Low red blood cell levels of deglycating enzymes in colorectal cancer patients

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AIM: To investigate Glyoxalase I and fructosamine-3-kinase (FN3K) activity in red blood cells from patients with colorectal adenomas and cancer.

METHODS: Thirty three consecutive subjects with one or more histologically confirmed colorectal adenomatous polyps, 16 colorectal cancer patients and a group of 11 control subjects with normal colonoscopy were included in the study. Glyoxalase I and FN3K activities were measured in red blood cells using a spectrophotometric and radiometric assay, respectively.

RESULTS: A significant reduction in both Glyoxalase I and FN3K activity was detected in patients with tumors compared to patients with adenomas and the controls. Erythrocyte Glyoxalase I activity in colorectal cancer was approximately 6 times lower than that detected in patients with adenoma (0.022 ± 0.01 mmol/min per milliliter vs 0.128 ± 0.19 mmol/min per milliliter of red blood cells, P = 0.003, Tukey’s test). FN3K activity in red blood cells from patients with colon cancer was approximately 2 times lower than that detected in adenoma patients (19.55 ± 6.4 pmol/min per milliliter vs 38.6 ± 31.7 pmol/min per milliliter of red blood cells, P = 0.04, Tukey’s test).

CONCLUSION: These findings suggest that deglycating enzymes may be involved in the malignant transformation of colon mucosa.

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Key words: Colorectal cancer; Enzymatic activity; Fructosamine-3-kinase; Glycation; Glyoxalase I

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INTRODUCTION

The enzymatic defense against glycation involves enzymatic activities, such as the glyoxalase system, amadoriase and fructosamine 3 kinase, which suppress the formation of glycation adducts and repair sites of early glycation [1].

Glyoxalase I together with Glyoxalase II constitutes the glyoxalase system, a ubiquitous detoxification pathway which protects against cellular damage caused by potent cytotoxic metabolites, such as methylglyoxal. Methylglyoxal is a physiological substrate, derived from glycolysis, via degradation of triose phosphate intermediates, lipid peroxidation, and fragmentation of glycated proteins [2].
As a highly reactive metabolite, methylglyoxal has a strong ability to cross-link with protein amino groups to form stable products called advanced glycation end products, and to attack guanine residues of DNA leading to DNA glycation[3]. The cytotoxicity of methylglyoxal is due to its mutagenic and antiproliferative properties and to its ability to trigger apoptosis[4], via oxidative signaling[5]. Experimental evidence shows that the glyoxalase system is involved in the regulation of cellular growth[6]. Altered expression of this system is involved in several human disorders, including cancer[7,8]. Over-expression of Glyoxalase I is associated with clinical multidrug resistance in tumors of high incidence and mortality, such as carcinomas of the lung, breast and prostate and Glyoxalase I inhibitors provide effective therapy for these tumors[9,10].

Fructosamine-3-kinase (FN3K) is an intracellular deglycating enzyme that phosphorylates fructosamines on the third carbon of their deoxyfructose moiety. The fructosamine 3-phosphates so formed are unstable and their spontaneous decomposition leads to the regeneration of the free amine[11,12]. This enzyme seems to catalyze a repair mechanism offering selective cell advantage.

We previously evaluated FN3K gene expression in colorectal cancer patients, and showed that FN3K gene expression was significantly lower in colon cancer tissue than in the corresponding surrounding normal mucosa[13]. Moreover, we found that FN3K activity is particularly downregulated in tumors located on the left side of the colon[14].

The adenoma-carcinoma sequence in the colon represents one of the most characterized models of human tumor progression. The transition from normal to malignant phenotype implies the activation of pathways that underlie aberrant clone expansion[15].

Alterations of metabolic pathways, such as changes in the balance between glycation and enzymatic anti-glycation defense, are considered to be crucial for sustaining tumor development[16]. The risk of colorectal adenoma increases with serum levels of fructosamine[17]. The decline in expression of deglycating enzymes may be the key to increased protein glycation in the tumor phenotype.

In this study, we evaluated the levels of Glyoxalase I and FN3K activity in red blood cells from patients with colorectal adenomas and cancer.

MATERIALS AND METHODS

Subjects

The study included thirty three consecutive subjects (18 males and 15 females, mean age 67.6 ± 11.7 years) with one or more histologically confirmed colorectal adenomatous polyps removed after complete endoscopy, and sixteen colorectal cancer patients (6 males and 10 females, mean age 68.1 ± 6.4 years) undergoing colon surgery. A group of eleven control subjects (6 males and 5 females, mean age 45 ± 5.8 years) with normal colonoscopy, performed in the same endoscopy unit during the same period, was also included.

