Nitric Oxide Levels and Lipid Peroxidation in Plasma of Patients with Gastric Cancer

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Background: The aim was to investigate the levels of malondialdehyde and total NO₂⁻ plus NO₃⁻ marker for NO⁻ generation in gastric carcinoma and to correlate their levels with the cancer stage.

Method: The pretreatment plasma samples were obtained from 38 patients with gastric cancer (seven patients at stage II, 19 at stage III and 12 at stage IV). Nitrite (NO₂⁻) and nitrate (NO₃⁻) levels, the end products of nitric oxide (NO⁻), were determined in these samples. NO₂⁻ was measured by using the Griess reaction and after enzymatic conversion of NO₃⁻ into NO₂⁻ by nitrate reductase, the resultant NO₂⁻ was also measured by the same method. Malondialdehyde (MDA), a lipid peroxidation marker, was measured by the thiobarbituric acid method.

Results: The levels of plasma MDA, NO⁻ and NO₃⁻ were significantly higher in patients with gastric cancer compared with the healthy control group. Higher levels of MDA, NO⁻ and NO₃⁻ were observed as the stage of the disease increased.

Conclusion: We found that increased NO⁻ production and MDA levels were present in plasma of patients with gastric cancer. These increases can be associated with the oxidant–antioxidant status in these patients.

Key words: lipid peroxidation – malondialdehyde – nitric oxide – gastric cancer – free radicals

INTRODUCTION

Gastric cancer is the second most common fatal malignancy in the world and is the cause of more than 750,000 deaths annually. In 1990, it was the fourteenth most frequent cause of death globally and, despite a general decline in its incidence, projections indicate that the annual number of new cases will increase significantly in the developing world during the next few decades as a result of adult population growth. Most gastric cancer is diagnosed at an advanced stage and survival is uniformly poor, usually no more than 15% at 5 years (1). Gastric cancer formation is a multifactorial process and possible mechanisms leading to gastric carcinogenesis have not yet been clarified. Therefore, this subject needs further molecular studies (2). Growing evidence indicates that reactive oxygen species (ROS) are associated with the different steps of carcinogenesis, either through structural DNA damage, interaction with oncogenes or tumor suppressor genes or immunological mechanisms (3).

For a variety of human cancers, chronic infection and inflammation have long been recognized as risk factors. It has been suggested that active oxygen species such as superoxide anion, hydrogen peroxide and hydroxyl radical generated in inflamed tissues can damage the target cells, resulting in DNA damage and being able to contribute to tumor development (4). There is growing evidence that nitric oxide (NO⁻), an unpaired electron and its derivatives produced by activated phagocytes may also play a role in the multistage carcinogenesis process (4–6). NO⁻ is known, together with other ROS, to induce cytotoxicity and cytostasis. Several studies on NO⁻ and H₂O₂-induced oxidative damage have cited similarities between the two chemicals in their enzymatic generation, chemical interaction with macromolecules and resulting cytotoxicity (7).

NO⁻ is an inorganic free radical gas produced from L-arginine by a family of isoenzymes called NO synthases (8,9). Two of them are constitutively expressed and a third is inducible by immunological stimuli. It is the NO⁻ released by the constitutive enzymes that acts as an important signaling molecule in the cardiovascular and nervous systems and NO⁻ released by the inducible NO synthase (iNOS) is generated for long periods by cells of the immune system among others and has been shown to be cytostatic/cytotoxic for tumor cells and a variety of microorganisms (9).
The role of NO in tumor biology is still poorly understood (9,10). Interactions of endothelial cells of the tumor vasculature, tumor-infiltrating immune cells such as T lymphocytes and macrophages and the tumor cells themselves regulate the growth of solid tumors. Most of these cellular components have been shown to generate NO in vitro (9). Thomsen and coworkers (11,12) showed that NOS was present in fresh human tumor tissue, where it was localized to tumor cells in gynecological cancers and to stroma of breast cancers. Subsequently, increased activity of NOS was reported in patients with human gastric cancer (13,14).

It is well known that NO possesses either antioxidant or pro-oxidant properties. It has been found that the concentrations of NO, under non-pathological conditions, are at nanomolar levels and, under conditions of oxidant injury, at micromolar levels (10,13,15).

