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COX-2 and iNOS in Human Colon Cancer

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ABSTRACT

A variety of possible prognostic factors was examined in colon cancer, however, a single marker was usually assessed, not in relation to other factors which were known to affect biological behaviour. Since a group of molecular markers may be more useful in tumour characteristics, examinations that analyze marker group expression might be important in the selection of therapy. The material collected by surgeons might be used prior to treatment to identify biomarkers, which predict susceptibility or resistance to a particular medication, or to determine molecular features of ovarian cancer, which is to be treated. It may provide procedures which will enable non-invasive evaluation of selected parameters facilitating more effective diagnosis.

KEYWORDS : colon cancer; angiogenesis; iNOS; COX-2

INTRODUCTION

Nitrogen oxide (II) (NO) is a particle of short half-life, however necessary to perform numerous physiological functions. It is produced by three different isoforms of nitrogen oxide synthase (NOS) [1].Activity ofthe neuronal and endothelial NO synthase is dependent on calcium ions, while theinducible isoform (iNOS)proves independent of such ions and may be subject to expression as a response to proinflammatory factors.It has been hypothesised that nitrogen oxide has a role in genesis of a neoplasm as it was noted that high level of nitrogen oxide and iNOS overexpression in the tissues are associated with the tumour growth and metastasis in *in vitro*models. Moreover, the studies performed pointed to elevated expression of iNOS in several types of human neoplasms [2,3]. Such overexpression is associatedwith malignant phenotype of a neoplasm and poor prognosis for the patient. It has been claimed that nitrogen oxide synthesis inhibitors may appear as potential chemopreventive and therapeutic factors [4].

The role of NO in tumour biology is complex as has both, stimulating and suppressing role in cellular processes, depending on such conditions as local NO concentration or presence of other regulatory compounds [5]. Expression of nitrogen oxide may enhance the tumour growth, metastasis and angiogenesis through stimulation of p53 gene and up-regulation of the vascular endothelial growth factor(VEGF) [6]. On the other hand, at high concentration, NO may show the cytostatic or cytotoxic effect upon the tumour cells through suppression of the cell growth cycle dependent on p53 and induction of apoptosis [7].Prognostic meaning of iNOS expression in tumours remains controversial[3,8,9].Overproduction of NO persisting over some longer period may effect in mutation and eventually contribute to the growth of tumour [10]. NO produced by the tumour cells enhances angiogenesis as the basic process oftumour growth and subsistence.

COX-2 is an enzyme, expressed in response to such stimuli as cytokines, growth factors or hormones. It is also a protein, the activity of which is manifested in inflammatory conditions.COX-2 has an important role in oncogenesis. It has been observed that overexpression in neoplasms stimulates the growth of vessels through stimulation of the vascular endothelial growth factor(VEGF), through expression ofthe arachidonic acid products, such as thromboxane A_2, prostaglandin E_2 (PGE2) and prostacyclin. COXincreases also the resistance to apoptosis and enhances survival of the vascular endothelial cells [11]. As a result, some tumours showing COX-2 expression manifest a more aggressivephenotype and clinical behaviour. Patients with tumours, showing COX-2 overexpression, tend to respond worse to standard therapies and prove to have shorter life expectancy. It has been indicated that COX-2 overexpression contributes to unfavourable prognosis in breast cancer[12], colon cancer [13]as well as in other solid tumours.The experimental studies pointed that COX-2 inhibitors block the tumour growth through a variety of mechanisms, in particular through the antiangiogenic and proapoptotic function [14].

COX-2 expression takes place in malignant tumours[15-17] as well as in those showing low malignancy potential[18,19].Significantly higher share of cells withpositive COX-2 expression was observed in serous tumours of low malignancy potential, as compared to mucous tumours of low malignancy potential[20],and significant correlation with the clinical stage of such tumours was observed [19,21].The results obtained so far have provedcontradictory with regard to COX-2 expression in benign tumours and in healthy tissues of an organ.

