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Cervical Cancer-Induced Structural Disorganization of Human Erythrocyte Membrane

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ABSTRACT

Cervical cancer is the fifth most common cancer of the world, and poses a major public health problem. This paper explores a *de novo* observation that cervical cancer induced oxidative stress is responsible for erythrocyte membrane disorganization in the patients of advanced stage of clinical progression of the disease. The study of carbonyl content, antioxidant enzymes, lipid peroxidation, membrane fluidity and SDS-PAGE of erythrocyte membrane protein has been conducted on 94 adult cervical cancer patients and an equal number of age and sex matched normal subjects. Lipid peroxidation of erythrocyte membrane is observed to be enhanced and antioxidant enzyme activity alters significantly in the pathologic samples. Increased membrane fluidity is indicated by analysis of fluorescence depolarization using 1,6 diphenyl-1,3,5 hexatriene compared to healthy controls. The transition temperature of membrane lipids from gel to sol phase transition is observed to be shifted from 35°C (control subjects) to 25°C (cervical cancer patients). Degradation of the spectrin band is evidenced in SDS-PAGE of the membrane protein profile of the diseased subjects. It can be elucidated that cervical cancer induces oxidative stress in erythrocytes which finally results in increased erythrocyte membrane fluidity, altered phase transition temperature and modified protein profile. 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 membranes and may be used for the diagnosis of cervical cancer.

Keywords: antioxidants, lipid peroxidation, spectrin

Abbreviations: CAT, catalase; **GPx**, glutathione peroxidase; **GSH**, reduced glutathione; **MDA**, malondialdehyde; **ROS**, reactive oxygen species; **SDS-PAGE**, sodium dodecyl sulfate-polyacryl-amide gel electrophoresis; **SEM**, standard error of mean; **SOD**, superoxide dismutase; **TBARS**, thiobarbituric acid reactive substances

INTRODUCTION

Cervical carcinoma, is the fifth most common cancer in women worldwide and in less developed countries, this type of cancer is the second most frequent in women (Parkin *et al.* 2005; Cervical Cancer and HPV- Information 2008; Tong *et al*. 2009). This is the main cause of cancer mortality in developing countries, posing a major public health problem (Pisani *et al.* 1999; Mandic *et al*. 2005). It is a malignant neoplastic disease that tends to begin slowly when there is disruption of the cervical epithelium. Histologically, 85-90% of cervical cancers are of squamous of cell origin, with most of the remainder being adenocarcinomas. Infections with the human papilloma virus (HPV) have been suggested as a major risk factor of cervical cancer (Dalstein *et al.* 2003), but an early age of intercourse, multiple sexual partners and low socio-economic status may also have a role in the disease pathogenesis (Gajalaksmi *et al.* 1996; Castellsagué and Muñoz 2003). Molecular mechanisms involved in progressive stages of cervical cancer, are quite unknown yet.

The erythrocyte membrane provides 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 superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) are the backbone of cellular antioxidant defense mechanisms (Naidu *et al.* 2007; Woźniak *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 following *in vitro* oxidative damage of human erythrocytes (Debouzy *et al.* 1992; Looi *et al.* 2008).

This study attempts to investigate disease-induced structural modification of the erythrocyte membrane in patients with cervical cancer. This work explores the status of antioxidant enzymes, lipid peroxidation, red blood cell membrane fluidity and gel electrophoresis pattern of the membrane cytoskeleton proteins which have never been reported earlier.

MATERIALS AND METHODS

Subjects

Ninety four newly-diagnosed female patients aged 20-40 yrs. with malignancy in the cervix region attending the Department of Gynecology and Obstetrics, Calcutta Medical College and Hospital, Kolkata were selected for the study. An equal number of age-matched healthy subjects were also selected. Informed consent was obtained from both the cervical cancer patients and the normal subjects before the study.

Common laboratory chemicals used were of analytical grade and purchased either from E. Merck or BDH. Fine chemicals were obtained from Cipla Company (Mumbai, Central, India).

