Histamine metabolism and histamine receptor expression pattern in human colorectal cancer

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INTRODUCTION

Histamine is one of the most important biogenic amines. In addition to the three well-known pharmacological functions such as the contraction of smooth muscles, the increase in vascular permeability, and the stimulation of gastric acid secretion, histamine has been known to play various roles in neurotransmission, immunomodulation and the regulation of cell proliferation. Development of molecular biology elucidated the enzymes involved in histamine metabolism including the enzyme histidine decarboxylase (HDC), and the histamine inactivating enzyme diamine oxidase (DAO). Different biological effects of histamine are mediated through the activation of specific histamine membrane receptor types (H1R, H2R, H3R and H4R). Histamine receptors are expressed in many malignant cell types and they can activate distinct signalling pathways. Nevertheless, the effect of histamine depends on cell type, the presence of different receptor subtypes and the activated signalling pathways. H1R is mainly expressed in the brain, endothelial cells, and smooth muscle cells, and is thought to play an important role in allergy. The main effect of H2R is the regulation of gastric acid secretion. Moreover, there are data supporting the role of both H1R and H2R in melanoma cell proliferation. The H3R is located mainly in the central nervous system as a presynaptic autoreceptor. Finally, the H4R is predominantly expressed in leukocytes, but, unlike to other histamine receptors, its functional relevance is not well understood yet.

The first reported evidence on the relevance of histamine in tumour development and progression came when the increase in histamine content, and HDC activity has been observed.Therefore, HDC can serve as a specific marker for the biosynthesis of histamine. High concentrations of histamine in tumours compared to the content of normal surrounding tissues has been demonstrated in diverse human malignancies such as melanoma, breast cancer, colon carcinoma, lymphomas, and leukaemia. In experimental tumour models the role of histamine has been established more accurately, furthermore histamine was demonstrated to be an autocrine growth factor. In vitro studies employing cell lines have demonstrated the presence of histamine receptors and the pathways through which histamine may contribute to the modulation of cell proliferation. Direct effect of histamine on tumour cells was found to act primarily

through autocrine loop, although paracrine effects of histamine released by tumor cells influencing immune responses has been proved to be significant as well.

The role of histamine in colorectal carcinogenesis has been reported. Histamine has been found to stimulate growth of colorectal cancer in vitro and in vivo. It was also shown that histamine stimulates growth of colon tumours by suppressing anti-tumour immunity. Significantly higher histamine content was measured in tumour tissue than in adjacent normal colonic mucosa. Elevation of histamine concentration in colorectal tumours is attributable to an increase in HDC and a decrease in DAO activity. Studies demonstrated that concentration of histamine in colon cancer is high enough to induce immunosuppression locally.

Colorectal cancer is a very serious public health problem. Polyps of the colon, especially tubular and villous adenomas may undergo malignant transformation. When localized to the bowel, colorectal cancer is highly treatable and often curable. Recurrence following radical surgery is a major problem, up to 50% of patients operated with colorectal cancer will develop locally recurrent or distant metastatic disease.

Based on experimental data, histamine has a dual activity both in melanoma and colon tumours. In these malignancies histamine acts as a growth factor, and also impairs the local immune response through Th2 polarization. From the clinical point of view, invasion and spreading of a malignant tumour are even more relevant processes than tumour growth itself. Several reports support the direct role of histamine in carcinogenesis and tumour progression, mainly via H1R and H2R. Histamine also influences the activity of cytotoxic T lymphocytes and NK cells, and modifies the cytokine production of other types of immune cells as well. Recently, Sander et al. (2006) have reported the histamine receptor distribution in human gastrointestinal tract. However, only limited data are available regarding the histamine receptor expression in colorectal tumours. Results of clinical trials have been showed that administration of H2R antagonists (cimetidine or ranitidine) improves survival of patients with colorectal cancer. We intended to study the histamine metabolism and to analyse the histamine receptor expression pattern in colon tumours and benign polyps. We examined human colon tumour samples, adenomas and carcinomas and we compared them to the normal colonic mucosa.