Colorectal carcinoma is one of the most common causes of death in neoplastic diseases worldwide. Despite a tremendous number of studies on the mechanisms underlying the tumour, no explicit indication of which of those and in what way play the key role in the tumour development. Colorectal carcinomamay depend on expression of cyclooxygenase. COX-2 inhibition reduces the tumour growth, increases apoptosis and is associated with reduced angiogenesis of the tumour. It has been observed, however, that animals showing COX deficiency are not protected against tumour development in the induced colon/anus cancer; it is concluded then that COX expression is not the main marker ofcancer development. At the same time it has been hypothesised that nitrogen oxide has an important role in tumour genesis, as high level of the oxide as well as iNOS overexpression were noted as associated with the tumour growth and metastasis in *in vitro* models.

In our study we attempted to accomplish the assumed objectives answering what is the expression of the evaluated proteins at G1, G2, G3 andG3Mgrades in human large intestine and establishing whether the tested biological material shows expression ofiNOSandCOX-2and if so, what is its level.

MATERIALS AND METHODS

The test scheme

The tests made use of paraffin blocks to prepare samples which were then stained immunohistochemically with specific antibodies for given epitopes.

The inclusion criteria were as follows:

The study comprised 18 patients with colorectal carcinoma at G1 grade, 16 patients at G2 grade, 13 patients at G3 grade and 12 pa-

tients at G3M grade. The patients required surgical treatment.

The control group (15 patients: 7 women, 8 men, median age 51 years) included the large intestine samples, taken for reasons other than the conditions specified above. The group included the hospitalized patients in whom colonoscopy showed normal intestine or only some single polyps.

The study has been approved by the Local Ethical Committee, Medical University of Silesia, no. KNW/0022/KB/55/10.

Immunohistochemical tests
Of each paraffin block five microscope slides were prepared, every fifth section selected. This allowed for an appropriate number of preparations in each of the test groups. A check assay was made while cutting the sections to ensure that a subsequent sectionis taken from the lesion.

In order to expose the antigens, the preparations were incubated in the water bath at95^0 C in TrisEDTA solution ofpH 9 during 30 minutes and then cooled for about 20 minutes. After cooling they were washed in PBS. Places of nonspecific antibody binding were blocked with1% solution of BSA in PBS, during 30 minutes at room temperature. After removal of BSA solution, respective primary antibodies (anti-iNOS and anti-COX-2, both polyclonal, rabbit) were placed over the sections. Incubation was performed during 22 hours at 4^0 C. On the following day, activity of the endogenous peroxidase was blocked by incubation in 0.3% (v/v) hydrogen peroxide in 0,1% solution of NaN_3 inPBS during 10 minutes. ABC technique was used to expose the bound antibodies. Applied to the sections were appropriately biotinylated secondary antibodies and next the avidin-biotinylated peroxidase complex (Vectastain Elite ABC Kit, Vector Laboratories).

Visualisation of the ABC complex employed the peroxidase substrate containing 3.3-diaminobenzidine (DAB) and hydrogen peroxide, following the manufacturers' protocol(Vector Laboratories). The preparations were stained with Gill hematoxylin, dehydrated and closed. Negative control was provided by sections where the primary antibody was replaced by rabbit IgG, at solution such as the primary antibody.The control was performed parallelly for each slide in order to reveal the nonspecific binding of the primary antibody.

The obtained Immunohistochemical reactions were evaluated under the light microscope. Evaluated were both, the cellular positioning of selected proteins and, thanks to computer analysis, their quantity. The results were statistically analysed.

Archiving
Photographic records were made with the use of a light microscope supplied with a digital camera. To assess intensity of the immunological response 10 images of each delivered reaction were made at magnification x200 andx400 (x20 or x40 lense and x10 eyepiece) using Nikon Eclipse E200microscope with Nikon DS-Fi1 digital camera.

Evaluation of the immunohistochemical response
A quantitative analysis was performed for the evaluated proteins in the sections tested. With the use of NIS-AR (Nikon) software, the optical density of microscope preparations was evaluated in areas showing the colour reaction to a selected protein, Measurement of the light wave absorption grade indicates the optical density of cells, in cytoplasm of which the antigen-antibody complex was discovered,pointing to the reaction product contents.

