INTRODUCTION

Cervical cancer, the third most common cancer of the world, is also the second most frequent in women (Parkin et al. 1992; Looi et al. 1992; Looi et al. 2005). It is a malignant neoplastic disease that tends to begin slowly, but advances rapidly. The study of carbonyl content, antioxidant enzymes, lipid peroxidation, membrane disorganization, and oxidative stress in erythrocytes which finally results in increased erythrocyte membrane fluidity, altered phase transition temperature and oxidative damage of human erythrocytes is an important area of research. The antioxidant enzymes provide an excellent model for studying the membrane biology and structure-function relations due to its simplicity and availability. Until now very little attention has been directed to the effects of cancer on the cytoskeleton of the erythrocyte membrane (Kopczynski et al. 1998). The antioxidant enzymes provide an excellent model for studying the membrane biology and structure-function relations due to its simplicity and availability. Until now very little attention has been directed to the effects of cancer on the cytoskeleton of the erythrocyte membrane (Kopczynski et al. 1998). The antioxidant enzymes, lipid peroxidation, spectrin, and reactive oxygen species (ROS) are the backbone of cellular antioxidant defense mechanisms (Naidu et al. 2007; Monnik et al. 2007). MDA (malondialdehyde) is an end product of erythrocyte membrane lipid peroxidation; accumulation of MDA may affect the lipid packing density and microviscosity of lipid bilayer which can be used as a characteristic signature of the red blood cell membrane and may be used for the diagnosis of cervical cancer. The current study attempts to investigate disease-induced modification of the erythrocyte membrane in vitro. This work explores the status of antioxidant enzymes, lipid peroxidation, red blood cell membrane carbonyl content, and protein profile in RBCs from cervical cancer patients. This is an original work reporting for the first time, the importance of the protein profile of RBC membrane which can be used as a characteristic signature of the red blood cell membrane and may be used for the diagnosis of cervical cancer.

METHODS AND MATERIALS

Subjects

Ninety-four newly diagnosed female patients aged 20–40 yrs with cervical cancer were selected for the study. The patients were divided into two groups: group A consisted of 47 patients with cervical cancer, and group B constituted of 47 age- and sex-matched normal subjects. Blood samples were collected by venous arm puncture from the patients with cervical cancer and normal subjects before the study. Blood samples were collected in 3 mL of the anticoagulant. EDTA was obtained from both the cervical cancer patients and the normal control subjects. Informed consent was obtained from all the subjects after the procedure was fully explained. Blood samples were centrifuged at 800 rpm for 15 min. The plasmas were stored for estimation of oxidative damage of human erythrocytes. Common laboratory chemicals used were of analytical grade or ultrapure. Plasma MDA was estimated by thiobarbituric acid reactive substances (TBARS). The lipid peroxidation density and microviscosity of lipid bilayer which can be used as a characteristic signature of the red blood cell membrane and may be used for the diagnosis of cervical cancer.

Keywords: antioxidants, lipid peroxidation, spectrin, MDA, malondialdehyde; ROS, reactive oxygen species; SOD, superoxide dismutase; CAT, catalase; GPx, glutathione peroxidase; SEM, standard error of mean; SOD, superoxide dismutase; CAT, catalase; GPx, glutathione peroxidase; SEM, standard error of mean; ROS, reactive oxygen species; E. Merck, BDH. Fine chemicals were obtained from Cipla Company (Mumbai, Central, India). Commercial chemicals were either from E. Merck or BDH. Fine chemicals were obtained from Cipla Company (Mumbai, Central, India).

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RESULTS

Among cervical cancer patients, 63% showed advanced stage of clinical progression of the disease. The study of carbonyl content, antioxidant enzymes, lipid peroxidation, membrane disorganization, and oxidative stress in erythrocytes which finally results in increased erythrocyte membrane fluidity and oxidized erythrocytes which may be used for the diagnosis of cervical cancer.

DISCUSSION

Cervical cancer is the primary cancer of the world, and poses a major public health problem. This paper explores the role of cancer in the disease pathogenesis (Gajalaksmi et al., 2006). A recent study by Dhar et al. (2006) has demonstrated that HPV type 16 is present in the cervical cancer tissues. The study of carbonyl content, antioxidant enzymes, lipid peroxidation, membrane disorganization, and oxidative stress in erythrocytes which finally results in increased erythrocyte membrane fluidity and oxidized erythrocytes which may be used for the diagnosis of cervical cancer.