AIMS

The evaluation of the role of histamine in colorectal cancer by studying the histamine metabolism, and distribution of histamine receptors.

Comparative analysis of histamine metabolism in colorectal adenocarcinoma, adenoma and normal mucosa samples. The analysis of the histamine content and presence of the key enzymes of histamine metabolism (HDC and DAO) in benign and malignant tumours of the colon, in comparison with normal adjacent mucosa.

Analysis of histamine receptor expression (H1R-H4R) in human colorectal cancer, adenoma and normal mucosa by quantitative reverse transcription-polymerase chain reaction, Western blot analysis and immunostaining.

Analysis of histamine receptor expression pattern in Dukes' classified and selected tumour samples.

Indirect immunhistochemical analysis of CD8+ T-lymphocytes in colorectal adenoma and adenocarcinoma samples.

MATERIAL AND METHODS

HDC indirect immunostaining in 40 colorectal carcinoma and 20 adenoma samples.

HDC Western blot analysis in 20 colorectal cancer and 10 adenoma samples compared to the normal mucosa.

DAO activity assessed in 20 colorectal cancer and 10 adenoma samples in comparison with the normal adjacent mucosa.

Histamine concentration measured by HPLC method in 20 tissue samples obtained from adenocarcinoma and 20 adenoma compared to the normal mucosa.

RT-PCR analysis of H1- and H2-receptors in 20 adenocarcinoma samples.

Western blot analysis of histamine receptor expression in 20 adenocarcinoma and 18 adenoma samples in comparison with the normal surrounding tissue.

Real time RT-PCR and Western blot analysis of histamine receptors in 40 Dukes' classified and selected colorectal cancer samples compared to the adjacent normal mucosa.

Histamine receptor (H1R, H2R, and H4R) indirect immunostaining performed in 12 adenocarcinoma samples and compared with the normal mucosa.

Evaluation of CD8+ T lymphocyte using indirect immunostaining in 12 carcinoma and 12 adenoma tissue samples.

Statistical methods

Real time PCR histamine receptor values of normal and tumor tissue samples were compared using *two-way repeated measurement ANOVA method* for statistical analysis. The same statistical method was used for the densitometrical analysis of Western blots results.

Western blot analysis of HDC protein expression in carcinoma, adenoma and normal tissue samples: two-way repeated measurement ANOVA for statistical analysis. The same method was used for the densitometrical analysis of Western blot results.

DAO enzym activity in adenocarcinoma, adenoma and normal samples: one-way ANOVA determination for statistical analysis followed by post hoc Tukey test.

Histamine content determination: Kruskal- Wallis test.

Histamine receptor expression determination (real time RT-PCR and densitometrical analysis): two-way repeated ANOVA determination for statistical analysis.

RESULTS

High histamine content has been found in experimental and human tumours. We intended to analyse the histamine metabolism in malignant and benign colorectal tumours. Our aims were to compare histamine metabolism in normal mucosa, adenoma and adenocarcinoma and its possible role in tumour promotion and progression. At the beginning of our experiments we measured histamine concentrations in these tissue samples by HPLC method. There were available literary there were available data regarding histamine content in colorectal cancer, but there were not any data on histamine levels in benign polyps of the colon. Some authors have published high histamine concentrations in human colorectal cancer, but the histamine content was not measured in adenoma. We have measured elevated histamine concentrations in examined malignant tissues, but we could not demonstrate any change in histamine concentrations in adenoma compared to the normal adjacent colonic mucosa.

For further analysis of histamine metabolism in adenocarcinoma and adenoma samples of the colon we examined the presence of HDC enzyme and DAO activity in these specimens. We analyzed expression of HDC by immunostaining method using paraffin embedded human malignant and benign colon tissue samples. In situ expression of HDC has not yet been studied due to the lack of specific anti-HDC antibody. The production of first anti-HDC antibody was developed in collaboration between our institute and Promega company. Expression of HDC was observed both in carcinoma and adenoma tissues. It was found that 90% of the adenocarcinoma samples were HDC positive, most of the tumours have shown high immunoreactivity. Like in other solid tumours, HDC staining was found in different cell types of the connective tissue, which are thought to be enterochromaffin-like- (ECL) and tumour infiltrating cells as well. Moreover, in most of the samples, the tumour cells themselves were also positive. In addition, in the stroma, the tumour infiltrating inflammatory cells (e.g. lymphocytes and mast cells) showed staining for HDC enzyme. Surprisingly, we observed high HDC immunoreactivity in all adenoma samples. Not only the epithelial and ECL cells, but also the infiltrating inflammatory cells occuring in high number in the stroma, were HDC positive. We would like to emphasize the importance of our results, that tumour cells themselves showed strong staining for HDC. These results

