Tumour angiogenesis is required for the growth of solid tumours and metastasis. The significance of solid tumour angiogenesis as a prognostic factor has been investigated in many human tumours including gastric carcinoma (Maeda et al, 1995; Araya et al, 1997). Among the various angiogenic factors, vascular endothelial growth factor (VEGF) has been regarded as the most likely candidate for the induction of angiogenesis in tumour growth (Senger et al, 1993).

Nitric oxide (NO) is a biologically active mediator derived from L-arginine, which is catalysed by NO synthase (NOS) (Moncada et al, 1991). NO produces multiple effects that can influence the outcome of metastasis and regulates vasodilatation which affect tumour cell arrest in capillaries (Dong et al, 1994). In recent years, some studies have shown that NOS is present in human tumour tissues, including gynaecological cancer (Thomsen et al, 1994), breast cancer (Thomsen et al, 1995), brain tumour (Cobbs et al, 1995), gastric cancer (Miles et al, 1996) and head and neck cancer (Gallo et al, 1998).

Although many studies have shown that VEGF was over-expressed by a variety of human cancer tissue, relatively little is known about the behaviour of VEGF in the circulating level in patients with gastric cancer. Moreover, previous investigations have reported that several murine and human tumour cell lines produce NO. However, still little is about the correlation between the NO and VEGF in the circulating levels.

The aim of the present study was to investigate serum concentrations of VEGF and nitrite as an estimate of in vivo nitric oxide in patients with gastric cancer as well as healthy individuals and to examine the influence of tumour stage on circulating levels of NO and VEGF.

MATERIALS AND METHODS

Study populations

The study consisted of 11 healthy individuals without any evidence of disease (e.g. liver dysfunction, diabetes etc.) and 37 patients with primary gastric carcinoma who were treated at our department. No patient had received chemotherapy or radiation therapy before surgery. None of the subjects had renal dysfunction, infection, or gastrointestinal bleeding. In addition, none of them received any antibiotics or vasoactive drugs before the sera were obtained.

The patients ranged in age from 32 to 81 years (mean age 61 years). There were 21 men and 16 women. To investigate whether NO and VEGF concentrations in serum could reflect the tumour stage, we classified the patients according to 1997 TNM classification (Sobin and Wittekind, 1997), and measured the VEGF and estimated NO concentrations in their serum. In addition, patients were categorized into three groups according to tumour grade. Patients’ characteristics are summarized in Table 1.
Laboratory assay

Sera were obtained from patients who had not undergone surgery. All patients and control subjects fasted for at least 12 h. Venous blood samples were drawn into a tube and allowed to clot for 20 min before centrifugation at 3000 rpm for 10 min. The serum samples were stored at –80°C until analysis. The VEGF levels in preoperative sera of patients and healthy donors were measured by using the quantitative sandwich immunoassay technique (Quantikine, R&D Systems, UK).

Griess reaction was the method of choice to evaluate serum nitrite/nitrate levels with the following modifications. In vivo NO concentration* estimation was done via analysis of nitrates (NO–3) in serum without reduction of nitrates (NO–2) step, as it has recently been suggested that inclusion of nitrates in the determination of in vivo NO* causes an overestimation of approximately 500% than when NO was measured by the haemoglobin reaction (Privat et al, 1997). Briefly, serum samples were deproteinized with 0.6 N perchloric acid and the supernatants were assayed for nitrite concentration through recording the absorbance at 540 nm.

\[
\text{NO}_{\text{in vivo}} = \left( \frac{\text{OD}_{\text{serum}} - \text{OD}_{\text{serum blank w/ H2O}}}{\text{F}} \right) \times \mu\text{M}
\]

F = 100 (conversion factor from \(A_{540}\) to \(\mu\text{M}\) nitrite from standard curve) due to the equations valid for oxygenated solutions (1)–(3) (Hevel and Marletta, 1994).
increased mean level of VEGF, 695.2 ± 311.4 pg ml⁻¹ and 759.3 ± 
400.5 pg ml⁻¹ respectively, compared with well-differentiated 
tumours (349.1 ± 247.3 pg ml⁻¹), but these differences were not 
found as statistically significant (P > 0.05). Also, there was no 
significant difference between men and women and between 
tumour locations (P > 0.05).

The distribution of the mean level of VEGF on the basis tumour 
stage was as follows: 166 ± 52.4 pg ml⁻¹ (range 122.6–242.3) in 
stage 1; 248.1 ± 107.7 pg ml⁻¹ (range 168.2–504) in stage 2; 658.6 
± 324 pg ml⁻¹ (range 209.6–1273) in stage 3; and 1233.6 ± 317.3 
pg ml⁻¹ (range 798.7–1777) in stage 4. It was seen that large 
tumour burden was associated with increased level of VEGF. 
The mean level of VEGF in sera from healthy individuals was 
94.5 ± 55.7 pg ml⁻¹ (range 36.1–218). A statistically significant 
difference in serum VEGF concentrations was found between the 
groups (P < 0.001) (Figure 1).

As shown in Figure 2, serum NO levels in patients with stage 1 
gastric carcinoma (mean 11 ± 2.9 μM, range 8–14) were 
significantly higher than those in healthy individuals (mean 8.5 ± 4.3 μM, 
range 2–11).

