Circulating Cytokines and Nitric Oxide are Involved in the Inhibition of Neutrophil Migration in Patients with Uterine Cervical Neoplasia

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Abstract

Aim: To verify if patients with cervical neoplasia produce mediators that reduce leukocyte function.

Methods: Control neutrophils incubated with normal serum or serum from pre-invasive or invasive neoplasia patients were assayed for chemotaxis. Mediators were assayed in serum and in leukocyte supernatants. Experiments were also performed in random patients after surgery.

Results: Neutrophils incubated with patient sera, but not normal sera, failed to migrate towards the chemoattractants. In invasive neoplasia compared to controls, IL-6 and IL-8, and IL-10 and TNF-α were elevated in serum and in neutrophil supernatants, respectively. Nitrite levels were elevated in mononuclear cell supernatants from patients than controls. After surgery, serum cytokine levels were reduced, mainly in pre-invasive patients. Neutrophils treated with serum from pre-invasive patients undergone surgery had restored migration.

Conclusion: Patients with cervical neoplasia produce mediators, predominantly induced by tumor cells, able to impair the inflammatory response at very early stages of disease.

Keywords: cervical cancer, tumor stage, neutrophil function, cytokine, nitric oxide
Introduction

Macrophages and lymphocytes have been the predominant focus of studies into the mechanisms involved in the elimination of tumor cells, but neutrophils also possess anti-tumor activity through the induction of apoptosis-inducing genes.\(^1\) In addition, these cells contain reactive oxygen species and proteinases that are capable of modifying tumor growth and invasiveness.\(^2\) Pre-clinical studies evaluating the treatment of advanced rectal carcinoma have demonstrated that granulocytes are the most effective cell population.\(^3\) The anti-tumor activity of alemtuzumab, a monoclonal antibody approved for the treatment of B-cell chronic lymphocytic leukemia, has been shown to be mediated by neutrophils, since increasing the number of circulating neutrophils enhanced the anti-tumor activity of the antibody.\(^4\)

Patients with cancer may present alterations in leukocyte function. Neutrophils obtained from patients with colorectal carcinoma had reduced phagocytic activity upon diagnosis compared to controls.\(^5\) In addition, patients with gynecologic cancer, including cervical cancer, had reduced production of superoxides at the initial stages that markedly evolved with the disease.\(^6\) In epithelial ovarian cancer patients, a defect in the anti-tumor function of macrophages has been reported.\(^7\) Multiple lineages of tumors, including breast cancer, colon cancer and melanoma, exhibit an interruption in the functional maturation of natural-killer-cells during tumor growth.\(^8\)

The mechanisms involved in the inhibition of leukocyte function in cancer patients may in part, be due to the presence of circulating factors.\(^9\)\(^-\)\(^11\) Circulating cytokines and chemokines reduce neutrophil adhesion to endothelial cells through the activation of the inducible nitric oxide synthase, which results in a reduction of neutrophil migration to the site of infection in models of sepsis.\(^12\) Moreover, plasma concentration of cytokines and nitric oxide (NO) is increased in human sepsis.\(^13\)

Previously, we had shown that a defect in neutrophil migration was associated with cancer progression in cervical neoplasia, which is the second most common cancer in women worldwide. A reduction in the capacity of neutrophil migration was evident in patients with invasive cervical cancer compared with patients at early stages of the disease and with controls. In addition, surgical elimination of tumors in patients with pre-invasive neoplasia resulted in an increase in neutrophil and mononuclear cell migration, suggesting that tumor cells may produce inhibitory soluble factors of leukocyte migration.\(^14\)\(^,\)\(^15\)

Based on these observations, we hypothesized that patients with uterine cervical neoplasia produce circulating factors with inhibitory effect on leukocyte function, apart from pre-invasive stage. To address this question, we evaluated whether there are alterations in the migratory capacity of normal blood neutrophils treated with sera from patients with cervical neoplasia. In addition, we quantified the systemic levels of mediators that may be involved in the inhibition of neutrophil migration in those patients. Finally, in order to confirm these findings, we assessed the same parameters after surgical treatment.

