The assessment of matrix metalloproteinase-9 expression and angiogenesis in colorectal cancer

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Abstract
Colorectal cancer (CRC) is one of the most frequent types of cancer in the world. Between tumor cells and the stroma mutual interconnections are established that favors the tumor development and metastasis. In this respect, the extracellular matrix is remodeled so that it may become totally different from a morphologic perspective than the stroma of the organ in which the tumor develops. Matrix metalloproteinases (MMPs) have an essential role in the remodeling of the tumor stroma. We assessed the expression of MMP-9 on a number of 31 stage III colorectal adenocarcinomas. Generally, MMP-9 had a high but inconstant expression in tumor cells. The highest expression was found in poorly and moderately differentiated carcinomas, with a lower expression in well-differentiated colorectal cancers. Occasionally, MMP-9 expression was identified also in peritumoral macrophages and in stromal cells. Metastasis-free lymph nodes had an intense positive reaction in both macrophages and lymphocytes. The intensely positive reaction was observed for the macrophages and lymphocytes in the tumor necrosis regions. The process of angiogenesis was generally correlated with the intensity of MMP-9 reaction.

Keywords: colorectal cancer, matrix metalloproteinase-9, angiogenesis, extracellular matrix, tumor stroma.

Introduction
Colorectal cancer (CRC) is one of the most frequent types of cancer in the world, with a high incidence and prevalence especially in the developed countries [1]. Based on the statistical data of 2008, CRC is the fourth main cause of mortality of all cancers worldwide after pulmonary, gastric, and hepatic cancer [2].

In the United States (US), in 2010, there have been 102 900 cases of colon cancer and 39 670 cases of rectal cancer, and approximately 51 370 patients died of CRC in the same year, accounting for around 9% of the total cancer deaths [3, 4]. Despite all efforts that have been made for an early diagnosis and for improving the therapeutic tools, CRC remains a big public health challenge even in developed countries [5]. For 2014, the epidemiologic studies predicted that the number of patients in US diagnosed with CRC will drop to 136 830 cases, and the mortality to 50 310 deaths, accounting for 8.3% of all cancer deaths [6]. In Western Europe, CRC is the second most frequent cause of cancer. In 2012, alone 447 000 new cases of colorectal cancer have been registered, and the number of CRC deaths in the same year was of 215 000 cases [2, 7].

The research on colon cancer was mainly concerning the tumor cell, with its histopathology, molecular profile, multiplication and metastasis ability, in order to create new profiles with prognostic value. There is also a sustained effort to define the molecular subtypes based on the genetic alterations that are specific to the different tumor cells, which would allow a predictable prognostic evaluation in the clinical practice [8–10].

During the last few years, in CRC as in most tumors a special attention has been paid to the study of interactions between the tumor cells and the surrounding stroma, which appears to be one of the key aspects in the mechanisms of neoplastic invasion and metastasis [11]. Recent studies underlined the importance of extracellular matrix (ECM) in maintaining the morphology of the tissue and in regulating the cellular behavior [12–14]. Another aspect that needs to be underlined is the fact that both in normal and neoplastic conditions there are regulatory cellular mechanisms that ensure specific dynamics to ECM, characterized by production, degradation and remodeling of ECM [15, 16].

The remodeling of ECM is essentially realized by controlling the expression and the activity of some enzymes, primarily of matrix metalloproteinases (MMPs) at different levels (transcriptional, translational and post-translational) [17]. ECM is composed of a high collection of biochemically distinct components, represented by proteins, glycoproteins, proteoglycans and polysaccharides with various physical and biochemical properties, various collagen molecules (collagen I, II, III, V, and IX) that are involved in maintaining the morphology of the tissue and in neoplastic invasion and metastasis [11]. Recent studies demonstrated that the extracellular matrix components, especially the collagen fibers, play a crucial role in the tumor invasion and metastasis [18, 19].

The neoplastic stromal compartment contains inflammatory cells, mast cells, macrophages, fibroblasts and myofibroblasts that facilitate the tumor growth and metastasis via the secretion of ECM degradation proteases, including MMPs. MMPs form a family of zinc-dependent...
endopeptidases, consisting of more than 21 human proteases. MMP-9 is secreted as a 92-kDa proenzyme, which, after activation, can degrade type IV collagen and denatured collagen molecules. It is why MMP-9 is also known as 92-kDa type IV collagenase/gelatinase. This implies that MMP-9 can degrade the basal membranes, which is often the first step of carcinoma invasion [20–22].

