[Frontiers in Bioscience 18, 1003-1016, June 1, 2013]

Creation of a suppressive microenvironment by macrophages and cancer-associated fibroblasts

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1. ABSTRACT

The cancer microenvironment makes up the stroma of the neoplasm and is the tissue that determines tumor growth, progression, and ability to initiate metastases. Because of the role that the cancer microenvironment plays in each stage of tumor development, knowledge about the interactions of the tumor with its microenvironment would seem to be of the utmost importance for developing new treatment strategies. The cancer microenvironment is created by the tissue surrounding the tumor cells and is composed of cells, extracellular matrix, and the proteins of the extracellular matrix. Although tumor cells are capable of penetrating the surrounding stroma, it is the tumor stroma that provides the necessary blood supply and growth factors for the tumor cells that condition tumor growth. In the present review we discuss the role of various cells like tumor-associated macrophages and cancer-associated fibroblasts, expressing RCAS1, B7-H4 molecules, and MT in creating the suppressive profile of the cancer microenvironment and in the cancer microenvironment remodeling that enables both local tumor spread and the creation of metastases.

2. INTRODUCTION

The cancer microenvironment makes up the stroma of the neoplasm and is the tissue that determines tumor growth, progression, and ability to initiate metastases. The tumor microenvironment can also restrict the access of therapeutic agents to the neoplasm; it can even alter the metabolism of these agents and participate in developing resistance to chemotherapy. Because of the role that the cancer microenvironment plays in each stage of tumor development, knowledge about the interactions of the tumor with its microenvironment would seem to be of the utmost importance for developing new treatment strategies aimed at restoring normal mechanisms of cellular control.

The malignant neoplasm modifies the cancer microenvironment through the induction and maintenance of chronic inflammation, also called cancer-accompanied inflammation. This inflammation is typified by excessive immune system cell infiltration. The next step in the development of cancer involves a change in the immune system cell profile and activity. This helps to create local immune tolerance for the tumor cells and is called a selective

immune suppression. It leads to the creation of the suppressive profile of the cancer microenvironment and involves the suppression of the antitumor immune response, including the change from the macrophage phenotype into tumor-associated macrophages. The alteration in the cancer microenvironment profile into a suppressive one also involves structural cells, such as fibroblasts and their transition into cancer-associated fibroblasts. These cells independently induce and maintain the chronic inflammation and the local immune tolerance for tumor cells. At the same time they induce cancer microenvironment remodeling through the phenomenon of epithelial to mesenchymal transition that enables the local and distant spread of the tumor.

The cancer microenvironment is created by the tissue surrounding the tumor cells and is composed of cells, extracellular matrix, and the proteins of the extracellular matrix. Many types of cells can be identified in the stroma, including endothelial cells and their precursors, pericytes, smooth muscle fibers, fibroblasts, tumor-associated fibroblasts, myofibroblasts, neutrophils, basophils, eosinophils, mast cells, lymphocytes T and B, and NK cells; additionally there are antigen-presenting cells such as macrophages and dendritic cells. The tumor stroma may also play an important role in the development of the tumor. Although tumor cells are capable of penetrating the surrounding stroma, it is the tumor stroma that provides the necessary blood supply and growth factors for the tumor cells that condition tumor growth. Some neoplasms use and modify the existing stroma for their growth while other tumors induce the development of new stroma. In sum, the tumor stroma is not a neoplasmatic tissue, but rather a tissue which has been modified by the tumor for the purpose of its own growth and development.

Many processes related to the inflammation and immune response that determine tumor growth but do not provide the effective antitumor immune response actually take place in the tumor microenvironment.

3. INFLAMMATION AND IMMUNE RESPONSE IN THE CANCER MICROENVIRONMENT

In the nineteenth century Rudolf Virchow observed that cancer grows in conditions of chronic inflammation and described the presence of leukocytes in tumor tissues (1,2). Immune system cells and their mediators are present within the cancer microenvironments of all types of malignant tumors (3). The development of the tumor is accompanied by chronic inflammation. Such inflammation induces carcinogenesis in some situations while in others it exerts an anti-cancer effect (3). Cancer-related inflammation is predominantly linked with non-specific innate immune response (4). The acquired, specific immune response also participates in the cancer-associated inflammation; on animal models it was observed that B lymphocytes also participated in carcinogenesis and in the anti-tumor immune response. In patients with cancer, specific anti-tumor antibodies (antibodies against tumor antigens) were observed (5,6). Moreover, it has been observed that the cancer microenvironment can restrict the activity of tumor-infiltrating lymphocytes (7). Both types of immune-response participate in inducing carcinogenesis and in inhibiting tumor growth. It has further been observed that congenital and acquired immune deficits are related to an increased incidence of malignant neoplasms, and this indicates that the immune system plays an important role in fighting cancer (8,9). Additionally, the relationship between cancer and the immune system seems to constitute a dynamic process. Initially, the immune system controls and eliminates the pre-neoplastic lesions and early malignant tumors (10). As the tumor grows, however, the cancer cells, following the exposure to the anti-tumor immune response, become resistant to the immune attack and acquire a new phenotype able to manipulate and alter immune system cell activity through the secretion of cytokines and chemokines (11). At this point, tumor-associated macrophages and B lymphocytes might interact, promoting tumor growth through the secretion of factors that activate cancer microenvironment remodeling, including angiogenesis.