Written informed consent was obtained from all the participants.

Measurement

Anthropometric measurements were obtained by the participants wearing scrub suits without shoes. Body weight was measured using a calibrated scale (Detecto; model 437). Standing height was measured with a vertical metal ruler. Body mass index (BMI) was calculated as weight in kilograms divided by the square of the height in meters (kg/m²).

Ficoll-Paque separation

Participants were fasted for 12 h prior to examination. Blood samples taken from the subjects by venous puncture were collected in tubes containing EthyleneDiamineTetraacetic Acid (K-EDTA) anticoagulant or a serum separator gel. Blood serum was shipped to the central laboratory for routine analyses. For in vitro isolation of erythrocytes, blood samples with K-EDTA were quickly layered on the Ficoll-Paque solution and centrifuged at 400 g for 40 min at 20C. The lymphocytes and plasma were then removed and the erythrocytes were recovered from the bottom layer and washed with 4-volumes of phosphate-buffered saline. Isolated red blood cells were stored at -80°C until assayed. All the analyses were performed within 6 mo.

Glyoxalase I activity assay

Glyoxalase I enzymatic activity was measured in frozen erythrocytes, according to the method described by Thornalley[18] with minor modifications. The frozen red blood cell pellet was lysed with 1 mL of 10 mmol/L TRIS-HCl, pH = 7.8, 1 mmol/L DTT, 1 μg/ml leupeptin and was well mixed. The samples were centrifuged for 10 min at 2000 g and the supernatant was used for the enzymatic activity assay. Aliquots of 50 μL of supernatant were incubated with 100 μL of reaction mix containing 7.9 mmol/L methylglyoxal, 1 mmol/L glutathione, 14.6 mmol/L magnesium sulfate and 182 mmol/L imidazole HCl, pH = 7.0. The activity of Glyoxalase I was determined by monitoring the increase in absorbance at 240 nm due to the formation of S-D-lactoylglutathione for 2 min at 25°C. One unit of activity was defined as the formation of 1 mmol of S-D-lactoylguthione/min per milliliter of blood red cells.

Fructosamine-3 kinase activity assay

FN3K enzymatic activity was measured in frozen erythrocytes lysed with 1 mL of 10 mmol/L TRIS-HCl, pH = 7.8, 1 mmol/L DTT and 1 μg/ml leupeptin. The samples were centrifuged for 10 min at 2000 g and 50 μL of supernatant were incubated with 100 μL of reaction mix [5 mmol/L glucose, 10 mmol/L Tris-HCl (pH 7.8), 1 mmol/L DTT, 1 μg/ml leupeptin, and 2 mmol/L D-[1-14C]-glucose (49.5 μCi/mmol) for 40 min at 37°C.

Subsequently, 30 μL aliquots of the samples were spotted on cation-exchange papers (P81; Whatman), which were washed three times with ice-cold 75 mmol/L H2PO4 and then once with alcohol and once with acetone. After drying, the papers were counted for radioactivity in the presence of a scintillator. FN3K activity was expressed as picomoles of incorporated D-[1-14C]-glucose/min per milliliter of red blood cells. Parallel samples were as-
sayed to evaluate total and non-specific radioactivity. The enzyme activity assay was validated using samples in the presence of the FN3K inhibitor, 1-deoxy-1-morpholino-fructose.

**Statistical analysis**

The mean and standard deviation were calculated for each group. Groups were compared using one-way analysis of variance and Tukey’s Multiple Comparison test. Differences in the means were considered statistically significant if the P-value was < 0.05. Analysis of covariance was used to model, controlling for glycemia, a potential variable confounder.

**RESULTS**

The clinical characteristics of all subjects studied are shown in Table 1. There was a weak increase in glycemia levels from the controls to the adenoma and cancer patients. No difference in mean BMI values between the groups was observed.

Table 2 summarizes the data for Glyoxalase I and FN3K activity levels detected in red blood cells from controls, adenoma and tumor-bearing patients.

There was a significant reduction in Glyoxalase I activity in red blood cells from patients with tumor compared to controls, and a trend in decreasing activity from controls to adenoma and cancer patients. Erythrocyte Glyoxalase I activity in colorectal cancer was about 6 times lower than that detected in patients with adenoma (0.022 ± 0.01 mmol/min per milliliter vs. 0.128 ± 0.19 mmol/min per milliliter of red blood cells, P = 0.003, Tukey’s test). A significant reduction in FN3K activity levels in erythrocytes from colon cancer patients with respect to controls and adenoma patients was also observed. FN3K activity in the red blood cells of patients with colon cancer was approximately 2 times lower than that detected in adenoma patients (19.55 ± 6.4 pmol/min per milliliter vs. 38.6 ± 31.7 pmol/min per milliliter of red blood cells, P = 0.04, Tukey’s test).