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REFERENCES

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Original Research Paper

Disorganization of Human Erythrocyte Membrane

Sagarika Roychowdhury, Arpita Chakraborty, Maitree Bhattacharyya

INTRODUCTION

Cervical cancer is the third most common cancer of the world, and poses a major public health problem. This paper explores the role of cancer in the disease pathogenesis (Gajalaksmi et al., 2006). A recent study by Dhar et al. (2006) has demonstrated that HPV type 16 is present in the cervical cancer tissues. The study of carbonyl content, antioxidant enzymes, lipid peroxidation, membrane disorganization, and oxidative stress in erythrocytes which finally results in increased erythrocyte membrane fluidity and oxidized erythrocytes which may be used for the diagnosis of cervical cancer.

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saline solution (pH 7.4). To determine the activity of RBC antioxidative enzymes, hemolysate was prepared by lysing a known volume of erythrocytes with cold hypotonic (10 mM) phosphate buffer (pH 7.4) by centrifugation at 15,000 × g for 40 min. The hemolysate was used for determination of antioxidant enzyme activity.

**Estimation of protein carbonyl content**

Protein carbonyl content was measured by reaction with 2,4-dinitrophenyl hydrazine (DNPH) following the method of Levin and Garland (Levin et al. 1990). The results were expressed as nmols of carbonyl groups/mg proteins using a molar extinction coefficient of 22,000 for DNPH derivatives.

**Specific activity of antioxidant enzymes**

Superoxide dismutase (SOD, EC 1.15.1.1) activity was assayed according to the method of Marklund (Marklund et al. 1974) by measuring the percent inhibition of pyrogallol auto-oxidation. One unit of superoxide dismutase was defined as that amount of enzyme required inhibiting the auto-oxidation of pyrogallol by 50%. The specific activity was expressed as units mg⁻¹ of protein. Catalase (EC 1.11.1.6) activity was assayed according to the method of Beers and Sizer (Beers et al. 1952). The absorbance was measured at 240 nm for 1 min to monitor the breakdown of H₂O₂. The concentration of H₂O₂ was calculated using a molar extinction coefficient 43.6 mol⁻¹ cm⁻¹. Specific activity of catalase was expressed as μmole of H₂O₂ decomposed min⁻¹ mg⁻¹ of hemolysate protein.

**Peroxidase activity of hemoglobin**

Glutathione peroxidase (EC 1.11.1.9) activity was assayed according to the method of Paglia and Valentine (Paglia et al. 1967). The specific activity was calculated using the extinction coefficient 6.22 milli mol⁻¹ cm⁻³ for NADPH system and was expressed as μmol NADPH oxidized mg⁻¹ hemolysate protein min⁻¹.

**Preparation of erythrocyte membranes**

Venous blood samples were processed (Vincent 1967) by washing with 10 volumes of isotonic NaCl. The packed cells were lysed in 20 volumes of 5 mM Tris-HCl buffer containing EDTA, pH 7.4 and the ghost membranes were pelleted by centrifugation at 14,000 rpm for 30 min. The pellets were washed once with NaCl and EDTA to yield a milk-white suspension, which was stored at 4°C and studied within 48 hrs of preparation.

**Lipid peroxidation**

Lipid peroxidation in erythrocyte membrane was determined by assaying malondialdehyde (MDA) formation (Sinnhuber 1958). One volume of erythrocyte membrane was added to two volumes of 10% TCA (trichloroacetic acid), mixed and centrifuged. 1% TBA (thiobarbituric acid) was added to the transferred supernatant and boiled. The absorbance was noted at 535 nm and expressed as nmol of MDA formed/mg of membrane protein, using a molar extinction coefficient 1.56 × 10⁴ M⁻¹ cm⁻¹.

**Fluidity measurement**

Fluorescence polarization was measured (Shinizky 1978) in a Hitachi spectro-fluorimeter (F-3010) equipped with polarizers using an excitation wavelength of 365 nm and emission wavelength of 430 nm. A stock solution of 2 mM probe in tetrahydrofuran (THF) was injected with rapid stirring into 1 ml of PBS (pH 7.4) at room temperature. In this experiment, the erythrocyte membrane (100-200 μg of membrane protein) was incubated in PBS buffer containing 1 μM DPPI (1,6-diphenyl-hexa-1,3,5-triene) suspension for 2 hrs at 37°C. The fluorescent anisotropy, r, was used to calculate the apparent microviscosity, η, in absolute units of poise. The temperature range 5 to 40°C was maintained using a circulator multi-temperature water-bath attached to the spectrofluorimeter. The temperature dependence of fluidity was expressed by plotting the anisotropy parameter.

