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Main Article

Cytokine levels in patients with Epstein–Barr virus associated laryngeal carcinoma

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Abstract

Objective: Some researchers have suggested that Epstein–Barr virus may play a role in the pathogenesis of laryngeal malignancies. In order to clarify the role of cytokines in this disease context, the current study aimed to determine the serum levels of cytokines in Epstein–Barr virus DNA positive patients with laryngeal carcinoma.

Subjects: The study included 10 patients with diagnosed laryngeal carcinoma and Epstein–Barr virus DNA positive tumour tissue samples. The control group comprised 10 Epstein–Barr virus DNA negative patients diagnosed with laryngeal carcinoma, 10 patients with acute Epstein–Barr virus infection and 10 healthy individuals.

Method: Serum cytokine levels were determined by enzyme-linked immunosorbent assay.

Results: The Epstein–Barr virus DNA positive and negative laryngeal carcinoma patients showed no differences regarding serum levels of the following cytokines: interleukins 1β, 2, 6 and 12, tumour necrosis factor α, and interferon γ. However, serum levels of interleukin 10 and transforming growth factor β1 were significantly higher in Epstein–Barr virus DNA positive laryngeal carcinoma patients compared with Epstein–Barr virus DNA negative laryngeal carcinoma patients ($p < 0.05$).

Conclusion: Our results suggest that the cytokines interleukin 10 and transforming growth factor β1 may act as growth factors in Epstein–Barr virus related laryngeal carcinoma. These cytokines may thus represent potential targets for molecular therapeutic treatment for laryngeal carcinoma; they may also be useful as indicators of disease prognosis.

Key words: Larynx Neoplasms; Cytokines; Polymerase Chain Reaction; Prognosis

Introduction

Head and neck cancers, including laryngeal tumours, have a high incidence and a complex pathogenesis.[1] The larynx is one of the sites most frequently affected by head and neck squamous cell carcinoma.[2] Some researchers have suggested that viruses may play a role in the pathogenesis of laryngeal malignancies. The association between human papilloma virus (HPV) and laryngeal squamous cell carcinoma has been well studied.[2] Recently, Epstein–Barr virus (EBV) has been proposed to also play a role in the pathogenesis of this tumour.[1]

Epstein–Barr virus is a member of the herpes virus family and is widespread in all areas of the world, infecting over 95 per cent of the adult population.[3] It is a persistent virus which is shed long after the primary infection.[4] Epstein–Barr virus was the first human virus to be directly implicated in carcinogenesis.[5] It is strongly associated with tumours of lymphoid and epithelial origin, such as Burkitt's lymphoma, Hodgkin's lymphoma and nasopharyngeal carcinoma (NPC).[6] In order to be oncogenic, EBV must maintain its viral genome within the cell, avoid killing the cell itself, and prevent the cell from becoming a target for immune-mediated destruction. Eventually, the virus must also activate cellular growth.[5] Even so, the pathogenesis of EBV-associated malignancies is not well understood, since affected patients are usually fairly immunocompetent.[7]

Following primary EBV infection, the response of immunological control mechanisms is important, as malfunction of these mechanisms may lead to reactivation of infection, or may prepare the ground for fulminant infection and contingent malignancies especially in immunosuppressed patients.[8] The immunological reaction to EBV infection is very complex. Vigorous humoral and cellular immune responses control the proliferation of EBV-infected cells in healthy virus carriers.[7] Cellular immune responses play a more significant role in EBV infection control than humoral immune responses.[9]

Cytokines are produced by a wide variety of cells. The anti-inflammatory cytokines comprise a series of immunoregulatory molecules that control the

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proinflammatory cytokine response. A dynamic balance exists between proinflammatory cytokines and anti-inflammatory components of the human immune system. Cytokines act in concert with specific cytokine inhibitors and soluble cytokine receptors to regulate the human immune response.[10] They play an important role in the regulation of immune and inflammatory responses during infection.[11]

Besides their role in the development of various infections, cytokines are considered to have an effect on the pathophysiology of acute EBV infection.[9] It is agreed that many EBV genes infect B cells persistently by regulating apoptotic signals and cytokine balance while causing no damage to the host.[12] Cytokines are thought to play an essential role in the gene expression of inhibitors of apoptosis proteins, which may affect the anti-apoptotic process.[13] It is also believed that cytokines which affect inflammation may act as mediators in the development of cancers caused by factors such as EBV.[14]

