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Modulatory Action of α-Tocopherol on Erythrocyte Membrane Adenosine Triphosphatase against Radiation Damage in Oral Cancer

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Abstract To investigate the possible effects of α-tocopherol on erythrocyte membrane adenosine triphosphatases against radiation damage in oral cancer patients. Adenosine triphosphatase activities were analysed in oral cancer patients before and after radiotherapy (at a dosage of 6000 cGY in five fractions per week for a period of six weeks) and after supplemented with α-tocopherol (400 IU per day for entire period of radiotherapy). The membrane bound enzymes such as Na^+/K^+-ATPase, Ca^{2+}-ATPase, Mg^{2+}-ATPase and some trace elements were altered in oral cancer patients before and after radiotherapy. Supplemented with α-tocopherol modulates the erythrocyte membrane which is damaged by radiotherapy which suggests that α-tocopherol protects the erythrocyte membrane from radiation damage in oral cancer patients.

Keywords ATPases · Biochemistry · Structure/function · Na^+/K^+-ATPase · Ion channels

Introduction

Oral cancer is the sixth most common cancer worldwide (Parkin et al. 1993). In India, oral cancer comprises 30–40% of the total malignancies and squamous cell carcinoma of the oral cavity is primarily a disease of older men with a long history of tobacco and/or alcohol use (Jayant and Notani 1991). Radiation therapy is the most commonly used therapeutic modality for inoperable oral cancer. The effects of radiation therapy are mediated by the production of free radicals. Membranes are vital for biological systems, and their integrity is essential for normal cell functions. Radiation-induced free radicals produce peroxidation of lipids, leading to structural and functional damage to cellular membranes (Purohit et al. 1980). The damage to membrane organization is an initial step in cell death. During radiotherapy, formation of hydroperoxides in membranes would result in the damage of membrane-bound enzymes. Accumulation of lipid peroxides induces hydrophilic moieties to enter membrane hydrophobic phase and, thus, alters membrane permeability and cell function (Maggio et al. 1997).

Na^+/K^+-ATPase is an important regulator of intracellular electrolyte levels in almost all mammalian cells. The Mg^{2+}-dependent P-type transport pump is responsible for maintaining the low intracellular Na^+/K^+ ratio that is essential for cell homeostasis and physiological function. It catalyzes the active uptake of K^+ and extrusion of Na^+ at the expense of hydrolyzing ATP with a stoichiometry of $3Na^+$ for $2K^+$. The active form of Na^+/K^+-ATPase is primarily composed of two noncovalently attached subunits, a 110 kDa catalytic α-subunit and a 45–55 kDa glycosylated β-subunit (Mobasheri et al. 2003). The active transport of Na^+/K^+ across the cell membrane is controlled by Na^+/K^+-ATPase, which is an integral membrane protein. Na^+/K^+-ATPase activity is responsible for a large part of the energy consumption that constitutes the basic metabolic rate (Mobasheri et al. 2003).

Ca^{2+} is a unique metal ion, with a role in the physiological and biochemical regulation of a variety of cellular

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functions (Gupta and Kale 1995). Ca^{2+}-ATPase was first reported in human red blood cells (Dunham and Glynn 1961; Schatzmann 1966). The plasma membrane Ca^{2+}-ATPase is an essential regulator of free intracellular calcium. To elucidate the contribution of plasma membrane Ca^{2+}-ATPase to oral carcinogenesis and genetic and epigenetic changes, we analyzed mRNA and protein expression in primary oral squamous cell carcinomas, oral premalignant lesions and colon cancer cell line differentiation (Kuo et al. 2009). Magnesium, an abundant cation in cells, is involved in many biological functions. ATP strongly binds to Mg^{2+} and exists as an Mg–ATP complex in cells. Moreover, it has been known that intracellular Mg^{2+} concentration changes in response to extracellular stimulus and, in some cases, intracellular Mg^{2+} change is accompanied by intracellular Ca^{2+} change (Gotoh et al. 1999). The Mg^{2+} stores in cells have not been identified yet, but there are three candidates: endoplasmic reticulum, mitochondria and Mg–ATP.

Determination of membrane-associated enzyme activities indicates changes in the membrane, under special nutritional or pathological conditions. Vitamin E behaves as a biological antioxidant and preserves membrane integrity and fluidity; it also protects membranes from oxidative injuries. Peroxidation of membrane lipids initiates a loss of membrane-bound enzyme activity and cell lysis (Chen et al. 2000). Therefore, the present investigation was undertaken to study the effects of α-tocopherol on membrane-bound enzymes in oral cancer patients before and after radiotherapy.

