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Involvement of oxidative stress in the pre-malignant and malignant states of cervical cancer in women

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Abstract

Objectives: To evaluate the potential role of oxidative stress in the evolution of cervical cancer, including its pre-malignant states.

Design and methods: Erythrocytes thiobarbituric acid reactive substances (TBARS) levels, plasma vitamin C and thiol content and total blood δ-ALA-D levels were estimated in 46 untreated cervical cancer and pre-malignant patients and in 46 age–sex-matched controls.

Results: Erythrocytes from patients, regardless of disease state, pre-malignant (low squamous intraepithelial lesion—LSIL and high squamous intraepithelial lesion—HSIL) or cancer, showed a significant 2–3 times increase in TBARS levels ($P < 0.01$). Plasma vitamin C was lower in the carcinoma group ($P < 0.01$). The reactivation index of δ-aminolevulinate dehydratase (δ-ALA-D) was higher in the patient group, when compared to control ($P < 0.01$).

Conclusion: LSIL, HSIL or cervical cancer can be associated with changes in 3 indicators of oxidative stress: increase in erythrocyte TBARS, ALA-D reactivation index and a decrease in vitamin C content, that may play an important role in carcinogenesis.

Keywords: Cervical cancer; Pre-malignant states; Oxidative stress; ALA-D; Vitamin C; TBARS

Introduction

Inadequate antioxidant protection or excessive production of reactive oxygen species creates a condition known as oxidative stress, which is thought to play an important role in the etiology of various disease and in aging [1,2]. Reactive oxygen species (ROS) are involved in the etiology of a variety of human diseases, and cumulative experimental evidence supports a role for them on the initiation and progression of carcinogenesis [1]. Enzymes including superoxide dismutase, glutathione peroxidase and catalase, as well as β-carotene, α-tocoferol and ascorbic acid constitute major defenses against oxidative damage and they are modified in a variety of pathological conditions [3]. Epidemiological studies have demonstrated a link between the consumption of diets high in fruits and vegetables containing high levels of antioxidant vitamins and a reduced incidence of degenerative diseases including many types of cancer [4].

Cervical carcinoma is an important cause of cancer mortality and morbidity, particularly in less developed countries and is one of the most common cancers afflicting women in the world [5,6]. It is a slow-evolution disease characterized by two pre-malignant states (low squamous intraepithelial lesion—LSIL; high squamous intraepithelial lesion—HSIL), which precede the carcinoma state. A variety of factors have been implicated in the development of cervical carcinoma; however, Human Papilloma Virus (HPV) infection is one of the most important causative factors of cervical carcinoma and causes damage to the DNA and other constituents of the cell [7–9].

δ-Aminolevulinate dehydratase (E.C. 4.2.1.24) catalyzes the synthesis of tetrapyrrolic compounds such as billins and hemes. ALA-D is a sulfhydryl enzyme which depends on Zn^{2+} ion

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binding to display full activity and is sensitive to situations associated with oxidative stress [10–12]. Furthermore, enzyme inhibition can lead to ALA accumulation in the blood, which in turn can intensify oxidative stress by generating carbon centered reactive species or by releasing iron from proteins such as ferritin [13].

The present study was designated to evaluate a possible involvement of oxidative stress during the progression of cervical carcinoma from the pre-malignant to the malignant state. Importantly, our results demonstrated that pre-malignant states are already associated with reduction of vitamin C and with an increase in the ALA-D reactivation index and in erythrocytes TBARS.

Materials and methods

Reagents

The reagents were obtained from Sigma and Merck.