Endogenous NO plays a dual role in specialized tissues and cells, where it is an essential physiological signaling molecule mediating various cell functions but also induces cytotoxic and mutagenic effects when present in excess. NO reacts rapidly with superoxide anion to form peroxynitrite, which may be cytotoxic by itself or easily decompose to the highly reactive and toxic hydroxyl radical and nitrogen dioxide (NO₂) (4):

$$\text{NO}^+ + \text{O}_2^- \rightarrow \text{ONOO}^-$$
$$\text{ONOO}^- + \text{H}^+ \rightarrow \text{ONO}^-$$
$$\text{ONO}^- \rightarrow \text{HO}^- + \text{NO}_2^-$$

It is well known that peroxynitrite is much more reactive than NO or superoxide, which will cause various chemical reactions in biological systems including nitrination of tyrosine residues of proteins, triggering of lipid peroxidation, inactivation of aconitases, inhibition of mitochondrial electron transport and oxidation of biological thiol compounds (16). NO could also be directly oxidized to NO₂⁻, which induces DNA damage. In addition, the reaction of NO with H₂O₂ has recently been shown to produce potentially cytotoxic singlet oxygen (4).

The process of lipid peroxidation is one of oxidative conversion of polyunsaturated fatty acids to products known as malondialdehyde (MDA) or lipid peroxides, which is the most studied, biologically relevant, free radical reaction (17). MDA itself, owing to its high cytotoxicity and inhibitory action on protective enzymes, is suggested to act as a tumor promoter and a co-carcinogenic agent (18). On the other hand, it is reported that lipid hydroperoxides decompose to yield reactive aldehydes, such as MDA and 4-hydroxynoneal. MDA is a well-characterized mutagen that reacts with deoxyguanosine to form a major endogenous adduct found in the DNA of human liver (19).

The aim of this study was to investigate the plasma nitrite (NO₂⁻), nitrate (NO₃⁻), total NO and MDA levels in the plasma of patients with gastric cancer and to correlate their levels with the disease stage.

### PATIENTS AND METHODS

#### PATIENTS

Thirty-eight patients (25 males, 13 females) with gastric cancer comprised the patient group and 24 healthy subjects (16 males, 8 females) were taken as control group, with the age range being 39–72 years (mean ± SD, 54.5 ± 11.2 years) for the patients and 38–69 years (mean ± SD, 51.9 ± 10.7 years) for the controls. Patients’ characteristics are shown in Table 1. Tumors were classified according to UICC criteria (20). Seven patients were in stage II, 19 in stage III and 12 in stage IV. Seven of the stage IV patients had distant organ–tissue metastases. Seven patients had peritoneal dissemination. Distant organ and lymph node involvement was documented by chest radiography, abdominal ultrasonography and computed tomography.

#### BLOOD SAMPLING

Gastric cancer patients and healthy control subjects were recruited into the study after obtaining their informed consent. Venous blood (10 ml) was taken from controls and patients. The blood samples were centrifuged and the plasma samples obtained were stored at −80°C until the analysis date.

#### BIOCHEMICAL MEASUREMENTS

The measurement of plasma NO is difficult because this radical is poorly soluble in water and has a short half-life in tissue (10–60 s), but its half-life may be as long as 4 min in the presence of oxygen. For these reasons, the NO itself can be determined only with difficulty and requires the handling of radioisotopes. In spite of this, the end products of the phenomenon, nitrate and nitrite, are preferentially used in clinical biochemistry (21,22). Plasma total nitrite + nitrate levels were measured with use of the Griess reagent as described previ-
Table 2. Levels (µmol/l) of MDA, NO$_3^-$, NO$_2^-$ and NO$^*$ in plasma of gastric cancer patients and control subject group (mean ± SD)