strongly support the hypothesis that colon cancer cells are capable of producing histamine.

As we mentioned, HDC positive cells in the stroma represented probably populations of tumour infiltrating cells, like lymphocytes and mast cells. Tumour infiltrating lymphocytes are known to play an essential role in the antitumoral cellular immune response. CD4+ T cells play a central role in orchestrating the immune response to cancer, their main role is to prime CD8+ cells with more potent cytotoxic abilities. Based on literature data we assessed the infiltration of CD4+ and CD8+ cells by immunohistochemistry. Both adenoma and adenocarcinoma samples were examined. The proportion of tumour-infiltrating T cells was high in adenoma, while in tumour specimens we could observe only negligible proportion of positive stained cells.

We continued our experiments with HDC protein analysis in carcinoma and adenoma tissues using Western blot method. We could detect the 53 kDa HDC protein isoforms. Malignant melanoma cell lines (M1/15) served as positive controlls because in these cell lines we measured high concentrations of HDC protein by Western blot method previously. We performed Western blot densitometry analysis as well. The HDC protein blot results of matched colon carcinoma and adenoma revealed the increased expression of HDC compared to the normal colonic mucosa. Previously other authors had shown high levels of HDC protein in colon carcinoma, but we were the first who demonsrated an elevated HDC protein activity in benign polyps of the colon.

In the gastrointestinal tract histamine inactivation occurs by direct oxidative deamination and the reaction is catalyzed by diamine-oxidase (DAO). As DAO activity has an important role in the metabolism of the histamine, we measured DAO enzyme activity in the investigated human colonic tissue specimens too. We found significantly decreased DAO activity in adenocarcinoma tissue in comparison with the normal colonic mucosa ($p<0,01$). These results were in concordance with data published on these issue by others. We concluded that elevated HDC activity and/or decreased DAO activity are responsible for high histamine concentrations in colon tumours. DAO activity of benign tumours has not been investigated by others yet. According to our results, in adenoma DAO activity is not increased despite the increased amount of HDC enzyme.

We have identified histamine receptor subtype expression in colon carcinoma, adenoma and normal mucosa. Several reports support the direct role of histamine in carcinogenesis and tumour progression, mainly via H1R and H2R. However, only limited data are available regarding the histamine receptor expression in colorectal tumours.

We examined protein expression of histamine receptors in intestinal tissue homogenates of biopsy samples, we used normal colonic mucosa as positive control. Previous analysis of human cell lines revealed that H1R, H2R, and H4R were expressed in the basophil KU-812F and the colon cancer HT29, HCT116 cell lines. In our experiments KU-812F basophil cell line was used as positive control for histamine receptors. H1R and H2R antibodies detected a protein band of approximately 50 kDa, while a specific band for H4R migrated at 75 kDa. Analyzing the human colon carcinoma cell lines, we found specific bands for histamine receptors at the expected weight of 50 kDa and 75 kDa. No bands were visible on the blots when peptide pre-absorbed primary antibodies were used as controls. The same results were obtained with H1R and H4R control peptide pre-absorbed primary antibodies.

We identified H1R, H2R and H4R subtype-specific bands by Western blot analysis of matched adenoma or adenocarcinoma and adjacent normal mucosa samples. H3R could be detected only in a few samples. At the same time Sander et al. (2006) reported the distribution of the H1R, H2R, and H4R receptors in the human gastrointestinal tract. They found an altered expression of these receptors in patients with inflammatory bowel disease and food allergies. They also concluded that H3R was not expressed in human intestinal tissue. Our results had been confirmed by receptor expression described by Sanders et al. that there is no H3R expression int he normal gastointestinalis mucosa. Cianchi et al. (2005) have reported the expression of H1R, H2R, and H4R mRNA in both colorectal cancer specimens and in adjacent normal colonic mucosa. However, the authors neither examined the distribution of these receptors, nor did they compare the receptor expression with respect to the normal mucosa.