Knowledge of the expression and functions of certain cytokines in relation to NPC may help explain the lack of an effective tumour-specific immune response in NPC patients, despite the induction of EBV-specific humoral and cytotoxic responses. The cytokines and growth factors known to be important in NPC include interleukins 6, 8 and 10 and transforming growth factor β1.[15] Since there may be a relationship between laryngeal carcinoma and EBV infection, the identification of factors which influence the development of laryngeal carcinoma may facilitate earlier diagnosis and better treatment of this tumour.

Our study aimed to determine cytokine serum levels in EBV DNA positive patients with laryngeal carcinoma, and to compare these results with those obtained from EBV DNA negative laryngeal carcinoma patients, patients with acute EBV infection and healthy individuals.

Materials and methods

Patients

The study included 10 patients who had presented initially with hoarseness, dyspnoea, voice changes, persistent cough, sore throat and dysphagia, who following biopsy had subsequently been diagnosed histopathologically with laryngeal carcinoma, and whose tumour tissue was found to be positive for Epstein–Barr virus (EBV) DNA using a real-time polymerase chain reaction method. All patients were male, and their mean age was 54.6 years. These 10 patients had not yet received any treatment.

To enable comparison, the study also included the following as controls: 10 patients with EBV DNA negative laryngeal carcinoma, 10 patients with acute EBV infection and 10 healthy individuals without EBV infection.

Sample collection

Tumour biopsies were taken from untreated laryngeal carcinoma patients. The tumour biopsies were excised, placed in phosphate-buffered saline and transported immediately to the laboratory, where they were stored in Eppendorf tubes at −80°C until analysed.

Heparinised venous blood samples were also obtained from patients and controls. These were centrifuged and the sera collected and stored at −80°C until analysed.

DNA extraction

Deoxyribonucleic acid was extracted from tumour tissue samples using the QIAamp® DNA mini kit (Qiagen, Hamburg, Germany), following the manufacturer's instructions.

Real-time quantative polymerase chain reaction

The EBV DNA load in the tumour tissue samples was determined by quantitative real-time polymerase chain reaction, using a Qiagen Artus EBV Rotor Gene kit. An aliquot of 20 µl of purified DNA, isolated from the tumour tissue sample, was used for amplification in a total reaction volume of 50 µl. The amplification reaction was performed in duplicate for each sample and standard. Amplification cycling was performed using the Rotor-Gene 6000 device (Corbett Research, Mortlake, NSW, Australia).

Data analysis was performed using the Rotor-Gene software, according to the manufacturer's instructions.

Enzyme-linked immunosorbent assay

Specific enzyme-linked immunosorbent assay techniques were used, according to the manufacturer's instructions (Biosource, Camarillo, California, USA), to determine the serum levels of the following cytokines: interleukins 1β, 2, 6, 10 and 12; interferon γ; tumour necrosis factor α; and transforming growth factor β1. Cytokine concentrations were determined spectrophotometrically. Absorbances were read at 450 nm (BioTek, Vermont, USA).

We constructed a standard curve using chemokine standards. The cytokine concentrations for unknown samples were calculated according to this standard curve.

Statistical methods

Results were analysed using one-way analysis of variance. The Bonferroni test was used as post hoc analysis. A level of $p < 0.05$ was considered significant.

Results

Table I shows the Epstein–Barr virus (EBV) DNA load in the tumour tissue samples of the 10 EBV DNA positive laryngeal carcinoma patients.

Serum levels of the proinflammatory cytokines interleukin (IL) 1β, IL-2, IL-12, tumour necrosis factor α and interferon γ were slightly higher in EBV DNA positive laryngeal carcinoma patients and in acute EBV infection patients, compared with the EBV DNA negative laryngeal carcinoma patients and the healthy individuals. However, this increase was not statistically significant ($p > 0.05$). Serum levels of IL-6 did not differ significantly amongst the four groups studied ($p > 0.05$).