Materials and Methods

Stage II, III and IV oral cancer patients admitted as inpatients in the Department of Oral Oncology, Government Arignar Anna Memorial Cancer Research Institute and Hospital, Kancheepuram, Tamil Nadu, India, were included in this cancer clinical trial. Histologically proven samples were taken for the present study. Oral cancer patients (age mean ± SD = 50 ± 15 years, 37 males and 23 females) had cancer at various sites, such as the cheek ($n = 20$), alveolus ($n = 13$), tongue ($n = 9$), floor of the mouth ($n = 8$), lip ($n = 5$), palate ($n = 2$) and retromolar trigone ($n = 3$). Some patients had a habit of chewing tobacco ($n = 28$), smoking tobacco ($n = 21$) or consuming alcohol ($n = 11$). Tumor staging was according to the TNM classification of the UICC: I ($n = 0$), II ($n = 10$), III ($n = 21$) and IVA ($n = 29$). Blood was collected from 35 normal healthy volunteers (19 males, 16 females). The mean age ± SD of the healthy group was 50 ± 12 years (Table 1). Normal controls were not suffering from any inflammatory diseases, had no illness and had taken no medication in the recent past. All patients gave informed consent prior to inclusion in the study, and the medical practitioner monitored the whole experiment. Studies were performed in accordance with the ethical standards of the hospital.

The present study consisted of four groups: group 1, healthy volunteers (control, $n = 35$); group 2, oral cancer patients ($n = 60$), randomly subdivided into two groups (i.e., groups 3 and 4); group 3, oral cancer patients treated with radiation alone ($n = 30$); and group 4, oral cancer patients treated with radiation and simultaneously supplemented with α-tocopherol ($n = 30$) during the entire period of radiotherapy. Radiation treatment was given with a telecobalt beam using anterior and lateral wedge pairs or lateral parallel portals (Gammatron-60CO, Theraton-780-60CO, Phoenix-60CO; Siemens, Malvern, PA) at a dosage of 6,000 cGy in five fractions per week for a period of 6 weeks. α-Tocopherol (Bio-E capsules; American Remedies, Chennai, India) was given at a dosage of 400 IU/day for 6 weeks (Vatassery et al. 1999) from the first day of radiation to the end of the treatment period in group 4 patients.

Table 1 Different demographic variables and stages of oral cavity cancer patients in the study samples

Demographic variables	Cancer patients, total ($n = 60$) (%)
Sex	
Male	37 (62)
Female	23 (38)
Age (years)	
Normal healthy controls	35
<45	11 (18)
45–60	33 (55)
>60	16 (27)
Site of occurrence	
Cheek	20 (34)
Alveolus	13 (22)
Tongue	9 (15)
Floor of the mouth	8 (13)
Lip	5 (8)
Palate	2 (3)
Retromolar trigone	3 (5)
Lifestyle habits	
Chewing tobacco	28 (47)
Smoking tobacco	21 (35)
Alcohol consumption	11 (18)
Tumor staging	
II	10 (17)
III	21 (35)
IVA	29 (48)

Isolation of RBC Membranes

An erythrocyte membrane was isolated according to the procedure of Dodge et al. (1963) with slight modification in buffer composition. Blood was collected with EDTA as an anticoagulant. Plasma was separated by centrifugation at 1,500×*g* for 15 min. Packed cells were washed with isotonic saline. After washing with saline, packed cells were listed by suspending in hypotonic Tris–HCl buffer (5 mM Tris and 15 mM sodium chloride) for 1 h. Lysed cells were centrifuged at 15,000×*g* for 30 min. The supernatant red fluid was decanted and the cell pellet washed with hypotonic buffer three times. Ghosts were colorless or pale pink, which led to further analysis. Ghosts prepared by this procedure contain <1% of the original cellular hemoglobin.

Assay of Na^+/K^+-ATP

Na^+/K^+-dependent ATPase activity was assayed by the method of Bonting (1970). Briefly, 0.2 ml of 50 mM $MgSO_4$, 600 mM NaCl, 50 mM KCl, 1 mM EDTA and 40 mM ATP were added to 1 ml of Tris–HCl buffer (90 mM, pH 7.5) and 0.2 ml of enzyme preparation and the reaction mixture was incubated for 15 min at 37°C. The reaction was arrested with 1 ml of 10% TCA and centrifuged at 1,500×*g* for 10 min, and the supernatant was used for estimation of phosphorus content by the method of Fisk and Subbarow (1925).

