Potential novel markers in IBD and CRC diagnostics. Are MMP-19 and RAGE promising candidates?

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Aims. Inflammatory bowel diseases and colorectal cancer are serious intestinal disorders with continuously increasing incidence. Many aspects of etiopathogenesis still remain unclear. There is an urgent need to improve early diagnostics and markers indicating the progression of the disease. The aim of our study was to analyze the expression of matrix metalloproteinase-19 (MMP-19), and the receptor for advanced glycation end-products (RAGE) in different cell subpopulations in inflammatory bowel diseases (IBD) and colorectal cancer (CRC) compared to the tissue in the vicinity of pathological processes.

Methods. Expression of both markers in epithelium, macrophages and vessels were evaluated in IBD and CRC groups. They were detected using immunohistochemistry in paraffin sections.

Results. There were significant differences between the expression of MMP-19 on macrophages and vessels among healthy and cancer tissues. In both, macrophages and vessels were significantly lower levels in cancer tissues. The expression of MMP-19 on vessels was also significantly different between peritumoral and cancer tissues (higher levels in peritumoral tissue). RAGE expression in macrophages was significantly different between healthy and cancer tissues and between peritumoral and cancer tissues. There was significantly lower expression in cancer tissues than in healthy and peritumoral tissues. Expression of RAGE in vessels was significantly different just in the comparison of healthy and peritumoral tissues (higher levels in healthy tissues).

Conclusion. Both markers seem to be promising potential auxiliary markers in IBD and CRC diagnostics. They can also improve evaluation of disease progression.

Key words: inflammatory bowel disease, colorectal cancer, matrix metalloproteinase, receptor of advanced glycation end-products

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INTRODUCTION

Inflammatory bowel diseases (IBD) and colorectal cancer (CRC) are clinical units with continuously increasing trends which are thought to be associated with western lifestyle. Despite several theses concerning this topic being published, many aspects of etiopathology still remain unclear. There is also a scope to improve early diagnostics and markers indicating progression of disease. Based on our previous research we selected two promising markers, matrix metalloproteinase-19 (MMP-19) and the receptor for advanced glycation end-products (RAGE) (ref.[1]).

Matrix metalloproteinases (MMPs) are known to play an important role in cell migration during tissue remodeling and embryonic development, and in some pathological conditions such as inflammation (arthritis) and tumor metastasis, require cell invasion and degradation of extracellular matrix (ECM) and basement membranes. MMPs secreted by connective-tissue cells are considered effectors of these degradative processes[2]. Generally, proteolysis of extracellular matrix (ECM) is supposed to be a key factor of the inflammatory response, modulating the cell-cell and cell-ECM interactions underlying both disease and repair processes[3]. Therefore, the role of MMPs in ECM turnover is one of the current topics supposed to identify MMPs as possible therapeutic targets in inflammatory diseases.

MMP19 belongs to the matrixin family of zinc dependent endopeptidases. It is widely present in various human tissues and plays a role in tissue remodeling, wound healing and epithelial cell migration[4]. MMP19 is known as a basement membrane-degrading enzyme, but it also digests some other ECM molecules[5]. Suppression of MMP7 and MMP19 by triptolide showed to inhibit the migration and invasion of ovarian cancer cells in vitro[6]. It was already presented that MMP19-/- mice developed diet induced obesity and reduced susceptibility to skin cancer[7].

MMP19 may possess an anti-angiogenic effect and earlier onset of tumoral angiogenesis in MMP19-/- mice was described[8]. MMP19 mediated anti-angiogenic effect in endothelial cells (EC) via generation of angiostatin-like fragments was also detected in vitro[9]. Chan et al.[10] observed association of MMP19 anti-angiogenic effect with its catalytic activity through the inhibition of VEGF production in nasopharyngeal carcinoma both in vitro and in vivo.