Statistical analysis
The results were introduced to the database to evaluate distribution of continuous variables in the tested groups applying the Kolmogrov-Smirnov test.Statistical significance of differences between variable medians in normal distribution were evaluated byt-Student test while variable medians in distribution other than normal employed U-Mann-Whitney test. In case more than two medians,two-way ANOVA variance analysis was used. Values were presented as arithmetic means ± standard deviation. The differences were considered statistically significant at significance levelp<0.05. The statistical analysis was performed with the use of Statisticasoftware by Stat Soft, USA.

RESULTS

Inducible cyclooxygenase (COX-2)
Control
Expression of this cyclooxygenase was well manifested in the control assays. Response to the enzyme was observed only in the stroma cells (Fig. 1)

Colorectal carcinoma
G1 grade
Evaluation of COX-2 expression in the tested structures of the neoplastic lesions of the large intestine pointed that optical density of the product of immunohistochemical response to this parameter was not remarkably high, scoring 135% of the control value (Fig. 1). It has been shown that expression of the protein was manifested in the stroma and in some individual cells of the adenoma (Fig. 2).

G2 grade
Analysis of cyclooxygenase-2 level in the tested structures showed that the general optical density of the product of immunohistochemical response to this protein was higher than that for G1 grade, scoring 115% of that level (Fig. 1). Expression of that enzyme was observed only in part of the adenocarcinoma cells (Fig. 2).

G3 grade
Evaluation of COX-2 level in the tested tissue structures showed that the general optical density of the product of immunohistochemical response to this enzyme was comparable to grade G2 (Fig. 1). Reaction to this isoform was revealed in both, tumour cells and those of the stroma (Fig. 2).

G3M grade
Analysis of the level of the assessed cyclooxygenase in the tested structures showed that the general optical density of the product of immunohistochemical response to this protein reached the values as for grade G2 (Fig. 1). Reaction to this enzyme was observed in the tumour and stroma cells (Fig. 2).

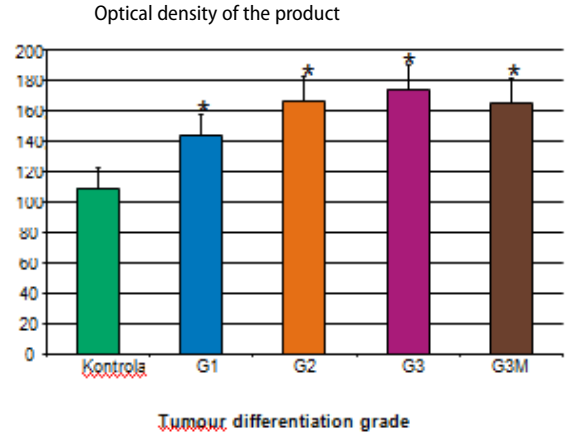

Figure 1.Optical density of the response product for COX-2 at different cell grades in human colorectal carcinoma
Letters correspond to statistically significant lesions for $p < 0.05$ between:

* - control and the cancer grade

Comparison
Evaluation of COX-2expression in colorectal carcinoma showed that the general optical density of the product of immunohistochemical response to this enzyme was slightly higher than the control value. It was observed that it scored from 130% of the control value at G1 grade, up to 160% at G3 grade. No statistically significant differences between particular grades were shown.

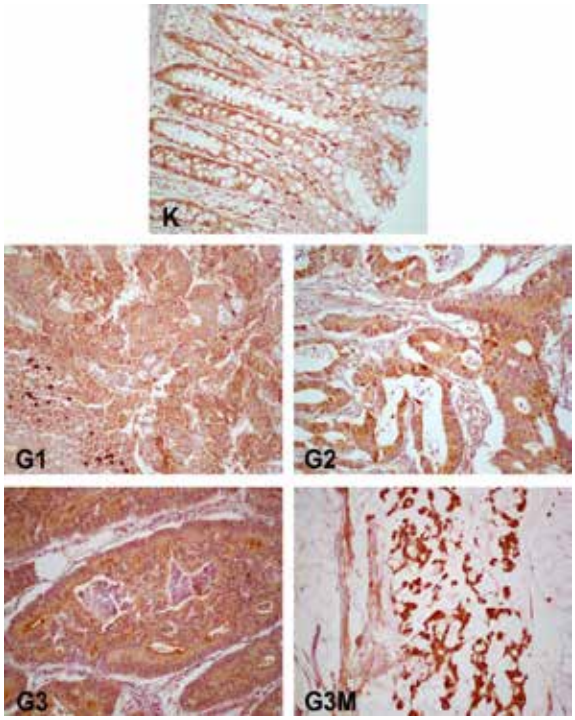

Figure 2. Immunohistochemical positioning of COX-2 at particular grades of cell differentiation in colorectal carcinoma. Magnification 200x