**Estimation of total protein content**

The amount of total protein content was estimated according to the method of Lowry et al. (1951). The standard protein Bovine Serum Albumin (BSA) was purchased from Sigma chemicals.

**SDS-PAGE**

The membrane protein profile was observed by SDS-PAGE in the discontinuous buffer system of Laemmli (1970), with an 8% separating gel and 3.5% of stacking gel under reducing conditions. Protein bands were visualized by staining with Coomassie Brilliant Blue. Densitometric profiles of stained SDS-PAGE gel was obtained with an Easy Gel Analysis Densitometer (Version 1.1). Protein bands were quantified in terms of total protein present in the profile. Membrane electrophoresis experiments for each subject were performed in duplicate.

**Statistical analysis**

The results are expressed as mean ± standard error. Differences between the groups were considered significant at P < 0.05. Groups of data were compared using the analysis of variance (ANOVA) followed by Scheffe’s method of multiple (Scheffe et al. 1959) comparisons. Statistical evaluation was performed by Statistica 6.0.

**RESULTS**

The baseline demographic characteristics like, age, body mass index, blood pressure, blood glucose, hemoglobin count of the subjects are presented in Table 1. Table 2 shows the level of carbonyl content, antioxidants in the circulation and in vitro lipid peroxidation in erythrocyte membranes of control and cervical cancer subjects indicating significant differences (P < 0.001) in the values for cervical cancer patients.

Anisotropy (r) values as a function of temperature has been recorded in Table 3. Fluorescence polarization was measured at range of temperature (40-5°C) in Hitachi spectrofluorimeter equipped with polarizer using an excitation wavelength of 365 nm and emission wavelength of 430 nm. Fluorescence anisotropy (Lakowicz 2006) was defined as $r = (I_{I+I})/[(I_{I+I} + 2I_{I})]$, where $I_{I}$ and $I_{I}$ are the fluorescence intensities detected through a polarizer oriented parallel and perpendicular respectively, to the direction of excited light. Viscosity is a function of temperature and for the liquid-like environment of the probe molecule, the functional relation is $\eta = \eta_0 e^{-K T}$ (Sheehan 2000), where $E$ is the activation energy. $E$ has been plotted against absolute temperature in Table 4 A maximum value of $E = 44.8 \times 10^{-2}$ Joules for control and $65.76 \times 10^{-2}$ Joules for cervix cancer patients. A significant reduction in the spectrum band (actin was used as a loading control) is seen in Fig. 1, where the gel pattern of erythrocyte membrane proteins of patients with cervical cancer is shown. No other significant qualitative change was evidenced except spectrin in protein fractions of erythrocyte membranes of cervical cancer subjects, though quantitative analysis (Fig. 2A, 2B) revealed decrease in some other membrane proteins of cancer patients.

<table>
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<th>Table 1 Baseline characteristics of the study subjects.</th>
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**Table 2**

Baseline characteristics of the study subjects.

| Age (Years) | 35 ± 2.9 | 42 ± 3.2 |
| Body mass index (Kg/m²) | 24.6 ± 2.3 | 18.6 ± 1.9 |
| Blood Pressure (mm Hg) | 120 ± 5.5/75 ± 3.2 | 95 ± 6.2/65 ± 2.8 |
| Blood glucose (mg/dl) | 98.5 ± 4.3 | 90 ± 3.8 |
| Haemoglobin(g/dl) | 12.6 ± 2.2 | 7.8 ± 1.8 |
| No of issues | < 3     | > 3       |

**Table 3**

Baseline characteristics of the study subjects.

| Number (n)  | 95      | 94             |
| Age (Years) | 35 ± 2.9 | 42 ± 3.2     |
| Body mass index (Kg/m²) | 24.6 ± 2.3 | 18.6 ± 1.9 |
| Blood Pressure (mm Hg) | 120 ± 5.5/75 ± 3.2 | 95 ± 6.2/65 ± 2.8 |
| Blood glucose (mg/dl) | 98.5 ± 4.3 | 90 ± 3.8    |
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| No of issues | < 3     | > 3            |

**Table 4**

Baseline characteristics of the study subjects.
DISCUSSION

In developing countries cervical cancer is common threat of mortality (Beeli et al., 2007; Choconta-Piraquive et al., 2010). The growing risk of cervical cancer in women in India (aged 0-64 years) is 2.4% compared to 1.3% for the world (Kapoor, 2008). Several ongoing research in this field revealed that free radical-mediated oxidative stress is directly implemented with disease pathogenesis of cervical cancer (Lee, 2005).

