

Lipid Peroxidation and Antioxidant Status in Human Cervical Carcinoma

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ABSTRACT: Reactive oxygen species (ROS), represented by superoxide, hydrogen peroxide and hydroxyl radicals, have been implicated in many diseases including cancer. ROS have been known to play an important role in the initiation and promotion of multistep carcinogenesis. The cellular antioxidants play a crucial role in protection against neoplastic disease. However, very little is known about the antioxidant defense in cervical carcinoma. This is addressed in the present study. Lipid peroxides, glutathione content and the activities of antioxidant enzymes, together with vitamin C and E content, were estimated in patients who had carcinoma of the cervix, and the values were compared with those of normal women. The results showed a remarkable reduction in the content of glutathione, vitamin E and C. Activities of glutathione peroxidase and superoxide dismutase were also reduced in cervical cancer compared to normal controls ($P < 0.001$). This reduction was more marked in late stages (III, IV) than in early stages (I, II) ($P < 0.001$). Glutathione was reduced more in poorly differentiated tumors (grade III) than in well and moderately differentiated ones (grade I, II) ($P < 0.05$). Levels of lipid peroxides were found to be significantly higher in malignant than in normal tissue samples and their levels were correlated with advanced clinical stage ($P < 0.001$). Our results suggest impaired antioxidant status in carcinoma of

the cervix. This impairment is related to tumor progression.

KEYWORDS: Cervical carcinoma, LP, GSH, SOD, GSH-Px, vitamin C, vitamin E

INTRODUCTION

The close relationship between free radical activity and malignancy has been well-documented [10]. An increase in reactive oxygen species (ROS) in the cell due to overproduction and/or inability to destroy them may lead to severe damage of cell molecules and structures. Among the biological consequences of this damage are mutations, chromosomal aberrations and carcinogenesis [9]. The range of antioxidant defences available within and outside the cell should be adequate to protect against oxidative damage. Therefore, a critical balance between free radical generation and antioxidant defences is required [3]. Intracellular antioxidants include the enzymes glutathione peroxidase (GSH-Px), superoxide dismutase (SOD) and catalase. Sacrificial antioxidants present in cell membranes include tocopherols (vitamin E), beta-carotenes and ubiquinone. Many that circulate in the blood are derived from diet, for example ascorbate ion (vitamin C) and lipophilic tocopherols [31]. Lipid peroxidation has a very important role in the initiation and promotion of cancer [5] and has been implicated in adverse tissue changes in cancer [19]. Thus, measuring lipid peroxides (LP) as thiobarbituric acid reactive substances (TBARS) provides an indirect measure of antioxidant deficit [31]. Tumor cells have been shown to have abnormal levels of antioxidant enzyme activities when compared with normal cells. However, enzyme activities also differ among individual tumors [27]. The current study

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investigates the alterations of LP and the activities of GSH-Px and SOD together with the endogenous concentration of glutathione (GSH), one of the most important antioxidants, as well as vitamin E and C in human cervical carcinoma.

MATERIALS AND METHODS

Patients

The study was conducted on patients suffering from cervical carcinoma treated in Obstetrics and Gynaecology Department, Ain Shams University Hospitals in the period from October 1996 through December 1998. Blood samples and tissue specimens were collected from 27 patients with cervical carcinoma in different stages. Control blood and tissue samples were obtained from 20 patients undergoing hysterectomy for non-malignant conditions. All patients were examined under anaesthesia to assess tumor size, parametrial or vaginal extension of the growth. The clinical stage was determined according to FIGO staging. Patients were treated either surgically, by radiotherapy or both. Patients with bulky tumors received neoadjuvant chemo-therapy before definitive treatment whether surgical or radiotherapy. Serum and surgically resected tissue samples were obtained before therapy and kept at -70°C till the time of analysis.

Processing of tissue samples

Tissues were rinsed in saline to remove blood then homogenized (20% w/v) in ice cold KCL (0.15 M) using an ultraturax T-25 homogenizer with 3–5 bursts 30s, each with 1 min pause, on ice. One aliquot of the homogenate was kept aside for estimation of LP, SOD activity and total proteins. The rest of homogenate was centrifuged at $100,000 \times g$ for one hour at 4°C using a Beckman ultracentrifuge L7 (Brae, USA). The supernatant (cytosolic fraction) was separated and used for estimation of GSH, GSH-Px and total proteins. The pellet (membrane fraction) was reconstituted in KCL and used for estimation of vitamin E.