There are two types of inflammation that occur in the human body; one is acute inflammation; which is short-lived and carries positive therapeutic consequences. The other is chronic inflammation which lasts for a long time and has destructive consequences that support cancer development and initiate metastases. Chronic inflammation is therefore a risk factor for developing many types of malignant neoplasms (e.g., cigarette smoking in cases of lung cancer, Helicobacter pylori infection and chronic gastritis in cases of gastric cancer, HPV virus infection and chronic cervicitis in cases of cervical cancer, HPV virus infection and chronic mucositis in cases of oral and pharyngeal cancer, HBV and HCV infections and chronic hepatitis in cases of hepatic cancer, etc.) (12).

The development of chronic inflammation is associated with hypoxia. Hypoxia is commonly found in solid tumors of various types and is connected with a decreased response to anti-cancer treatment, malignant progression, local invasion, and distant metastases. Hypoxia-inducible factor (HIF-1) is a transcription factor commonly induced by inflammatory mediators, including cytokines, hormones (insulin and insulin like growth factor 1 and 2 (IGF-1, IGF-2), and vasoactive peptides (angiotensin II) (13,14). Cytokines, such as IL-β and TNF-α, induce HIF-1α activity in the hepatocellular cancer cell line. Additionally, HIF-1 stimulates the expression of genes controlling inflammation, including erythropoietin, VEGF, VEGF-receptor, iNOS (inducible NO synthase), COX-2, glycolytic enzymes, and others in order to induce oxidative stress (15). HIF-1 is a main regulator of the adaptation of cells to oxidative stress, controlling the alteration from aerobic to anaerobic metabolism, inducing the glycolytic phenotype in cancer cells and enabling the access to energy and survival (15). The activation of HIF-1 in cancer cells induces various types of mechanisms that activate angiogenesis, glycolysis, secretion of growth factors (PDGF- platelet derived growth factor and EGF- epidermal growth factor) (platelet derived growth factor, epidermal growth factor), TGF-β (transforming growth factor), IGF-2, and proteins that participate in tumor invasion. Moreover, hypoxia inhibits the expression of adhesion proteins enabling the detachment of single cells and their migration(16,17). Macrophages typically accumulate in the hypoxemic region of a tumor. This microenvironment induces

the adaptation of cells to hypoxemia and the change in their phenotype; for instance, the experimental removal of HIF-1 from these cells increased their cytotoxicity. Hypoxia selectively induces the expression of CXCR4 receptor (chemokine receptor CXC), influencing the migration of the cancer cells (18).

Chronic inflammation also induces the reactive oxygen and nitrogen species, thus generating oxidative stress in the cancer microenvironment. Oxidative stress is a condition of disturbed tissue homeostasis between the activity of reactive oxygen and nitrogen species and the processes of detoxification and tissue repair. Reactive oxygen species develop in all cells during the physiological process of respiration and react with the most important structures and molecules in the cell changing their biological function. The disturbance of homeostasis between the reactive oxygen species and the mechanisms of cell repair and detoxification lead to tissue destruction and the development of a malignant tumor and its progression (19).

4. CELLS IN THE CANCER MICROENVIRONMENT

The infiltration of immune system cells and angiogenesis constitute the cancer microenvironment's response to the tumor and together they play the most important role in tumor progression. Initially, the excessive proliferation of cancer cells leads to local hypoxemia and necrosis, inducing the cancer microenvironment cells to secrete growth factors and cytokines (colony-stimulating factor CSF-1, granulocyte-monocyte-colony stimulating factor GM-CSF, transforming growth factor TGF-β, and chemokines CCL2, CCL7, CCL3, and CCL4) that chemo-attract the monocytes and macrophages (20). In response to this, macrophages release growth factors that determine tumor behavior (such as an increase of tumor activity) and activate endothelium, all of which support cancer-related inflammation (21). Macrophages/monocytes infiltrating the tumor microenvironment release VEGF-C, basic fibroblast growth factor bFGF, TNF, HGF, factors from EGF family, and platelet-derived growth factor PDG as well as chemokines CXCL12 and IL-8 (20,22).

4.1. Macrophages in the cancer microenvironment

The role of tumor-infiltrating macrophages (TIMs, TAM- tumor associated macrophages, TEM- tumor educated macrophages) in cancer development seems to be a dual one. On the one hand, these cells can stimulate tumor growth; on the other hand, they can control tumor rejection (23). A positive correlation has been found between the number of tumor-infiltrating macrophages and a poor prognosis for cases of various types of malignant tumors. Moreover, macrophages are present within the tumor stroma from the earliest stages of the tumor's development, alongside hyperplasia and atypical cells appearance (24, 25); this confirms their role in the initiation of tumor development. The tumor and its stroma express chemo-attractive factors for macrophages while macrophages secrete growth and the pro-angiogenic factors (26) mentioned above, thus influencing the cancer microenvironment and modifying tumor growth (27). The suppression of macrophage infiltration in the tumor correlates with the inhibition of tumor growth in vivo (28) while the induction of cytokines activating macrophages promotes macrophage infiltration of the tumor and the promotion of tumor growth (29,30). Macrophages/monocytes that infiltrate the tumor and its stroma are stimulated by the stroma and acquire one of two phenotypes, M1 or M2 (21). The M1 phenotype is related to antigen-presenting cell activity in inflammation and the activity against infection, while phenotype M2 is associated with tissue remodeling and the pro-angiogenic activity of these cells. The M2 phenotype seems to be crucial for tumor development and is related to IL-12lowIL-10high and the production of TGF-β.

