Mitochondrial Lipid Peroxides and Antioxidant Enzymes in Colorectal Adenocarcinoma Tissues

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Lipid peroxide levels and superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), and glutathione transferase (GST) activities were investigated in mitochondrial fractions obtained from tumorous and nontumorous colorectal tissues of fourteen patients with colon and rectum cancer. Histopathological evaluations, including type, stage, necrosis and lymphocyte infiltration were also performed for each patient. The activities of SOD, GSH-Px and GST were increased significantly, but lipid peroxide levels remained unchanged in mitochondria obtained from tumors compared to adjacent normal tissues of subjects with colorectal cancer. When the patients were grouped according to their histopathological evaluation, such as type, stage, necrosis and lymphocyte infiltration, no relationship was observed between the histopathological results and the mitochondrial lipid peroxidation or antioxidant enzyme activities.

Key words: Lipid peroxides — Antioxidant enzymes — Mitochondria — Colorectal cancer

The oxidant-antioxidant balance within tissues is thought to contribute to the development and progression of cancer.1–3 Although changes in lipid peroxidation and antioxidant enzyme systems in different tissues due to malignant processes have been described in both human cancer and experimental animal models of cancer, the relation of the increase in reactive oxygen species with changes in antioxidant enzymes is not well understood.4 It has been thought that these changes are not tumor-specific, but are tissue-dependent and/or dependent on the tumor stage.5

Colorectal cancer is one of the most common human malignancies. There are limited, but conflicting reports in the literature on the prooxidant-antioxidant balance in cancerous tissues5–11 as well as plasma and erythrocytes12–15 of patients with colorectal cancer. In our previous study, lipid peroxide and glutathione (GSH) levels and the activities of superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) were found to be increased, while glutathione transferase (GST) remained unchanged in post-mitochondrial fractions of tumors compared to adjacent normal tissues of patients with colorectal cancer, but a definite relation between histopathological results and lipid peroxidation or antioxidant systems was not observed.16

Mitochondria are extremely susceptible to oxidative stress.17 It has been reported that mitochondrial integrity and function are important in carcinogenesis.18–20 Further, mitochondrial lipid peroxidation and mitochondrial SOD (MnSOD) activity are influenced by dietary factors such as diets high in fat or iron in the early period of experimental colon carcinogenesis.20, 21 However, there is no report on mitochondrial oxidant-prooxidant balance in human colorectal cancer. Therefore, we investigated malondialdehyde (MDA), the end product of lipid peroxidation, and the activities of SOD, GSH-Px and GST in the mitochondrial fraction of cancerous and adjacent normal tissues of patients with colorectal cancer. The relationships between the histopathological results and mitochondrial lipid peroxidation and antioxidant enzymes were also studied.

MATERIALS AND METHODS

The investigation was carried out on fourteen colorectal cancer patients (6 men and 8 women, mean age±SD, 57.2±9.59 years) who had undergone operation at the Department of General Surgery.

Malignant and normal tissue specimens were immediately transported to the laboratory in ice and cleaned by washing with ice-cold 0.9% NaCl. Tumor-free tissues were taken from areas at least 10 cm away from the tumor site and used as normal colorectal tissue. Fourteen paired samples were analyzed and histopathologically confirmed as adenocarcinoma at the Department of Pathology.

Preparation of mitochondrial fractions Tissue samples were homogenized in a buffer containing 220 mM mannitol, 70 mM sucrose, 10 mM HEPES, 0.25 mM EGTA, 1 mg/ml bovine serum albumin, pH 7.4 at 0°C in a Potter Elvehjem tissue homogenizer. Mitochondria were obtained by sequential centrifugation. The homogenates (10% w/v)
were centrifuged at 600g for 10 min at below 4°C, and the resultant supernatant fractions were centrifuged at 10 000g for 20 min. The pellets were washed with ice-cold homogenizing buffer and 0.15 M KCl to eliminate contamination and were suspended in 0.15 M KCl.\(^\text{22)}\)

**Determination of lipid peroxidation** Lipid peroxidation in mitochondria was evaluated by the fluorometric method based on the reaction of MDA and thiobarbituric acid.\(^\text{23)}\) Results were expressed as nmol MDA per mg protein. The breakdown product of 1,1,3,3-tetraethoxypropane was used as a standard.

**Determination of MnSOD activity** MnSOD was determined on the basis of its ability to increase the effect of riboflavin sensitized photooxidation of o-dianisidine.\(^\text{24)}\) In that assay, 4 mM potassium cyanide is added to inhibit CuZnSOD. Enzyme activity was calculated using the SOD standard and expressed as U/mg protein.