Our presumed hypothesis regarding changed histamine metabolism in adenoma which could be a transition to the dramatic alterions in carcinoma histamine's metabolism, was not verified. We could not find neither changed HDC and DAO

activity, nor elevation of histamine levels in adenoma compared to the normal mucosa. According to these results, in turn we focused on the investigation of cancer tissues. Based on Western blot results we performed real-time RT- PCR, Western blot and immunohistochemical analysis of Dukes' classified and selected, matched normal mucosa and adenocarcinoma samples.

Histamine receptor mRNA expression analysis showed the same results, we could identify 3 receptors, both in carcinoma and normal colonic mucosa tissue, H1R, H2R, and H4R. We found that in tumours H1R and H4R mRNA expression levels were significatly reduced ($p<0.001$), while no significant change was observed in H2R mRNA expression level. Lack of H3R mRNA expression was characteristic for most of the samples, and only three of the 38 samples (7.9%) expressed H3R. In Western blot analysis of carcinoma and normal mucosa samples were probed with H1R, H2R, and H4R antibodies. Both adenocarcinoma and normal colonic mucosa showed a specific band that migrated at the expected molecular weight. We have not found significant difference in histamine receptor expression according to Dukes' stages of tumour groups. Each group had a similar receptor expression pattern.

Western blot densitometry was performed to analyze blots of matched colon carcinoma and normal mucosa samples. Blots, simultaneously developed on the same X-ray film (2 blots/film), were used in the study, and for comparative analysis, receptor protein levels were standardized to β-actin bands. Analysis of the blots revealed significantly decreased levels of H4R protein in colon carcinoma compared to the controls ($p<0.01$). Dowregulation of H1R expression showed similar, but not significant trend ($p=0.06$). Interestingly, regarding H2R levels, there were no substantial difference between normal mucosa and tumours ($p=0.36$). Western blot findings further supported histamine receptor mRNA expression results, we showed significantly reduced H4R levels in tumours in comparison with normal mucosa. These altered expressions in tumour tissue are just opposite to the results found in inflammatory bowel diseases and food allergy where H1R and H2R were upregulated. The important findings of real time RT-PCR and Western blot analysis have not shown significant changes in histamine receptor expression and Dukes' stages of tumours.

In order to analyze histamine receptor expression in further details, immunohistochemical staining of paraffin embedded colon cancer samples was

performed. The staining patterns of H1R, H2R, and H4R corresponded to the observed mRNA expression. H3R immunohistochemical analysis were not carried out because no detectable mRNA expressions were present in most samples. All types of negative controls demonstrated negligible background staining. In normal tissue samples enterocytes of colon have shown an extensive immunostaining for the histamine receptors (H1R, H2R and H4R). Some non-epithelial mucosal cells had strong positive staining for H1R and H2R, while less pronounced staining was detectable for H4R in other cell types of mucosa. In submucosa weak H1R and H2R positive staining was observed in some connective tissue cells. Although according to the published data, H4R expression was found predominantly in leukocytes, we detected positive H4R staining in colon enterocytes. Less pronounced, sporadic staining of connective tissue cells was present in submucosa.

Regarding the tumour tissue samples, we found different staining patterns of histamine receptors. H1R, H2R and H4R stainings were detected in epithelial cells of all tumours, but immunostaining appeared less pronounced in the cancer samples than in normal tissues. In the stroma of the tumours sporadic H1R, H2R and H4R positive staining were seen.

