Microscopic aspects of angiogenesis and lymphangiogenesis in oral squamous cell carcinoma

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Abstract
Despite various great scientific and financial efforts, head and neck carcinomas represent a public health problem, being the eighth cause of cancer death worldwide. The rate of tumor growth, its local expansion, as well as the metastasis of cancerous cells depend on the tumor vascularization, on the ability of blood vessels to provide a constant supply of nourishing substances and oxygen and to eliminate the residual products resulted from tumor growth. That is why angiogenesis and lymphogenesis are considered to be essential processes within the neoplastic process. The assessment of tumoral neoformed blood vessels in oral squamous carcinomas, using the CD34 antibody, showed a significant growth of the microvascular density, the average number being 504.66±177.65 vessels/mm². The diameter of angiogenesis vessels varied between 3.42 and 121.27 μm. The density of lymphogenesis vessels was 508.78±235.93 vessels/mm², while the diameter varied from 2.82 to 165.28 μm. Both angiogenesis and lymphogenesis vessels were more numerous in the areas where the inflammatory infiltrate was more abundant, which suggests that chronic inflammation plays the part of a promoter factor of neoplastic lesions.

Keywords: angiogenesis, lymphogenesis, precursor endothelial cells, CD34, D2-40.

Introduction
In spite of the significant scientific and financial efforts, despite its localization in an easy to examine area, head and neck cancer represents one of the major public health problems all over the world. The clinical and statistical data in recent years showed that head and neck carcinomas represent the eighth cause of cancer death worldwide [1]. Its incidence, over 300 000 new cases every year [2], varies a lot from one region to another over the world map. While in North America and the European Union head and neck cancer represents 3–4%, in South Asia and Africa the same disease represents approximately 8–10% of all types of cancer [3].

Oral carcinogenesis is a multifactorial, quite complex process-taking place when the epithelial cells are affected by various genetic alterations [4]. The main etiopathogenic factors in oral carcinogenesis are considered to be alcohol intake and cigarette smoke. Chronic exposure to carcinogens determines genetic abnormalities in the cells of oral mucosa. When these genetic abnormalities determine the activation of proto-oncogenes and the inactivation of tumor suppressor genes, the cells modify their rhythm of growth and multiplication. These cellular populations have a marked tendency of accumulating additional genetic abnormalities, due to their genomic instability. In the end, these abnormal cells acquire a malignant phenotype, they lose their normal ability of differentiation and invade the basic membrane, having a local destructive effect and leading to regional metastasis.

The rate of tumor growth, its local expansion, as well as the metastasis of cancer cells depend on the tumor vascularization, on the ability of blood vessels to provide a constant supply of nourishing substances and oxygen and to eliminate the residual products resulted from tumor growth. Without any inherent vascular network, incipient carcinomas cannot grow over 2 mm³ [5, 6]. After the tumor reaches a size of 1–2 mm, it triggers the formation of new blood vessels necessary for its further growth [7].

Tumor angiogenesis, like the physiological one, is the process of creating new blood vessels starting from the already existing ones, either by recruiting precursor endothelial cells, or by multiplying the endothelial cells of the already existing capillaries. It represents an essential element in tumor development, and also in disseminating the cancerous cells towards other tissues.
and organs [8, 9]. Tumor angiogenesis differs from normal vascularization as it determines the formation of blood vessels with an altered morphology [10].

Lymphangiogenesis represents a dynamic process during embryogenesis, but largely absent after birth, under normal physiological circumstances [11]. Under pathological circumstances, such as inflammation, tissue repair and tumor growth, lymphangiogenesis has got a major contribution to the formation of new lymphatic vessels, by proliferating and germinating the endothelial cells within the pre-existent lymphatic vessels [12–14].

In the present study, we proposed to assess some aspects of the angiogenesis and lymphogenesis processes in oral squamous carcinomas.

Materials and Methods

The study was performed on a number of 115 carcinomas taken from patients hospitalized within the Clinic of Oral and Maxillofacial Surgery of the Clinical Emergency Hospital of Craiova, Romania, between 2008 and 2012, subjected to various surgical interventions prone to radicality. The age of the patients was between 1 and 87 years, with an average value of 63±14.45 years. The localization of the tumors was: at the level of inferior lip (n=47), mandible (n=31), buccal plate (n=16), tongue (n=12), superior lip (n=6) and superior maxilla (n=3).

Immediately after the surgery, the resection samples were fixed in neutral formalin and introduced in paraffin, using the classic histological technique. The histopathological study was performed on samples stained with Hematoxylin–Eosin and trichromic Goldner–Szekely.