Biochemical analysis

Lipid peroxide levels in the homogenate were estimated by determining the TBARS following the procedure of Esterbauer et al [11]. The samples were heated with thiobarbituric acid under acidic conditions and the pink color formed was read at 532 nm. The lipid peroxide content was expressed as n moles of malondialdehyde (TBARS) per gm tissue.

Protein concentrations in cytosols and homogenates were estimated according to Bradford [4].

Total reduced glutathione (GSH) was determined in the cytosol based on the reaction with 5,5'-dithiobis-2 nitrobenzoic acid (DTNB) reagent to give a compound that absorbs light at 412 nm [26]. GSH was expressed as $\mu\text{g}/\text{mg}$ protein.

The activities of SOD in the homogenate and GSH-Px in the cytosol were assayed using Ransel KIT (Randox, Lab. Ltd., Ardmore, Crumlin, Co. Antrim, UK, BT29 4QY). Their activities were expressed as U/min/mg protein. The method for measurement of SOD activity [28] in tissue homogenate employs xanthine and xanthine oxidase to generate superoxide radicals which react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (I.N.T) to form formazon dye. The SOD activity is then measured by the degree of inhibition of this reaction.

GSH-Px activity in tissue cytosols is based on GSH oxidation by cumene hydroperoxide catalysed by cytosolic GSH-Px activity. In the presence of GSH-reductase and NADPH the oxidized GSH (GSSG) is immediately converted to the reduced form with concomitant oxidation of NADPH to NADP^+ [23]. The decrease in absorbance at 340 nm is measured.

Vitamin E in membranes was estimated by the method of Hashim and Schultringer [13] where dipyriddy, and ferric chloride were added to an aliquot of the upper heptane to produce an orange color.

Ascorbic acid (vitamin C) in the serum was determined by the method of Aye-Kyaw et al [1]. The method is a simple precipitation of proteins

and extraction of vitamin C by acid phosphotungstate and the blue color developed was measured at 700 nm. Vitamin C was expressed as $\mu\text{g/ml}$ serum.

Statistical analysis

Biochemical values were expressed as mean \pm standard deviation for patients and control separately. The statistical difference was analysed using student 't' test and 'P' values were expressed. One way analysis (ANOVA) was done to compare values of different parameters at different stages and grades of carcinoma. Correlation analysis was also performed. All analyses were performed using Statistical Package for the Social Sciences (SPSS) software.

RESULTS

Patients with cervical cancer were clinically staged by the FIGO staging system, they were distributed as following: 6 stage I, 7 stage II, 8 stage III, and 6 stage IV. Histopathological examination of cervical carcinoma tissues revealed squamous cell carcinoma in 21 cases (77.8%), and adenocarcinoma in 6 cases (22.2%). Clinicopathological, and biomedical data are listed in Table 1.

Table 2 depicts the levels of lipid peroxides (LP), glutathione (GSH), the activities of antioxidant scavenging enzymes, glutathione peroxidase (GSH-Px) and superoxide dismutase (SOD) and vitamin E and C contents in patients with cervical carcinoma and in controls. Our data revealed significant reduction in tissue content of GSH, and vitamin E and also in the activities of GSH-Px and SOD in patients with cervical carcinoma versus controls ($P < 0.001$). Serum levels of vitamin C were also reduced in patients with carcinoma compared to the control group. Lipid peroxides were significantly elevated in cervical carcinoma compared to control group ($P < 0.001$). Considering the different stages of cervical carcinoma, GSH content and the activities of GSH-Px and SOD

exhibited progressive decrease while LP exhibited progressive increase through stages I, II, III and IV (Tables 3 and 4). Moreover, GSH levels showed significant reduction in poorly differentiated tumors, grade III (mean \pm SD: 5.1 ± 0.95) compared to well and moderately differentiated, grade I, II (mean \pm SD: 7.4 ± 3.0) as evidenced by student t test ($t = 2.58$, $P = 0.016$). In stage I patients, membrane vitamin E remained unaltered. However, the vitamin showed progressive decrease in stages II, III and IV (Table 5). Vitamin C was not altered in early stages (I, II) of cervical carcinoma and its level decreased in stages III and IV (Table 5). In cervical carcinoma, LP levels were inversely correlated with GSH-Px activity ($r = -0.55$, $P = 0.03$) and vitamin E content ($r = 0.71$, $P < 0.001$). GSH content was correlated with GSH-Px activity ($r = 0.73$, $P < 0.001$) and with both vitamin E ($r = 0.67$, $P < 0.001$) and vitamin C ($r = 0.76$, $P < 0.001$). Vitamin C was correlated with GSH-Px activity ($r = 0.071$, $P < 0.001$) and with vitamin E content ($r = 0.67$, $P < 0.001$). Vitamin E was correlated with GSH-Px activity ($r = 0.67$, $P < 0.001$).