Samples

Blood samples were collected by venous arm puncture from the cervical cancer patients pre-operatively into heparinized tubes. Blood plasma was separated by centrifugation of blood sample at 3000 × *g* for 15 min. The plasma was stored for estimation of protein carbonyl content. The buffy coat was discarded and the packed RBCs were washed with twice volume isotonic (15 mM)

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saline solution (pH 7.4). To determine the activity of RBC antioxidant enzymes, hemolysate was prepared by lysing a known volume of erythrocytes with cold hypotonic (10 mM) phosphate buffer (pH 7.4) by centrifugation at 15000 × *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 nmoles 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 H_2O_2. The concentration of H_2O_2 was calculated using a molar extinction coefficient 43.6 mol^{-1} cm^{-1}. Specific activity of catalase was expressed as µmole of H_2O_2 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^{-1} 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-HCI 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 1.56×10^5 M^{-1} cm^{-1}.

Fluidity measurement

Fluorescence polarization was measured (Shinitzky 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 DPH (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 circulatory multi-temperature water-bath attached to the spectrofluorimeter. The temperature dependence of fluidity was expressed by plotting the anisotropy parameter.

Table 1 Baseline characteristics of the study subjects.

Particulars	Control	Cervical cancer
Number (n)	95	94
Age (Years)	35 ± 2.9	42 ± 3.2
Body mass index (Kg/m^2)	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(gm%)	12.6 ± 2.2	7.8 ± 1.8
No of issues	< 3	> 3

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 $r = (I_{II}-I_I)/(I_{II} + 2I_I)$, where I_{II} 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 $\eta = \eta_0 e^{E/KT}$ (Sheehan 2000), where E is the activation energy. E has been plotted against absolute temperature in **Table 4** A maximum value of E was 44.8×10^{-22} Joules for control and 65.76×10^{-22} Joules for cervix cancer patients.

Significant reduction in the spectrin 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 2 The level of carbonyl content, antioxidants in the circulation and *in vitro* lipid peroxidation.

Type of patients	Carbonyl content [a]Units/ mg of plasma	Glutathione peroxidase [b]Units/ mg of hemolysate protein	Superoxide dismutase [c]Units/ mg of hemolysate protein	Catalase [d]Units/ mg of hemolysate protein	Lipid peroxidation in membrane [e]Units/ mg of membrane protein
Control	2.85 ± 0.15	6.72 ± 0.11	2.75 ± 0.175	5.6 ± 0.467	1.6 ± 0.068
Cervical cancer	3.45 ± 0.20	3.56 ± 0.119 *	1.37 ± 0.127 *	3.26 ± 0.282 *	3.02 ± 0.195 *

Comparative study of the carbonyl content, activity of glutathione peroxidase, superoxide dismutase, catalase and amount of MDA produced (mean ± SEM, n =94). *Significantly different from healthy subjects: p< 0.001, n = total number of subjects in each group.[a] nanomol of carbonyl formed/ mg of plasma protein, [b]µmoles of NADPH oxidized/min/mg of hemolysate protein, [c]amount of enzyme required to inhibit the auto-oxidation of pyrogallol by 50%, [d] nmoles of H_2O_2 decomposed min^{-1}, [e]nmoles of MDA formed/ mg of membrane protein.

Table 3 Representation of fluorescence anisotropy (r) as a function of temperature (5-40ºC) in DPH-probed erythrocyte membrane. Erythrocyte membrane containing 200µg of protein (n = 94).

Temperature (ºC)	5	10	15	20	25	30	35	40
Control	0.285 ± 0.009	0.280 ± 0.008	0.270 ± 0.007	0.260 ± 0.008	0.190 ± 0.009	0.230 ± 0.007	0.280 ± 0.006	0.270 ± 0.007
Cervical cancer	0.301 ± 0.008	0.257 ± 0.008	0.265 ± 0.009	0.267 ± 0.006	0.310 ± 0.006	0.280 ± 0.005	0.230 ± 0.006	0.250 ± 0.005

Table 4 Representation of activation energy (E)[a] as a function of temperature in DPH- probed erythrocyte membrane.