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TABLE I

EBV DNA LOAD IN TUMOUR TISSUE FROM EBV DNA POSITIVE LARYNGEAL CARCINOMA PATIENTS

Pt no	Viral load (copies/ml)
1	1×10^5
2	4×10^3
3	1×10^3
4	1×10^4
5	2.5×10^3
6	3.8×10^3
7	1.4×10^4
8	2.7×10^3
9	3.1×10^4
10	3.9×10^3

EBV = Epstein–Barr virus; pt no = patient number

Serum levels of the anti-inflammatory cytokines IL-10 and transforming growth factor β1 were found to be higher only in the EBV DNA positive laryngeal carcinoma patients, and this increase was statistically significant compared with the other three groups ($p < 0.05$). Furthermore, we observed no increase in the serum levels of these two cytokines in the EBV DNA negative laryngeal carcinoma patients, the acute EBV infection patients or the healthy individuals (Figure 1).

We observed no significant correlation between the EBV DNA load of the EBV DNA positive laryngeal carcinoma patients' tissue samples and any of the cytokines studied.

Discussion

Laryngeal squamous cell carcinoma is the most frequent head and neck cancer. The molecular mechanisms of laryngeal carcinoma pathogenesis and progression have yet to be fully explained.[16]

Epstein–Barr virus (EBV) has been aetiologically associated with several disease conditions, including various malignancies and lymphoproliferative disorders.[17] Although the role of EBV infection in the development of laryngeal carcinoma has not been clearly determined, it is thought to be associated in some way. There is a close association between EBV infection and nasopharyngeal carcinoma (NPC), which strongly suggests the possibility of an association with laryngeal carcinoma.[15]

Cytokines are produced by a wide variety of cells and tissues, and play an important role in cell differentiation, growth, and the regulation of immune and inflammatory responses.[17] They also play an important role in the pathogenesis of EBV infection. Epstein–Barr virus interacts with immune cells, and can cause changes in cytokine activation and host immune response balance.[4] Thus, changes in cytokine levels during EBV-associated diseases may play a role in disease pathogenesis and symptom development.[8]

Several factors are considered to be important in the pathogenesis of EBV-associated tumours. Chua *et al.* have reported that EBV infection may act as an alternative anti-apoptotic mechanism in NPC.[13] It is assumed that EBV infection and cytokines both affect anti-apoptotic mechanisms and may thus be instrumental in the development of carcinoma.