As a result of the interaction between the tumor and macrophages, macrophages release cytokines, chemokines, growth factors, and activity factors (such as GM-CSF, IL-8, and EGF), which in turn increase immune system cell infiltration to the area of the tumor stroma, aggravating the inflammatory response in the cancer microenvironment (1). Chemokines play an important role in coordinating the stromal response to tumor growth. They polarize the response of the immune system cells to the tumor, regulate the type of cellular infiltration, and initiate angiogenesis. The receptors for chemokines were identified on cancer cells and their ligands were demonstrated in primary malignant tumors and metastatic cells which together suggest the important role of chemokines in tumor growth and the development of metastases (1).

The participation of macrophages in the process of angiogenesis is also dual. On the one hand, macrophages produce pro-angiogenic factors; on the other hand, they secrete anti-angiogenic factors which inhibit angiogenesis and destroy the integrity of the blood vessels. The interaction between the tumor and macrophages induces the pro-angiogenic function in macrophages, and the accumulation of macrophages is associated with the secretion of VEGF and PDGF (1). The hypoxemic regions of the tumor induce the migration of macrophages and evoke the pro-angiogenic program in these cells. Among the factors undergoing expression in macrophages under the hypoxemia, the following were identified: VEGF, TNF-α, bFGF, CXCL8 (IL-8), and glycolytic enzymes, the transcription of which is controlled by HIF-1 and HIF-2 transcription factors (31-33). Macrophages infiltrating the cancer stroma along with directly acting factors secreted by tumor cells (e.g., CXCL12 acts as a chemokine for various endothelial cells typified by CXCR4, IL-8-CXCL8, VEGF, and bFGF) regulate the process of angiogenesis in another mode as well. Macrophages also control the lymphangiogenesis process in the stroma and lymphangiogenesis is regulated by VEGF-C and VEGF-D acting through the receptor VEGFR3. Recently, it has been shown that VEGF-A chemo-attracts monocytes and increases lymphangiogenesis through the induction of monocyte infiltration (31-35). In cervical cancer, the production of VEGF-C by macrophages plays an important role in lymphangiogenesis and cancer dissemination through the lymphatic route. Additionally, macrophages induce angiogenesis in the cancer stroma through the production of thymidine phosphorylase (TP), the pro-angiogenic factor stimulating the migration of endothelial cells in vitro, and

its high expression has been correlated with tumor neovascularization (34,35).

Local tumor development depends on the controlled degradation of the extracellular matrix. It has been shown that macrophages participate in initiating the creation of metastases and an increased number typically indicates a poor prognosis. Furthermore, genetic studies on mice have demonstrated that a decreased number of macrophages correlates with a lower incidence of metastases (36). Macrophages are characterized by proteolytic activity that results in the degradation of the basal membrane in pre-invasive cancer (in situ) and enables the spread of local cancer cells to the microenvironment (37).

In sum, macrophages express factors that modulate cell proliferation, angiogenesis, and the degradation of connective tissue. These cells are also able to stimulate the creation of the new cancer microenvironment by secreting PDGF, acting together with TGF-β secreted by the tumor cells (37). Macrophages release the matrix metaloproteinases, MMP-2 and MMP-9 that degrade the extra-cellular matrix, as well as MMP activators, such as chemokines, and other factors that facilitate matrix degradation and cancer cell invasion and migration (TGF-β, PDGF, IL-6, and tissue type plasminogen activators u-PA and t-PA) (38-41). Macrophages also secrete factors that encourage cancer cells to home the tissue (e.g., EGF), while cancer cells release factors that chemoattract macrophages (e.g., M-CSF or macrophage colony stimulating factor) (24-43).

4.1.1. B7-H4 positive macrophages

Antigen-presenting cells are important for initiating and maintaining the tumor-associated antigen-specific T-cell immunity. Tumor-infiltrating macrophages significantly counter the number of other antigen-presenting cells within the cancer microenvironment (25,37,44-46). In mice it was found that macrophages associated with the tumor were involved in promoting tumor growth and had direct metastatic action on cancer cells (25, 37, 44-46). B7-H4 (B7x and B7S1) has recently been described as a member of B7 molecules co-stimulating T lymphocytes, a negative regulator of T-cell response. This molecule inhibits T-cell proliferation, cell cycle progression, and the production of cytokines in vitro. Antigenic specific T-cell responses in mice are disturbed by B7-H4 protein; in humans, however, the expression, regulation, and function of B7-H4 protein remain unknown. Kryczek et al. have demonstrated the presence of B7-H4-positive macrophages in the cancer microenvironment in patients with ovarian cancer and have confirmed the suppressive activity of these cells, which is comparable to that of Treg cells. Moreover, B7-H4 macrophages interact with CD4+Treg cells, and CD4+Treg cells stimulated B7-H4 expression in macrophages, enabling the suppressive function of these cells. Through Il-6 and IL-10 cytokine secretion, the cancer microenvironment stimulated B7-H4 expression by macrophages. Additionally, Treg cells induce Il-10 production by APC which stimulates the suppressive activity of macrophages through B7-H4 (44, 47-48). In our study, B7-H4-positive macrophages were identified in almost all the patients with uterine cervical carcinoma. A significantly higher number of B7-H4-positive cancer cells were identified in the tumor front of those patients in whom lymph node metastases were present than those patients without such metastases. A significant increase in B7-H4-positive macrophage infiltration within the tumor microenvironment was observed in those patients who did have lymph node metastases (49).