Angiogenesis is one of the important factors in IBD pathogenesis[11]. Angiogenesis is a complex process of formation of new capillaries. The de novo organization of ECs into new vessels in the absence of pre-existing vascular system occurs only in early embryogenesis[12]. The second type of angiogenesis is interesting for IBD research. It represents continued expansion of the vascular tree because of ECs sprouting from existing vessels in avascular regions of the embryo and is repeated many times in mature organism most commonly after injury during wound healing and during tumor metastasis. The patterning of the vascular tree is a complex process and a cascade of many factors determining the site of sprouting and the route of migrating ECs during angiogenic are essential[12,13].

Inflammation including IBD is considered to promote angiogenesis in several ways. Under inflammatory conditions, tissues are typically hypoxic, and hypoxia can induce angiogenesis through up-regulation of vascular endothelial growth factor (VEGF), fibroblast growth factor-1 (FGF-1), tumor necrosis factor alpha (TNF-α), hypoxia-inducible factor-1 (HIF-1), and some other factors. Extravasated plasma fibrinogen also stimulates angiogenesis. Immune cells, such as macrophages, lymphocytes, mast cells or fibroblasts and some other cells produce pro-angiogenic factors and therefore support vessel growth. Finally, increased blood flow itself can stimulate angiogenesis through shear stress on the endothelium[14].

The receptor of advanced glycation end-products (RAGE) is a transmembrane multiligand binding protein that belongs to the immunoglobulin protein family[15]. In non-pathological conditions advanced glycation end-products are normally produced in the body and accumulate by age. Their enhanced formation, accumulation, and presence of their receptor (RAGE) is reported to be associated with natural ageing as well as chronic inflammation[16]. RAGE pathway is also involved in the pathologies of different cancers[15].

RAGE is expressed at a very low level in various cell types in non-pathological conditions. Increased expression is detected in a range of cell types and tissues in pathological conditions (diabetes, neurodegenerative disorders, and autoimmune/inflammatory conditions) (ref.[17]). RAGE is functionally designed as a scavenger receptor playing a role in immune reactions at sites of tissue destruction. RAGE is a type I transmembrane protein. It consists of three extracellular immunoglobulin-like domains (V, C1, and C2), a single transmembrane domain and a short cytoplasmic tail (it is mentioned to be important in signal transduction). RAGE can bind molecules HMGB1, S100b, calgranulins and others. Activation of RAGE induces several intracellular intermediaries including NF-kB, MAPKs, PI3K/Akt, Rho GTPases, Jak/STAT, and Src family kinases[18].

Except membrane-bound full-length RAGE (flRAGE) there are also soluble forms (sRAGE) in circulation. Two main mechanisms of producing sRAGE were identified. First of them represents alternative splicing to remove the transmembrane region. Endogenous secretory RAGE (esRAGE) are produced by this mechanism. The second mechanism is the proteolytical cleavage from the cell membrane by which cleaved (cRAGE) extracellular forms are produced. sRAGE have the same binding specificity as flRAGEhey may operate as a "decoy". They can bind pro-inflammatory ligands preventing them from reaching and activating the membrane RAGE (ref.[19]).

The aim of our study was to analyze the expression of MMP-19 and RAGE on different cell subpopulations in two biologically important intestinal lesions – IBD and colorectal cancer – compared to the tissue in the vicinity of pathological processes.

MATERIAL AND METHODS

Cohort of patients and harvested material

The presented study was conducted in University Hospital Bulovka in Prague. It had been approved by the Ethics Committee of the hospital before it commenced. Patients signed an informed consent form before the surgical procedure.

The experimental cohort contains 20 samples from patients with Crohn's disease (15 fibrostenosing type, 5 fistulous (penetrating) type), 9 samples from patients suffering from ulcerative colitis and 58 samples from patients with colorectal cancer. 33 samples from heathy areas of intestine were included for comparison as controls samples. They were obtained from patients with non-malignant, non-inflammatory disease (diverticulitis/diverticulosis in steady state of disease, intestinal ischemia).