Inducible synthase of nitrogen oxide (iNOS)

Control

The level of expression of the evaluated synthase of nitrogen oxide in the control group was very weak as compared to the assessed colorectal carcinoma. It has been shown that expression of this protein was manifested in individual stroma cells and partly in the intestine crypt cells (Fig.3). No expression of the receptor was observed in enterocytes or mucocytes.

Colorectal carcinoma

G1 grade

Evaluation of iNOS expression in the assessed neoplastic lesions of the large intestine showed high optical density of the product of immunohistochemical response to this parameter, scoring 200%of the control value(Fig. 3). Expression of this protein was shown in the stroma and in the adenoma cells (Fig. 4).

G2 grade

Evaluation of the assessed synthase in the tested structures showed that the general optical density of the product of immunohistochemical response to this protein was remarkably higherthan values observed at G1 grade (Fig. 3). Expression of this isoform was noted only in part of the adenoma cells (Fig. 4).

G3 grade

Evaluation ofiNOS level in the assessed tissue structures pointed to the general optical density of the product of immunohistochemical response to this factor, corresponding to the values observed at G2 grade(Fig. 3). Reaction to this isoform was shown not only in the tumour cells but also in the stroma (Fig. 4).

G3M grade

Evaluation of the synthase level in the assessed structures showed that the general optical density of the product of immunohistochemical response to this protein was slightly higher than values observed at G3 grade (Fig. 3). Also in this case, reaction to the isoform was confirmed in the tumour and stroma cells (Fig. 4).

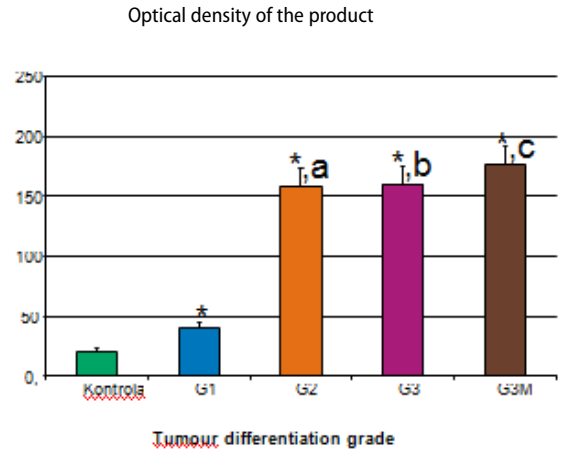

Figure 3. Optical density of the product of response toiNOS at particular grades of cell differentiation in human colorectal carcinoma

Letters correspond to statistically significant changes for $p < 0.05$ between:

* - control and the tumour stage

a - grade G1 and grade G2

b - grade G1 and grade G3

c - grade G1 and grade G3M

Comparison

Evaluation ofiNOS expression in colorectal carcinoma showed that the general optical density of the product of the immunohistochemical response to the receptor was markedly higher than the control values. The scores proved 2-fold higher than those at G1 grade and at least 8-fold higher than values recorded for the remaining large intestine tumour grades. Statistically significant differences were observed between grades G1and G2, G3 and G3M.