Assay of Ca^{2+}-ATP

Ca^{2+}-dependent ATPase activity was assayed by the method of Hjerten and Pan (1983). Briefly, 0.1 ml of 50 mM $CaCl_2$, 10 mM ATP and enzyme preparation were added to 1 ml of Tris–HCl buffer (125 mM, pH 8.0) and the reaction mixture was incubated for 15 min at 37°C. The reaction was arrested with 1 ml of 10% TCA and centrifuged at 1,500×*g* for 10 min, and the supernatant was used for estimation of phosphorus content by the method of Fisk and Subbarow (1925).

Assay of Mg^{2+}-ATP

Mg^{2+}-dependent ATPase activity was assayed by the method of White and Ralston (1980). Briefly, 0.1 ml of 25 mM $MgCl_2$, 10 mM ATP and enzyme preparation were added to 1 ml of Tris–HCl buffer (375 mM, pH 7.6) and the reaction mixture was incubated for 15 min at 37°C. The reaction was arrested with 1 ml of 10% TCA and centrifuged at 1,500×*g* for 10 min, and the supernatant was used for estimation of phosphorus content by the method of Fisk and Subbarow (1925).

Activity of Total ATP

Total ATP activity was determined by the method of Evans (1969). Briefly, 0.1 ml of 100 mM $MgSO_4$, NaCl, KCl, $CaCl_2$, 1 mM ATP and enzyme preparation were added to 1.5 ml of Tris–HCl buffer (100 mM, pH 7.4) and the reaction mixture was incubated for 15 min at 37°C. The reaction was arrested with 1 ml of 10% TCA and centrifuged at 1,500×*g* for 10 min, and the supernatant was used for estimation of phosphorus content by the method of Fisk and Subbarow (1925). Membrane protein was estimated according to the method of Lowry et al. (1951). Enzyme activity in erythrocyte membranes was expressed in micromoles of Pi liberated per hour per milligram protein.

Analysis of Trace Elements

Calcium, magnesium, sodium and potassium were estimated using the Perkin-Elmer (Norwalk, CT) 2380 atomic absorption spectrophotometer.

Results

The demographic variables of the patients are given in Table 1. The activities of Na^+/K^+-ATPase, Ca^{2+}-ATPase (Fig. 1), Mg^{2+}-ATPase and total ATPases (Fig. 2) were significantly decreased ($P < 0.001$) in oral cancer and radiation-treated patients. Alterations in these levels were noticed in the α-tocopherol-supplemented group.

Levels of sodium and magnesium were significantly decreased in oral cancer patients ($P < 0.01$) and radiation-treated patients ($P < 0.001$), whereas potassium and calcium

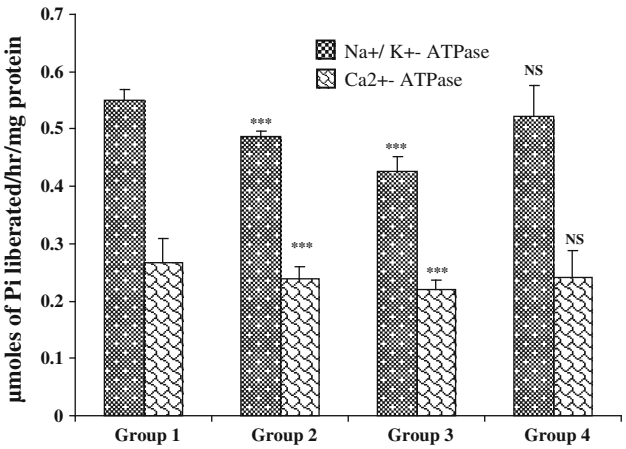

Fig. 1 Activities of Na^+/K^+-ATPase and Ca^{2+}-ATPase in experimental groups. *Group 1* healthy control; *group 2* oral cancer patients before radiation treatment; *group 3* oral cancer patients treated with radiotherapy; *group 4* α-tocopherol supplementation. Statistically significant values are compared with group 1 and expressed as ***$P < 0.001$; *NS* nonsignificant

