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Rapid Letter

Increased Myeloperoxidase and Lipid Peroxide-Modified Protein in Gynecological Malignancies

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ABSTRACT

Oxidative stress has been implicated in several diseases, including cancer. Oxidants induce oncogenes and their products associated with cell growth. Even though epidemiological studies implicate oxidants in promoting cancer, there is still a lack of *in vivo* evidence for the same. In this study, we measured the levels of myeloperoxidase (MPO), an enzyme associated with oxidation and autoantibodies to lipid peroxide-modified protein (LOOH-RSA), in the plasma of subjects with gynecological cancers. The gynecological cancer subjects ($n = 201$) had higher plasma MPO and LOOH-RSA levels compared with control subjects ($n = 60$). Immunohistochemical analysis of tissues revealed that immunostaining for MPO and LOOH-RSA was higher in cancer tissues compared with controls. The staining was specific to cell types and not ubiquitously present. Neutrophils, monocytes/macrophages, and natural killer cells have been proposed to play a role in cancer promotion and progression. This study proposes a role for oxidative stress and especially MPO in cancer. Antioxid. Redox Signal. 3, 1139–1146.

INTRODUCTION

THE ACTIVATION OF CELLULAR OXIDASES and oxygen radical generating systems is thought to lead to the oxidation of various cell-surface proteins and lipids and to damage cellular DNA (12). Such oxidative processes have been implicated in malignant transformation of cells through various mechanisms. Despite controversies, studies are accumulating on the potential benefits of antioxidants in the suppression of tumorigenesis (3).

In vitro cell culture studies have also suggested that oxidative stress may play a role in signaling and malignant transformation of cells (1, 18). Accordingly, antioxidants appear to prevent promalignant cellular changes induced by oxidative stress (17).

Clinical studies, on the other hand, have been limited in delineating the role of antioxidants in the prevention of malignant transformation and in determining the presence of various antioxidants and antioxidant enzymes in plasma and tissues of patients (8). The outcome of these studies has been often inconclusive and sometimes even negative, if not even suggestive of a worsened disease outcome. This can be attributed to various reasons that may include trials on very advanced cases, the etiology of such malignancies, lack of availability of antioxidants at the required sites, or a suboptimal ability to assess therapeutic responses.

Moreover, cell culture studies on apoptosis seem to suggest that oxidants promote and antioxidants decrease apoptosis (20). Taken together, these studies might indicate that other approaches to define the role of oxidation and cancer are needed. Specifically, inducers and

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Table 1. Characteristics of the Subjects

	Ovarian cancer	*Cervical cancer*	*Endometrial cancer*	*Benign*
Number	31	20	27	60
Age, yr (median range)	60.4 (33–85)	42.6 (18–74)	64.9 (47–83)	48.3 (22–84)
Premenopausal	5	6	9	42
Postmenopausal	26	15	18	18
Stage	NA = 3 I = 5 II = 3 III = 12 IV = 8	NA = 7 I = 3 II = 5 III = 3 IV = 2	NA = 3 I = 8 II = 6 III = 6 IV = 4	
Histology	Serous papillary = 12 Endometroid = 2 Granulosa = 3 Metastatic = 3 MMMT = 4 Borderline = 2 Mucinars = 5	Squamous = 12 Adenocarcinoma = 2 CMV = 6	Adenocarcinoma = 11 Serous papillary = 8 Clear cell = 3 Sarcoma = 2 MMMT = 3	Leiomyoma = 20 Cyst = 14 Endo = 16 Fibroma = 5 Vulvitis = 5

CMV, cytomegalovirus; MMMT, malignant mixed Mullerian tumor; NA, not applicable.

markers of oxidative stress need to be demonstrated in a variety of cancers, particularly if specific oxidation targets are considered potential targets for intervention.

