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CLINICAL SCIENCE

Interferon-gamma and interleukin-10 production by mononuclear cells from patients with advanced head and neck cancer

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OBJECTIVE: This study aims to evaluate the production of interferon-gamma and interleukin-10 by stimulated peripheral blood mononuclear cells isolated from patients with supraglottic laryngeal cancer before and after surgical treatment.

METHODS: Fourteen patients with advanced supraglottic laryngeal cancer were studied. Cultures of peripheral blood mononuclear cells isolated during the preoperative and late postoperative periods were stimulated with concanavalin A and Bacille Calmette-Guérin, and the supernatant concentrations of interferon-gamma and interleukin-10 were measured.

RESULTS: For non-stimulated cultures, the interferon-gamma levels produced by the preoperative period and the late postoperative period cultures were lower than the levels produced by the control group cultures. The interferon-gamma levels after stimulation with concanavalin A were higher in the late postoperative period cultures than in the preoperative evaluation cultures. Stimulation with Bacille Calmette-Guérin led to the production of similar levels of interferon-gamma and interleukin-10 by all cultures; thus, stimulation increased the levels of interferon-gamma produced by both the preoperative and postoperative cultures relative to the levels produced by the corresponding unstimulated cultures.

CONCLUSION: Patients with advanced supraglottic laryngeal cancer exhibit an *in vitro* deficiency in interferon-gamma secretion by mononuclear cells. Stimulated cells seem to recover this function during the postoperative period.

KEYWORDS: BCG; Cytokines; Interferon-γ; Interleukin-10; Head and Neck cancer.

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INTRODUCTION

Although the mechanism of action of Bacille Calmette-Guérin (BCG) has not been fully elucidated, BCG is currently regarded as the most successful cancer immunotherapy (1). BCG seems to enhance the cellular immune response in a nonspecific manner by activating macrophages and lymphocytes. Intravesical immunotherapy is clinically well established as a treatment for superficial bladder cancer, but it remains an experimental treatment for other solid tumors, such as laryngeal cancer (2).

Cytotoxic $CD8^+$ T cells play an important role in the control of tumor cells (3). The immune process, which culminates in the lysis of tumor cells, often begins with dendritic cells, which detect the tumor antigens presented by Major Histocompatibility Complex (MHC) molecules expressed on tumor cell surfaces and then phagocytose the presenting cells. These dendritic cells activate lymphocytes, starting the cytotoxic process and tumor cell lysis. This entire cell interaction mechanism is coordinated by different cytokines (4). The proinflammatory activities of cytokines such as interferon-gamma (IFN-γ) are associated with Th1 T lymphocyte differentiation. IFN-γ is thought to be associated with anti-tumoral cellular immunity. However, immune response activation involving cytokines such as interleukin-10 (IL-10) can promote Th2 T lymphocyte differentiation and lead to a predominantly humoral immune response (5).

Although BCG has been used as a successful immunotherapy for cancer, few studies have investigated cytokine release by BCG-activated immune cells. In a previous study, we evaluated the production of TNF-α and IL-6 in the supernatant of adherent cells cultured from peripheral blood mononuclear cells (PBMCs) isolated from patients with supraglottic laryngeal cancer before and after surgical

No potential conflict of interest was reported.

treatment. We found that BCG is able to modulate the immune response of these patients (6). In this study, we evaluated the production of IFN-γ and IL-10 by cultured PBMCs isolated from patients with advanced supraglottic laryngeal cancer. PBMCs were isolated during the pre-operative period (PREOP) and the late postoperative (LP) period.

METHODS

Fourteen patients with stage III or IV supraglottic laryngeal carcinoma were selected at the outpatient clinic of the University Hospital of the Ribeirão Preto School of Medicine, São Paulo University. Each tumor was staged according to the TNM classification. Patients with infectious and/or inflammatory diseases detected by blood and urine tests and chest X-rays and patients using immunosuppressive medications were excluded. Surgical treatment consisted of total or partial laryngectomy according to lesion stage. T3 stage patients underwent total or supraglottic laryngectomy, and T4 stage patients were treated with total laryngectomy. Blood samples were collected from all patients during the PREOP period and at 236 ± 18 days after surgery (LP).