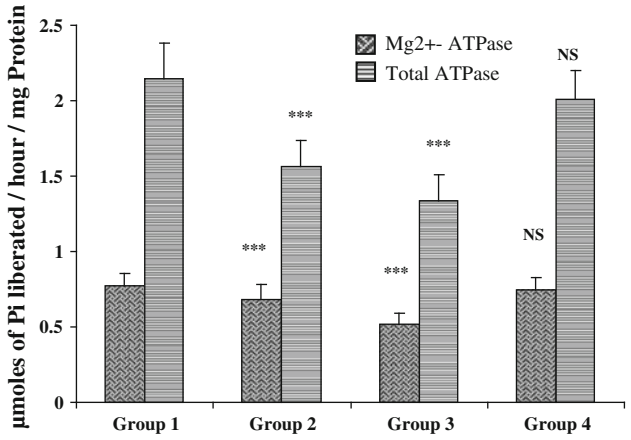

Fig. 2 Activities of Mg^{2+}-ATPase and total ATPase in experimental groups. *Group 1* healthy control; *group 2* oral cancer patients before radiation treatment; *group 3* oral cancer patients treated with radiotherapy; *group 4* α-tocopherol supplementation. Statistically significant values are compared with group 1 and expressed as ***$P < 0.001$; *NS* nonsignificant

levels were significantly increased in oral cancer ($P < 0.05$) and radiation-treated patients ($P < 0.01$) compared to normal healthy individuals. In group 4 patients these levels were maintained at near control values (Table 2).

Discussion

The erythrocyte has frequently been used to study radiation-induced membrane damage in oral cancer. Na^+/K^+-ATPase activity is responsible for a large part of the energy consumption that constitutes the basic metabolic enzyme activity and indicates a change in the membrane under special nutritional (Bonting 1970) or pathological (Masugi et al. 1988) conditions. Peroxidation of membrane lipid initiates a loss of membrane-bound enzyme activity and alters membrane permeability and cell function (Verma and Nair 1999). Active transport of Na^+ and K^+ across the cell membrane is controlled by Na^+/K^+-ATPase, which is an integral membrane protein. Red cell membrane Na^+/K^+-ATPase activity is considered an index of Na^+ pump action which restores the normal ionic concentration inside and outside the cells, thus assuming an important role in regulating the osmotic balance of red cells. The reduced activity of Na^+/K^+-ATPase indicates changes in the membrane under pathological conditions (Kometiani et al. 2005).

The activity of Na^+/K^+-ATPase was decreased in group 2 and group 3 patients, which was attributed to an increase in membrane cholesterol (Papahadjopoulos et al. 1973). Diminished red cell Na^+/K^+-ATPase activity correlates well with decreased red cell life span (Duran et al. 2007). A protein moiety of Na^+/K^+-ATPase has been modified by free radicals during radiotherapy in oral cancer patients. It is well known that as a result of oxidative stress the membrane loses this component, resulting in decreased erythrocyte deformability (Haas et al. 2000) and cell lysis. Another possible explanation is that removal of Na^+ depolarizes the membrane, causing increased permeability to Ca^{2+} as well as release of membrane-bound calcium (Saito et al. 2007). Mg^{2+} dependence on Ca^{2+} release from the sarcoplasmic reticulum induced by sevoflurane or halothane in skeletal muscle from humans susceptible to malignant hyperthermia has been reported (Duke et al. 2006).

Activities of Ca^{2+}-ATPase were decreased in oral cancer patients before and after radiotherapy, and this might be due to Ca^{2+}-ATPase being inhibited by lipid peroxidation or reactive oxygen species formed during these pathological conditions. The controlled Ca^{2+} translocation via Ca^{2+}-ATPase becomes inoperative due to inactivation of the enzyme, probably by oxidation of critical thiol groups after GSH depletion (McNeil et al. 1999). GSH depletion was noticed before and after radiotherapy in oral cancer patients (Chitra and Shyamaladevi 2008). In addition, since Ca^{2+} is known to regulate several biochemical processes, it is quite possible that some of these might also be involved in the modification of lipid peroxidation (Saito et al. 2006; Haugen and Drevon 2007). Diminished membrane Na^+/K^+-ATPase and Ca^{2+}-ATPase activities might be due to decreased Mg^{2+} levels in cancer patients (Mobasheri et al. 2003).