Indeed, in the past, many studies focused on the detection of products of oxidation such as aldehydes, thiobarbituric acid reactive substances (TBARS), the hydroxy products derived from lipid peroxides (LOOH), or even intact lipid peroxides. These products are transient and highly reactive. Thus, analysis of these products often led to questionable results

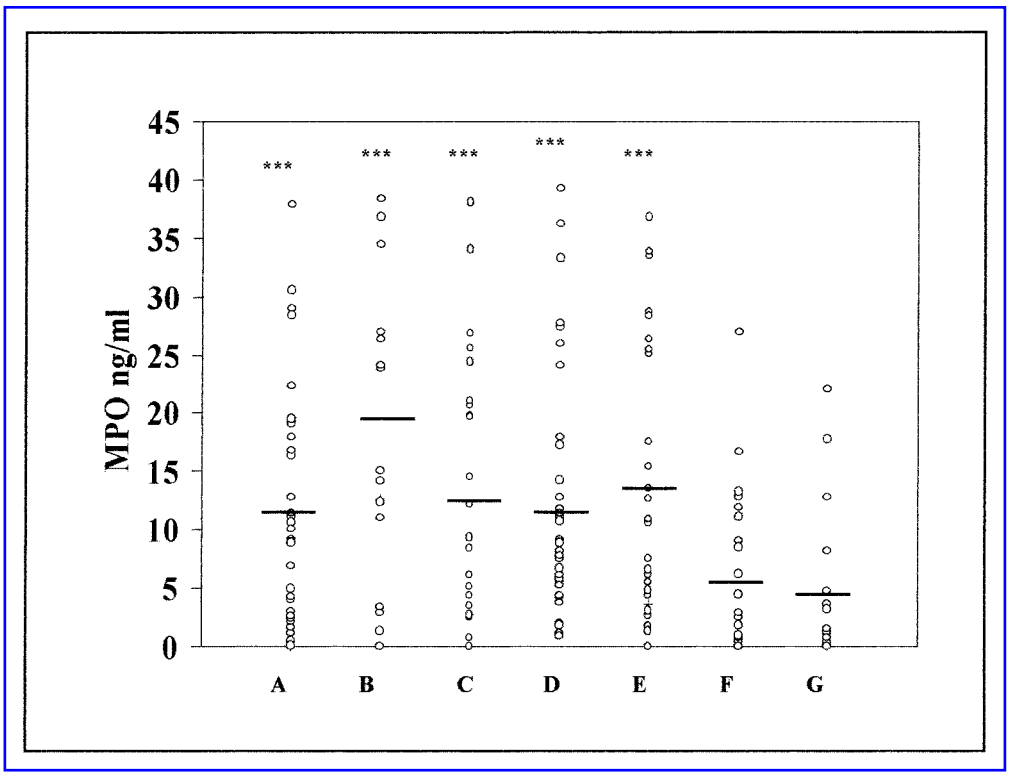

FIG. 1. Plasma MPO protein levels in gynecological malignancies. Plasma MPO levels were detected using ELISA in the cancer subjects (A, ovarian; B, cervical; C, endometrial; D, benign; and E, leiomyoma) and controls (F, postmenopausal; and G, premenopausal). Significance is shown at the following level: ***$p < 0.001$.

(6). Recently, it was shown that LOOH-modified proteins are highly antigenic and result in the generation of autoantibody (9, 14). Such autoantibodies have played a significant role in the elucidation of a role for oxidative stress in cardiovascular disease (10).

In this study, we document the presence of myeloperoxidase (MPO), as a marker of oxidation (2), in cancers related to the female reproductive system. We also demonstrate increased levels of autoantibodies and immunoreactivity to oxidatively modified proteins in cancer compared with control subjects.

As MPO represents an important constituent of neutrophils and monocytes and their recruitment into reproductive tissues is influenced by estrogen (11), our studies also point out a clear distinction between hormone dependent cancers and other types of malignancy in relation to oxidative stress.

MATERIALS AND METHODS

Subject population

A Human Investigations Committee approved protocol was developed that involved collection of heparinized fresh venous blood from volunteers and from patients subsequently diagnosed with a gynecologic malignancy. A total of 261 subjects were included in this study, and the tumor characteristics of the cancer patients are listed in Table 1. There were 60 control and 201 study subjects, of whom 99