<table>
<thead>
<tr>
<th></th>
<th>MDA</th>
<th>NO$_3^-$</th>
<th>NO$_2^-$</th>
<th>Total NO$^*$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control group (n = 24)</td>
<td>5.4 ± 1.1</td>
<td>32.9 ± 7.7</td>
<td>83.4 ± 21.3</td>
<td>116.5 ± 22.6</td>
</tr>
<tr>
<td>Patient groups</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stage II (n = 7)</td>
<td>6.7 ± 1.0$^a$</td>
<td>34.3 ± 7.8</td>
<td>136.0 ± 16.8$^b$</td>
<td>170.3 ± 23.0$^b$</td>
</tr>
<tr>
<td>Stage III (n = 19)</td>
<td>7.7 ± 1.5$^b$</td>
<td>36.5 ± 8.6</td>
<td>149.2 ± 23.2$^b$</td>
<td>184.1 ± 26.9$^b$</td>
</tr>
<tr>
<td>Stage IV (n = 12)</td>
<td>7.8 ± 1.3$^b$</td>
<td>37.6 ± 6.3</td>
<td>178.9 ± 25.6$^{b,c,e}$</td>
<td>212.9 ± 19.1$^{b,c,e}$</td>
</tr>
<tr>
<td>Total (n = 38)</td>
<td>7.6 ± 1.4$^b$</td>
<td>36.5 ± 7.6</td>
<td>155.0 ± 24.5$^b$</td>
<td>190.7 ± 28.4$^b$</td>
</tr>
</tbody>
</table>

* P < 0.01, $^a$P < 0.001, when compared with control group.

* P < 0.01, $^b$P < 0.001, when compared with stage II.

* P < 0.01, $^c$P < 0.001, when compared with stage III.

Table 3. Pearson’s rank correlation coefficients between plasma MDA, NO$_3^-$, NO$_2^-$ and NO$^*$ levels in gastric cancer patients

<table>
<thead>
<tr>
<th></th>
<th>Stage II</th>
<th>Stage III</th>
<th>Stage IV</th>
<th>Total group</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA–NO$_3^-$</td>
<td>0.535</td>
<td>N.S.</td>
<td>0.226</td>
<td>N.S.</td>
</tr>
<tr>
<td>MDA–NO$_2^-$</td>
<td>0.777</td>
<td>0.04</td>
<td>0.570</td>
<td>0.011</td>
</tr>
<tr>
<td>MDA–NO$^*$</td>
<td>0.752</td>
<td>0.051</td>
<td>0.493</td>
<td>0.032</td>
</tr>
</tbody>
</table>

N.S.: not significant.

Statistical Analysis

The findings were expressed as the mean ± SD. Statistical and correlation analyses were undertaken using the Mann–Whitney U-test and Pearson’s rank correlation test, respectively. A P value <0.05 was accepted as statistically significant. SPSS (for Windows, Version 10.0) was used for statistical analyses.

RESULTS

Our findings on the assessed parameters and correlations between parameters in gastric cancer and healthy control subjects are shown in Tables 2 and 3, respectively (Fig. 1). Mean plasma NO$_3^-$, NO$_2^-$ and MDA levels were found to be lowest in the control group and highest in the stage IV patients. As seen in Table 2, as the stage of the disease increased, higher levels of plasma NO$_3^-$, NO$_2^-$ and MDA were determined. Plasma NO$_3^-$, NO$_2^-$ and MDA levels in the patients of each stage were compared with those of control groups. For MDA, the difference between the control group and stage II group was at the P < 0.01 level and that between the controls and the stage III and IV groups at the P < 0.001 level. For NO$_3^-$ and NO$_2^-$, the difference between control group and stage II, III and IV groups was at the P < 0.001 level. The difference in plasma NO$_2^-$ between stage II and stage IV groups was at the P < 0.001 level. The difference in plasma NO$_3^-$, NO$_2^-$ and MDA between total patient group and control group was at the P < 0.001 level. Plasma NO$_2^-$ levels were of no significance in all comparisons. There was no statistically significant difference in the parameters when the patients were grouped with respect to metastasis. Table 3 summarizes the correlations between the parameters.