Table 2 Levels of electrolytes in experimental groups

	Sodium (meq/l)	Potassium (meq/l)	Calcium (meq/l)	Magnesium (meq/l)
Group 1	145.9 ± 12.9	4.52 ± 0.34	9.40 ± 0.92	2.64 ± 0.18
Group 2	141.7 ± 13.8**	4.96 ± 0.35*	11.0 ± 1.32*	2.33 ± 0.21**
Group 3	129.2 ± 12.6***	5.08 ± 0.41**	11.7 ± 1.28**	1.94 ± 0.17***
Group 4	143.2 ± 14.1^{NS}	4.82 ± 0.39^{NS}	10.6 ± 1.04*	2.12 ± 0.20*

Group 1 healthy control; *group 2* oral cancer patients before radiation treatment; *group 3* oral cancer patients treated with radiotherapy; *group 4* α-tocopherol supplementation

Statistically significant values are compared with group 1 and expressed as * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; *NS* nonsignificant

Vitamin E is presumed to be associated with intracellular membranes. A randomized trial of antioxidant vitamins reported prevention of acute adverse effects of radiation therapy in head and neck cancer patients (Ladas et al. 2004; Coleman et al. 2004). The alterations in the activities of Na^+/K^+-ATPase and Ca^{2+}-ATPase in group 4 patients could be due to the ability of α-tocopherol to protect the "SH" group from oxidative damage through inhibition of membrane lipid peroxidation (Bairati et al. 2005). Thus, vitamin E, which prevents lipid peroxidation in erythrocyte membranes, can retain the phospholipid composition of the membrane, which in turn can maintain the activities of membrane-bound enzymes in α-tocopherol-supplemented patients. Vitamin E, which inhibits the activation of phospholipase A_2, could have maintained the membrane phospholipid content and thereby restored the activities of membrane ion pumps (Kumar et al. 2002). Vitamin E improves Na^+/K^+-ATPase and Ca^{2+}-ATPase activities and thereby increases intracellular Mg^{2+} levels (Lourdes et al. 2009) and Mg^{2+}-ATPase activity in group 4 patients.

Potassium levels were elevated in oral cancer and radiation-treated patients. Cells with increased sensitivity to oxidative stress are more susceptible to damage by ionizing radiation than normal cells, causing a significant potassium leakage (Geetha et al. 1989). Radiation-induced hemolysis causes increased potassium release (Inouye et al. 1979). Potassium was maintained at control levels in group 4 patients, possibly due to α-tocopherol inhibiting both hemolysis and potassium release from erythrocytes (Inouye et al. 1979).

Hypercalcemia is a late manifestation of advanced squamous cell carcinoma of the head and neck and is an ominous prognostic sign (Dorman et al. 1998). The levels of calcium in serum were increased in oral cancer and radiation-treated patients. Ca^{2+} is a unique metal ion that plays an important role in the physiological and biochemical regulation of a variety of cellular functions involved in cell damage and cell death (Gupta and Kale 1995). Radiation increases plasma calcium levels initially, with subsequent diminution, while plasma Mg^{2+} decreases levels (Sarkar et al. 1982). α-Tocopherol is able to maintain bone calcium content (Norazlina et al. 2000). Ca^{2+} levels were maintained in group 4 patients.

Decreased serum Mg^{2+} levels were observed in oral cancer and radiation-treated patients. Cancer induces Mg^{2+} disturbances, which cause Mg^{2+} loading in tumor tissue, possibly due to Mg^{2+} mobilization through blood cells (Mak et al. 1994). A decrease in serum Mg^{2+} has been demonstrated to enhance platelet reactivity. Radiation enteritis increases fecal Mg^{2+} loss, cancer cachexia and urinary Mg^{2+} loss (Durlach 1988). Mg^{2+} may inhibit blood glutathione (Durlach 1988). The lowered Mg^{2+} levels may also have played an important role in the low levels of blood glutathione in radiation-treated patients (Chitra and Shyamaladevi 2008). α-Tocopherol protects mucosa against acute radiation-induced enteritis (Empey et al. 1992) and, thus, could prevent magnesium loss. The present study correlates well with the above findings after α-tocopherol treatment in oral cancer patients undergoing radiotherapy. This protection afforded to RBCs may have a number of physiological implications with respect to RBC function. Hence, the present findings show that vitamin E has a modulatory effect on membrane-bound enzymes in radiation-treated oral cancer patients.

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