Subjects

Forty-six patients positive for cervical pre-malignant (low squamous intraepithelial lesion—LSIL group, $n = 15$, mean age 37.9 ± 9.8, range 20–54 years; high squamous intraepithelial lesion—HSIL, $n = 20$, mean age 37.7 ± 12.8, range 19–62 years) or malignant lesion ($n = 11$, mean age 44.8 ± 10.9, range 30–64 years) in the Papanicolaou test, without any previous treatment for the lesion, were selected for the study. They were all patients of the 'Hospital Universitário' from the 'Universidade Federal de Santa Maria', RS, Brazil. The group of cases is heterogeneous and the majority of cases of cervical intraepithelial neoplasia will not progress to cervical cancer and most of these lesions are caused by transient HPV infection [8]. Forty-six healthy female volunteers served as control (mean age 39.1 ± 10.6) and were age-matched with patients. Statistical analysis revealed no significant difference in the age of the experimental groups (t test, $P = 0.38$). The control subjects were free from any prior gynecologic disease or dysfunction and had a negative Papanicolaou smear (within 1 year). Patients and the control subjects had no concomitant disease such as diabetes mellitus, liver disease and rheumatoid arthritis. All the patients and control subjects declared that they were nonsmokers and did not consume vitamin or mineral supplements. Since we have no idea about the variability of the data of analyzed biochemical parameters, we initially designed the study to be a case-control study; however, after data collection, we observed that the variation among control subjects was low. Consequently, data were analyzed considering control subjects and patients in different stages of the diseases as different experimental groups (stage of diseases was considered as a fixed factor).

A questionnaire and a consent form were obtained from both patient and control subjects. The present study was approved by the Human Ethical Committee of the Universidade Federal de Santa Maria, protocol number 001/03.

Sample collection

Blood (7 mL) was collected by venous arm puncture in heparinized vacutainer tubes and the plasma and cells were separated by centrifugation at 1000×g for 12 min. Blood was collected between 8 and 10 a.m. and all the subjects were fasted for at least 12 h. Samples were obtained from April 2002 to November 2003.

Protein was precipitated from both the erythrocytes and plasma using 40% trichloroacetic acid (TCA). The plasma to be analyzed for vitamin C and protein thiol groups and the erythrocytes for TBARS evaluation were frozen for less than 3 weeks at −20°C. A preliminary study from our laboratory indicated that these parameters did not vary in 4 weeks of storage. δ-ALA-D activity was assayed in fresh total blood within 3 h after collection.

Biochemical analysis

Lipid peroxidation was estimated by measurement of thiobarbituric acid reactive substances (TBARS) in erythrocytes according to the method of Lappena et al. [14] using 1% phosphoric acid and 0.6% of thiobarbituric acid (TBA). However, no butylated hydroxytoluene (BHT) was added to the assay because we observed no differences in MDA levels of the same samples incubated with or without BHT. This is probably due to the presence of sodium dodecyl sulfate (SDS) that, in our hands, abolished the increase of TBARS during the heating step (unpublished observations). The pink chromogen produced by the reaction of thiobarbituric acid with malondialdehyde, a secondary product of lipid peroxidation, was measured spectrophotometrically at 532 nm.

Plasma vitamin C was estimated as described by Galley et al. [15] with minor modifications [16]. In this technique, dehydroascorbic acid is coupled with 2,4-dinitrophenylhydrazine (DNPH) and, when treated with sulfuric acid, forms an orange red compound measured at 520 nm.

δ-Aminolevulinate dehydratase (E.C. 2.4.1.24) activity was assayed in the total blood by the method of Berlin and Schaller [17] by measuring the rate of porphobilinogen (PBG) formation in 1 h at 37°C, in the presence and absence of the reductor agent dithiothreitol (DTT-2 mM final concentration). The enzyme reaction was initiated after 10 min of pre-incubation. The reaction was started by adding δ-aminolevulinic acid (ALA) to a final concentration of 4 mM in a phosphate buffered solution, and incubation was carried out for 1 h at 37°C and the reaction product was measured at 555 nm. The reactivation index was estimated using: ABS · ALA-D/DTT − ABS · ALA-D without DTT ÷ ABS · ALA-D/DTT × 100. This parameter seems to be a more reliable indicator of enzyme oxidation [18].

Protein thiols were assayed in plasma by the method of Ellman modified by Jacques-Silva et al. [16], which consisted in the reduction of 5,5′-dithio(bis-nithrobenzoic) acid (DTNB) in pH 7.4, measured at 412 nm.

Statistical analysis was taken by the commercial SPSS package for Windows©. One-way ANOVA followed by Bonferroni post hoc test comparisons were used. Comparisons