Oxidative stress is imposed on cells as a result of one of the three factors: an increase in oxidant generation, a decrease in antioxidant protection, or a failure to repair oxidative damage (Srivastava, 2009). Increased oxidative stress is associated with a variety of pathological conditions resulting in irreversible cell damage, which has also been observed to be associated with human cancer and animal cancer models (Chen et al., 2000). Decreased activity of catalase indicates the erythrocyte to be in stressed condition, when complete removal of H2O2 is not possible. Decreased antioxidant protection has been evidenced in this work as reduced enzymatic activities of GPx, SOD, and CAT is observed in the red blood cell of cervical cancer patients compared to healthy controls (Table 2). Similar observation was also reported by Balasubramanijyan et al. (1994) and Kumar et al. (1995) in cervical as well as in invasive cancer. GPx utilizes the reducing equivalents of glutathione to reduce H2O2; and it is possibly the main mechanisms for protection against the deleterious effects of hydroperoxides (Saroja et al., 2000; Pejic et al., 2006); Catalase plays a major role for protecting erythrocyte from oxidative stress. Low levels of SOD and CAT described for tumors are regarded as markers of malignant-transformation. Thus low levels of GPx, and SOD in the circulation of cervical cancer patients possibly account for increased utilization of these enzymes to scavenge lipid peroxides (Manaharan et al., 2004). This can justify for the accumulation of superoxide anion, a highly diffusible and potent oxidizing radical capable of traversing through membranes, causing deleterious effects at sites far from tumor.

Lipid peroxidation is an important indicator of membrane damage, which serves to promote irreversible dys-
function of essential cellular components and ultimately triggers accidental cell death and necrosis (Kolwattowski et al. 1999; Bartsch 2006). Observed enhancement of lipid peroxides (Table 2) in cervical cancer patients is closely associated with the decline in GSH, SOD and CAT activity. A high concentration of MDA in the erythrocyte leads to membrane ‘gap’ formation (Lubin 1972) and malignant lymphoma (Abou-Seif et al. 2000). Diminished activities of peroxidase scavenger enzymes observed in cervical cancer patients supports the idea that free radical species could result in unscheduled oxidation of proteins. An increase in plasma RCD (DNP=reactive carbonyl derivatives) was revealed by elevated protein carbonyl content in the plasma of the subjects (Table 1).

Erythrocyte membrane is a supra-molecular structure with many molecules organized through non-covalent interactions into a higher order structure and emergent properties. Table 3 represents the anisotropy curve of erythrocyte membrane in cervical cancer patients compared to control subjects within the temperature range 5-40°C. The degree of fluidity of a membrane depends on temperature and membrane composition. At low temperature, lipids are in a gel-crystalline state, while lipids are restricted in their mobility. As temperature is increased, there is a phase transition into a liquid-crystalline state, with an increase in fluidity (Bhosle 2002; Karp 2002). Table 4 describes the change of activation energy of the erythrocyte membrane and as the temperature is lowered, control and diseased erythrocytes differ at their transition temperatures from solid to gel phase, the values being 35 and 25°C, respectively.

Fig. 1 represents the SDS-PAGE of erythrocyte membrane proteins in diseased and control subjects. Haest suggested that spectrin plays an important role in the maintenance of phospholipids asymmetry in human erythrocyte membranes (Haest et al. 1978). It joins the membrane phospholipids by ankyrin, actin and 4.1-band protein and affects hydrophobic zone lipid bilayer settlement (Manno et al. 1995; Baines 2010). Quantitative value of spectrin is observed to be decreased significantly as evidenced by densitometry analysis of erythrocyte membrane proteins (Fig. 2A, 2B). Quantitative changes of membrane proteins in erythrocyte cytoskeleton may affect the normal membrane organization and erythrocyte stability (Baines 2009). It can be attributed from our experiments that cytoskeletal protein, spectrin is the most adversely degraded in diseased condition. A generalized decrease of other cytoskeletal proteins has also been witnessed that confirms the fact that erythrocyte membrane is subjected to stress conditions in cervical cancer.

This study unfolds the fact that, elevated MDA production and insufficient antioxidant potential is responsible for the increased erythrocyte membrane fluidity (decreased fluorescence anisotropy) and alteration in phase transition temperature in the RBC of cervical cancer subjects. Experiments also explore the unique finding, that cervical cancer induced oxidative stress results in significantly decreased quantity of the heterodimeric protein spectrin in red blood cell membrane cytoskeleton. Thus we infer that dynamic behaviour of erythrocyte bilayer membrane is altered by cervical cancer. The concentration of spectrin protein may be identified as a characteristic signature of bilayer membrane of a specific source and so that can be used as a marker for diagnosis of the disease.

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