**Determination of GSH-Px activity** GSH-Px activity was measured in mitochondrial fractions using cumene hydroperoxide as the substrate.\(^\text{25)}\)

**Determination of GST activity** GST activity was assayed by the spectrophotometric method using 1-chloro-2,4-dinitrobenzene (CDNB) as the substrate.\(^\text{26)}\) Results were calculated by using the absorption coefficient (9.6 mM\(^{-1}\) cm\(^{-1}\)) of the product formed by the conjugation of GSH and CDNB. The enzyme activity was expressed as nmol/min/mg protein. Protein concentrations were measured by the method described.\(^\text{27)}\)

**Statistical analysis** Data were expressed as the means±SD and the statistical analyses were performed by using the Wilcoxon matched-pairs signed-ranks and Mann-Whitney U-Wilcoxon rank sum W tests.

### RESULTS

Mitochondrial SOD, GSH-Px and GST activities were found to be increased (30.9%, 75.7% and 213.6%, respectively) in malignant tissues of patients with colorectal cancer, but mitochondrial MDA levels remained unchanged (Table I). However, considerable interindividual variations were found in these parameters (Figs. 1–4). There was no difference between men and women for each parameter (data not shown).

The histological type of the cancer, stage of TNM classification, and extent of necrotic areas within the specimens are shown in Table II. When the patients were grouped according to their histological evaluation, we

| Parameter | Normal tissues (n=14) | Malignant tissues (n=14) |
|-----------|-----------------------|-------------------------|
| MDA (nmol/mg protein) | 0.81±0.36 | 0.83±0.28 |
| MnSOD (U/mg protein) | 6.02±3.61 | 7.88±4.96** |
| GSH-Px (nmol/min/mg protein) | 8.20±4.02 | 14.41±7.65** |
| GST (nmol/min/mg protein) | 3.61±2.01 | 11.32±5.35* |

* P<0.001, ** P<0.01, *** P<0.05 as compared to normal tissue.
observed no statistically significant relationship between the histopathological results and the mitochondrial lipid peroxidation or antioxidant systems (Table III).

DISCUSSION

Biochemical and immunochemical studies have suggested that oxidative stress plays an important role in carcinogenesis. Protection of cell constituents from oxidative stress can be accomplished through enzymatic and non-enzymatic mechanisms. Enzymatic defence mainly consists of SOD, catalase, and GSH-Px. In eukaryotic cells two forms of SOD are present; one contains both Cu and Zn (CuZnSOD), and the other contains Mn (MnSOD). The CuZnSOD is found mainly in the cytosol, and the MnSOD resides in the matrix of mitochondria. SOD catalyzes the dismutation of superoxide anions to hydrogen peroxide, which is metabolized by catalase and GSH-Px.

Mitochondria are the powerhouse of the cell and any alteration of their structure and function will have drastic consequences for the cells. Various tumors and tumor cell lines may exhibit abnormalities in mitochondrial morphology, enzymatic activity and transport mechanisms, and impaired energy metabolism in cancer cells may be related to altered mitochondrial structure and function. Mitochondria are one of the main sources of reactive oxygen species (ROS) production, and in the absence of adequate protection, ROS cause damage to target proteins, lipids and DNA of this subcellular organelle. About 1–2% of inhaled O₂ may be converted to superoxide anion in mitochondria. MnSOD and GSH-Px are important enzymes in the protection of mitochondria from oxidative stress, since they do not have catalase activity. Mitochondria are one of the main sources of reactive oxygen species (ROS) production, and in the absence of adequate protection, ROS cause damage to target proteins, lipids and DNA of this subcellular organelle. About 1–2% of inhaled O₂ may be converted to superoxide anion in mitochondria. MnSOD and GSH-Px are important enzymes in the protection of mitochondria from oxidative stress, since they do not have catalase activity.

Table II. Histopathological Evaluation of the Specimens with Regard to Histopathological Type, Stage, Necrosis and Lymphocyte Infiltration

| Patients | Histological type | TNM stage | Necrosis | Lymphocyte infiltration |
|----------|-------------------|-----------|----------|-------------------------|
| 1        | M                 | IIA       | +        | +                       |
| 2        | W                 | IIA       | −        | −                       |
| 3        | M                 | IIA       | −        | −                       |
| 4        | M                 | IIA       | +        | +                       |
| 5        | M                 | IIA       | +        | +                       |
| 6        | M                 | IV        | −        | −                       |
| 7        | M                 | I         | −        | −                       |
| 8        | M                 | IIB       | +        | −                       |
| 9        | W                 | I         | −        | −                       |
| 10       | W                 | IIB       | +        | +                       |
| 11       | M                 | IIB       | +        | +                       |
| 12       | M                 | IIB       | +        | +                       |
| 13       | M                 | IIA       | −        | −                       |
| 14       | M                 | IIB       | +        | +                       |