Patients and Methods

Patients

Women attending the Outpatient Gynecologic Service of the Federal University of Triângulo Mineiro (UFTM) who had been diagnosed with cervical neoplasia were considered for this study. Patients were not included if they had previously received treatment or were under current use with immunosuppressors. The study protocol was approved by the UFTM Human Subject Use Committee and written informed consent was obtained from each patient.

Patients without clinical and cytological findings were considered normal subjects (controls). The final cytological and anatomopathological diagnosis followed the International Federation of Gynaecology and Obstetrics (FIGO) classification. Accordingly, patients were diagnosed as having cervical intraepithelial neoplasia (CIN) grade 3 (pre-invasive neoplasia group) or invasive carcinoma (stage IB1 to IVB, invasive group). Randomly selected patients within the pre-invasive or invasive groups were treated by cold knife conization or Wherteim-Meigs surgery, respectively, and re-evaluated for the inhibitory effect of serum on neutrophil migration and for the production of mediators approximately 30 to 60 d after treatment.

Blood collection

Peripheral venous blood was collected from the controls and all patients upon diagnosis and then in randomly selected patients from the pre-invasive or invasive groups on follow-up 30 to 60 d
after treatment. One 5 mL sample was collected with heparine (100 IU/mL) for purification of leukocytes and one 5 mL sample was collected without an anticoagulant for assessment of cytokines and NO levels. The blood was centrifuged at 180 × g for 15 min and the sera was stored at −70 °C for future assessment.

Isolation of neutrophils and mononuclear cells
Blood neutrophils and mononuclear cells from patients with cervical neoplasia or healthy volunteers were isolated using a Histopaque gradient. Viable cells (greater than 95% as determined by trypan blue exclusion) were washed three times with RPMI medium (Sigma Chemical, St. Louis, MO) with centrifugation at 180 × g for 10 min after each wash, and then resuspended in RPMI for cell culture or in RPMI containing 0.01% bovine serum albumin (RPMI-BSA) for the neutrophil chemotaxis assay.

Pretreatment of healthy neutrophils with patient sera
Healthy neutrophils were pre-incubated for 30 min at 37 °C and 5% CO₂ with sera (diluted to 0.5% to 50% v/v in RPMI-BSA) obtained from normal donors or with sera from patients from the pre-invasive or invasive groups. In a set of experiments, control neutrophils were also incubated with sera obtained from pre-invasive patients after surgical treatment. After incubation, the neutrophils were assayed for chemotaxis.

Neutrophil migration assay
Chemotaxis was assayed in 48-well microchemotaxis chambers (Neuro Probe, Cabin John, MD, USA) containing 5-µm PVP-free polycarbonate filters. Briefly, 28 µL of the chemoattractants (10⁻⁷ M, Sigma) N-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP), LTB₄, or IL-8 were diluted in RPMI-BSA and added to individual bottom compartments of the chamber. RPMI medium alone was used as a control for random migration. Fifty µL of the neutrophils (10⁶ cells/mL) that were previously treated with control or patient sera was added to the top compartment. The chamber was incubated for 1 h at 37 °C and 5% CO₂ and then the filter was removed, fixed and stained with the Hema 3 Stain set (Biochemical Sciences, Bridgeport, NJ). The number of neutrophils that had migrated to the lower side of the filter was counted (100× objective) in four randomly selected fields. Each sample was assayed in triplicate. The results were expressed as the absolute number of neutrophils per field.

Supernatants obtained from cultured leukocytes
Cultured neutrophils and mononuclear cells (10⁶ cells/mL) obtained from the controls and randomly selected patients before and after treatment were stimulated with endotoxin (1 µg/mL of LPS from E.coli) and incubated at 37 °C and 5% CO₂ for 24 h (neutrophils) or 48 h (mononuclear cells). The cells were then centrifuged at 180 × g for 10 min and the supernatants were stored at −70 °C for future cytokine and nitrite determination.

