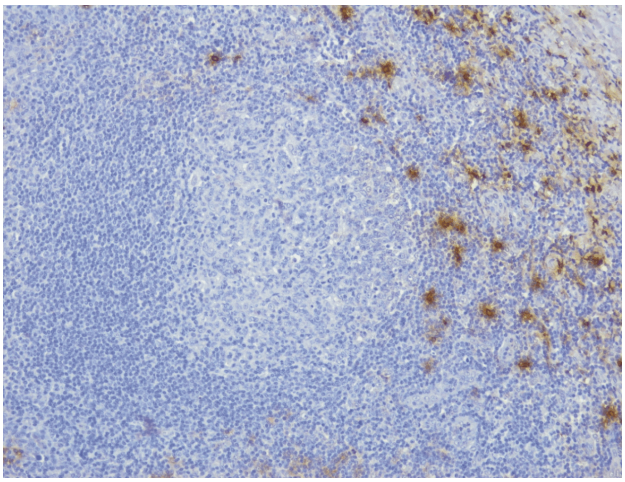

Figure 8 – ***Intensely-reactive mast cells placed at the edge of a lymphoid follicle in chronic tonsillitis. Anti-tryptase immunostaining, ×100.***

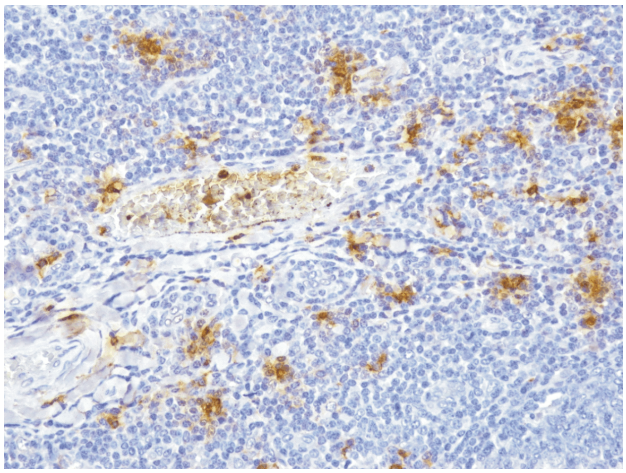

Figure 9 – ***Microscopic image of chronic tonsilitis, with numerous mast cells preponderantly distributed at a perivascular level. Anti-tryptase immunostaining, ×200.***

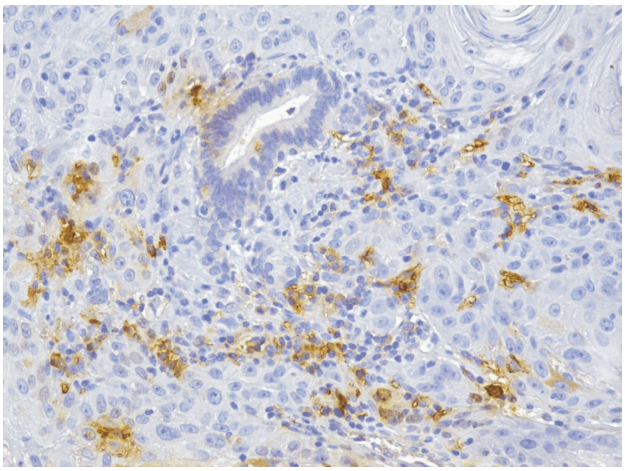

Figure 10 – ***Well-differentiated tonsil carcinoma with moderate mast cell reaction at tumoral proliferation front. Anti-tryptase immunostaining, ×200.***

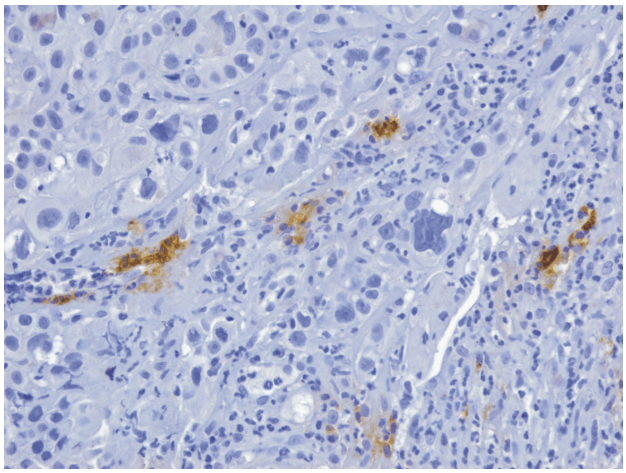

Figure 11 – ***Moderately-differentiated tonsil carcinoma with rare mast cells present in stroma. Anti-tryptase immunostaining, ×200.***

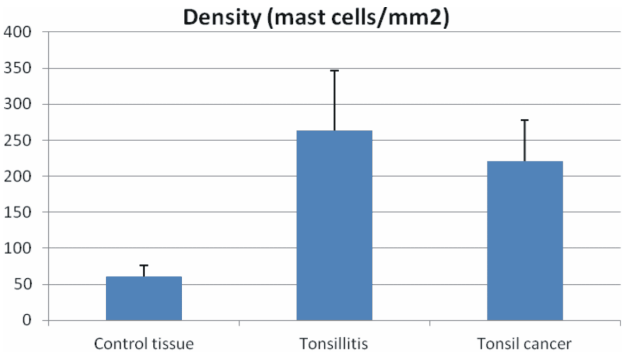

Figure 12 – ***Evaluation of the mast cells maximum density in normal tonsil stroma, in chronic tonsillitis and in tonsil cancer.***

Discussion

Nowadays, cancer represents one of the major public health problems in the whole world. In 2008, around 12.7 million people were diagnosed with different forms of cancer and around 7.6 million people died because of it [15]. As far as the tonsil carcinoma with squamous cells is regarded, it appears to be one of the most frequent localization of the oropharyngeal tumors in the U.S.A., where it represents around 15–20% of the intra-oral and oropharyngeal carcinomas. It is also interesting that the incidence of the tonsil carcinomas has grown with 2–3% every year, between 1973–1995, in Caucasian and Afro-American men, respectively, while the incidence of cancer, in other oral locations, remained constant [16].