4.1.2. RCAS1-positive macrophages

RCAS1 (receptor-binding cancer antigen expressed on SiSo cells) is a II type trans-membrane protein with a gene EBAG9 or estrogen receptor-binding fragment-associated antigen 9, located at 8q23gen (51). This protein was first described by Sonoda et al. in 1996 on cervical cancer cells. It has been demonstrated that RCAS1 is a ligand for the putative receptor expressed by lymphocytes T, B, and NK cell. It has been shown both in vitro and in vivo that the interaction of RCAS1 with the receptor inhibits the growth of receptor-expressing cells and induces their apoptosis through the activation of FADD and the caspase pathway (51-52). It was noted that RCAS1 is secreted to the supernatant in the cancer cell line as a soluble form of RCAS1 (sRCAS1) during ectodomain shedding (53-55). It was determined that sRCAS1 was able to inhibit the growth of receptor expressing cells and induce their apoptosis; it thus possessed the same biological function as a membrane form (53-55). The level of sRCAS1 increased in the sera of patients with cervical and head and neck cancers as the tumor progressed (53-56); in patients with head and neck cancer, it was observed that the sRCAS1 level decreased in blood sera following surgical treatment and increased again in those patients with a recurrence of the disease (56). RCAS1 protein is therefore responsible for tumor escape from host immunological surveillance and participates in the creation of immune tolerance for the tumor cells and maternal immune tolerance for fetal antigens during pregnancy (51-64). RCAS1 expression has been found in various malignant neoplasms, and it is significantly higher in patients with advanced tumors, high tumor grade, and the presence of lymph node metastases, and in cases with a poor prognosis (51-64). It has furthermore been suggested that in patients with cervical cancer RCAS1 participates in cancer microenvironment remodeling (53-55).

RCAS1-positive macrophages were first described in cervical cancer tissue (51); later, these cells were also detected in the bone marrow where they play an important regulative role in erythropoiesis through the expression of RCAS1 (65-67). It has been shown that RCAS1 is expressed by activated monocytes or stimulated by lipopolysaccharide (65). RCAS1-positive macrophages have been observed in various types of hepatitis, including acute viral hepatitis, chronic viral hepatitis, primary cirrhosis, and immune-mediated hepatitis. RCAS1-positive macrophages have been identified in patients with very high ALT (alanine aminotransferase) and surrounding massive hepatocellular necrosis. The observed RCAS1-positive macrophages indicated that these cells might represent the M2 phenotype of macrophages and might play a regulatory role in chronic inflammation. Chronic inflammation may induce the expression of RCAS1 on macrophages so that the M2 phenotype of a suppressive activity is induced in turn (65-67). The interaction of RCAS1 protein with the tumor and its microenvironment is presented in Figure 1. In our previous

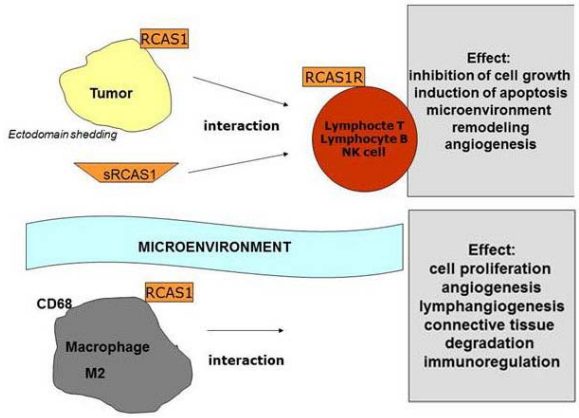

Figure 1. The interaction of RCAS1 protein between the tumor and its microenvironment

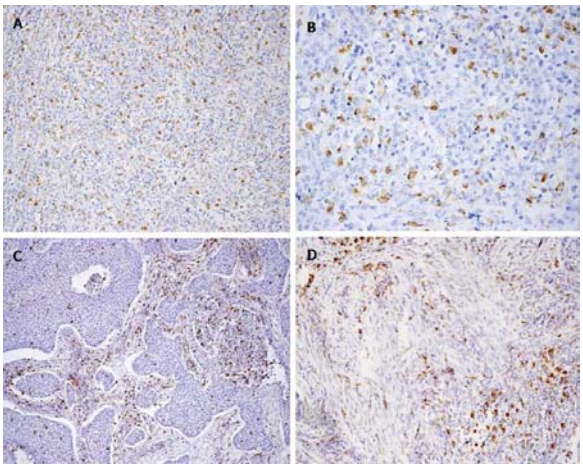

Figure 2. CD 68 positive cells in malignant B-cell lymphoma of the palatine tonsils. A-intensive infiltration of CD68 positive cells (magnification 20x), B- the same cells in magnification 40x, C-intensive infiltration of CD68 positive cells in the tumor tissue and in the stroma (magnification 20x), D- CD68 positive cells in the stroma magnification 40x).

