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Original Article

Monitoring of Serum Nitric Oxide in Patients with Acute Leukemia

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Abstract

Nitric oxide (NO) is a molecule required for many physiological functions, produced from L-arginine by NO synthases (NOS). It is a free radical, producing many reactive intermediates that account for its bioactivity. Sustained induction of the inducible form of NOS (iNOS) in chronic inflammation may be mutagenic, through NO-mediated DNA damage or hindrance to DNA repair, and thus potentially carcinogenic. Due to the short half-life of NO, usually its end products (nitrate or nitrite) are measured as an index of NO production. There is evidence that expression of iNOS in tumor cells, including acute myeloid leukemia and chronic lymphocytic leukemia increased. In this study, the levels of nitrate and nitrite (nitric oxide products) in the serum of patients with acute leukemia were determined.

The serum levels of these compounds were measured in 40 acute leukemia patients. The results of serum nitrite and nitrate of patients were compared with corresponding values obtained in 40 healthy volunteers.

These results indicate that patients with acute leukemia had a significant increase in the serum level of nitrite and nitrate.

Keywords: Nitric oxide; Nitrate; Nitrite; Leukemia.

Introduction

Nitric oxide (NO) is a free radical molecule, that at physiological levels is associated with neurotransmission and vasodilatation and at higher levels has tumoricidal and bactericidal effects (1, 2). In the cell mediated immune responses, NO is produced in macrophages, neutrophils and lymphocytes (3, 4). NO is produced through the oxidation of L-arginine to L-citruline, by the enzyme nitric oxide synthase (NOS). Three isoforms of NOS exist, namely the constitutive forms, endothelial NOS (eNOS/NOS2) and neuronal NOS (nNOS/ bNOS/NOS1), and the cytokine inducible NOS (iNOS/NOS2) (5-7). NOS are a unique family of P_{450}-type hemoproteins which use NADPH, FAD, FMN, heme and tetrahydrobiopterin as co-factors (8). The constitutive NOS (cNOS) forms are constitutively active and regulated by calcium and calmodulin (9). This class produces

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pico – to nano – mole of NO for short periods, in response to receptor stimulation such as acetylcholine or shear stress (10). The inducible NOS, contains calmodulin as an integral component and is not affected by external calcium concentration. This enzyme releases high levels of NO for extended periods of time (11). Inducible NOS is a cytosolic enzyme of many cells, such as macrophages, endothelial cells, condrocytes, hepatocytes, synoviocytes and smooth muscle cells (10). Inducible NOS is induced by inflammatory stimuli (12) and NO produced by this enzyme is a vital component of the tumouricidal and fungicidal apparatus of macrophages (13). The expression of iNOS is regulated by the balance of cytokines in the micro-environment, for example, transforming, growth factor β and interlukin-10 inhibit iNOS expression in macrophage (10). There is evidence that increased amounts of blood nitrate can be detected *in-vivo* during infections (14), following cytokine administration (15), sepsis (16), ulcerative colitis (17), arthritis (18), multiple sclerosis (19), type I diabetes (20) and a variety of rheumatic diseases, including systemic lupus erythematosus, sjogren's syndrome, vasculitis, osteoarthritis and rheumatoid arthritis (21). Significant activity of inducible NOS has been reported in tumor cells, including acute and chronic leukaemic cells (22). Thus, in the present study, measured the serum concentration of nitric oxide metabolites (nitrate and nitrite) was measured in patients with acute leukemia.

Experimental

Materials

All the chemicals used were obtained from Sigma Chemical Company (Germany), except for cadmium granules which were purchased from Aldrich (Dorset, UK) and centriflo membrane filter cones which were used for the deproteinization of serum from Amicon (Stonehouse, Glos; UK).

Subjects

This study included 40 healthy male adults aged between 20 to 40 years (mean of 33 ± 6.19) as the control group and 40 male adult patients suffering from acute leukemia (regardless of the type of acute leukemia) aged between 20 to 40 years (mean of 32 ± 10.71 years) as the study group.

Sample preparation

Venous blood samples (6-7 ml) were obtained from all patients and controls. The samples were collected in plain tubes and serum was separated. The sera were kept at -20°C until the day of analysis.

Methods

For plotting the calibration curve from the stock solution of sodium nitrite (1000 µm), different concentrations of nitrite from 0.78 to 50 µm were prepared. Then to each tube 105 µl of the mixture, with a ratio 1:1 containing sulphanilamide solution (58 mmol/l in 3 M HCl) and naphthylethylenediamine solution (772 µmol/l), were added and mixed vigorously by vortexing. After 15 min absorbance of the solutions were taken using a Perkin-Elmer UV/Vis Lambda 2 Spectrophotometer at 543 nm, against distilled water as the blank (23, 24). It is necessary to use protein free samples for nitrite assay. Hence, samples were deproteinized by centrifugation, using the centriflo membrane cones type CF-25. Two ml of serum were pipetted into each cone and centrifuged at 920×g for 35 min. Filtrates were used for nitrite assay. To one ml of the filtrate, 105 µl of sulphanilamide and naphthylethylenediamin (1:1) mixture were added. Absorbance of the samples was taken at 543 nm and its nitrite concentration determined using the calibration curve (24). For measurement of nitrate, samples were deproteinized, using the Somogyi's method (25).This method is a rapid, simple and inexpensive procedure for the precipitation of blood proteins (25). To one ml of serum, 8 ml distilled water, 0.5 ml zinc sulphate (10%) and 0.5 ml NaOH (0.5 N) were added. Samples were incubated for five min at room temperature and then centrifuged at 4000×g for ten min (25). For measurement of nitrate, copper coated cadmium granules were used to convert nitrate to nitrite. For this step, cadmium granules previously stored in 0.1 M sulphuric acid were washed by swirling with distilled water. Then a solution of copper sulphate (15 mmol/l in 0.2 mol/l glycine buffer, pH 9.7) was used to coat

the granules by submerging them for two min. Cadmium granules were drained and dried over tissue paper, and used within five min. To reduce nitrate to nitrite, 0.5 ml of deproteinized samples were added to labeled tubes, followed by the addition of 0.5 ml of glycine buffer (0.2 mol/l, pH 9.7) and 2-3 g of copper coated cadmium granules. The tubes were shaken for 15 min by vortexing. After the reduction step, 0.5 ml of the sample was transferred to an appropriate labeled tube, followed by the addition of 0.5 ml of freshly prepared color reagent (a mixture of sulphanilamide and naphthylenediamine with a ratio of 1:1), and then the nitrite levels determined using the assay method described above (24).