During the last decades of the XXth century, it was concluded that angiogenesis is an essential agent for tumor development and metastasis. This process is favored by the synthesis and release of some essential factors of angiogenesis, especially the vascular endothelial growth factor (VEGF), as well as proteolytic enzymes such as MMPs (particularly MMP-9), by the endothelial cells, stromal cells, the leukocytes invading the tumor, etc. [23].

Because MMP-9 appears to also stimulate the processes of tumor angiogenesis, in our study we aimed to evaluate the expression of MMP-9 and the angiogenesis in colorectal cancer.

Materials and Methods

This study was carried out on 31 stage III colorectal adenocarcinomas, operated throughout 2014 in 1st Surgical Department of the Emergency County Hospital of Craiova, Romania. After the surgical procedure, tumor samples of 2/2 cm have been taken and fixed in 10% buffered formalin solution at room temperature for 72 hours. The biological material was embedded in paraffin according to the protocol used in the surgical pathology departments. The sectioning of the biological samples was preformed using Microm HM350 rotary microtome equipped with section transfer unit on water bath. For the histological study, the classical staining with Hematoxylin–Eosin (HE) and Goldner–Szekely (GS) trichromic was used.

For the immunohistochemistry study, 4 μm thick sections were cut and collected on poly-L-Lysine covered blades. After deparaffinization and hydration, the histological sections were incubated for 30 minutes in a 3% hydrogen peroxide solution, washed in tap water for 5 minutes, and boiled in the microwave in sodium citrate, pH 6, for 21 minutes (seven cycles of 3 minutes each) for antigen retrieval.

After boiling, the sections were allowed to cool down for 15 minutes, then the slides were washed in phosphate-buffered saline solution for 5 minutes followed by the blocking step of non-specific sites in 2% skim milk for 30 minutes. The sections were incubated with the primary antibodies for 18 hours (overnight) in the refrigerator at 4°C. The next day the secondary biotinylated antibody was added for 30 minutes followed by Streptavidin HPR application for 30 minutes. The signal was detected with 3,3′-diaminobenzidine (DAB) (Dako) then the Hematoxylin counterstaining, alcohol dehydration, xylene clarification and the slides were coverslipped in DPX (Fluka). In our study, we used the following antibodies: MMP-9 (A0150, 1:100, Dako), CD34 class II (M7165, 1:50, Dako).

Results

In our study, the classical histological stains allowed us to notice that there are major stromal changes in CRC, with a high variation from one patient to another and from one region to another inside the same tumor. These aspects indicate that on one hand the tumor cells are capable to synthesize and to release a multitude of molecular mediators, which stimulate the stromal cells and create a tumoral stroma that allows the development and the metastasis of tumor cells. The matrix metalloproteinases are involved in the creation of the tumoral stroma by the remodeling of ECM according to the tumor cell development requirements.

Besides the stromal cells, the inflammatory cells are also involved in creating the tumoral stroma, thus constituting the body’s response (highly complex and variable) to “neoplastic aggression”. The histopathological aspects of the tumoral stroma in the CRC cases evaluated in this study varied from a loose type stroma, with numerous fibroblastic and myofibroblastic-type cells (Figure 1) to a dense stroma, rich in fibrillary, desmoplastic-like collagen (Figure 2). The desmoplastic stroma was one of the most frequently encountered types of tumoral stroma in our specimens.

Sometimes, both tumoral and peritumoral stroma were infiltrated with inflammatory cells, particularly with lymphocytes, plasma cells and macrophages (Figure 3). These microscopic aspects make us believe that tumor cells are able to stimulate local fibroblasts and myofibroblasts to synthesize certain components of ECM. Our microscopic findings reveal the complex relationships between tumor cells and surrounding connective stroma.