The ANOVA test also showed a significant difference in serum 
NO concentrations among stage 1, stage 2 (mean 20.4 ± 4.5 μM, 
range 13–27), stage 3 (mean 36.8 ± 7.4 μM, range 26–53) and 
stage 4 disease (mean 55.6 ± 17.5 μM, range 38–95) (P < 0.001). 
On the other hand, the serum nitrite level was higher in grade 3 
tumour patients (mean 40 ± 14.8 μM) compared with grade 1 
(mean 26.6 ± 10.8 μM) or grade 2 tumour patients (mean 32 ± 24.3 
μM). But, these differences were not statistically significant 
(P > 0.05). Moreover, there was no significant difference with 
respect to patient gender and tumour location. In addition, when 
we correlated serum VEGF level with serum NO concentration, 
we found that the increased VEGF level was statistically associ-
ated with an elevated nitrite level (r = 0.8 and P < 0.0001).

By ROC analysis, the sensitivity and the specificity for VEGF 
and NO were computed as 91.9% and 81.8%; 89.2% and 90.9% 
respectively. Area under the curves of VEGF and NO were also compared. 
For all tests, a P-value less than 0.05 was accepted as significant.

The statistical analysis was performed by Statistical Package 
for Social Sciences (SPSS for MS Windows Release 5.0, Chicago, 
IL, USA).

RESULTS

Two major histological types considered in this study were ade-
ocarcinoma, diagnosed in 28 patients, and mucinous carcinoma, 
defined as when a mucinous component was evident in more than 
50% of the tumour section in nine patients.

Tumours were staged by TNM classification. Accordingly, there 
were four patients in stage 1 (T1 N0 M0 in one, T1 N1 M0 in two, 
T2 N0 M0 in one), eight in stage 2 (T2 N1 M0 in four, T3 N0 M0 
in four), 15 in stage 3 (T3 N1 M0 in six, T3 N2 M0 in eight, 
T4 N0 M0 in one) and ten in stage 4 (T3 N3 M0 in four, T4 N2 M0 
in two, T4 N2 M1 in two, T4 N3 M1 in two). The distribution of 
patients according to the degree of histological differentiation 
of the tumour was as follows: well-differentiated tumours (G1) in six 
patients, moderately differentiated (G2) in 12, poorly differenti-
tiated tumours (G3) in 19 patients.

No significant correlation was seen between histological type of 
the tumour and serum VEGF concentrations. Moderately differenti-
tiated and poorly differentiated tumours were related to an
shown that serum VEGF level was significantly elevated in some population of cancer patients including those of gastric cancer compared with control subjects (Yamamoto et al, 1996).

In the present study, we determined serum VEGF concentrations in patients with gastric carcinoma as well as healthy individuals. Here we reported our findings from serum samples from different tumour stages of human gastric cancer. Elevated serum levels of VEGF were found in advanced disease patients when compared to early-stage disease and healthy individuals. This suggests that high VEGF level is related to advanced tumour stage. Although the number of patients studied was small, we suggest that VEGF assay may be useful in predicting the advanced gastric carcinoma.

Important steps in angiogenesis include invasion of the vascular wall of the parent vessel by activated endothelial cells which then proliferate, migrate and form the lumen of the new vessel (Fidler and Ellis, 1994). The role of NO in tumour angiogenesis is not sufficiently understood. Although NO was first identified in endothelial cells, it is well known to be generated by a variety of solid human tumours. Previous investigations have shown NO to cause increased tumour blood flow, oedema and vascular permeability (Nathan and Xie, 1994; Fukumura et al, 1997). NO production by tumour cells may influence tumour growth and metastasis (Pipili-Synetos et al, 1993; Jenkins et al, 1995). The functions of NO in tumours potentially facilitate tumour growth. Moreover, tumour blood flow and vessel diameter of experimental tumours in mice decreased after the treatment with NOS inhibitors (Andrade et al, 1992; Thomsen et al, 1997).

NOS activity was observed in solid human tumour tissues, including gynaecological cancer (Thomsen et al, 1994), breast cancer (Thomsen et al, 1995), brain tumour (Cobbs et al, 1995), gastric cancer (Miles et al, 1996) and head and neck cancer (Gallo et al, 1998). Furthermore, this enzyme activity in the tumour tissues correlated with grade.

In a recent study, it was suggested that the increased plasma nitrite/nitrate levels were correlated to tumour volume in patients with hepatocellular carcinoma (Moriyama et al, 1997). In the present study, we have estimated serum NO as nitrite in gastric cancer patients and control subjects. We have investigated the effect of tumour stage on the production of NO in sera of gastric cancer patients. A striking relationship was found between serum NO levels and tumour stage (P < 0.001). Although the serum NO level was elevated in high-grade tumour compared with low-grade tumour, this difference was found to be statistically insignificant. The slightly lower serum NO concentrations were observed in stage I disease; however, the highest serum level of NO was found in stage 4 tumour. Serum NO levels were higher in patients with advanced gastric cancer and were also positively correlated with increased VEGF levels.

Recently, Gallo et al (1998) reported an interesting analysis of NO production and angiogenesis in 27 patients with head and neck cancer. Their results revealed a strong positive correlation between the expression of NOS and tumour angiogenesis and tumour progression. To our knowledge, there has been no report about the relationship between NO and VEGF concentration in sera in gastric cancer patients. We therefore examined whether serum NO levels correlate with serum VEGF levels.

In this study, we show that an increase in NO levels is found in gastric cancer, is associated with elevated VEGF levels and correlates with tumour stage. This positive correlation between tumour stage and serum NO and VEGF levels in patients with gastric cancer suggests that NO might play a role in the biology of the gastric cancer. The relationship observed between tumour stage, VEGF and NO may be due to ongoing neovascularization associated with increasing tumour burden. It needs further studies to elucidate a precise role for the NO pathway in tumour angiogenesis. In addition, antiangiogenetic therapy has been investigated and may be a new treatment for metastatic disease in the future (Folkman, 1997). Accordingly, it is also recommended that the monitoring of VEGF and NO levels in the circulation may be useful to assess the anti-angiogenetic therapy.

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