DISCUSSION

Reactive oxygen species (ROS) production by oxidative toxins from many sources may account for at least some of their carcinogenic actions. ROS can oxidize any vulnerable molecular species, and since DNA is one of these, oxidative stress can lead to cancer. The variability of individual response could be due to different antioxidant status at the time, or over the period of exposure as well as genetic factors [31]. Various studies in the literature have demonstrated that tumors from different organs have wide variations in their antioxidant status. Moreover, the link between lipid peroxidation (LP) and ROS has also given rise to much controversy. On the other hand, LP has been reported to be elevated in neoplastic tissues [5]. The increase in LP has been related to the pathogenesis of many diseases such as aging, atherosclerosis and carcinogenesis [7,32]. In the

Table 1
Clinicopathological parameters, and biochemical data of the studied groups

VitE µg/g tissue	VitC µg/ml serum	SOD U/mg protein	GSHPX U/mg protein	GSH µg/mg protein	MDA nM/g tissue	Grade	Stage	Pathology	Type	Age	No
10.98	17.30	0.48	0.47	11.50	29.20	.	.		Normal	42	1
11.53	13.90	0.42	0.54	15.44	26.40	.	.		Normal	36	2
8.20	17.30	0.35	0.52	16.07	37.95	.	.		Normal	45	3
9.63	14.75	0.35	0.45	9.90	22.50	.	.		Normal	48	4
11.03	20.25	0.6	0.45	11.50	34.10	.	.		Normal	42	5
6.98	20.40	0.63	0.57	13.56	21.20	.	.		Normal	49	6
12.50	22.10	0.42	0.54	13.72	34.40	.	.		Normal	43	7
9.78	16.00	0.42	0.49	16.07	37.70	.	.		Normal	42	8
12.60	22.20	0.71	0.53	18.55	21.30	.	.		Normal	58	9
7.54	19.00	0.61	0.54	12.09	30.24	.	.		Normal	37	10
7.14	21.90	0.62	0.57	14.89	22.30	.	.		Normal	72	11
11.95	15.12	0.35	0.51	12.78	29.40	.	.		Normal	54	12
11.54	20.00	0.63	0.53	15.35	21.90	.	.		Normal	65	13
10.53	19.60	0.51	0.53	10.03	23.00	.	.		Normal	45	14
7.55	14.90	0.54	0.48	10.96	22.00	.	.		Normal	50	15
7.13	14.45	0.55	0.51	11.30	24.10	.	.		Normal	56	16
10.50	19.95	0.36	0.43	16.40	25.20	.	.		Normal	43	17
9.49	19.15	0.53	0.53	14.80	31.20	.	.		Normal	45	18
10.79	16.18	0.49	0.53	11.30	32.00	.	.		Normal	48	19
8.49	18.13	0.51	0.49	11.90	34.00	.	.		Normal	51	20
6.93	17.08	0.28	0.37	9.34	38.10	1	I	SCC	Cancer	39	21
8.10	14.00	0.22	0.49	7.60	40.80	1	I	AC	Cancer	45	22
7.67	23.00	0.39	0.55	12.60	42.16	2	I	AC	Cancer	52	23
10.67	13.55	0.39	0.31	6.90	35.40	1	I	SCC	Cancer	53	24
7.45	17.90	0.40	0.38	10.46	38.19	1	I	SCC	Cancer	38	25
8.20	17.35	0.24	0.43	7.82	36.80	2	I	SCC	Cancer	41	26
6.33	20.00	0.20	0.30	6.40	41.98	1	II	SCC	Cancer	38	27
7.95	12.40	0.24	0.44	9.95	38.14	2	II	AC	Cancer	47	28
7.34	14.40	0.28	0.34	11.75	36.51	2	II	SCC	Cancer	50	29
6.20	13.60	0.37	0.33	6.85	47.17	2	II	SCC	Cancer	65	30
7.69	14.80	0.27	0.41	5.65	34.10	3	II	SCC	Cancer	55	31
6.80	13.40	0.29	0.36	9.90	35.05	1	II	SCC	Cancer	50	32
6.70	16.80	0.27	0.35	9.58	40.19	2	II	AC	Cancer	50	33
5.75	12.90	0.23	0.32	6.60	44.32	3	III	SCC	Cancer	46	34