Thirteen healthy age- and sex-matched individuals were recruited as the control group. The presence of neoplasms in the control volunteers was ruled out by detailed physical investigation. The study was approved by the Ethics Committee of the University Hospital of the Ribeirão Preto School of Medicine, São Paulo University, and all patients gave written informed consent prior to participation in the study.

Measurement of the cytokine concentrations

Blood samples were collected from the peripheral vein under sterile conditions during both the PREOP and LP periods from patients with laryngeal cancer. Blood staples were collected only once from the control subjects. Peripheral blood mononuclear cells (PBMCs) were isolated with a Ficoll-Hypaque gradient, and their viability was determined by 2% Trypan Blue exclusion. Cells were kept at a concentration of $2.0x10^5$ cells/mL in 12.5% bovine fetal serum. Non-stimulated and stimulated cultures were evaluated; stimulated cells were exposed to 10 μg/mL of concanavalin A (ConA) or 20 μg/mL of BCG. After 72 h of culture at 37℃ in a humid environment containing nearly 5% CO_2, the supernatant was collected, and the IL-10 and IFN-γ levels were measured (7).

Human monoclonal IL-10 and IFN-γ antibodies were used [Pharmingen International (Life Science Research), San Diego, CA, USA] as the capture antibodies, and biotinylated antihuman antibodies against the analyzed cytokines (Pharmingen) were used as the detecting antibodies. Binding was detected with peroxidase-labeled streptavidin (DAKO, Glostrup, Denmark) and *O*-phenylenediamine-2HCl/substrate (OPD, Sigma, St. Louis, MO, USA). The intra-assay and inter-assay variation levels were below 10%. The detection limits of the cytokine ELISAs were 78 pg/mL and 39 pg/mL for IFN-γ and IL-10, respectively.

The results are reported as the median (*M*), mean (*x*) and standard deviation (SD). GraphPad Prism (San Diego, CA, USA) was used for statistical analysis. Between-group comparisons were performed using the Mann-Whitney and Wilcoxon tests, and $p<0.05$ was considered statistically significant.

RESULTS

Demographic data for the patients and controls are shown in Table 1. All patients smoked an average of 20 ± 8.5 (x ± DP) cigarettes/day for 44.5 ± 10 years. Eight patients received adjuvant radiotherapy 27.1 ± 8 days after surgery, and the post-operative evaluation was conducted at least six months after the completion of the radiotherapy. No patient exhibited evidence of tumor recurrence at the time of blood sample collection for LP evaluation, as determined by endoscopy and radiography. All patients had stopped smoking by the time of the postoperative evaluation. Thirteen healthy control subjects were selected, ten men and three women. The control subjects ranged in age from 40 to 67 years (52 ± 9.3). No significant differences were observed between the controls and the patients.

The IFN-γ levels in the PREOP and LP BCG-stimulated cultures were similar to those in the control group cultures, whereas, the IFN-γ levels were lower in the non-stimulated PREOP and LP cultures than in the non-stimulated control cultures (Figure 1). For the control cultures, the IFN-γ level was 154.86 (32.32 – 192.8) pg/ml in the non-stimulated cultures and 239.9 (139.83 – 1,718.08) pg/ml in the BCG-stimulate cultures. A higher elevation index was observed in patients at both the PREOP and LP timepoints, with increases from 27.63 (1.12 – 140.25) pg/ml to 116.19 (5.5 – 3,030.86) pg/ml and from 41.47 (30.03 – 218.09) pg/ml to 186.47 (27.74 – 1,459.73) pg/ml, respectively (Table 2).

The IFN-γ levels in the supernatants of non-stimulated PBMC cultures were lower for the PREOP and LP cultures than for the control cultures ($p = 0.001$, $p = 0.04$, respectively). However, when the cultures were stimulated with ConA, there were no significant differences between the control cultures and either the PREOP or LP cultures. Higher levels of IFN-γ were observed in the supernatants of the ConA-stimulated LP cultures than in those of the ConA-stimulated PREOP cultures ($p = 0.005$) (Figure 1).