studies, RCAS1-expression and RCAS1-positive macrophages have been observed in various types of malignant neoplasms and their microenvironments as well as in conditions of chronic inflammation, such as chronic tonsillitis and nasal polyps (68-75)). The expression of RCAS1 was identified in both squamous cell carcinoma and adenocarcinoma cells from the tissues of patients with head and neck, cervical, and ovarian cancer; it was also found in hydatidiform mole tissue and in the cancer microenvironments of these patients. The tissue remodeling of the cancer microenvironment was marked by the vimentin and MT expression, and the suppressive cancer microenvironment was confirmed by a decreased number of TIL with lower immunoreactivity of such antigens as CD56 and CD57; moreover, this cancer microenvironment was infiltrated by RCAS1-expressing macrophages (68-73). In our studies on head and neck cancer in both histological types of tumors (squamous carcinoma and adenocarcinoma), the number of RCAS1-positive macrophages infiltrating the cancer microenvironment was significantly higher in patients with the presence of lymph node metastases than in patients without such metastases (68-73). Moreover, we detected tumor-infiltrating macrophages and RCAS1-positive macrophages infiltrating the tumor and stroma of malignant B-cell lymphomas originating from palatine tonsils (Figure 2, Figure 3). In patients with hydatiform mole, RCAS1 immunoreactivity was identified in both trophoblast and decidual cells as well as in the stroma. Significantly lower RCAS1 levels were found in those patients who were treated by surgery alone than in the patients who also required chemotherapy. Since strongly RCAS1-positive macrophages were found dispersed in the stroma, we concluded that RCAS1 staining might provide information about the intensity of the immune suppressive microenvironment in the molar lesion and endometrium (72) and could serve as a marker of the need for more aggressive treatment. In ovarian cancer, the cancer microenvironment was characterized by the presence of the excessive infiltration of RCAS1-positive macrophages. A statistically significantly higher number of RCAS1-positive macrophages were identified in patients with the presence of lymph node metastases than in patients without such metastases. Moreover, the cytoplasmic RCAS1 expression and the number of RCAS1-positive macrophages was higher in the border part of the tumor (which was defined as a younger part of the tumor with signs of dynamic growth) derived from the patients with the lymph node metastases in comparison to those patients without such metastases (71). Furthermore, RCAS1-positive macrophages were identified in the cancer microenvironment of all the patients in the study with uterine cervical carcinoma. No correlation, however, was seen between the presence of these cells and the particular stage of the disease. A correlation might be observed if patients with operable cervical cancer and with less advanced stages of the disease (I and II) were to be included in the study (49-50).

In patients who had had their palatine tonsils removed due to chronic tonsillitis, RCAS1 immunoreactivity was detected in the crypts epithelium, a very specialized tissue responsible for immune interactions with foreign antigens (Figure 4). Single epithelial exfoliated cells and macrophages positive for RCAS1 were also observed in the crypts lumen. Nasal polyps are a symptom of chronic rhinitis and sinusitis, and the presence of RCAS1-positive macrophages has been noted within the stroma of such polyps (69-70, 74-75). These observations would seem to confirm a very important immunoregulatory role for RCAS1-positive macrophages in various types of clinical situations. It seems that RCAS1-positive macrophages belong to the main regulatory mechanisms of the immune system.

We would therefore propose that RCAS1-positive macrophages represent the M2 macrophage phenotype, participate in microenvironment remodeling, enable the local and distant spread of the tumor, and negatively regulate the anti-tumor immune response, and so are responsible for the creation of the suppressive cancer microenvironment in these tumors.

In sum, tumor-associated macrophages, expressing RCAS1, B7-H4 molecules, and inhibiting the anti-tumor immune response, are all responsible for the development of the suppressive phenotype of the cancer microenvironment and are also related to poor prognosis.

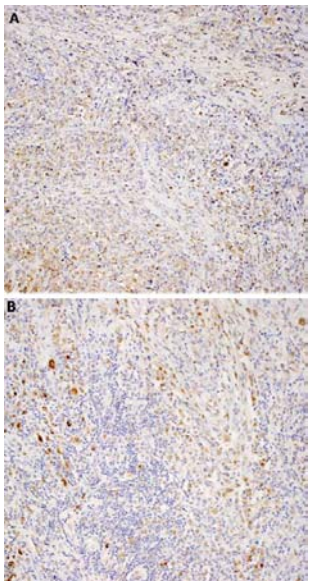

Figure 3. RCAS1 immunoreactivity in the malignant B-cell lymphoma of palatine tonsils in the tumor and the stroma tissue. Numerous RCAS1-positive macrophages are present in the stroma. A-magnification 20x, B-magnification 40x.

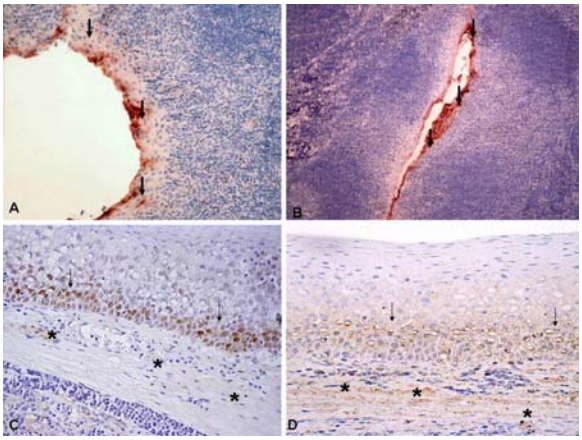

Figure 4. RCAS1 immunoreactivity in healthy epithelium.