The tumor growth and metastasis processes require the presence of a vast/ extensive network of blood vessels, involving the creation of new blood vessels through angiogenesis, but also the continuous remodeling of tumoral stroma that would allow the development of such a vascular network. Therefore, by means of immunohistochemistry, we assessed the MMP-9 reaction as well as the process of angiogenesis for both colon adenocarcinoma and lymph node metastases.

In our study, we determined that MMP-9 is synthesized especially by the tumor cells (Figure 4). However, not all tumor cells had a positive immunohistochemical reaction for MMP-9. Moderately and poorly differentiated adenocarcinomas had a more intense reaction for MMP-9 in both primary tumor and lymph node metastases (Figures 5 and 6), while some well differentiated colon adenocarcinomas were negative for MMP-9. It seems interesting that sometimes a number of stromal cells (probably fibroblasts) had a positive reaction for MMP-9 (Figures 7 and 8).

An intensely positive reaction for MMP-9 was observed in some macrophages from the tumoral stroma, especially at the tumor invasion margins (Figure 9). The same intense reaction was observed in the macrophages of the metastases-free lymph nodes (Figures 10–12), but also in the invaded lymph nodes. In the invaded lymph nodes, there were some lymphocytes alongside the macrophages, which were intensely positive for MMP-9 (Figure 13). A highly positive reaction for MMP-9 was observed in the inflammatory cells (probably macrophages and lymphocytes) from the tumor necrosis regions (Figure 14). These microscopic details allow us to consider that some stromal cells, but macrophages as well, and even a number of lymphocytes, are capable of being involved in the altering of tumoral stroma by MMP synthesis.

Concerning the relation between MMP-9 expression and the process of angiogenesis, we observed that in most cases where MMP-9 was well expressed, the process of
angiogenesis was also intense (Figures 15 and 16). However, there were also cases when MMP-9 expression was intense, and the angiogenesis was moderate (Figures 17 and 18). For the invaded lymph nodes, in which MMP-9 expression was moderate or intense, neoangiogenesis was far lower. This aspect could be explained by the reduced vascularization of the lymph nodes, but also because the metastatic tumor cells in their early phases of development (until the tumor reaches a diameter of 1 mm) are feeding by diffusion from the vessels of the invaded organ. Only in a more advanced phase, the tumor develops its own vascular network connected to the original vessels of the invaded organ.

Figure 1 – Well-differentiated colon adenocarcinoma with loose-type stroma, rich in fibroblasts. HE staining, ×100.

Figure 2 – Poorly differentiated colon adenocarcinoma with rich collagen stroma. GS trichromic staining, ×100.

Figure 3 – Peritumoral stroma infiltrated with lymphocytic type cells. GS trichromic staining, ×100.

Figure 4 – Moderately differentiated colon adenocarcinoma with intense MMP-9 reaction of the tumor cells. Immunostaining with anti-MMP-9 antibody, ×200.

Figure 5 – Overview image of a lymph node with tumor metastases and positive MMP-9 reaction. Immunostaining with anti-MMP-9 antibody, ×100.

Figure 6 – Lymph node metastasis with low positive reaction for MMP-9 (detail from the previous image). Immunostaining with anti-MMP-9 antibody, ×200.
Figure 7 – Poorly differentiated colon adenocarcinoma with low reaction for MMP-9. MMP-9 highly positive cells in the stroma. Immunostaining with anti-MMP-9 antibody, ×100.

Figure 8 – Tumor stroma with MMP-9 highly positive cells (detail from the previous image). Immunostaining with anti-MMP-9 antibody, ×400.

Figure 9 – Poorly differentiated colon adenocarcinoma with a weak reaction to MMP-9 in tumor cells but intense reaction in the stromal macrophages. Immunostaining with anti-MMP-9 antibody, ×400.

Figure 10 – Peritumoral lymph node, with no metastases, with positive reaction for MMP-9 in the macrophages of the germinal center. Immunostaining with anti-MMP-9 antibody, ×100.

Figure 11 – Peritumoral lymph node with intense MMP-9 reaction in the macrophages from the interfollicular lymphoid infiltrate. Immunostaining with anti-MMP-9 antibody, ×100.

Figure 12 – Interfollicular macrophages with intense reaction for MMP-9 (detail from the previous image). Immunostaining with anti-MMP-9 antibody, ×400.
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Figure 13 – Peritumoral lymph node with metastases and intensely positive MMP-9 reaction in both macrophages and certain lymphocytes. Immunostaining with anti-MMP-9 antibody, ×200.