Statistical data show that in Sweden and Finland, as well, a growing incidence of the tonsil cancer can be noticed, in spite of the reduced tendency of smoking incidence [1, 17]. These statistical data show the importance of some thorough studies that are performed in almost every country in the world regarding the genetical processes, cellular and molecular biology involved in the etiopathogenesis of the neoplastic disease. Although during the last years new information has appeared about molecular biology trying to solve the process of the oropharyngeal carcinogenesis [18], there still is various unknown data of this pathology, which explains their tendency of incidence growing.

In the last 40 years, special attention was given to tumoral angiogenesis processes, after Folkman J (1971) confirmed that angiogenesis is an essential process in the tumoral development and metastases [19].

In our study, we evaluated the microvascular density in the tonsil carcinoma stroma with squamous cells, compared to the microvascular density of chronic tonsillitis and normal tonsil stroma. We noticed that in tonsil carcinomas the number of blood vessels doubled compared to the number of vessels in normal tonsil stroma, while in the chronic inflammatory processes the growth of vascular density was insignificant. Besides the great number of vessels on the surface unit, we remarked the fact that the vascular area in carcinomas significantly grew up to three times compared to the vascular area in normal tonsil stroma and up to two times compared to the vascular area in chronic tonsillitis. These latter aspects indicate the fact that the tumoral vessels are even more numerous and have a greater caliber. That is

why we consider, just like other authors [20], that the vascular modifications in tumoral stroma favor the rapid growth of the tumor by bringing a plus of oxygen and nutritive substances, but they favor the metastasis of the tumoral cells, too, because of their wide lumen and the grown blood flow. According to some authors [21, 22], the tumoral metastasis is also due to the fact that the angiogenesis vessels at the level of the solid tumors is extremely permeable and they allow the tumoral cells to easily enter in the circulatory torrent compared to the mature vessels. The importance of the tumoral vessels quantification is also useful to evaluate the prognosis of the affection [22], although, sometimes, the clinical significance of the microvascular density is a controversial one [23, 24]. In spite of all these facts, the molecular therapy regarding the inhibition of the angiogenesis represents a start justifying research continuity regarding the tumoral angiogenesis.

The appearance of tumoral vessels is an extremely complex process, intermediated by more stimulatory and inhibitor molecules, that are released by the tumoral cells as well as by the host cells, some of them having a stimulatory effect of angiogenesis, others having an inhibitor effect [25]. Among the host cells that produce and release angiogenic factors, there are also the mast cells. They are real unicellular glands that synthesize and release a multitude of angiogenic factors, like histamine, heparin, chymase, the vascular endothelium growth factor (VEGF), etc. [25].

In our study, we noticed a significant growth of mast cells in the tumoral stroma of the carcinoma with squamous cells at the level of the palatine tonsils, compared to normal tonsil stroma. Still, in chronic tonsillitis, there could be remarked the presence of a greater number of mast cells, on the surface unit, than in the tumoral stroma, though the microvascular density was greater in the tumoral stroma.

These immunohistochemical aspects are explained through the fact that in the tumoral angiogenesis process, there interfere more factors secreted by a multitude of cells, whose input to the development of the tumoral vascular system is still difficult to evaluate. It is also possible for the mast cells to synthesize and to secrete different quantities of inflammatory mediators during the chronic inflammatory and the neoplastic processes.

Our observation that the number of the mast cells in the tumoral stroma is variable according to the grade of differentiation of the carcinoma is according to the observations of Coussens LM *et al.* (1999), that demonstrated on an animal sample that the stroma of the slightly-differentiated carcinoma lacked mast cells [26].

The role of mast cells in the tumoral progression is extremely controversial. It is well known the fact that mast cells, together with other inflammatory cells, favor the development of angiogenesis vessels and thus, implicitly, the tumor development, while other studies indicate the fact that mast cells can participate in the process of tumoral rejection by producing molecules of interleukin type (IL-1, IL-4, IL-6) and the tumoral necrosis factor (TNF-alpha) that destroy the tumoral cells [27].

Conclusions

In tonsil carcinoma with squamous cells, there could be noticed an ununiform growing of the number and caliber of the blood vessels, compared to the blood vessels in normal tonsil stroma and in chronic inflammatory lesions. The greatest volume of blood vessels was identified at the progression front of the tumoral lesion, where associated with a lymphocytic and macrophage chronic infiltrate. The microvascular density in the carcinoma with squamous cells of palatine tonsillitis was bigger than the microvascular density of chronic tonsillitis. The maximum number of blood vessels in tumoral lesions as well as their area, quantified through the "hot spot" technique, on the surface unit, was two times greater than in chronic tonsillitis. The number of mast cells was significantly greater in chronic tonsillitis as well as in tonsil carcinoma, but the reaction of these cells in inflammatory affections was more intense than in neoplastic lesions.

Acknowledgments

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Corresponding author
Carmen Aurelia Mogoantă, MD, PhD, Department of ENT, University of Medicine and Pharmacy of Craiova, 2 Petru Rareş Street, 200349 Craiova, Romania; Phone +40728–020 623, e-mail: carmen_mogo@yahoo.com

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