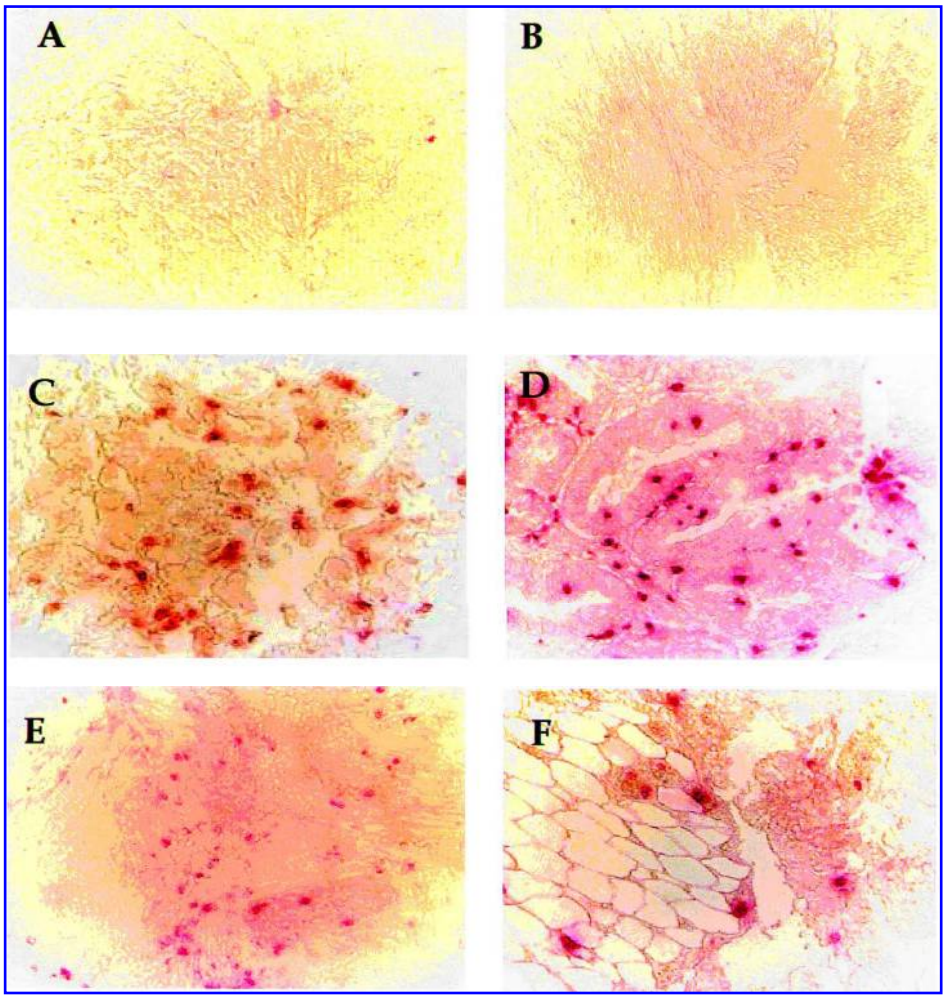

FIG. 2. Immunostaining of MPO protein in gynecological tumor tissues. Immunostaining using MPO antibody was performed in various gynecological tumor tissues. (**A**) Normal ovary; (**B**) normal uterus; (**C**) ovarian cancer; (**D**) endometrial cancer; (**E**) cervical cancer; (**F**) breast cancer.

had benign diseases (leiomyoma, cyst, fibroma, endometriosis) and 102 had malignancies (endometrial, cervical, vulvar, and ovarian) based on final pathology.

Materials

Linoleic acid, soybean lipoxygenase, rabbit serum albumin (RSA), etc. were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). The enzyme-linked immunosorbent assay (ELISA) kits for MPO were purchased from R & D systems (Minneapolis, MN, U.S.A.).

Preparation of LOOH-modified RSA

Linoleic acid was converted into 13-hydroperoxy linoleate by treatment with soybean lipoxygenase (5). Linoleic acid hydroperoxide (LOOH) was immediately reacted with immunoglobulin-free RSA and incubated at 37°C for 2 days. The product was extracted to remove unreacted LOOH and then washed with ice-cold acetone. The final product, LOOH-modified RSA, was soluble in aqueous buffers. The generation of LOOH, as well as the modification of the protein, was performed in the absence of any added metals to limit the formation of aldehydes (9).

ELISA assay for LOOH-modified antibody

Ninety-six-well plates coated with 100 μl of a 5 μg/ml solution of LOOH-modified RSA were incubated at 37°C overnight. Plates were washed with 1× phosphate-buffered saline (PBS) and blocked for 2 h with 100 μl of 3% RSA in PBS. After blocking, the plates were washed with 1 × PBS, and 100 μl of the patients' plasma (1–100 diluted in PBS) was added to each well and incubated at 37°C for 1 h. After washing, anti-human IgG conjugated with alkaline phosphatase (1–10,000 dilution) was added to each well and incubated for 1 h. After washing, 100 μl of *p*-nitrophenyl phosphate was added to each well and incubated at 37°C. The plates were read at OD 405 nm after 30 min. Results were expressed as optical density at 405 nm.