Determination of serum NO metabolites and nitrite in culture supernatants
The nitrate concentration in serum samples from controls and patients with cervical neoplasia was assayed by enzymatically reducing nitrate. Briefly, 50 µL of samples were incubated with the same volume of reductase buffer (0.1 M potassium phosphate with 1 mM nicotinamide adenine dinucleotide, and 4 units of nitrate reductase/mL) for 20 h at 37 °C. A standard nitrate curve was obtained by incubating sodium nitrate (10–200 µM) with the buffer. The total amount of nitrite as well as the amount of nitrite in the culture supernatants was then determined using the Griess method. The samples were incubated with the same volume of Griess reagent (1% sulphanilamide and 0.1% naphthylethylenediamine dihydrochloride in 5% phosphoric acid). The absorbance at 550 nm was determined by using a multiwell plate reader. The results were reported as the concentration of nitrate plus nitrite (µM NO₃ + NO₂) for serum samples or concentration of nitrite for supernatants.

Cytokine measurements
The concentrations of cytokines in the blood samples and in samples obtained from leukocyte supernatants were determined by ELISA. Briefly, flat-bottomed 96-well microtiter plates were coated with an anti-cytokine antibody (50 µL/well) diluted in coating buffer (150 pg/mL for IL-6, 100 pg/mL for IL-8 or...
500 pg/mL for IL-10 and TNF-α) and incubated overnight. The plates were then washed and non-specific binding was blocked with 1% BSA for 120 min at 37 °C. Non-diluted samples and standards were loaded onto the plates. Recombinant human TNF-α, IL-6, IL-8 and IL-10 standard curves were used to calculate the cytokine levels. The plates were thoroughly washed and then incubated with the appropriate biotinylated monoclonal anti-cytokine antibody for 1 h. The plates were washed, incubated with avidin peroxidase (diluted 1:5000) for 30 min and washed again. The substrate (0.4 mg of o-phenylenediamine dihydrochloride (Sigma, St. Louis, MO, USA) and 0.4 µL of hydrogen peroxide (Merck, Rio de Janeiro, RJ, Brazil) in 1 mL of substrate buffer) was added and the reaction was stopped with 1 M H2SO4. The optical density was measured on a plate reader at 490 nm. The results were expressed as pg of cytokine per mL and the optical density of the samples was compared to the standard curves.

Statistical analysis
Data were analyzed using SigmaStat 2.03 software. Differences between two unpaired groups were tested using Student t-test or Mann-Whitney, according to the distribution. For multiple comparisons, either the analysis of variance (ANOVA) followed by the Tukey test, or the Kruskal-Wallis followed by Dunn were performed, according to normal or non-normal distribution, respectively. The data obtained before and after treatment were compared by paired t-test (normal) or Wilcoxon (non-normal). Statistical significance was set at P < 0.05.

Results
Study population
Forty patients with cervical neoplasia were enrolled in this study. Eighteen (45%) patients were diagnosed with pre-invasive neoplasia (CIN group) and 22 patients (55%) were diagnosed with invasive neoplasia. The mean age (±SD) of the patients was 45.2 ± 14.2 yr old, and the CIN group had a lower age (39.3 ± 13.8, range of 23–67 yr) than the invasive group (50.1 ± 12.9, range of 26–71 yr) (P < 0.05, Student t-test). Controls comprised 36 healthy adult volunteer women, with a mean age of 42.4 ± 10.2 yr (range 26–65 yr).

Patient sera dose-dependently inhibited the migration of healthy neutrophils
To investigate the presence of circulating inhibitory mediators on neutrophil migration in patients with cervical neoplasia, control neutrophils were incubated with sera obtained from patients before treatment (n = 5 from each group) and the capacity of the neutrophils to migrate toward the chemotactants was assayed. The control group consisted of healthy neutrophils treated with normal heterologous serum (n = 7). Healthy neutrophils incubated with patient sera had a dose-dependent reduction in migration to all of the chemotactants tested and reached statistical significance at a 50% serum concentration for fMLP and IL-8 in both groups, compared to 0.5% of patient sera (data not shown). Importantly, control neutrophils incubated with a 50% concentration of healthy serum displayed significant migration to all chemotactants compared to the RPMI control, whereas the same treatment with patient sera completely inhibited neutrophil migration to fMLP, LTB4, and IL-8 (Fig. 1).