For the immunohistochemical study, from the same biological material, there were performed histological sections with a thickness of 3 μm, by using a rotary microtome (Microm HM350), equipped with a system of water transfer of the histopathological sections (STS, Microm). The sections were collected on poly-L-Lysine covered blades, and kept in a thermostat at 37°C for 24 hours for increasing the adherence of the biological material. After deparaffinization and hydration, the biological material was hatched for 30 minutes in a 1% oxygenated water solution (hydrogen peroxide). Afterwards, the sections were washed in tap water and boiled in a citrate solution pH 6 for 20 minutes for antigen demasking. After boiling, they were left to cool down for 15 minutes; then they were washed in a phosphate buffered saline (PBS), followed by the stage of blocking the nonspecific antibodies. After washing, the sections were incubated overnight in a 5% normal goat serum (NGS), to reduce the non-specific background. Then, the sections were washed and incubated for 1 hour with primary antibodies (Table 1). The sections were washed in oxygenated water solution (hydrogen peroxide). Afterwards, the sections were washed in tap water and boiled in a citrate solution pH 6 for 20 minutes for antigen demasking. After boiling, they were left to cool down for 15 minutes; then they were washed in a phosphate buffered saline (PBS), followed by the stage of blocking the nonspecific antibodies. After washing, the sections were incubated overnight in a 5% normal goat serum (NGS), to reduce the non-specific background. Then, the sections were washed and incubated for 1 hour with primary antibodies (Table 1). For the immunohistochemical study, the following antibodies were used: CD34 (Dako) for highlighting endothelial cells. For an accurate assessment of the tumor angiogenesis process, more precisely of the microvascular density, we acquired 10 images with a 20× microscopic objective from various areas of tumor lesions from well-differentiated, moderately-differentiated and poorly-differentiated squamous carcinomas, subsequently analyzed by using the ImageProPlus software in order to obtain the number and sizes of angiogenesis vessels. Then, this data was introduced in an Excel program for obtaining comparable data.

In our study, young blood vessels from the tumor stroma had a completely scattered localization, most of the time being identified around the islands of tumor parenchyma. The vessel diameter varied quite a lot from one area to another within the same tumor. Most of the angiogenesis vessels had reduced sizes, being represented by immature neoformed capillaries, with the wall formed only by CD34+ endothelial cells. The diameter of the angiogenesis vessels varied from 3.42 to 121.27 μm, while the average number was 504.66±177.65 vessels/mm² (Figures 3 and 4). Unfortunately, the usual histological stainings, even though they allowed the highlighting of angiogenesis vessels, they did not provide us with enough details for a more accurate study of the angiogenesis and lymphogenesis processes. The emergence of immunohistochemistry techniques led to a better investigation of these processes.

The study of the angiogenesis vessels was performed by using the anti-CD34 antibody, which marked young endothelial cells. For an accurate assessment of the tumor angiogenesis process, more precisely of the microvascular density, we acquired 10 images with a 20× microscopic objective from various areas of tumor lesions from well-differentiated, moderately-differentiated and poorly-differentiated squamous carcinomas, subsequently analyzed by using the ImageProPlus software in order to obtain the number and sizes of angiogenesis vessels. Then, this data was introduced in an Excel program for obtaining comparable data.

The analysis of histopathological and immunohistochemical samples was performed under a Nikon Eclipse 55i microscope (Apiderg, Romania). The microscopic images were captured, stored and analyzed by using the ProPlus 7 AMS Image Soft (Cybernetic Media, Inc., Buckinghamshire, UK).

Results

The histopathological study of the tumor lesions is compulsory for establishing the positive and differential diagnosis, the differentiating degree of tumors and for assessing the prognosis factors. In our case, the histopathological study of squamous cell carcinomas allowed us to identify the lesion type and the differentiating degree. Thus, out of a total number of 115 squamous carcinoma cases, 61 were well-differentiated squamous carcinomas, 36 moderately-differentiated squamous carcinomas and 18 cases of poorly-differentiated squamous carcinomas (Figures 1 and 2).
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carcinomas there is high number of lymphatic vessels, located nearby the tumor parenchyma. The number and dimensions of lymphangiogenesis vessels were extremely variable from one tumor to another and from one area to another within the same tumor. Therefore, the average number of lymphatic vessels was 508.78±235.93/mm², the minimum diameter was 2.82 µm, while the maximum diameter was 165.28 µm (Figures 5 and 6).

Figure 1 – Image of well-differentiated squamous cell carcinoma from a lip tumor. HE staining, ×40.

Figure 2 – Poorly-differentiated buccal plate carcinoma with cellular pleomorphism, abundant inflammatory infiltrate and angiogenesis vessels in the stroma. HE staining, ×100.