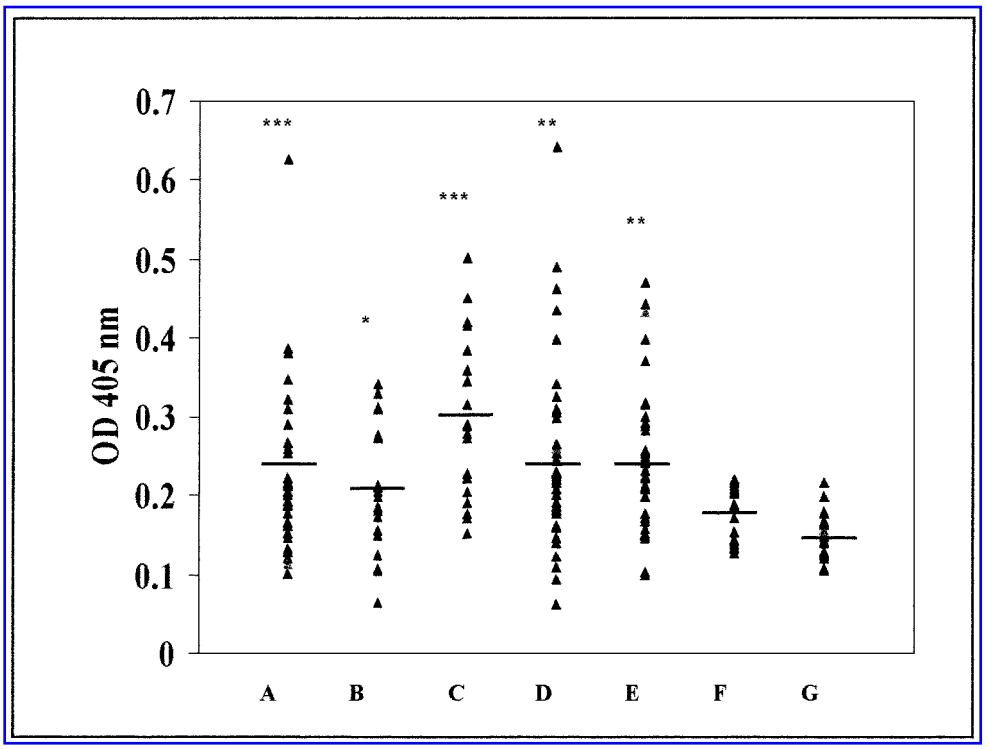

FIG. 3. Plasma autoantibody levels of LOOH-RSA in gynecological malignancies. Plasma autoantibody levels to LOOH-RSA were detected using ELISA in the cancer subjects (A, ovarian; B, cervical; C, endometrial; D, benign; and E, leiomyoma) and controls (F, postmenopausal; and G, premenopausal). Significance is shown at the following levels: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

ELISA assay for plasma MPO protein

An ELISA, following the manufacturer's instructions, was used to measure plasma MPO protein levels (R & D). Results were expressed as nanograms of MPO protein per milliliter.

Immunohistochemistry for MPO and LOOH-RSA in tissues

Tissues fixed in formal sucrose were sectioned and incubated with 1:100 dilution of rabbit anti-human MPO antibody (or rabbit antibody to LOOH-RSA). For the negative control, primary antibody was omitted. After washing, tissue sections, including the negative control, were incubated with secondary antibody conjugated with alkaline phosphatase for 2 h. Sections were washed three times with PBS, and Fast Red was added as chromogen in conjunction with Naphthol AS-TR phosphate. Immunostaining was visualized under the microscope, and the results were analyzed.

Statistical analysis

Student's t test was used to analyze any differences between groups. The results are given as means $\pm$ SD. Statistical analysis was performed using GB stat software. A p value of <0.05 was considered statistically significant.