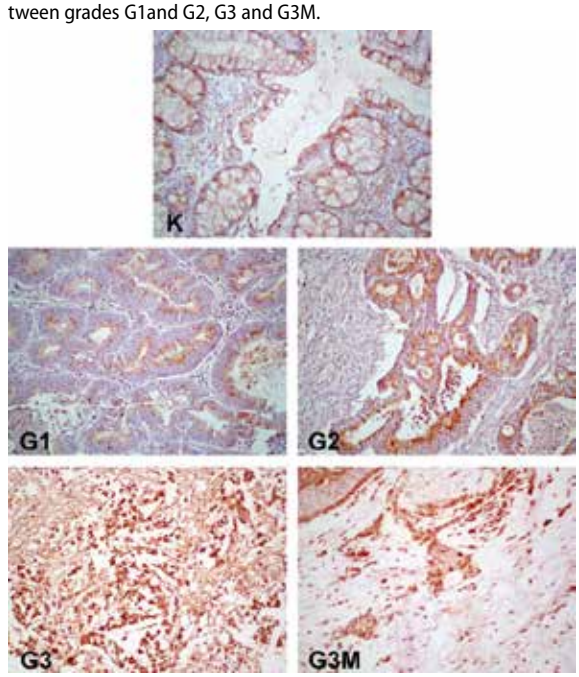

Figure 4. Immunohistochemical positioning of iNOS at particular grades of cell differentiation in colorectal carcinoma. Magnification 200x

DISCUSSION

Cyclooxygenase-2 (COX-2) is an enzyme, showing high activity in ar-

eas affected by inflammatory conditions. Correlation was proved between activity of this enzymatic protein and proliferation of certain neoplastic conditions, including colorectal adenocarcinoma [22].Recently, the role of COX-2 in tumour processes of epithelial genesis has also been proved. Elevated COX-2 activity is remarkably associated with distant metastasis and poor prognosis upon multivariate analysis [23].

An important role of iNOS in tumour genesis is activation of COX-2. Our study attempted at evaluation of COX-2 expression in colorectal carcinoma, ranging between the benign stage (G1) and the malignant grades (G3 and G3M).

It has been shown recently that elevated COX-2 level is correlated with worse prognosis for patients with certain forms of tumour, including colorectal carcinoma. COX-2 activation effects in increased vascular permeability and inhibition of apoptosis. Positive correlation has also been shown between COX-2 expression and iNOS. Shorter, free of the disease, survival periods of patients with elevated COX-2 expression, suggest that addition ofinhibitors, specific for this cyclooxygenase,to cancer therapy may turn it more efficient.

The role of COX-2 in neoplasia has been shown upon numerous studies. Overexpression of cyclooxygenase-2 in tumour cells enhances cell division, alters cell adhesion, increases cell mobility, inhibits apoptosis and induces angiogenesis. It has been shown that COX-2 inhibitors tone up the growth of cells in some tumours, while some reports suggest that chronic inflammatory condition may contribute to cancer genesis. It has been known that COX-2 overexpression is associated with aggressive and invasive potential of tumour cells, through several mechanisms. One of those, modulated by COX-2 throughout cancer genesis, is angiogenesis, most probably triggered by elevated production of proangiogenic factors, such as VEGF.

Although the mechanisms underlying COX-2 role in cancer genesis have not been fully recognized, it has been proved that the enzyme participates in tumour angiogenesis, reduction of apoptosis, proliferation of tumour cells and elevation of the metastatic potential of neoplastic cells through activation of metalloproteinase-2. Many results of studies on COX-2 expression in tumours associated with clinical and pathological changes, pointed to COX-2 expression affecting the patient's life expectancy prognosis.

Results of the recent study are found among the group of literature reports indicating tumour progression which did not correspond to elevated expression of the discussed cyclooxygenase. Such observations regarded equally the tumours of all grades, which is in a way unique. In principle, COX-2 expression was on the same level, yet markedly higher than in the controls. Therefore, our observations mean that COX-2 expression may not be accepted as an independent prognostic factor in evaluation of progression in colorectal carcinoma.

Laboratory studies by Athanassiadouet al. [24]also failed to prove that COX-2 could appear as an independent prognostic variable, which is consistent with other published results, although an independent prognostic value of COX-2 was suggested e.g. by Denkert et al. [25]. Nevertheless, some statistical analyses pointed to strong correlation between the elevated staining pattern of COX-2 and some undesirable clinical and pathological parameters, such as elevated tumour grade, increased malignancy and shorter survival time. Similar observations were reported earlier by a few authors which suggests that COX-2 overexpressionmay bea marker of some more aggressive clinical behaviour, worse prognosis in colorectal carcinoma, as well as resistance to radio- and chemotherapy[24].