W, well differentiated; M, moderately differentiated.
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Table III. Lipid Peroxide Levels and Activities of MnSOD, GSH-Px and GST in Mitochondria of Malignant Colorectal Tissues of Patients Grouped According to Histological Parameters Such as Histological Type, TNM Stage, Necrosis and Lymphocyte Infiltration (Mean±SD)

|                             | MDA (nmol/mg protein) | MnSOD (U/mg protein) | GSH-Px (nmol/min/mg protein) | GST (nmol/min/mg protein) |
|-----------------------------|-----------------------|----------------------|------------------------------|---------------------------|
| **Histological type**       |                       |                      |                              |                           |
| W (n=3)                     | 0.67±0.46             | 7.05±4.23            | 9.66±1.98                    | 9.16±7.21                 |
| M (n=11)                    | 0.85±0.35             | 8.08±5.31            | 15.71±8.16                   | 11.91±5.00                |
|                             | U=10.5; ns            | U=13.0; ns           | U=8.0; ns                    | U=12.0; ns                |
| **TNM stage**               |                       |                      |                              |                           |
| I and II (n=6)              | 0.66±0.29             | 9.14±7.12            | 14.89±10.40                  | 13.06±5.00                |
| III and IV (n=8)            | 0.93±0.38             | 6.94±2.66            | 14.05±5.57                   | 13.07±5.01                |
|                             | U=12.0; ns            | U=23.0; ns           | U=20.0; ns                   | U=13.0; ns                |
| **Necrosis**                |                       |                      |                              |                           |
| + (n=8)                     | 0.74±0.38             | 6.36±3.53            | 13.54±8.96                   | 9.45±4.25                 |
| − (n=6)                     | 0.89±0.35             | 9.91±6.14            | 15.58±6.07                   | 13.8±6.01                 |
|                             | U=16.5; ns            | U=17.0; ns           | U=14.0; ns                   | U=13.0; ns                |
| **Lymphocyte infiltration** |                       |                      |                              |                           |
| + (n=8)                     | 0.77±0.37             | 8.77±6.02            | 14.81±8.77                   | 10.02±4.43                |
| − (n=6)                     | 0.86±0.38             | 6.71±3.20            | 13.88±6.62                   | 13.06±6.37                |
|                             | U=18.5; ns            | U=18.0; ns           | U=24.0; ns                   | U=17.0; ns                |

Statistical evaluation was made by using the Mann-Whitney U-Wilcoxon rank sum W test.
W, well differentiated; M, moderately differentiated.
ns, non significant.

activity in colorectal malignant tissues are also contradictory. Increases\(^{10}\) and decreases\(^{5,9}\) in MnSOD activities were reported. Although there is no report on mitochondrial GSH-Px activity, decreased\(^{11}\), increased\(^{7,16}\) and unchanged\(^{5}\) cytosolic GSH-Px activities were observed in malignant tissues of patients with colorectal cancer.

In our study, mitochondrial MnSOD activity was found to be increased in malignant tissues of patients with colorectal cancer. This result indicates an increase in superoxide radical formation. However, since GSH-Px and SOD were also increased, mitochondria may be protected from oxidative stress produced by excess O\(_2\) and H\(_2\)O\(_2\). The results of this study, except for MDA, are in accordance with those of our previous study\(^{16}\) which was carried out in post-mitochondrial fractions of colorectal adenocarcinoma tissues. Indeed, mitochondrial MDA levels did not alter in malignant tissues of patients with colorectal cancer. On the other hand, mitochondrial GST was also determined in this study. GSTs are a group of enzymes capable of conjugating GSH with structurally diverse electrophilic compounds. Some of these enzymes are also known to exhibit GSH-Px activity and reduce lipid hydroperoxides.\(^{33}\)

It has been reported that several properties of mitochondrial GST are similar to those of microsomal GST.\(^{33,34}\) Since microsomal GSTs, in contrast to cytosolic GSTs, are activated by ROS due to sulfhydryl group oxidation\(^{33}\), increased mitochondrial GST activity may be related to an increase in ROS production in the malignant tissue.

In conclusion, our findings may indicate that mitochondria are protected by adaptive increases in antioxidant enzyme activities against oxidative stress in malignant tissues of patients with colorectal cancer. The cause of increases in activities of all antioxidant enzymes in the tissues of colorectal adenocarcinoma and their relation to carcinogenesis are not clear and require further studies.

**ACKNOWLEDGMENTS**

This work was supported by the Research Fund of the University of Istanbul. Project number Ö-536/081298.

(Received February 29, 2000/Revised July 21, 2000/2nd Revised August 17, 2000/Accepted August 24, 2000)
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