Figure 14 – Well-differentiated colon adenocarcinoma with tumor necrosis area in which numerous MMP-9 intensely positive inflammatory cells can be observed. Immunostaining with anti-MMP-9 antibody, ×100.

Figure 15 – Moderately differentiated colon adenocarcinoma with intense MMP-9 reaction. Immunostaining with anti-MMP-9 antibody, ×200.

Figure 16 – Colon adenocarcinoma with intense vascularization (same case as in previous image). Immunostaining with anti-CD34 antibody, ×200.

Figure 17 – Poorly differentiated colon adenocarcinoma with intense MMP-9 reaction in tumor cells and some of the stromal cells. Immunostaining with anti-MMP-9 antibody, ×200.

Figure 18 – Poorly differentiated colon adenocarcinoma with moderate angiogenesis processes. Immunostaining with anti-CD34 antibody, ×200.
Discussion

In our study, we evaluated the MMP9-expressing cells in colorectal cancer and we correlated the expression of this marker to the process of angiogenesis, starting from the finding that in order to develop and metastasize, the tumor cell creates a particular stroma, totally different of that of the host organ. It is well known that tumorigenesis begins by the altering of cellular genetic material. These genetic modifications are altering the functionality of several proteins that are involved in the proliferation, survival, migration and cellular invasion [24]. Following these genetic modifications, the tumor cells are uncontrollably developing and multiplying [25].

During the first stages of tumor development, the neoplastic cells are using the stroma of the host tissue, but afterwards, the tumor development requires important dynamic modifications of the stromal microenvironment [26]. Stromal modifications in cancer are similar to those consecutive to the reaction induced by any aggression and consists in the augmentation of the number of fibroblastic and myofibroblastic cells, ECM growth, structuring of an intrinsic vascular network with a particular structure, production and secretion of growth factors [27]. Following these modifications, the tumor stroma plays an important role in cancer progression, since bidirectional interactions are established between the neoplastic cells and the stroma. It was found that the interactions between the cancerous cells and the surrounding stroma are essential in the mechanism of tumor cell invasion and metastasizing [11].

It must be mentioned that ECM remodeling is an active, continuous process that unfolds during tumor progression. On one hand, ECM serves as a support for the survival and proliferation of cancerous cells, and, on the other hand, this functions as a barrier, which opposes tumor cell dissemination [28].

There is a multitude of enzymes that are involved in the process of tumor stroma remodeling, of which MMPs play an essential role. In addition, they have been widely reported as crucial factors in the migration and invasion of tumor cells [29, 30]. Among MMPs, MMP-9 appears to play a key role in colon cancer progression and metastasis [31, 32].

In our study, the most intense reaction to MMP-9 was observed in the tumor cells, which makes us believe that these cells are the main source of MMP-9 involved in ECM remodeling. The synthesis and excretion of the proteolytic enzymes by the cancerous cells allows them to destroy the matrix barriers surrounding the neoplastic cells and the tumor as a whole, followed by invasion in the adjacent connective tissues. The fact that the intensity of the reaction to MMP-9 of the tumor cells varied widely from one case to another and from one type of tumor to another indicates that the synthesis and excretion of MMP-9 by the neoplastic cells is regulated by multiple factors, some of them still unknown.

A large number of studies have proven that the expression of MMP-9 is elevated in colon adenocarcinoma, compared to normal mucosa [33–37]. By using real-time polymerase chain reaction (RT-PCR) Herszényi et al. (2008) [38] showed that there is a significantly higher level of MMP-9 in colon tumors than in normal colon mucosa. The presence of elevated levels of MMP in colon cancer cells explains their ability to degrade all the components of ECM and to allow tumor invasion. The presence of a high level of MMP-9 allows the neoplastic cells to degrade the type IV collagen of the basement membranes, including the basement membrane of blood vessels, facilitating the metastasizing process.