Figure 3 – Angiogenesis vessel network in a moderately-differentiated tongue carcinoma, with a main localization around the islands of cancer cells. Anti-CD34 antibody immunostaining, ×100.

Figure 4 – Large angiogenesis vessels identified into a lip carcinoma. Anti-CD34 antibody immunostaining, ×40.

Figure 5 – Microscopic image with numerous lymphangiogenesis vessels localized within the invasion area of the tumor, associated with an abundant chronic inflammatory infiltrate mainly formed of lymphocytes. D2-40 immunostaining, ×100.

Figure 6 – Lymphatic vessels of various dimensions within the tumor stroma of a moderately-differentiated tongue carcinoma. D2-40 immunostaining, ×100.
Lymphatic vessels appeared more numerous at the tumor invasion area, where the chronic inflammatory process was more abundant. Also, there could not be noticed any significant differences of the lymphangiogenesis process in comparison to the differentiation degree of squamous carcinomas.

Discussion

At present, angiogenesis is considered an essential process in tumor development. The study of tumor angiogenesis has become of great importance in the ’70s, after Folkman et al. (1971) [15] isolated a soluble tumor factor out of solid human and animal tumors, which presented mitogenic properties for the capillary endothelium, thus inducing the growth of new capillaries. Ever since, numerous studies have shown that in solid tumors there appears an abnormal vascularization.

In our study, we identified a high number of CD34+ angiogenesis vessels, with an uneven trajectory, variable caliber, mainly localized around the islands of tumor cells within the tumor parenchyma. The localization of neoformation vessels nearby tumor cells has the effect of a better supply of oxygen and nourishing substances of tumor cells, necessary for a high rate of growth and multiplication of tumor cells. According to some authors [16, 17], the localization of blood vessels nearby the tumor, alongside a better supply with nourishing substances, increases the risk of tumor cell metastazation, as newly formed vessels have a high permeability.

In our study, there could be observed that the majority of neoformation vessels had a reduced caliber, being formed of capillaries structured in a one-row CD34+ cells. We consider that the lack of pericytes in the structure of tumor angiogenesis vessels determines their immaturity, characterized by the emergence of uneven caliber and discontinuous membrane vessels. Under normal circumstances, pericytes play a stabilizing role for blood vessels (metarterioles, capillaries, post-capillary venules), the lack of these cells leading to aneurysmal blood vessels (metarterioles, capillaries, post-capillary venules), the lack of these cells leading to aneurysmal deformations of microvessels, parietal discontinuities, hemorrhagic suffusions or perivascular edema [18]. More and more studies have shown that between the two processes.

The process of tumor lymphangiogenesis has been less studied. The relatively recent identification of various molecular markers specific to lymphatic endothelial cells and some factors promoting the increase of lymphatic vessels, have brought new data for a more accurate understanding of lymphatic vascularization, both under normal physiological circumstances and under pathological ones [11]. Under pathological circumstances, lymphangiogenesis has a major contribution to the formation of new lymphatic vessels, through the proliferation and germination of endothelial cells from pre-existent lymphatic vessels [14]. The relative contribution to the formation of new vessels of progenitor circulatory endothelial cells still remains unclear [24]. As well as angiogenesis, tumor lymphangiogenesis is induced by the lymphangiogenic growth factors produced and secreted even by the tumor cells, and by the stromal cells, tumor infiltrated macrophages or activated blood platelets, too [25, 26].

In our study, we remarked a high number of angiogenesis and lymphangiogenesis vessels in the areas where there previously existed an abundant chronic infiltrate within the tumor stroma. We consider that the cells in the chronic inflammatory infiltrate secrete a series of soluble factors that contribute to the stimulation of the two processes.

Our observations are in accordance with the ones of other researchers who sustain that the cells of the inborn immune system (T-lymphocytes natural killer, macrophages, mastocytes) play an important part in promoting the development of cancer and especially of the angiogenesis and lymphangiogenesis processes [27, 28].

Conclusions

The angiogenesis and lymphangiogenesis vessels of oral squamous cell carcinomas had quite various sizes, from one tumor to another and from one to another within the same tumor, which shows that the proangiogenic and lymphangiogenic factors act unevenly upon the endothelial cells. The microvascular density was also extremely varied, both in the angiogenesis process and in the lymphangiogenesis one. The correlation between the intensity of angiogenesis and lymphangiogenesis processes with the intensity of the chronic inflammatory
infiltrate in the tumor stroma shows the part played by the immune system cells in the tumor development.

Contribution Note
All authors have equally contributed to the realization of the article.

References

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Received: January 23, 2013
Accepted: September 17, 2013