Earlier studies indicated that regular administration of non-steroid anti-inflammatory drugs, which inhibit COX-2 activity in a non-selective manner,remarkably reduced the risk of development of colorectal carcinoma. Cyclooxygenase 2 has been commonly recognized as an excellent objective in prevention of colorectal carcinoma, not only for the evidence supporting its role in tumour progression, but also because extensive availability of COX-2 inhibitors and safe upon common use [26].Therefore, new cancer therapies should also aim at the discussed cyclooxygenase. At least some of the performed experimental studies and clinical assays have suggested great prognostic and clinical value of certain COX-2 inhibitors, not only in chemotherapy but also in cancer chemoprevention [26]. COX-2 inhibitors are also likely to appear as useful factors in treatment of at least some groups of patients with the colorectal carcinoma.This is why further, wide-scoped studies are required to assess the prognostic validity of the marker.

The prognostic value of iNOS expression in neoplastic conditions remains controversial due to scarce number of studies on colorectal carcinoma, which would evaluate the prognostic role ofiNOS. It has been proved that iNOS expression is associated with the tumour angiogenesis. Studies of many neoplastic conditions reported on iNOS overexpression, includinghead and neck planoepithelial carcinomas, cholangiocarcinoma,mammary cancer or brain tumours. It has been suggested that the loss of iNOS expression occurs at early stage of pathogenesis in colon cancer [27].The role of NO and iNOS in the tumour growthhas been disputable. High NO concentration induces apoptosis, yet at low concentration, it stimulates the tumour growth through induction of angiogenesis [27].Higher expression of iNOS was observed in low grade carcinomas, however no correlation was with microvascularization density was found. Higher grade carcinomas showed low iNOS expression level, while the mean survival time of patients with low iNOS expression was shorter than in those with carcinomas of high iNOS expression. This could be due to stimulation of apoptosis upon high iNOS expression at early carcinogenesis stage and simultaneously, to progression of the tumour at reduced iNOS and NO expression, leading to inhibition of the anti-proliferatoryfunction.

Better understanding of molecular changes underlying development of carcinoma could appear helpful to design more effective therapies which would hopefully improvethe treatment indices and the patients survival rates.For example, higher expression of iNOS has been observed in colonic carcinomas, lung cancer, oropharyngeal tumours, mammary cancer as well as those of the central nervous system [28].A growing number of reports has recently revealed that iNOS overexpression is manifested in numerous malignant conditions and is strictly associated with aggressive behaviour of the tumour and poor prognosis for the patients.Despite some evidence which could allow for a hypothesis of iNOS appearing as a marker of malignant carcinomas,many authors would not consider it a marker of malignant conditions exclusively [29,30].

Our results are in line with such understanding. On the one hand, changes observed between carcinomas from G2 grade are too small to be regarded as a sole marker, on the other, however, the observed changes, at least in G1 tumours as compared to the controls and higher grades, may be a cornerstoneto suggestthatiNOS changes should be evaluated while establishing the treatment prognosis. In our opinion, evaluation of iNOS expression in malignant carcinomas, along with assessment of changes in expression of other factors, could be a basis for prognostic evaluations. Such studies should by all means continue.

It may be stated , that iNOS expression correlates with the tumourdifferentiation range, hypothesising then that no relation between NO release and iNOS expression indicates different types of cells participating in iNOS expression and points that NO synthesis control and synthase induction may as such deliver valuable strategies to prevent benign tissue transition into a malignant tumour.

Positioning of iNOS expression in carcinoma cell cytoplasm in the evaluated cases was consistent with earlier reports on colorectal carcinoma [31].Activity of iNOS has been position in both, malignant and benign tissues. The literature available shows that each of the histological types of carcinoma seems to be associated with remarkable morphological and molecular changes.Therefore, particular genes and molecular pathways as well as their role in progression of different histological types of carcinoma may differ significantly.

It has been suggested that iNOS expression may appear as an independent prognostic factor, despite the fact that in many studies iNOS expression was not regarded as a sufficient prognostic marker for multivariate analysis and elevated expression had no additional prognostic value. So far, it has only been proved that clinical and pathological factors, such as the tumour grade and stage, have independent prognostic meaning in carcinomas [32].

CONCLUSION

COX-2 expression did not depend on the stage of colon cancer, whereas the expression of inducible nitrogen oxide synthase significantly increased along with a decrease in cellular differentiation.

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