In some cases of this study, we noticed of colon adenocarcinoma fibroblastic stromal cells and peritumoral macrophages with positive reaction to MMP-9. We believe that they too are involved in degrading and remodeling the stromal matrix thus facilitating tumor growth. According to some authors [38, 39] the cellular stromal compartment, which comprises the inflammatory cells and fibroblasts, is facilitating the invasion and metastasis trough the secretion of extracellular proteases, including MMP. Other authors have also found much higher levels of MMP-9 in tumor tissue compared to normal tissue in a variety of cancers, including colon cancer [38]. Several studies have found much higher plasmatic levels of MMP-9 in patients with colorectal cancer compared to healthy subjects [40, 41]. These data confirm that high levels of MMP are synthesized and secreted in colorectal cancer.

The intense positive reaction to MMP-9 of the macrophages in the metastases-free peritumoral lymph nodes raises the question whether this is a physiological reaction or these macrophages are preparing the stroma for a potential tumor metastasis. The fact that the reaction to MMP-9 was highly positive in the invaded lymph nodes and in the areas of tumor necrosis makes us believe that other cells can synthesize and secrete MMP, and facilitates the structural altering of the stroma. We believe that the stimulation of the inflammatory cells (macrophages, lymphocytes, granulocytes) and local fibroblasts to synthesize MMP-9 is realized by “soluble messengers” still unknown.

The altering of ECM together with the synthesis of other components leads to profound architectural modifications, as indicated by us, to the emergence of a desmoplastic stroma. According to some studies [33], more than 50% of the colorectal cancers have an extended desmoplastic stroma due to a stromal cell and, primarily, a fibroblast and macrophage stimulation by the tumor cells.

The importance of emphasizing the MMP-9 activity seems to have a prognostic value as well. Several studies have confirmed that MMP-9 expression can be used as a prognostic marker, in the sense that patients with high levels of MMP-9 have a poorer prognosis, a reduced survival rate and a higher recurrence risk [42, 43]. Likewise, several immunohistochemistry studies have demonstrated that a higher MMP-9 expression is correlated with the presence of a deep, infiltrating tumor, lymph node invasion or distant metastases [44, 45].

In this study, the expression of MMP-9 mostly correlated with the process of angiogenesis, meaning that the cancers with a high MMP-9 expression had a large number of angiogenic blood vessels. However, we noticed a high variability of angiogenic blood vessels from one type of tumor to another and from one area to another inside the same type of tumor, in the sense that well-differentiated adenocarcinomas had areas with poor vascularization or, conversely, with intense vascularization even if MMP-9 expression was constant. This immunohistochemical aspect
indicates that the angiogenesis is a complex process, coordinated by multiple cellular and humoral factors.

Overall, angiogenesis is influenced by numerous factors, some of them acting as proangiogenics, and others as antiangiogenics. It seems that in carcinogenesis the balance between these two groups of factors is lost, with the proangiogenic factors prevailing [46]. It seems that in colorectal cancer the neovascularization is induced by hypoxia, which stimulates the production of (proangiogenic) factors [47].

The relation between MMP-9 and the process of angiogenesis is still far from being entirely known. Recent studies have shown that there are specific molecular pathways, which regulate the complex links between VEGF and MMP for the process of angiogenesis. It seems that MMP-9 is an essential component in these interactions, as the activation of MMP-9 leads to an overexpression of VEGF-A [23]. Other studies conducted on MMP-9-free genetic mice have indicated a breakdown in the tumor vascularization by reducing the recruitment of pericytes and the substantial inhibition of tumor metastatic process [48, 49].

All these data led to the introduction of MMP-9 as a therapeutic target in cancer.

Conclusions

In colorectal cancer, MMP-9 generally had an increased but inconstant expression in cancer cells. The strongest expression was found in moderately and poorly differentiated cancers and a lower expression in well differentiated colorectal cancers. Occasionally, MMP-9 expression was identified in peritumoral macrophages and stromal cells. The lymph nodes without metastasis showed an intensely positive reaction for MMP-9 in macrophages, while metastatic lymph nodes showed intense reaction both in macrophages and lymphocytes. Intense positive reaction was observed in macrophages and lymphocytes in the regions of tumor necrosis. Overall, the processes of angiogenesis were correlated with the intensity of the reaction to the MMP-9.

Conflict of interests

The authors declare that they have no conflict of interests.

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