The differences in enzymatic activities among the control, adenoma and cancer groups were controlled for fasting glycemia using analysis of covariance. The absolute value of association did not decrease; there was only an increase in standard error of the coefficients and consequently a decrease in the P-value of the null hypothesis. In any case, the inverse association of both enzymes with cancer was still statistically significant (two tails, P < 0.05).

**DISCUSSION**

This study provides evidence of a role for the deglycating enzymes, Glyoxalase I and FN3K, in colorectal cancer development. The trend of decreased Glyoxalase I activity from the controls to adenoma and cancer patients strengthens the association between this deglycating enzyme and colon cancer, and suggests that its decrease in activity can support the evolution of the malignant process.

The glyoxalase system has received considerable attention regarding its possible relationship with cancer. In an animal model of carcinogenesis, an appreciable decrease in rat liver glyoxalase activity was found after the development of hepatoma[21]. Some authors also showed that glyoxalase activity in the blood of tumor-bearing animals was much lower than glyoxalase activity in the blood of normal animals[22]. The presence of Glyoxalase I gene polymorphism, which may result in a decrease in glyoxalase activity, increases breast cancer risk[23]. This gene polymorphism seems to have a role not only in the development of breast cancer, but also in the progression of neoplasia[23].

Recently, a significant reduction in FN3K gene expression was detected in colorectal cancer with respect to normal pair-matched tissue[15,16], suggesting that decreased FN3K expression is related to the malignant phenotype. This study also suggests that there are functional modifications of FN3K in colon cancer development, since reduced FN3K activity is detectable in the progression from adenoma to cancer.

Epidemiological studies clearly indicate that the risk of several types of cancer (including pancreas, liver, breast, colorectal, and urinary tract) is increased in diabetic patients[24,25]. Higher levels of serum glucose were present in our patients with colon cancer compared to those with adenoma and the controls. The inverse association of both enzymes with cancer was maintained after controlling for fasting glycemia. However, we doubt the necessity to control for glycemia, because it may be part of the causal chain between the enzymes and the neoplasia, as an intermediate variable. Hyperglycemia may correlate with the development of adenoma and invasive colon cancer[26], and non-enzymatic glycation is one of the principal

**Table 1** Clinical characteristics of subjects enrolled in the study (mean ± SD)

|                      | Control subjects | Adenoma patients | Cancer patients |
|----------------------|------------------|------------------|----------------|
| n                    | 11               | 33               | 16             |
| Age (yr)             | 45 ± 5.8         | 67.6 ± 11.7      | 68.1 ± 6.4     |
| Female/male          | 5/6              | 15/18            | 10/6           |
| Glycemia (mmol/L)    | 4.87 ± 0.2       | 5.82 ± 1.2       | 7.06 ± 2.4     |
| BMI (kg/m²)          | 25.6 ± 7.7       | 27.8 ± 6.3       | 27.1 ± 3.1     |

BMI: Body mass index.

**Table 2** Red blood cell levels of Glyoxalase I and fructosamine-3-kinase activity in controls, adenoma and tumor-bearing patients (mean ± SD)

|                      | Glyoxalase I | FN3K  |
|----------------------|--------------|-------|
| Controls (n = 11)    | 0.173 ± 0.25 | 29.05 ± 14.6 |
| Adenomas (n = 33)    | 0.128 ± 0.19a| 38.6 ± 31.7  |
| Tumors (n = 16)      | 0.022 ± 0.01a| 19.55 ± 6.45a|

\(P < 0.05,\) Tukey’s test. The glyoxalase enzymatic activity is expressed as mmol/min per milliliter of red blood cells and the enzymatic activity of FN3K is expressed as pmol/min per milliliter of red blood cells. FN3K: Fructosamine-3-kinase.
mechanisms by which hyperglycemia contributes to cellular damage[27].

The enzymatic defense against glycation suppresses damage to biological macromolecules, if this defense in normal physiological states is at a low level, cellular injury occurs. Glycation proteins with associated functional impairment of repair enzymes has a critical role in the activation of pathways of cellular transformation[18,20].

The findings of this study provide evidence that deglycating enzymes may be involved in increasing the risk of precancerous lesions and malignant transformation of colon mucosa.

Further studies on a large cohort of patients with colorectal cancer will be designed to translate our findings into clinical practice, allowing the development of a fast and accurate blood test to diagnose colorectal cancer at different stages.

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