An additional part of our experiments was focused on comparing CRC group. Tissues from different areas (healthy, peritumoral, tumoral) were compared.

Immunohistochemistry

Formalin fixed tissue samples were embedded in paraffin 2 μm sections were routinely stained in hematoxylin and eosin (HE) for initial morphological evaluation. A selected area of the sample containing both, pathological process and non-affected tissue were used for further analysis. The following primary antibodies were used for immunohistochemistry: Human/Mouse/Rat RAGE Antibody (polyclonal goat IgG, R&D Systems, 1:100) (R&D Systems, Minneapolis, USA), Human MMP-19 Antibody (polyclonal goat IgG, R&D Systems, 1:100) (R&D Systems, Minneapolis, USA).

After deparaffination and rehydration EnVision™ FLEX Target Retrieval Solution, High pH (Dako, Glostrup, Denmark) was applied to retrieve antigen. The endogenous peroxidase activity was quenched using EnVision™ FLEX Peroxidase-Blocking Reagent (Dako, Glostrup, Denmark).

Unspecific binding was blocked using 2% (w/v) BSA solution for 20 min at RT. Primary antibodies were applied for 1h at RT. The SuperVision I Single Species HRP-Polymer Goat (DCS Innovative Dignostik-systeme, Hamburg, Germany) was applied for detection, followed by hematoxyllin-eosin counterstaining. All slides were mounted in Aquatex™ (Merck-Millipore, Darmstadt, Germany).

The expression of selected markers was evaluated in epithelium, on macrophages and vessels using modified scoring according to Remmele[20] by two histopathologists. The epithelial average value of staining for both markers was calculated by counting at least 100 epithelial cells multiplied by their staining level on two random sites of each slide. Not less than 20 macrophages were observed to set an average value of staining. The level of staining of vessels, ranging from larger calibers to capillaries, was evaluated and average value was set (Fig. 1, 2).

Statistical analysis was conducted using a SPSS software. Mann-Whitney Test was applied. Differences were considered significant at $P<0.05$.

RESULTS

The first phase of the experiment presented no expression of epithelial MMP-19 in the IBD group in both small intestine and colon samples compared to the control group (healthy or just mild inflammatory tissues), where positivity was detected in 60% of colon samples and 62.5% of small intestine samples.

The second phase of our research focused predominantly on the CRC group. First, we compared the CRC group with controls. Expression of MMP-19 in epithelium was significantly lower in the CRC group than in the control groups. On the contrary, there was a significantly higher expression of MMP-19 in macrophages compared to the control samples (Fig. 3, Tables 1–3). No significant changes were observed in vessels (Tables 1–3).

Expression of RAGE in epithelium was also significantly lower in CRC groups than controls. There were no significant changes in expression of RAGE in macrophages and vessels between CRC and control groups (Tables 1–3).

In the following experiments, we also compared results within the CRC group. This means we compared tissues according to the site of harvesting (healthy tissue, peritumoral tissue, cancer tissue). There were significant differences between the expression of MMP-19 in macrophages and vessels between healthy and cancer tissues. Significantly lower levels were detected in macrophages and vessels in cancer tissues. The expression of MMP-19 in vessels was also significantly different between peritumoral and cancer tissues (higher levels in peritumoral tissue).