4.2. Cancer-associated fibroblasts

Activated fibroblasts, that is, the myofibroblasts of the cancer microenvironment, have been classified as cancer-associated fibroblasts (CAFs) because the number of these cells increases significantly in various types of malignant neoplasms. Myofibroblasts have features similar to those of both smooth muscle fibers and fibroblasts, and significantly induce the growth and differentiation of cells during embryogenesis, wound healing, and other processes of tissue remodeling (79). Various cells can differentiate into myofibroblasts, not only fibroblasts, but also the smooth muscle fibers of vessels, pericytes, bone marrow precursor cells, and even cancer cells. PDGF secreted by the tumor stimulates fibroblast proliferation, while TGF-β released from macrophages chemoattracts fibroblasts in lower concentrations, while in higher concentrations it induces their transdifferentiation into myofibroblasts. Furthermore, tumor cells themselves express TGF-β (80). Myofibroblasts appear shortly before the cancer invasion, degrade the basal membrane and extracellular matrix through the secretion of serine proteases, matrix metalloproteinase, and urokinase activator of plasminogen. Myofibroblasts additionally express IGF and HGF/SF (hepatocyte growth factor/scatter factor), inducing the survival and migration of the cells and the expression of pro-angiogenic factors (FGF-2 and VEGF) and pro-inflammatory cytokines (IL-1, IL-6, and IL-8 i TNF-α). Myofibroblasts not only stimulate their own migration to the tumor site, they also induce the survival, proliferation, invasion of cancer cells, and angiogenesis, thus enhancing the ability of the tumor to grow and create metastases (81-83). Microenvironment fibroblasts participate in deciding whether the cancer cells proliferate, infiltrate the surrounding tissues, and metastasize.

In our previous studies, cancer-associated fibroblasts were identified in the cancer microenvironment of head and neck squamous cell carcinomas and adenocarcinomas; these cells expressed a strong level of vimentin (69-70). Statistically significantly higher vimentin immunoreactivity levels were observed in the tumor stroma in patients with advanced tumor size (T3 and T4) in head and neck squamous cell carcinoma. Cancer-associated fibroblasts (CAFs) in the cancer microenvironment in both histological types of tumors of the head and neck were observed to express metallothionein. Metallothioneins are a family of low-molecular weight proteins (6 kDa) with a high affinity for divalent metals, such as zinc and copper, as well as toxic metals, such as cadmium and mercury (84-85). Human metallothioneins include four isoforms: MT-1 and MT-2, which are widely expressed in tissues, and MT-3 and MT-4, which are present exclusively in specialized cells (84). The ability to bind the metal ions is linked to the biological role of this protein, including protection against metal toxicity, reservoir of zinc and copper for metaloenzymes during the apoptosis process, the production of transcription factors, and protection against oxidative stress (84). MTs may also play an important role in the proliferation and differentiation of cells (86). It has been established that MT expression in the cytoplasm helps to protect against cytotoxicity, while its expression in the nucleus protects against genotoxicity (84, 86-87). Genotoxicity concerns the acquisition of cells of the malignant phenotype, as a result of mutations important in the carcinogenesis process. Cytotoxicity is important in the interaction of cancer cells with immune system cells. MT expression was observed in various types of malignant neoplasms and in cancer microenvironments as well as in healthy tissues adjacent to cancer nests. That the MT expression in healthy epithelia was localized to the basal part of the epithelium, which comprises intensively dividing cells responsible for its renewal, was demonstrated in various studies, while in the more superficial layers of the epithelium which were composed of well-differentiated cells, MT expression was not found (88-91) (Figure 5). MT expression was also observed in tumor-adjacent tissue, epithelium, and even in tumors without MT expression (88-93). Moreover, MT expression was found in healthy thyroid cartilage adjacent to laryngeal cancer, in healthy vessels, and glandular epithelium in the tumor vicinity. It has been suggested that migrating tumor cells may stimulate cancer microenvironment cells to respond (Figure 5) (88, 92-93). The cancer microenvironment

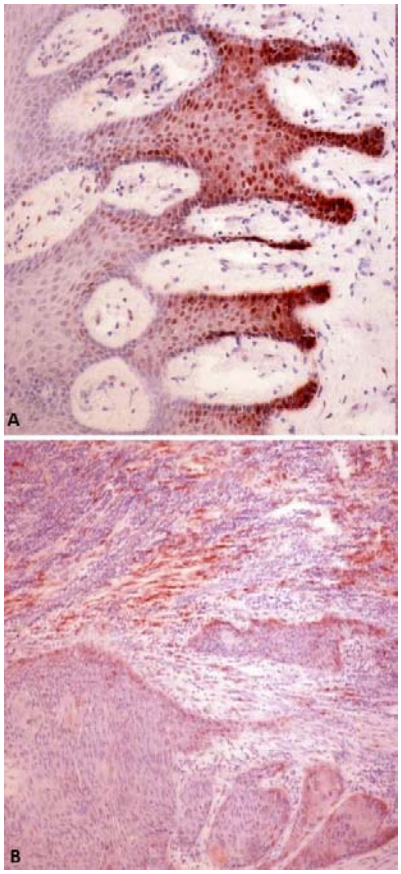

Figure 5. MT immunoreactivity: A-healthy epithelium of upper respiratory tract mucosa, B- MT positive fibroblasts in the stroma of head and neck squamous cell carcinoma.

expressed MT as well, and it has been shown that MT expression in the cancer microenvironment may be related to an increased degree of tumor invasiveness and local tumor spread. Moreover, the level of expression was found to be significantly higher in patients with the presence of lymph node metastases in comparison to those patients without such metastases (88). MT expression in the cancer microenvironment may also be related to the increasing infiltration of immune system cells. The response of the tumor stroma with MT expression may be the result of increasing resistance to immune-induced apoptosis, as MT plays a protective role against apoptosis. Because of MT's complex function, which includes anti-apoptotic, pro-proliferative, and immunomodulating properties, the local expression of MT may play a critical role in cancer invasion through CAFs. The immunomodulating role of MT expression has also been observed in nasal polyps, which are a symptom of chronic rhinosinusitis. Additionally, MT expression has been found in the stroma of nasal polyps, which is the area of excessive immune cell infiltration. There were differences in MT expression between the polyps depending on the type of the predominant immune cell infiltration. The presence of MT expression in the nasal polyp microenvironment seems to confirm its important immunoregulatory role as well as its participation in the maintenance of chronic inflammation (88, 94).