The present study was conducted to identify histamine receptor subtype expression in colon carcinoma, adenoma and normal mucosa. The role of H1R and H2R in different experimental tumour models is well established, but the relevance of H3R and H4R has not been clarified yet. Moreover, only limited data on the histamine receptor expression in colorectal tumours are available. Our findings confirmed that H1R, H2R and H4R are expressed in the human colorectal tract, both in normal tissue and colorectal tumor specimens. To the best of our knowledge, we demonstrated the expression of these histamine receptors in adenoma and human colorectal cancer at protein level for the first time. We also described the histamine receptor expression pattern in neoplastic colon tissue compared to the normal mucosa. Our experiments showed a significantly reduced H1R and H4R mRNA expression in colorectal cancer, regardless of the grade or Dukes' classification of the tumours. The H2R protein expression were similar in tumours and in normal colon mucosa. These altered expressions in cancer tissue are just opposite to the results found in inflammatory bowel diseases and food allergy, where H1R and H2R were upregulated. Results of

protein expression analysis also support these observations, since a significantly reduced H4R expression was found in tumours compared to their normal mucosal levels. We obtained, similar but not significant changes in the expression of H1R protein. In 10% (2/20) of the samples H1R protein expression proved to be very low, while in the majority of the cases moderately decreased values were measured. The immunohistochemical determination revealed that all 3 histamine receptor types (H1R, H2R, and H4R) were expressed on normal enterocytes and were present in adenocarcinoma cells as well. The variability in histamine receptor expression was less pronounced in tumour cells than in the normal epithelium. This also supports the fact of altered histamine receptor expression in tumours.

Histamine exerts multifunctional effects on the immune response, angiogenesis and metastasis formation. Histamine has an impact on cytokine production, (e.g. IL-1, TNF-α, IL-10 and IL-12), such as on the maturation of monocytes, dendritic cells and T-lymphocytes, and acts on actin polymerisation and chemotaxis as well. These actions of histamine are mediated through the four histamine receptor subtypes. Histamine has been demonstrated to shift local T helper 1 (Th1)/ T helper 2 (Th2) balance**,** and acts on immunoglobulin synthesis too. According to the original theory, histamine enhances the Th2 type cytokine production. Our present results suggest that the downregulated expression of H1R and H4R in colon tumours could produce a favorable microenvironment for tumour cell growth due to the H2R-mediated negative regulation of Th1 and Th2 lymphocyte response. It is well known that vascular endothelial growth factor (VEGF) is one of the main factors in tumour angiogenesis. There is an evidence that histamine, released by mast cells or tumour cells, could influence the angiogenesis through a H2R-mediated pathway. The H2R antagonist, cimetidine, showed antimetastatic potential in tumour bearing mice by decreasing E-selectin expression. Based on these results, histamine influences angiogenesis and metastatic spreading mainly through H2R. Decreased H1R expression, accompanied by unchanged H2R expression in colon tumour, could act towards the direction of tumour angiogenesis and metastasis formation. Our results suggest that histamine receptor profile in neoplastic tissue is markedly different to the normal one. Additional experiments are required to clarify which cell types are primarily responsible for the reduced H1R and H4R expression in human colon tumours, because in normal colonic mucosa, different cells

such as enterocytes, T lymphocytes, monocytes, mast cells, basophils and fibroblasts could express H1R, H2R and H4R. These details could help us in answering the question whether the direct or indirect effects of histamine, or both, mediated by histamine receptors, are responsible for tumour progression in colon carcinoma. Moreover, if the downregulation of H1R and H4R has a relevance in tumour formation, H1R and/or H4R agonists together with H2R antagonists should be tested in experimental tumour models in order to shift the histamine-mediated processes in the direction of tumour inhibition. We plan to use H4R as well as H1R KO mice for these experiments

CONCLUSIONS, NEW SCIENTIFIC RESULTS

Although there were previous literary data available about the presence and activity of HDC enzyme in colorectal tumours, the in situ identification of intracellular HDC protein could not be realized, attributable to the lack of anti-HDC antibody. Anti- HDC protein antibody was first prepared by the Institute of Genetics, Cell Biology and Immunbiology, and the product itself is patented by the Institute and the Promega company. **Immunohistochemical identification of HDC enzyme in colorectal adenoma and carcinoma was examined by our team for the first time. Our results demonstrated considerable differences regarding the presence and activity of HDC enzyme in malignant and benign tumours in relation with the normal tissues. Based on the HDC positivity of tumours cells we concluded and demonstrated that the malignant colon tumour cells are capable of producing HDC enzyme.**