Statistical analysis

Data are expressed as mean±S.E. Statistical significance was evaluated by the student's t-test. Differences were considered significant at $p \leq 0.05$.

Results And Disccusion

The calibration curve for nitrite could be seen in figure 1. Serum nitrite level for the control group was 7.5 ± 0.44 µmol/l, whereas this level for the patient group was 12.5 ± 0.18 µmol/l (Table 1). The mean nitrate level value obtained for the control group was 17 ± 0.35 µmol/l, while the value for the patient group was 21.7 ± 0.18 µmol/l (Table 1). The levels of nitrite and nitrate increased significantly ($p<0.001$) in the serum of patients with acute leukemia.

Leukemia is a type of cancer that starts in the bone marrow, but in most cases quickly moves into the blood. The exact cause of leukemia is still unknown. Scientists suspect that viral, genetic, environmental or immunological factors may be involved (26). In vivo studies suggest that nitric oxide (NO) plays an important role in the control of tumor growth (27). Despite the cytotoxic and cytostatic properties of NO in the tumoricidal activity of the immune system, studies have

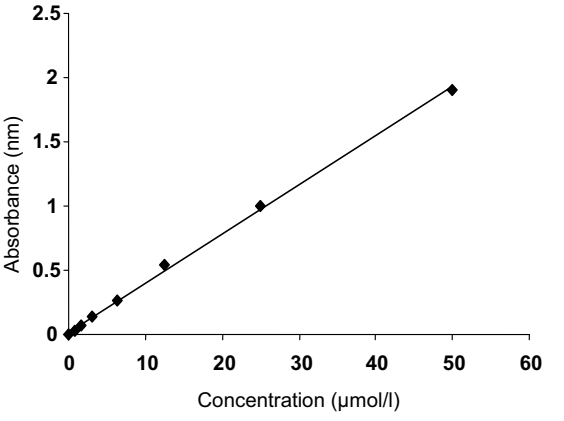

Figure 1. Calibration curve of nitrite at 543 nm (n=?).

indicated that NO can be an important mediator of tumor growth (28-30). In acute and chronic leukemia, the clonal accumulation of B tumoral cells appears as a consequence of prolonged survival due to the inhibition of apoptotic cell death rather than increased proliferation (22, 31). Recently it has been reported that inducible NO synthase (iNOS) protein can be detected in the cytoplasm of B chronic lymphocytic leukemia cells. It is proposed that this should provide further insights into the mechanism controlling proliferation and apoptosis in these tumor cells (32). The regulation of iNOS expression is complex and occurs at multiple levels, and includes transcriptional and post-transcriptional mechanisms (33). The human iNOS gene corresponds to a single 37 kb genomic DNA fragment and consists of 26 exons and 25 introns located on chromosome 17 (34). Interlukin-4 (IL-4) can prevent spontaneous apoptosis of cultured B chronic lymphocytic leukemia cells and IL-4 and interferon gamma (IFN-γ) can induce iNOS expression in these cells (35). Inducible NOS expression is typically regulated in a synergistic manner by a combination of inducers of the NF-κß pathway and by interferon-Jak-STAT pathways. The iNOS promoter contains several IFN-γ activation sites (GAS elements) regulated by signal transducer and activator of transcription-1 (STAT-1) and STAT-6 (36). Recent reports have demonstrated that STAT-1

Table 1. The nitrite and nitrate levels in the serum of patients with acute leukemia.

Group	Number	Nitrite (µmol/l) Mean±S.E.	Nitrate (µmol/l) Mean±S.E.
Control	40	7.52±0.44	17.1±0.35
Patient	40	12.36±0.18*	21.68±0.18*

*P<0.001 (Significant P value)

is activated and STAT-6 nuclear translocation is induced by IL-4 and control iNOS expression at the transcription level (37, 38).

In the present study the serum concentration of nitric oxide products (nitrite and nitrate) of patients with acute leukemia, regardless of the type of disease, was analyzed. All patients had higher levels of nitrite and nitrate, compared to the control group. Comparison of nitrite and nitrate levels in these patients with the control group showed an increase by approximately 39% and 21%, respectively.

Based on previous studies, a potent increase in the IL-4 level of T-cells obtained from chronic lymphocytic leukemia patients has also been reported (39). This was the result of iNOS expression and nitric oxide levels (37-39).

Tetrahydrobiopterin, a cofactor for NO synthase, is produced when the cells involved in cellular immunity are activated (40). Furthermore, it has been reported that urine concentrations of neopterin, an intermediate product of tetrahydrobiopterin, changes according to immunological conditions of the host (41). Urine neopterin levels were remarkably elevated at patients with malignant lymphoma, acute myelocytic leukemia and multiple myeloma. Therefore, the serum NO levels were also elevated in these patients (42).

The previous works and present results indicate that the measurement of nitric oxide (NO) could be a diagnostic, as well as prognostic, tool during the treatment of patients with acute leukemia.

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