The IL-10 levels in the supernatants of the ConA- and BCG-stimulated cultures of cells from patients in the PREOP and LP periods were also similar to in the levels for the control group cultures. The IL-10 level was undetectable in non-stimulated cultures (Table 2).

DISCUSSION

The impairment of immunological defense mechanisms may play an important role in cancer pathogenesis. Decreased *in vitro* lymphocytic function, lower numbers and percentages of lymphocytes and impaired lymphoproliferation have been reported in many cancer studies (8-12). Many researchers have examined different aspects of the immune behavior of head and neck cancer; however, few studies have examined supraglottic laryngeal cancer specifically. This attention is justified by the local behavior,

Table 1 - Sex, age and tumor stage (TNM classification; American Joint Committee on Cancer) of the patients and controls.

	Patients	Control Group
Sex	12*M*:2*F*	10*M*:3*F*
Age (years)	58.5 (SD = 9)	52 (SD = 9.3)
Stage (TNM)	7 stage III/ 7 stage IV	-

M: male, *F:* female, *SD:* Standard Deviation.

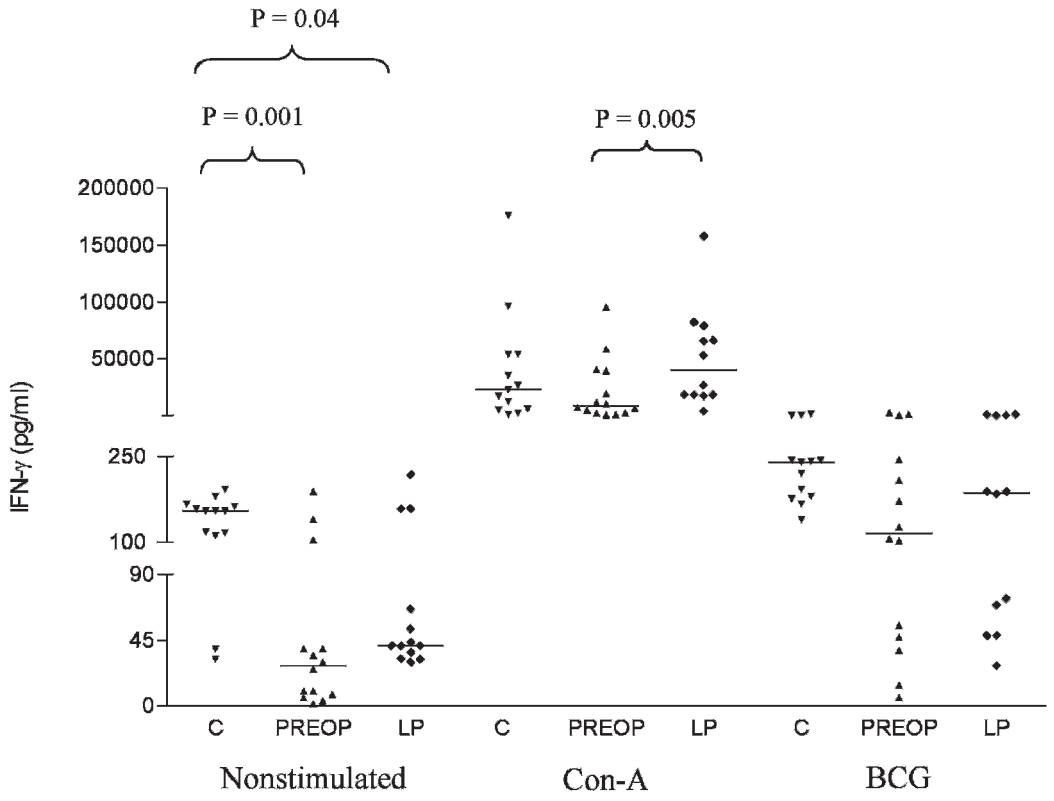

Figure 1 - Control (C), Preoperative (PREOP) and Late Postoperative (LP) IFN-γ (pg/ml) levels in the supernatant of non-stimulated lymphocytes cultures and in cultures stimulated with Con-A and BCG.

regional dissemination capability and potential participation of the immune system in cancer prognosis in this type of cancer (13).