Serum cytokine and nitrate concentration
To investigate the systemic production of mediators, serum samples were obtained from controls and patients with cervical neoplasia at the same time as the collection of blood for the migration assay. Serum IL-6 and IL-8 concentrations were significantly higher in cervical neoplasia patients than in controls (Fig. 2, panels B and C), whereas TNF-α levels approached the threshold of statistical significance (Fig. 2, panel A). Higher levels of IL-6 and IL-8 were also detected in the invasive group compared to controls (Fig. 2, panels B and C).

The concentrations (µM) of NO metabolites (means ± SD of NO2 + NO3) in serum did not differ between the controls (n = 29, 26.5 ± 14.5) and patients (n = 22, 28.1 ± 16.2), or between the CIN (n = 13, 31.8 ± 17.7) and invasive (n = 9, 25.4 ± 16.2) groups.

Production of cytokines and nitrite by cultured leukocytes
To estimate the production of inflammatory mediators by circulating leukocytes, neutrophil and mononuclear cells obtained from controls and patients with
Cervical neoplasia were cultured and the supernatants were collected. The concentrations of TNF-α and IL-10 were higher in the neutrophil supernatants of patients with invasive cervical neoplasia than the controls (panels A and C, $P < 0.05$). The IL-6 levels did not differ between groups (Fig. 3).

The levels of nitrite (means ± SD µM NO₂) were significantly reduced in neutrophil supernatants from patients ($n = 11, 6.9 ± 1.1$), including both the CIN ($n = 6, 6.5 ± 1.1$) and invasive ($n = 5, 7.5 ± 0.7$) groups compared to controls ($n = 7, 10.5 ± 2.1$). In opposite, higher levels of nitrite were found in the supernatants of mononuclear cells from patients ($n = 8, 11.1 ± 1.3$) including the CIN ($n = 5, 11.5 ± 1.5$) and invasive ($n = 3, 10.3 ± 0.5$) groups compared to controls ($n = 8, 2.6 ± 0.8$) ($P < 0.01$, ANOVA followed by Tukey test).

**Effect of surgical treatment on the production of mediators and on neutrophil migration**

To evaluate the effect of surgical removal of the neoplasia on the production of inflammatory mediators and on the inhibitory effect of patient sera on neutrophil migration, these parameters were re-evaluated in some of the randomized patients after surgery. We first assessed if the concentrations of the mediators that were elevated in the serum from cervical neoplasia patients were altered after surgery. Figure 4 shows that the levels of all of the mediators tested were significantly reduced after surgery ($P < 0.05$, paired $t$-test) except for TNF-α ($P = 0.052$). When tumor stage was considered, the concentration of IL-6 ($P < 0.05$), IL-8 ($P = 0.076$) and NO metabolites ($P < 0.05$) were reduced after surgery in the CIN group, while IL-10 ($P = 0.073$) had lower levels after treatment in invasive group (paired $t$-test).

The concentrations of TNF-α, IL-6, and IL-10 in the neutrophil supernatants did not differ between patients before and after surgery ($n = 6, 3$ CIN and 3 invasive patients). The nitrite levels in the supernatants of neutrophils and mononuclear cells also were not different between patients considering surgery ($n = 6, 3$ CIN and 3 invasive patients) (data not shown).

We next hypothesized that a reduction in the production of soluble circulating factors would be reflected in the restoration of neutrophil migration after treatment. Therefore, we tested the effect of sera, from the same five patients from the CIN group that were evaluated at pretreatment, on neutrophil migration. Neutrophils incubated with serum samples taken after...
surgery exhibited significant migration towards all the chemoattractants tested and at all serum concentrations compared to RPMI (Fig. 5, panel B), while migration was reduced when control neutrophils were incubated with sera obtained from CIN patients before treatment, most evident when 5% and 50% serum concentration was used (Fig. 5, panel A).