Cancer-associated fibroblasts have also been observed to express other important molecules that may determine the nature of their participation in creating the local suppressive microenvironment. For example, the presence of RCAS1 and B7-H4 positive CAFs was found in the cancer microenvironment of patients suffering from cervical cancer. The number of these cells in the cancer microenvironment did not, however, correlate with the stage of the disease, probably because these tumors were all in I and II stage (and hence operable). By contrast, in patients with ovarian cancer, the cancer microenvironment was characterized by the presence of RCAS1-positive carcinoma-associated fibroblasts, and this presence was more pronounced in the border part of the tumor (which is defined as a younger part having signs of dynamic growth) derived from those patients with the presence of lymph node metastases in comparison to those without such metastases.

All these findings would seem to confirm that CAFs and TAMs expressing MT, RCAS1, and B7-H4 molecules participate in creating the suppressive profile of the cancer microenvironment thus enabling both local tumor spread and the creation of metastases.

Beginning with basal membrane disruption, the tumor stimulates the remodeling of its own microenvironment in order to enable local spread as well as the creation of distant metastases. The development of those abilities that enable cells to detach from the main tumor, such as cell adhesion, invasion, extracellular matrix degradation, and extravasation with homing of distant tissues and organs, results in the creation of metastases. The acquisition of this phenotype is related to the phenomenon of epithelial to mesenchymal transition (EMT)(94-96).

5. Epithelial to mesenchymal transition in the cancer microenvironment

The integrity of the tissue and its functions are enabled by proper cell-to-cell contact and communication. The importance of cell-to-cell adhesion is illustrated by the phenomenon of anoikis - that is, cell death caused (97) by the loss of interaction with other cells or between the cell and its microenvironment. This phenomenon therefore indicates that cell adhesion impacts cell survival (98-99). In vivo normal cells detached from the microenvironment are not able to home other tissues or to develop into an ectopic growth. Unlike normal cells, however, cancer cells become resistant to anoikis because of the various alterations both in adhesion protein expression and in the interactions between cells. The expression of proteins regulating apoptosis facilitates the survival of detached single cells and so their survival in ectopic localizations (100). The creation of distant metastases is preceded by a disturbance in cell-to-cell adhesion as well as the loss of the integrity of the primary tumor (101-102). The proteins responsible for the intracellular communication are cell adhesion molecules (CAMs). These are glycoprotein receptors that constitute the integral part of the cell membrane

(103) . CAMs condition the interactions between cells, their exchange of information, their control, and their localization in the proper tissue. Among the members of the family of CAMs, the most important are immunoglobulin superfamily proteins, selectins, integrins, adresins (CD34, Gly-CAM-1, MAdCAM-1), cadherins, and CD44 molecules (103). Cadherins are glycoproteins with two domains, transmembrane and extracellular. The extracellular domain has a calcium-binding locus, which is a site for binding with another molecule from the same class (104) to form a mechanical junction of adherence. Cadherins bind to the actine cytoskeleton of another cell through the adhesion complex including catenins (□-, β-, □- and p120-catenin). They also participate in signal transduction (105) and their main role is to create intracellular junctions. E-cadherin is the most important protein for cell polarization and the organization of the epithelium. In various types of malignant epithelial neoplasms, the intracellular adhesion carried by E-cadherin disintegrates as the tumor progresses and correlates with tumor grade and a poor prognosis (106) . Intermediate filaments are the chief components of the cytoskeleton; they seem to play an important biological role given their abundance and because expression changes correlate with alterations in cell behavior. Among the intermediate filaments, four groups of proteins are selected with respect to their structure, including: type I-keratins, type II-cytokreatins, type III-vimentin, and type IV-neurofilaments. Vimentin is a 54 kDa protein connected with cell organelles, elements of cytoskeleton, and membrane adhesion factors which reflect the integrity of vimentin in their cell structure and function. Vimentin is present in various types of cells, including fibroblasts, endothelial cells, macrophages, neutrophils, and lymphocytes. It plays many important biological roles in physiological development and is expressed in adult mesenchymal cells of the central nervous system and muscles (107). Vimentin expression was observed in rat's and monkey's testes in Sertoli's cells where the level decreased with age, leading to weakened vimentin filaments. This in turn resulted in the impairment of the function of these cells and a disturbance in Sertoli cell development (108). Vimentin overexpression accompanies immune-mediated diseases and graft rejection. Vimentin expression levels have also been observed to be significantly increased in the endothelium of patients following the renal graft rejection in comparison to those patients in whom the graft was not performed. Vimentin is constitutively expressed in endothelial cells; it might be overexpressed in response to stress during the procedure of renal graft or following the graft rejection (109). As has been shown, vimentin is related to cholesterol transport during the steroidogenesis (107). It facilitates the development of the placenta and trophoblast invasion and its overexpression has been observed in invasive cells during placental development (107). The accumulation of vimentin in cells is a highly organized and dynamic process. It has been shown that vimentin may not only be apparent in the form of filaments, but also in non-filamental forms, unconnected with the membrane form of aggregates in cell cytoplasm, changing their shapes constantly, connecting and detaching, shortening and becoming longer (107, 110-112). In sum, it must be stressed that an increase in the level of vimentin expression correlates in many studies with an increase in cell migration and invasive properties both in physiological and pathological situations, such as in malignant neoplasms. Additionally, vimentin is a mesenchymal marker of the EMT process (107).