RAGE expression in macrophages was significantly different between healthy and cancer tissues and between peritumoral and cancer tissues. There was a significantly

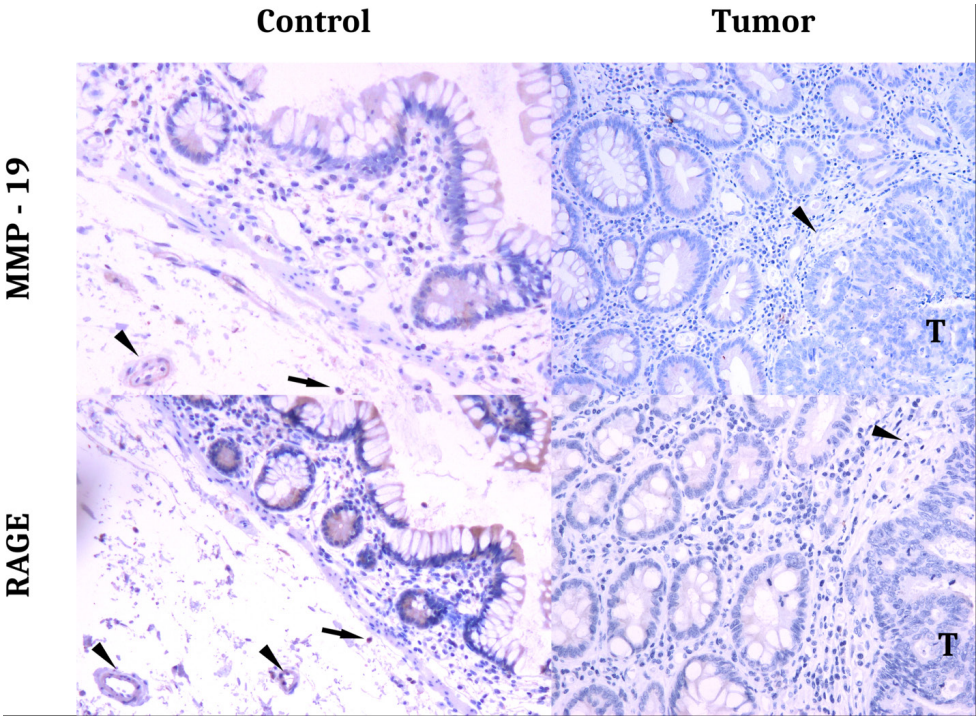

Fig. 1. Differences in MMP-19 and RAGE expression in healthy tissues in control group with tissues in proximity of tumor (letter T) in tumor group. Mild to moderate intensity of staining in epithelia, macrophages (arrows) and vessels (arrowheads) in control group compared to decreased intensity of staining in epithelia proximal to tumor as well as complete loss of staining in macrophages and blood vessels. Original magnification 200x.