RESULTS

Increased plasma MPO protein in cancer subjects

MPO protein levels were measured in the plasma of cancer and control subjects using a commercial ELISA kit. As seen in Fig. 1, there

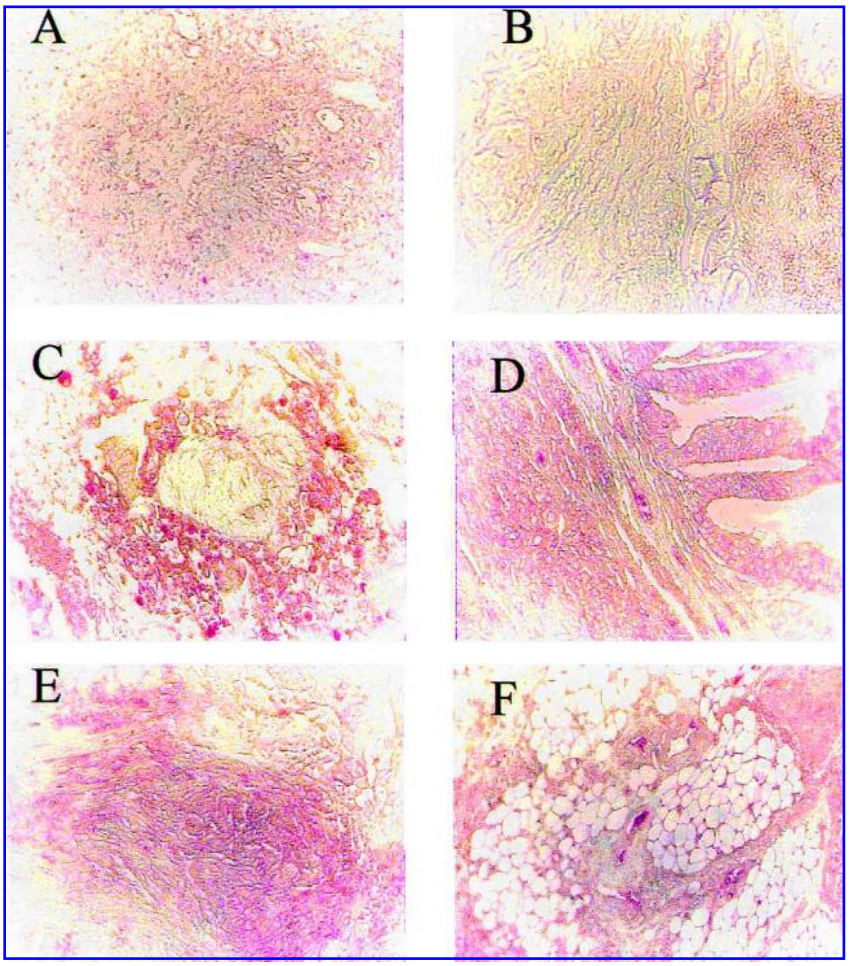

FIG. 4. Immunostaining of LOOH-RSA in gynecological tumor tissues. Immunostaining using LOOH-RSA antibody was performed in various gynecological tumor tissues. (**A**) Normal ovary; (**B**) normal uterus; (**C**) ovarian cancer; (**D**) endometrial cancer; (**E**) cervical cancer; (**F**) breast cancer.

was a significant level of MPO in the cancer subjects compared with controls. The MPO protein levels were different in different cancer subjects. As MPO is a constituent of neutrophils or monocytes, this increase in plasma MPO levels may suggest increased degranulation of these cells. Others and we have shown that estrogen increases the degranulation of monocytes and neutrophils (11, 15). This degranulation has also been shown to be oxidation- related. Therefore, this increase may also suggest an oxidation related hormonal response in these subjects.

Increased MPO protein in tumor tissues

As seen in Fig. 2, immunohistochemistry of the tumor tissues revealed higher immunostaining of MPO in tumor tissues compared with control tissues. This staining was very cell-specific and not ubiquitously present. This again suggests neutrophil or monocyte infiltration into tissues. Whether this infiltration is a response to an inflammatory condition or due to oxidative stress is still not known. However, studies have shown an increase in MPO protein and gene expression in arterial tissues due to oxidation (7).

Increased autoantibody to LOOH-RSA in cancer subjects compared with controls

LOOH-modified protiens are generated *in vivo* due to sustained oxidative stress. Others and we have shown an increase in autoantibodies to LOOH-modified proteins in disease conditions (13, 19). We have also shown that these autoantibody levels could be decreased by antioxidant supplementation, suggesting that the generation of these antibodies is oxidation-mediated (16). In the present study, we measured the autoantibodies to LOOH-RSA in the plasma of the subjects and controls using the ELISA method. As seen in Fig. 3, the cancer subjects had higher levels of autoantibodies compared with the controls. The presence of these autoantibodies suggests a sustained oxidation in these subjects.