The Gries reagent consists of sulfanilamide and N-(1-naphthyl)ethylenediamine. The method is based on a two-step process. The first step is the conversion of nitrite to nitrite using nitrate reductase. The second step is the addition of Griess reagent, which converts nitrite into a deep-purple azo compound; photometric measurement of the absorbance at 540 nm due to this azochromophore accurately determines the nitrite concentration (sodium nitrate is used as a standard). Protein interference was eliminated by treatment of the reacted samples with zinc sulfate and centrifugation for 5 min at 10 000 g. MDA, an end product of lipid peroxidation, reacts with thiobarbituric acid (TBA) to form a colored substance. Measurement of MDA by TBA reactivity is the most widely used method for assessing lipid peroxidation. MDA was estimated according to the modified method of Satoh (24). To 0.5 ml plasma was added 0.5 ml of 35% TCA. After vortex mixing, 0.5 ml of Tris–HCl buffer (50 mM; pH 7.4) was added, followed by further mixing and incubation at room temperature for 10 min. A 1 ml volume of 0.75% TBA in 2 M Na$_2$SO$_4$ was added and then the mixture was heated at 100 °C for 45 min. After cooling, 1 ml of 70% TCA was added and the mixture was vortex mixed and then centrifuged at 950 g for 10 min. The absorbance of the supernatant was measured at 532 nm. Total TBA-reactive materials were expressed as MDA, using a molar absorptivity for MDA of 1.56×10$^5$ cm$^{-1}$ M$^{-1}$. The results were expressed as µmol/l.
DISCUSSION

NO\(^{\cdot}\) is a multifunctional species that is implicated in a wide variety of physiological and pathological processes (3,9). NO\(_{2}^{\cdot}\) and NO\(_{3}^{\cdot}\) in plasma reflect the level of NO\(^{\cdot}\) formation. Various studies showed that NOS activity and NO\(^{\cdot}\) synthesis were high in tumor tissue and in plasma (13–16,25–29). The progression of a tumor might be related to the oxidant–antioxidant status (21,30,31). To our knowledge, plasma NO\(^{\cdot}\) and MDA levels are reported together in gastric cancer for the first time in this study. Our results showed that nitrite, nitrate, NO\(^{\cdot}\) and MDA levels were higher in gastric cancer than in the control group.

It has been claimed that MDA acts as a tumor promoter and co-carcinogenic agent because of its high cytotoxicity and inhibitory action on protective enzymes (18). There are controversial results on this subject in the literature. For example, decreased plasma MDA (21,31), thiobarbituric acid reactive substances (TBARS) (32) and tissue MDA (33) levels have been reported in patients with breast cancer. Gerber et al. (27) reported that plasma MDA levels decreased with increasing tumor size and progression in breast cancer. However, Huang et al. (34) reported significantly increased lipid peroxidation, measured as MDA, in the serum of breast cancer patients. On the other hand, Seven et al. (18) and Samir and el Kohly (17), in their studies on patients with laryngeal carcinoma, reported that plasma MDA levels were significantly increased compared with healthy controls. Nagini et al. (35) found that MDA levels were lower in tissue with oral squamous cell carcinoma than in the control group. Increased serum MDA levels were reported in patients with gastric cancer (36). Haklar et al. (25) reported significantly increased NO\(^{\cdot}\) in tissues in patients with colon tumors. Our results for MDA and NO\(^{\cdot}\) levels in plasma of gastric cancer patients are in agreement with those obtained by Choi et al. (36), Alagöl et al. (21) and Haklar et al. (25).

In conclusion, we found that increased NO\(^{\cdot}\) production and MDA levels were present in plasma of patients with gastric cancer, which can be related to an alteration in oxidant–antioxidant status. Since NO\(^{\cdot}\) seems to have a dual role in tumor progression, high concentrations of NO\(^{\cdot}\) for long periods could result in damage to DNA, leading to mutations and cancer. One should take into consideration that the levels of NO\(_{2}^{\cdot}\) and NO\(_{3}^{\cdot}\) are also influenced by dietary factors in addition to endogenous synthesis and that further studies to clarify this issue are necessary. Moreover, the role of NO\(^{\cdot}\) and nitrous compounds in carcinogenesis is still under discussion. Further studies are needed.

References

7. Abu-Shakra A, McQueen ET, Cunnigham ML. Rapid analysis of base-pair substitutions induced by mutagenic drugs through their oxygen radical or epoxide derivatives. Mutat Res 2000;470:11–8.