The phenomenon of epithelial to mesenchymal transition is characterized on the molecular level by changes in the expression of epithelial markers and by an increase in proteins related to migration and invasion. In this way, the metastasizing cells share many similarities with the cells undergoing EMT, and it is thought that EMT participates in the progression of cancer. EMT is defined as a situation where a cell loses its stable, polarized, non-migrative properties and takes on fibroblastic, migrational abilities with typical mesenchymal features. Such cells lose their polarization, and alterations in the cell-to-cell and cell to the extracellular matrix adhesion lead to their increased mobility (107).

EMT occurs physiologically in such tissue processes as embryonic development, tissue remodeling, wound healing, and inflammation (113). Various in vitro studies have indicated that both morphologically and on the molecular level, the changes in cancer cells necessary at the early stages to create metastases mimic the physiological EMT process (113). The factors inducing the EMT process are the growth factors secreted by cancer cells and their microenvironment: fibroblast growth factor-2 (FGF-2) and transforming growth factor-β (TGF-β). During EMT, epithelial cells progressively redistribute and decrease the expression of proteins specific for the apex and basis of the cell, such as adhesion molecules, including E-cadherins. The re-expression of mesenchymal proteins, such as vimentin and N-cadherin, has also been observed. These changes lead to the disintegration of intracellular junctions and the acquisition of the cell mobility needed for invasion (114).

The gene for E-cadherin is a tumor-suppressor gene inhibiting the creation of metastases. The inhibition of E-cadherin expression is observed during transcription through transcription-inhibiting factors (Snail1, Snail2 and others) (1115-116). The expression of inhibiting factors is regulated by TGF-β, FGF, EGF, Stat3, and NFκB (117-118). As a result of transcription suppression, factors inducing E-cadherin expression are recognized in various cancer cells under hypermethylation status (119). E-cadherin can also be inhibited at a protein level. The tyrosine kinase receptors, such as epithelial growth factor receptor (EGFR), c-Met, insulin-like growth factor receptor 1 (IGFR-1), and fibroblast growth factor receptor (FGFR) induce the phosphorylation of E-cadherin and catenins, leading to their degradation(120-121). Other factors, such as proteases, MMP-9, TGF-β, and HGF/SF are able to cut E-cadherin and destroy the intracellular contacts that it mediates (122). The most recent studies show that mesenchymal cadherins, especially N-cadherin, increase cancer cell motility and their migration counters E-cadherin action (123). In fact, cancer cell invasion induced by N-cadherin may exceed the pro-adhesive E-cadherin activity. These observations would seem to confirm the existence of a cadherin switch stimulating the change from epithelial into mesenchymal cadherins and encouraging the transformation from non-invasive to invasive tumor phenotype. Moreover, it has been shown that N-cadherin increases the ability of tumor cells to migrate by inducing the transduction of signals through the stimulation of FGF receptor (121-122).

It has been established that EMT may be induced in cancer cell lines in response to such growth factors as HGF, TGF-□, and EGF (123). The transition to the mesenchymal phenotype is related to more aggressive tumor behavior and a higher degree of invasiveness. Squamous-cell carcinoma cells obtained from metastatic cells in lymph nodes in vitro exhibited greater proliferative ability and motility than the primary tongue cancer cells in vivo. It was shown that cancer cells obtained from the metastatic lymph nodes had a higher level of vimentin expression than that observed in the primary cancer cells. Moreover, the vimentin expression level increased under EGF and TGF-□ factors when applied either together or independently. Cancer cells with lower levels of vimentin expression had decreased proliferative potential and were about 70% smaller than the control cells. These findings indicate that it might be possible to reverse the mesenchymal phenotype of cells by blocking vimentin expression, which causes the re-expression of epithelial markers and results in lower tumor aggressiveness (124). Moreover, it was confirmed that active Src (a potential factor inducing the EMT process; while tyrosine kinase is the aim for growth factors), together with decreased E-cadherin and increased vimentin expression levels, participated in inducing EMT transformation (125). In head and neck cancer, a correlation between the invasive type of growth and poorly differentiated cancers was noted (125-126). The EMT phenomenon in this study was determined by the high level of vimentin expression, low level of E-cadherin expression, and high degree of Src tyrosine kinase activity. In our studies, vimentin expression was observed in both head and neck squamous-cell carcinoma and its microenvironment and it characterized the invasive, mesenchymal phenotype. It was furthermore observed that the vimentin expression level increased with tumor size; no correlations, however, were observed between the vimentin expression level and the presence of lymph node metastases or the tumor grade (69-70).

In sum, tumor-associated macrophages and cancer-associated fibroblasts, expressing RCAS1, B7-H4 molecules, and MT participate in creating the suppressive profile of the cancer microenvironment and in the cancer microenvironment remodeling that enables both local tumor spread and the creation of metastases.

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Key Words: Cancer, Neoplasms, Tumor, Microenvironment, Suppressive, Macophage, Fibroblast, Review

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