Table 1
(continued)

Vit E µg/g tissue	Vit C µg/ml serum	SOD U/mg protein	GSH PX U/mg protein	GSH µg/mg protein	MDA nM/g tissue	Grade	Stage	Pathology	Type	Age	No
5.00	12.49	0.14	0.24	3.40	53.19	2	III	SCC	Cancer	55	35
5.25	7.83	0.18	0.33	3.70	37.10	3	III	SCC	Cancer	50	36
6.38	11.00	0.08	0.27	5.80	38.00	1	III	AC	Cancer	65	37
5.55	11.80	0.16	0.32	4.60	43.79	1	III	SCC	Cancer	60	38
5.45	9.40	0.17	0.28	4.38	37.81	2	III	SCC	Cancer	45	39
6.40	12.20	0.14	0.30	5.82	46.21	3	III	SCC	Cancer	52	40
5.45	11.43	0.15	0.28	4.50	43.38	2	III	SCC	Cancer	47	41
4.00	6.95	0.06	0.27	3.00	43.54	2	IV	AC	Cancer	55	42
5.15	7.12	0.08	0.25	4.90	54.90	3	IV	SCC	Cancer	60	43
4.00	6.95	0.07	0.26	4.80	52.00	3	IV	SCC	Cancer	58	44
3.86	6.75	0.07	0.24	3.10	53.14	2	IV	SCC	Cancer	48	45
4.97	6.55	0.09	0.26	4.60	46.24	3	IV	SCC	Cancer	52	46
4.00	7.35	0.07	0.25	4.20	56.88	2	IV	SCC	Cancer	51	47

SCC: Squamous cell carcinoma

AC: Adenocarcinoma

Table 2
LP and antioxidant status in uterine cervical tissues compared to control

Parameter studied	Normal control (n = 20)	Cervical carcinoma (n = 27)
LP (nM MDA/g tissue)	28.00 ± 5.7	42.13 ± 6.0*
GSH (µg/mg protein)	13.4 ± 2.4	6.72 ± 2.68*
GSH-Px (U/mg protein)	0.51 ± 0.04	0.33 ± 0.08*
SOD (U/mg protein)	0.5 ± 0.11	0.21 ± 0.1*
Vitamin E (µg/g tissue)	9.79 ± 1.0	6.37 ± 1.47*
Vitamin C (µg/ml serum)	18.13 ± 2.7	12.89 ± 4.07*

* $P < 0.001$: control versus carcinoma. The values are expressed as mean ± SD.

Table 3
Lipid peroxides (MDA), and glutathione (GSH) content in control and different stages of uterine cervical carcinoma

Groups	Number	MDA (nM/g tissue) mean ± SD	GSH (µg/mg protein) mean ± SD
Control	20	28.00 ± 5.7	13.39 ± 2.4
Stage I	6	38.58 ± 2.51*	9.12 ± 2.1*
Stage II	7	39.02 ± 4.54*	8.58 ± 2.27*
Stage III	8	42.98 ± 5.39*	4.85 ± 1.12 ^a
Stage IV	6	51.12 ± 5.17 ^{ab}	4.1 ± 0.85 ^a

* $P < 0.001$: each stage versus control; ^a $P < 0.001$: each stage versus stage I,II; ^b $P < 0.001$: each stage versus stage I, II, III.

Table 4
Activity of glutathione peroxidase (GSH-Px) and superoxide dismutase (SOD) in control and different stages of uterine cervical carcinoma

Groups	Number	GSH-Px (U/min/mgprotein) mean ± SD	SOD (U/min/mg protein) mean ± SD
Control	20	0.51 ± 0.04	0.51 ± 0.11
Stage I	6	0.42 ± 0.09*	0.32 ± 0.08*
Stage II	7	0.36 ± 0.05*	0.27 ± 0.05*
Stage III	8	0.29 ± 0.03 ^{ab}	0.157 ± 0.04 ^a
Stage IV	6	0.25 ± 0.01 ^a	0.07 ± 0.01 ^a

* $P < 0.001$: each stage versus control; ^a $P < 0.001$: each stage versus stage I,II; ^b $P < 0.001$: each stage versus stage I.