Discussion
The present study demonstrated that circulating mediators impair neutrophil function in patients with uterine cervical neoplasia, apart from the pre-invasive stage, compared to healthy controls. We observed a complete failure in the migration of neutrophils treated with the highest concentration of sera obtained from cervical neoplasia patients at both the pre-invasive and invasive stages in response to all chemoattractants tested. Important, normal sera did not affect neutrophil migration capacity when used at the same concentration as the patient sera. These data indicate that circulating inhibitory factors may negatively affect neutrophil migration in cervical neoplasia. In agreement with our findings, a previous study showed that sera from patients with head and neck cancer impaired the function of normal monocytes. Moreover, sera from breast cancer patients that had received chemotherapy had
Circulating mediators inhibit the neutrophil migration in cervical neoplasia patients

Figure 4. Concentrations of TNF-α (panel A), IL-6 (panel B), IL-8 (panel C), IL-10 (panel D), and NO metabolites (panel E) in serum samples from patients with cervical neoplasia (left pair of bars) pre-invasive (center pair of bars) and the invasive (right pair of bars) groups who had samples collected at diagnosis (before treatment) and upon follow-up at 30 to 60 d after surgery.

Notes: The 25th and 75th percentiles are represented by a bar centered around the median and the 10th and 90th percentiles are represented by error bars. Patients (total group): *P = 0.052, *P < 0.05 compared to pre-treatment (Wilcoxon and paired t-test); Pre-invasive group: *P = 0.076, *P < 0.05 compared to pre-treatment (paired t-test); Invasive group: *P = 0.073 compared to pre-treatment (paired t-test).

Figure 5. Sera obtained from patients with pre-invasive cervical neoplasia after surgery restored the migration of control neutrophils.

Notes: The box plots indicate the number of emigrated healthy neutrophils, incubated for 30 min in sera (0.5 to 50% concentration) of patients with pre-invasive cervical neoplasia (n = 5), on diagnosis (panel A) and 30 to 60 days after surgery (panel B) in response to RPMI (random migration) or to the chemoattractants (10^-7 M) fMLP, LTB4, and IL-8. The 25th and 75th percentiles are represented by a bar centered about the median, the 10th and 90th percentiles are error bars. *P < 0.05 compared to the respective RPMI (Kruskal-Wallis followed by Dunn).

Neutrophils express receptors necessary for the recognition and elimination of tumor cells, and are rich in granules that contain defensins, proteins with high toxic potential against various tumor cells. But although activated neutrophils perform antitumor activity, in some tumor types they can promote tumor metastasis, so, the role of neutrophil in tumor microenvironment may change depending on tumor type and tumor stage. Our present results support the hypothesis that neutrophils may function early in the host response against tumor, since patient sera exhibited an inhibitory effect on neutrophil migration apart from the pre-invasive stage.

Elevated concentrations of TNF-α, IL-6, IL-8, IL-10, and NO are involved in the impairment of neutrophil migration in endotoxemia and sepsis. Therefore, we investigated the systemic levels of those mediators and the specific production by leukocytes. The inhibitory effect of patient sera on the migration of normal neutrophils was accompanied by higher serum levels of IL-6 and IL-8 in patients with invasive neoplasia. A neutrophil migration assay using patient sera pretreated with neutralizing antibody to the specific cytokines would help to identify which cytokine(s) mainly mediated the inhibition of neutrophil migration.
Plasma IL-6 levels were elevated in ovarian cancer patients at advanced stage compared with tumors of low malignity. In patients with head and neck carcinoma, IL-6 serum levels were significantly increased compared to healthy controls and these levels increased as the tumor stage progressed. In patients with ovarian cancer, elevated IL-8 levels were associated with poor prognosis. Moreover, in advanced tumors, the higher serum levels of IL-10 correlated with poorly differentiated or undifferentiated tumors. The serum levels of NO metabolites did not differ between groups, although previous studies had detected elevated NO serum levels in patients with cervical cancer. However, failure to detect a circulating mediator at a specific time point does not rule out the possibility that neutrophil migration is affected by the factor released at earlier time points.

Neutrophils obtained from patients with invasive cervical neoplasia produced higher concentrations of TNF-α and IL-10 than controls. In agreement, patients with advanced cervical cancer had increased levels of IL-4 and IL-10 in the supernatants of mononuclear cells compared to controls. However, a differential production was observed for NO in the patient samples of leukocyte supernatants. A reduction in the nitrite level was detected in neutrophils, while an elevation was seen in mononuclear cells compared to the controls. A previous study showed that higher levels of nitrite were accompanied by reduced antioxidant activity in the supernatants of leukocytes from patients with gastric cancer.