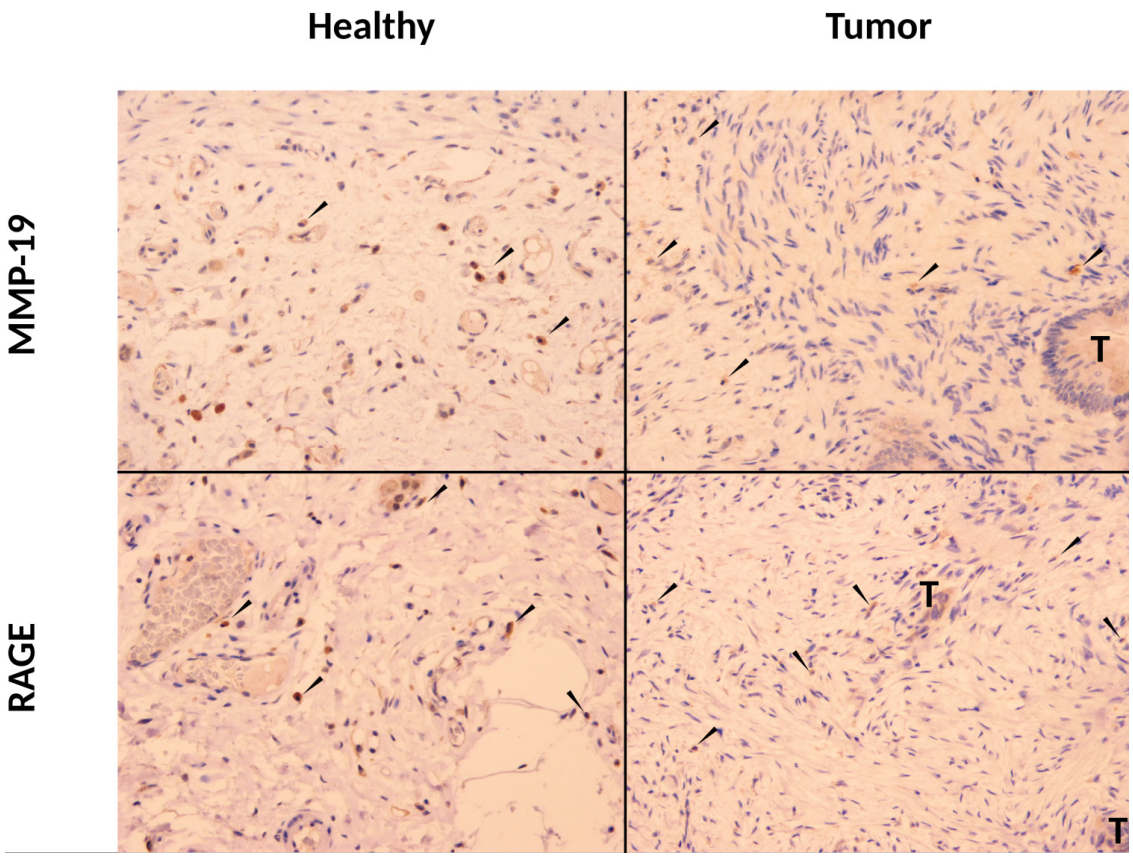

Fig. 2. Microphotographs represent MMP-19 and RAGE expression of macrophages in healthy tissue and tumor. Macrophages (arrowheads) exhibit diffuse strong reaction (2+ to 3+) in healthy tissue compared to overall weak granulate expression in tumor's proximity.

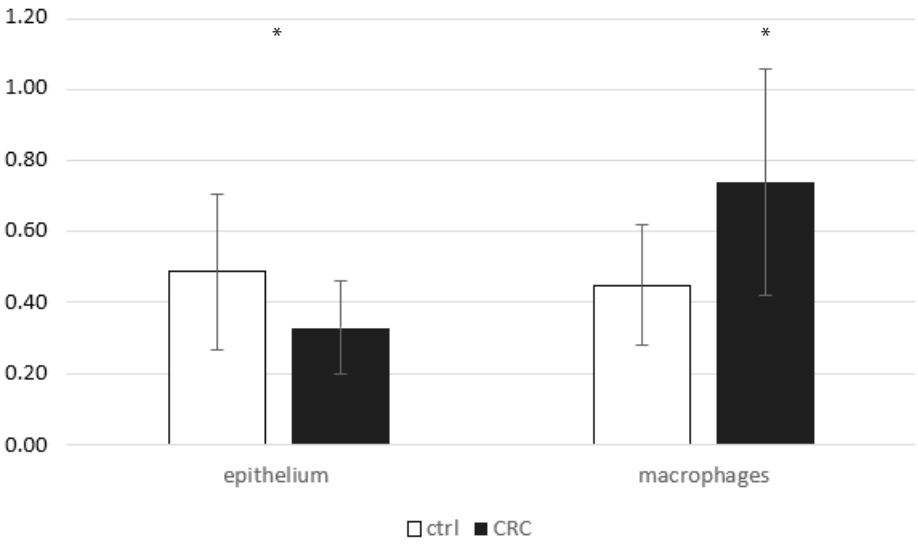

Fig. 3. Expression of MMP-19 in epithelium and macrophages. Compared CRC group to controls; 58 samples of CRC and 33 control samples were included; * P<0.05 (Mann-Whitney Test).

lower expression in cancer tissues than in healthy and peritumoral tissues. The expression of RAGE on vessels was significantly different just in the comparison of healthy and peritumoral tissues (higher levels in healthy tissues). However, this result could be a bit skewed because the overall expression of RAGE in vessels was simply rare (Tables 1–3).