We have shown (like other authors) that histamine metabolism is markedly changed in colorectal tumours. **We concluded that increased level of histidine decarboxylase and decreased DAO activity are responsible for increased histamine concentrations in malignant tumours.**

We demonstrated for first time unchanged levels of histamine, and histamine metabolizing enzymes (HDC and DAO) in the adenoma specimens compared to the normal colonic mucosa. We concluded that in adenoma tissue neither changed histamine levels nor diamino-oxidase activity were detectable. **In adenomas we could not observe the markedly altered histamine metabolism which is characteristic for malignant tumours. Consequently, the activity of the histamin metabolising enzyme is different in malignant tumours and normal tissues, and this enzyme pattern is identical in benign (ie. still not transformed) adenomas (polyps) and normal tissues.**

In humans, there are limited data available in the literature regarding expression and distribution of histamine receptors (HR) within colorectal tumours, moreover they have

not been examined in adenoma yet. **According to our results in malignant and benign tumours of the colon and normal colonic mucosa 3 receptors are expressed H1R, H2R, and H4R. We were the first who characterized the histamine receptor expression in adenoma at protein level.**

Protein level expression of histamin receptors in adenocarcinoma has been already demonstrated by other investigators. **We were the first who showed an altered histamine expression profile (decreased H1R and H4R, unchanged H2R) in comparison to the normal colonic mucosa. This change is characteristic to all tumours independent of the Dukes' stages.**

The immnuhistochemical detection of histamin receptors in malignant colorectal specimens should be regarded as a new scientific result as well. **Our results demonstrate, that, in addition to the normal colonic mucosa tissue, histamin receptors are expressed in colorectal cancer as well.**

SUMMARY

It was repeatedly demonstrated that histamine synthesis in tumours is significantly increased compared to the surrounding normal tissues, such as in breast cancer, colon carcinoma, malignant melanoma and hematological malignancies. Previous research has revealed the local production of histamine in colon tumours, and the significance of histamine in the development and progression of cancer. The local effect of histamine is largely determined by the actual histamine receptor (H1R-H4R) expression pattern. It has been shown that in experimental carcinomas endogenous histamine may act as an autocrine growth factor by stimulating tumour cell proliferation via H2 receptors. In humans, several clinical trials have been performed with H2 receptor antagonists as additive treatment to surgical resection, with contradictory results so far. Colon adenomas represent an increased risk for developing colon cancer, it is well documented that adenomatous polyps may undergo malignant transformation.

We intented to analyse the histamine metabolism and receptor expression profile in human colorectal carcinoma. In our experiments we perfomed the comparative analysis of histamine metabolism and receptor expression in colorectal cancer, adenoma and normal colonic mucosa. Our results showed an elevated histamine concentration, increased HDC activity and decreased DAO content in adenocarcinoma in comparison to the normal mucosa and adenoma. Based on our original hypothesis we expected to find an altered histamine metabolism in adenomas suggesting a transition to the metabolic pattern found in carcinomas. In contrast we could not demonstrate any altered metabolism in the benign laesions of the colon. The analysis of histamine receptor expression profile showed a marked difference in malignant tumours and normal mucosa. A well defined distribution and expression pattern of receptors was found in selected tumour samples, namely significantly downregulated H1R and H4R, with unchanged H2R expression. These results are just opposite to the results found in normal colonic mucosa and inflammatory bowel disease published by others. In addition to these results there were no difference between the receptor expression profile according to different Dukes' stages. The role of histamine with respect to the intracellular mechanisms involved in the colon tumour development and progression

has not been clarified yet. More details are needed to clarify the role of histamine and its receptors as prognostic factors in the treatment of colorectal cancer patients.

PUBLICATIONS AND POSTER PRESENTATIONS

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2. **Boér K**. Docetaxel az emlőrák adjuváns kezelésében. (2002) Pathology and Oncology Research Suppl, 2: 20-25.
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