Table 5
Levels of vitamin C and E in control and different stages of uterine cervical carcinoma

Groups	Number	Vitamin C (µg/ml) mean ± SD	Vitamin E (µg/g tissue) mean ± SD
Control	20	18.13 ± 2.7	9.79 ± 1.88
Stage I	6	17.15 ± 3.4	8.17 ± 1.31
Stage II	7	15.06 ± 2.58	7.00 ± 0.67*
Stage III	8	11.13 ± 1.71 ^{ab}	5.65 ± 0.5*
Stage IV	6	6.95 ± 0.28 ^a	4.33 ± 0.57 ^{ab}

* $P < 0.001$: each stage versus control; ^a $P < 0.001$: each stage versus stage I,II; ^b $P < 0.001$: each stage versus stage I.

present investigation, the observed increase in LP, the end product of lipid peroxidation, in cervical carcinoma tissues compared to controls may be due to extensive tissue damage or decrease in the antioxidant defence mechanisms. Increased levels of LP in tumor tissues and in the sera of patients with cervical carcinoma have been reported [2,3,8]. It has been suggested that increase in peroxidation would cause degeneration of tissues and that LP formed at the primary sites, would be transferred through the circulation to other tissues and provoke damage by propagating the process of lipid peroxidation [18]. The negative correlation between LP levels and vitamin E content as well as GSH-Px activity in cervical carcinoma indicates that increased lipid peroxidation could be a result of poor antioxidant defences in tumor cells.

Glutathione is a tripeptide which plays a vital role in the protection of cellular constituents against oxidative damage. GSH alone or in conjunction with other proteins can protect the cells against lipid peroxidation [29]. The tissue GSH level was low in all stages of malignancy compared to controls which agrees with previous reports [2,3]. Corrocher et al. [9] have reported that a diminished level of GSH leads to overproduction of free radicals and consequently, neoplastic transformation. It has been stressed that supplementation of GSH to mice bearing experimental hepatoma leads to a remarkable improvement and finally disappearance of tumors [20,21]. Following this approach, Kawano et al. [16] successfully treated with a large dose of GSH a patient with inoperable hepatoma.

Glutathione peroxidase (GSH-Px) is a selenium-dependant enzyme which catalyses the detoxification of hydrogen peroxide and other oxidants through glutathione [30]. GSH-Px activity was found to be decreased in cervical carcinoma and this decrease was correlated to advanced stage. Decreased activities of GSH-Px were reported in experimental carcinogenesis [22,4], in human hepatoma [6,9] and in cervical carcinoma [2,3]. The low activity of GSH-Px might increase the accumulation of free radicals which, in turn, may induce the neoplastic process. Further, it is possible that the scavenger

system is impaired due to reduced synthesis of glutathione peroxidase due to carcinogenesis. The low availability of its substrate, GSH, may be a reason for the decreased activity of the enzymes, given the significant correlation between the two values. It has been also reported that GSH-Px activity is inactivated by increased production of free radicals [9].

Superoxide dismutase (SOD) catalyzes cell defence reactions, against the potentially harmful effects of superoxide anion generated by a wide variety of biological process [3]. We observed marked reduction in SOD activity in carcinoma of the uterine cervix as compared to control tissues. The decrease in SOD activity might be attributed to reduced synthesis. Depressed SOD activity and other defense enzymes in tumors could be responsible for the increased peroxidation products generated by tumor cells [2]. These observations coincide with studies reported for patients with malignant lymphoma and acute myeloid leukemia [25] and with cervical carcinoma [2,3]. Depressed SOD activities were also seen in different organs in Ehrlich carcinoma bearing mice [17] and also in few examples of cell lines when compared to normal tissues [12].

Vitamins C and E are powerful antioxidants, free radical scavengers and well-known inhibitors of lipid peroxidation [15]. Jayashree and Sukla [14] have reported that serum ascorbic acid levels were diminished in cancer patients and supplementation of this vitamin could inhibit the growth of transplantable sarcoma. Diminished levels of vitamin E and C were also observed in sera of patients suffering from cervical carcinoma compared to controls [3]. These findings have been supported in the present study as regards vitamin E and C levels and the decreased antioxidants may be one of the factors responsible for progression of cervical carcinoma. Although the precise mechanisms by which oxidative stress can produce cancer are still difficult to establish, our findings suggest that oxidative stress in the form of elevated levels of lipid peroxides together with impaired antioxidant defence mechanisms may play a role in the etiology and progression of cervical carcinoma.

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