DISCUSSION

We observed a significantly lower expression of MMP-19 in IBD group in comparison to non-inflamed tissue. This result has a very promising importance for clinical practice since positivity of MMP-19 could be a good tool for exclusion of IBD diagnosis. However, more investiga-

Table 1. Percentage of positive cells in samples.

	Controls	Healthy	Cancer samples peritumoral	Cancer
MMP-19 epithelium	100.00	84.62	84.21	80.00
MMP-19 macrophages	51.52	85.71	69.57	61.90
MMP-19 vessels	30.30	59.18	52.17	19.05
RAGE epithelium	100.00	94.87	86.84	85.71
RAGE macrophages	60.61	77.55	78.26	47.62
RAGE vessels	0.00	14.29	2.17	7.14

Table 2. Expression of MMP-19 and RAGE. Compared CRC group to controls.

	Epithelium	Macrophages	Vessels
MMP-19	0.025*	0.050*	0.342
RAGE	0.051*	0.846	1.000

*P<0.05.

Table 3. Expression of MMP-19 and RAGE within the CRC group.

MMP-19	Epithelium	Macrophages	Vessels
Healthy vs. cancer	0.648	0.017*	<0.001**
Healthy vs. peritumoral	0.297	0.386	0.251
Peritumoral vs. cancer	0.799	0.166	0.001**
RAGE	Epithelium	Macrophages	Vessels
Healthy vs. cancer	0.828	<0.001**	0.113
Healthy vs. peritumoral	0.356	0.146	0.014*
Peritumoral vs. cancer	0.482	0.008**	0.307

*P<0.05, ** P<0.01.

tion should be provided to confirm this important observation.

In CRC group we detected a higher expression of MMP-19 in epithelium and a lower expression in macrophages compared to the controls. Based on these results we hypothesized that the ratio of positivity of MMP-19 in epithelium and macrophages could serve as an auxiliary diagnostic and/or prognostic marker. However, more experiments to confirm this hypothesis are needed.

We revealed a significantly lower expression of RAGE in epithelium in the CRC group in comparison to the controls. Therefore, this marker also seems to be promising in CRC diagnostics.

Comparison within the CRC group revealed differences in expression of MMP-19 in macrophages and vessels between healthy and cancer tissues. Lower levels of MMP-19 in vessels in cancer tissues probably corresponds with the previously stated presumed anti-angiogenic effect of MMP-19 (ref.[8]).

RAGE expression in macrophages was significantly lower in cancer tissues than in healthy and peritumoral tissues. This result could indicate that RAGE expression in macrophages decreases with progression of the disease. It suggests that RAGE in macrophages could potentially serve as an auxiliary marker of CRC progression.

Current results are partially different in comparison to our previous research[1]. Resented study included more probands and its main focus was on cancer patients compared to previous study that focused predominantly on IBD. There were reverse ratios of samples. Now more CRC samples were included whereas previous study included more IBD samples. Another possible reason is that in previous research we used antibodies of our own production. We chose commonly available antibodies in current study as our aim was also to evaluate whether detection of mentioned markers could be potentially used in routine practice. Also, there were slightly different experimental designs as currently we focused also on comparison within the sample not just among groups. It meant we compared also healthy area, peritumoral and tumoral tissue in the same sample.

We formed a hypothesis that measurement of sRAGE could be beneficial for evaluation of disease progression. Especially the ratio of sRAGE and expression of RAGE in tissues could also have a predictive value in that respect[19]. Thus, research in this field would be desirable.

CONCLUSION

Based on the presented results we can conclude that both, MMP-19 and RAGE seem to be promising auxiliary markers in IBD and CRC diagnostics. Our results also indicate that those markers could be beneficial in the evaluation of the disease progression. However, further studies are needed to confirm our hypotheses.

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