Increased immunostaining of LOOH-RSA in tumor tissues

We have earlier shown an increased immunostaining of LOOH-RSA in the atherosclerotic artery (10). This was due to sustained oxidation in the tissue level. Similar, immunostaining of tissues, as seen in Fig. 4, showed increased staining in tumor tissues compared with controls.

DISCUSSION

Oxidation is a phenomenon that is ubiquitous and accounts for various pathologic human condition (4). Studies imply a benefit to treatment with antioxidants, both in reduction of expression of disease and when used in chemoprevention (8).

Our data show that there is an increased level of circulating autoantibody to LOOH-modified proteins and MPO protein in the plasma of patients with gynecologic malignancies. Animal studies and studies in subjects undergoing ovarian hyperstimulation for *in vitro* fertilization have suggested that the increased availability of estrogens might induce leukocyte recruitment and degranulation and the release of enzymes such as MPO (11, 15). MPO has been implicated in the induction of lipid peroxidation. Its increased presence in the plasma might signify a role for this enzyme and estrogens in the oxidative stress observed in these subjects. We also observed increased immunostaining of MPO in the tumor tissues. MPO is a true component of monocytes/neutrophils. Differentiating macrophages also express increased gene expression dominant in MPO levels. The increased immunostaining may be due to leukocyte infiltration into tumor tissues. It is possible that plasma MPO contributes to the oxidative stress or the presence of leukocytes and, actually, MPO in tumor tissues may be a better predictor of the oxidative stress. Earlier (7) studies have shown leukocyte infiltration in the atherosclerotic artery due to oxidation. The presence of MPO may also suggest chemotactic recruitment of neutrophils in the tumor tissue. Breast cancer and uterine endometrium respond to estrogen. Others and we have shown that estrogens increase neutrophil MPO (11, 15). Thus, the presence of MPO particularly in its hormone responsiveness might be a causative agent. We speculate that there may be an estrogen-dependent myeloid response

playing a role in these oxidative events. Conversely, this might also suggest an inflammatory response accompanying malignancy.

Lipid peroxidation may further support the role of MPO in oxidation. MPO is not active without the presence of LOOHs. In the presence of LOOHs, MPO can generate several oxidants such as chlorotyrosine that are commonly used markers of MPO (7). Traditionally, TBARS and other products are measured, which are less sensitive and require polyenoic acid formation, and also represent short-term oxidation, because they get cleared from the tissue. LOOHs, however, accumulate in the tissue long-term. Thus, its presence may suggest long-term oxidation. Although short-term markers of oxidation as well as differences in antioxidant levels in plasma may not represent oxidation in tumor tissue, the circulating autoantibody levels represent response to oxidative stress in the tissue. The increase in autoantibodies to oxidatively modified proteins and its decrease by antioxidant supplementation have been well documented in the literature (16). The novel approach used in this study avoids the pitfalls encountered in the past in quantifying cumulative oxidative stress.

A significant increase in autoantibodies suggests not only an ongoing oxidative process, but also the potential use of this marker to determine the efficacy of antioxidants in controlling oxidation. However, unless the oxidative stress reflects the oxidation, specific to tumor tissue, a decrease in the titer of autoantibody levels may not reflect any antioxidant benefit in controlling tumor progression.

In summary, this study provides direct evidence for one of the potential inducers of oxidation in hormone-dependent tumor tissues and direct evidence of long-term oxidative stress in cancer. Whereas most clinical trials centered on smoking-associated lung cancer or breast cancer and used agents such as vitamin E and β-carotene and antihormones, our studies suggest that antioxidant or specific inhibitors of enzyme action need to be considered. Furthermore, this method may also be valuable in serving as a clinically available parameter for future therapeutic and chemopreventive trials.

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ABBREVIATIONS

ELISA, enzyme-linked immunosorbent assay; LOOH, lipid peroxide; LOOH-RSA, lipid peroxide-modified protein; MPO, myeloperoxidase; PBS, phosphate-buffered saline; RSA, rabbit serum albumin; TBARS, thiobarbituric acid reactive substances.

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