IFN-γ is the first detectable cytokine at the immunization site after stimulation with protein antigen and plays a critical role in the activation and regulation of the immune response (14). It is a Th1-specific cytokine and promotes the Th1 response and inhibits the Th2 response. Moreover, IFN-γ activates macrophages, leading to the increased production of pro-inflammatory cytokines, including TNF-α, in response to different stimuli.

IFN-γ was expressed at lower levels in non-stimulated patient cultures (PREOP and LP) than in control group cultures, confirming that the immune system is impaired in these patients. When the cultures were stimulated with ConA, the secretion of this cytokine was improved, and the increase was greater in the LP group than in the PREOP group. The higher levels of IFN-γ secretion by the LP cultures than by the PREOP cultures may be associated with improved nutritional status in the LP period or due to a reduction in smoking. Although the effects of smoking and alcohol abuse on supraglottic laryngeal cancer are not fully clear, some authors have demonstrated that smoking can affect some cytokines, mainly IL-4 (15).

Lymphocyte cultures from controls and patients with laryngeal cancer were stimulated with BCG (Table 2). BCG induced a more effective increase in IFN-γ production in patient cells (4x increase) than in control cells (1.5x increase). As IFN-γ has a potent anti-tumor activity, these data suggest that BCG should be tested further *in vivo*.

Kim (16) observed that *in vitro,* IL-10 inhibited the destruction of squamous cell carcinoma tumor cells by peritumoral lymphocytes. IL-10 inhibits macrophage differentiation and, moreover, inhibits antigen presentation to CD8 T lymphocytes, preventing the effective destruction of tumor cells. Thus, IL-10 acts as a local immunosuppressive factor,

Table 2 - Control, preoperative (PREOP), and late postoperative (LP) IL-10 and IFN-γ levels in non-stimulated cell cultures and in cultures stimulated with ConA and BCG.

	IFN-γ (pg/ml)			IL-10 (pg/ml)		
	Nonstimulated cultures	ConA Stimulation	BCG Stimulation	Nonstimulated cultures	ConA Stimulation	BCG Stimulation
Control	154.86 (32.32-192.8)	23,111.03 (1,577.56-176,197.9)	239.9 (139.83-1,718.08)	ND	1,093.48 (119.42-2,222.62)	78.63 (0.9-314.62)
PREOP	27.63 (1.12-140.25)	8,976.98 (887.49-95,346.54)	116.19 (5.5-3,030.86)	ND	1,318.25 (362.85-6,262.97)	392.73 (17.84-624.13)
LP	41.47 (30.03-218.09)	40,091.64 (4,314.92-157,944.7)	186.47 (27.74-1,459.73)	ND	1,374.09 (739.97-3,714.12)	151.73 (17.38-580.91)

Data are reported as the median and (range). ND – not detectable.

allowing the tumor to escape from the immune system (17,18). In this study, the level of IL-10 production by non-adherent cells was similar to that of the control cells. This result indicates that the primary activity of IL-10 is not associated with the cellular response. The true role of IL-10 in the immunoregulation of cancer remains controversial (19-21).

Although the influence of IFN-γ levels on patient prognosis has not been examined, further studies that demonstrate the relationship between activity levels and angiogenic factors (22) and that evaluate other variables that can alter the capacity of the immune system, such as alcohol consumption, poor nutrition and smoking, may support the clinical application of BCG in selected patients. Ultimately, BCG may help modify disease progression in terms of survival and prognosis parameters.

In conclusion, patients with advanced supraglottic laryngeal cancer exhibited an *in vitro* deficiency in IFN-γ secretion by mononuclear cells. Stimulated cells seem to recover this function in the postoperative period.

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AUTHOR CONTRIBUTIONS

Conti-Freitas LC made substantial contributions to the conception and design of the study; participated in the acquisition and interpretation of the data and was involved in the drafting of the manuscript. Foss-Freitas MC participated in the drafting of the manuscript and performed the statistical analysis. Mamede RCM participated in the study conception and design and in the data interpretation. Foss NT conceived the study, participated in its design and coordination, and helped to draft the manuscript. All authors read and approved the final manuscript.

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