Absolute temperature[b]	3.19	3.25	3.3	3.36	3.41	3.47	3.53
Control	40 ± 1.1	45 ± 1.4	17 ± 0.9	4 ± 0.5	21 ± 0.9	24 ± 0.9	19.5 ± 0.7
Cervical cancer	48 ± 0.9	15.8 ± 1.0	47 ± 1.7	68 ± 1.9	22 ± 1.0	15.5 ± 0.6	13 ± 0.5

[a] The unit of activation energy is expressed as Joules (value x 10^{22}). [b]Absolute temperature (ºK) and expressed as 1/T x 10^3(K^{-1}).

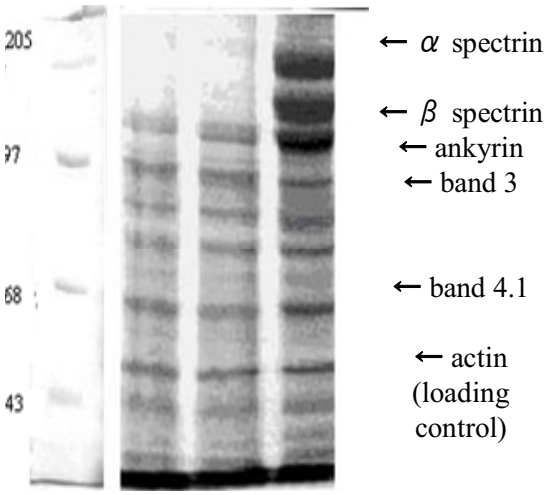

Fig. 1 SDS-polyacrylamide (8%) gel electrophoresis of erythrocyte membrane proteins. 100 µg of protein was applied to each lane. Gel was stained with Coomassie Blue. A and B: erythrocyte membrane proteins of cervical cancer; C: normal control erythrocyte membrane proteins, M: Marker Protein. Actin protein is present in all the experimental sets (cervical cancer and control) in equal amount and considered as loading control. The respective proportion of spectrin protein is calculated and normalized based on the amount of loading control.

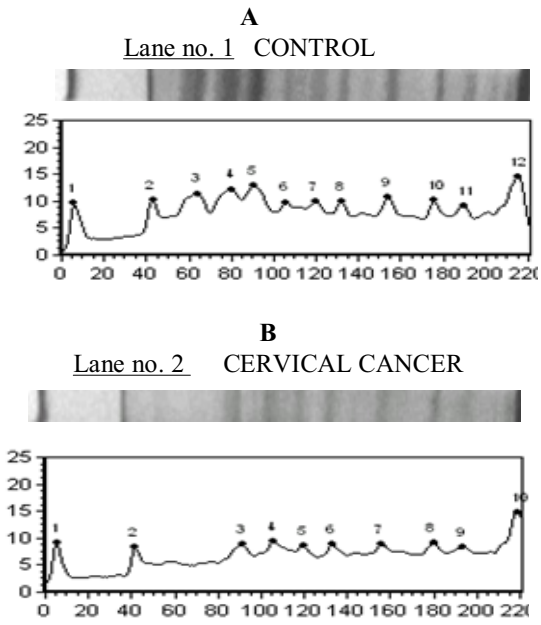

Fig. 2 Densitometry scan of the SDS-PAGE protein separations. The peaks represent the following proteins: (**A**) Control: 3, Spectrin (α); 4, Spectrin (β); 5, Ankyrin; 6, band 3; 8, band 4.1; 11, Actin. (**B**) Patient: 3, Ankyrin; 4, band 3; 6, band 4.1; 9, Actin.

DISCUSSION

In developing countries cervical cancer is common threat of mortality (Beevi *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 H_2O_2 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 Balasubramaniyan *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 H_2O_2 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 GP_x 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 indication of membrane damage, which serves to promote irreversible dys-

function of essential cellular components and ultimately triggers accidental cell death and necrosis (Kolwaltowski *et al.* 1999; Bartsch 2006). Observed enhancement of lipid peroxides (**Table 2**) in cervical cancer patients is closely associated with the decline in GP_x, SOD and CAT activity. A high concentration of MDA in the erythrocyte leads to membrane 'gap' formation (Lubin *et al.* 1981) and raises cell susceptibility to hemolysis. Elevated erythrocyte membrane lipid peroxidation in relation to disease pathophysiology has been reported in certain hemolytic anemias (Shohet 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 (DNPH-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, when 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 the disease.

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