The tumor microenvironment controls leukocyte migration and other functions after these cells arrive at the tumor site. Considering the higher levels of cytokines in sera and in the supernatants of cultured leukocytes detected in samples from patients of invasive group compared to controls, it is suggested that tumor cells promote an immune response involving the production of Th1 and Th2 cytokines. To further confirm whether tumor cells could have an effect on the production of mediators, and on the inhibitory activity of patient sera, we investigated these parameters after surgical elimination of the tumor in randomly selected patients.

Neutrophil migration was only assayed in patients with pre-invasive neoplasia and all of them were tested before surgery. Significant neutrophil migration toward all chemoattractants was observed for control neutrophils incubated with patient sera obtained after surgery. These data strongly suggest that the tumor cells produce and/or stimulate the production of inhibitory mediator(s) of neutrophil migration. The mediators probably included TNF-α, IL-6, IL-8, IL-10, and NO, since the removal of tumor significantly reduced the production of these factors and restored neutrophil migration capacity in the host response. The specific production of mediators by systemic leukocytes was not altered, which supported the hypothesis that the tumor cells had a role in the production of soluble factors.

In patients with ovarian cancer treated with chemotherapy, a decrease in IL-4 and IL-10 levels was observed in responders than non-responders. In patients with advanced ovarian cancer, serum IL-10 levels were significantly reduced within 8 d after surgery. Our data suggest that no individual factor was responsible for the impairment in neutrophil migration, since different mediators were elevated at diagnosis and were altered after surgery.

Tumors produce chemokines that recruit leukocytes. However, a deficiency in promoting an inflammatory response at locations other than the tumor site exists. It has been hypothesized that circulating chemokines may desensitize leukocytes to migrate or that the tumor may produce anti-inflammatory factors. Our data support the hypothesis that tumor cells produce soluble inhibitory factors of neutrophil migration that may also affect leukocyte function when present in the circulation.

Other factors, which were not evaluated in this study, may also have a role in the impairment of leukocyte function in cancer patients. Acute phase proteins, such as alpha-1-acid glycoprotein (AGP) and haptoglobin, may have increased production in cancer as a reaction to injury. It was reported that AGP is detected in sera of severely septic patients and is able to inhibit neutrophil migration through a NO-dependent mechanism.

In conclusion, patients with cervical neoplasia produce circulating inhibitory mediators of neutrophil migration, apart from the pre-invasive stages. We presented indirect evidence that those mediators
could be cytokines and NO. The results also indicate that tumor cells act early to impair the innate immune response. These findings may contribute to the development of new therapeutic strategies in cancer.

**Author Contributions**

Conceived and designed the experiments: DC Micheli, EFC Murta and BM Tavares-Murta; Analysed the data: B.M. Tavares-Murta; Wrote the first draft of the manuscript: B.M. Tavares-Murta; Contributed to the writing of the manuscript: DC Micheli, EFC Murta and BM Tavares-Murta; Agree with manuscript results and conclusions: DC Micheli, PC Fernandes-Jr, ID Nomelini, JCG Cruvinel, EFC Murta and BM Tavares-Murta; Made critical revisions and approved final version: DC Micheli, PC Fernandes-Jr, ID Nomelini, JCG Cruvinel, EFC Murta and BM Tavares-Murta; Jointly developed the structure and arguments for the paper: DC Micheli, PC Fernandes-Jr, ID Nomelini, JCG Cruvinel, EFC Murta and BM Tavares-Murta; Made critical revisions and approved final version: DC Micheli, PC Fernandes-Jr, ID Nomelini, JCG Cruvinel, EFC Murta and BM Tavares-Murta; All authors reviewed and approved of the final manuscript.

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As a requirement of publication author(s) have provided to the publisher signed confirmation of compliance with legal and ethical obligations including but not limited to the following: authorship and contributorship, conflicts of interest, privacy and confidentiality and (where applicable) protection of human and animal research subjects. The authors have read and confirmed their agreement with the ICMJE authorship and conflict of interest criteria. The authors have also confirmed that this article is unique and not under consideration or published in any other publication, and that they have permission from rights holders to reproduce any copyrighted material. Any disclosures are made in this section. The external blind peer reviewers report no conflicts of interest.

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