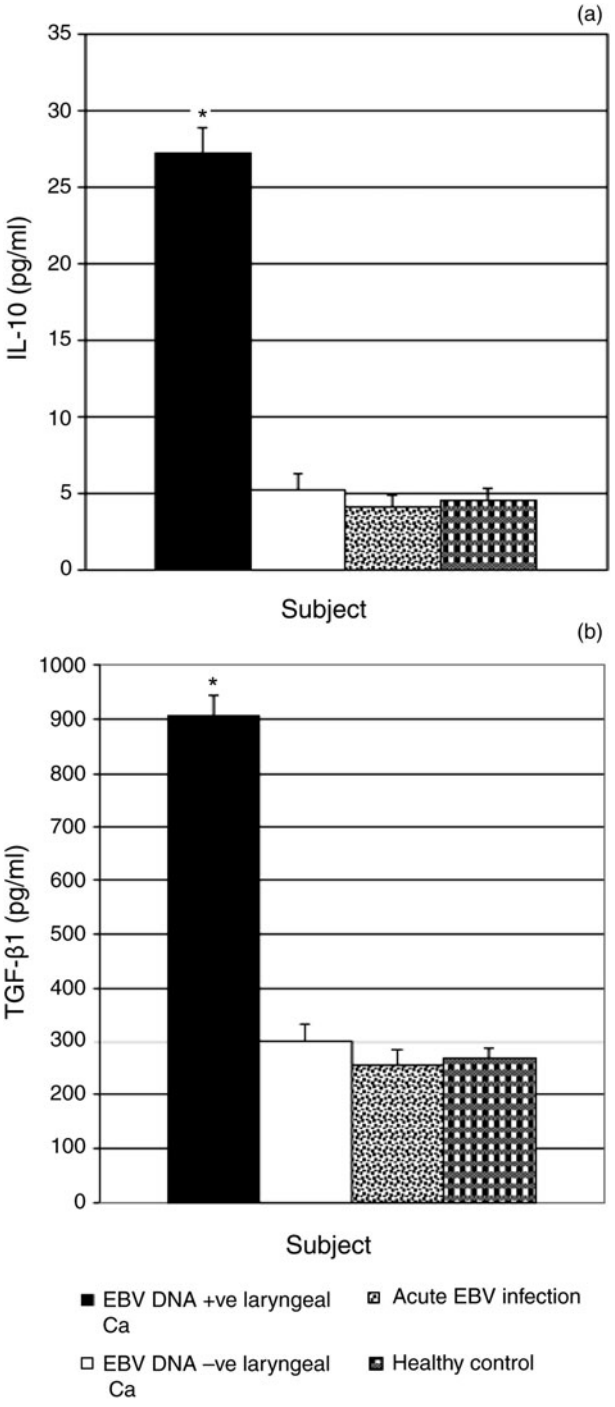

FIG. 1

Serum levels of interleukin (IL) 10 and transforming growth factor (TGF) β1 in the groups tested. Bars indicate means and standard errors of the mean. *Significant difference at $p < 0.05$. EBV = Epstein–Barr virus

Moreover, cytokines may be secreted to destroy the anti-tumour immune response in cases of malignancy.[17] The fact that cytokines are effective in this respect in many cases suggests that they may play an important role in EBV-associated disease. Cytokines may also be potentially useful as novel biomarkers in cases of EBV-associated cancer, with prognostic and therapeutic implications.

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Our study aimed to determine the serum cytokine levels in patients with EBV DNA positive laryngeal carcinoma. Of the cytokines analysed, only interleukin (IL) 10 and transforming growth factor β1 were present at significantly higher levels in EBV DNA positive laryngeal carcinoma patients, compared with the other groups tested. Furthermore, serum levels of these two cytokines were not increased in the control groups tested (i.e. patients with EBV DNA negative laryngeal carcinoma, patients with acute EBV infection and healthy individuals).

Transforming growth factor β1 and IL-10 are immunosuppressive cytokines and act synergistically.[17] This synergism may lead to an additive effect during tumour development.

Ho *et al.* reported that certain cytokines, such as IL-10, may be expressed in EBV-positive lymphomas.[18] They proposed that such cytokine environments may enhance EBV infection and contribute to tumourigenesis. Jebreel *et al.* reported that head and neck squamous cell carcinoma patients had higher IL-10 levels than controls.[19] In agreement with our own findings, Budiani *et al.* reported that serum IL-10 levels were significantly increased in patients with EBV-associated NPC, suggesting that IL-10 may play a role in the development of NPC.[20]

Li *et al.* analysed the serum levels of T-helper type 1 cytokines (i.e. interferon γ, IL-2 and tumour necrosis factor α) and T-helper type 2 cytokines (i.e. IL-4, IL-6 and IL-10) in patients with NPC and controls. They reported that interferon γ, IL-6 and IL-10 were present in the sera of the majority of patients, and that serum levels of these cytokines were significantly increased in patients compared with controls.[7]

Interleukin 10 is a cytokine which possesses immunosuppressive activity. It is mainly produced via T-helper type two cells. Ogino *et al.* suggested that EBV-infected NPC cells may activate an immunoescape mechanism involving FasL upregulation associated with IL-10 expression.[21] They also reported that IL-10 acts not only as an immunosuppressive but also as a growth factor for malignant cells.

Transforming growth factor β1 is also an immunosuppressive cytokine, and can induce immunoglobulin A switch and EBV replication in latently infected cells.[12,22] Xu *et al.* found that transforming growth factor β1 serum levels were significantly increased in patients with EBV-associated, undifferentiated and poorly differentiated NPC, suggesting involvement of this cytokine in the pathogenesis of EBV-associated tumours. They believed that such an increase in transforming growth factor β1 levels may result from enhanced EBV replication, either alone or in associated with EBV production from tumour cells.[17] Other authors have reported that high levels of transforming growth factor β1 may play a unique role in the pathogenesis of head and neck cancers.[23]

Our study findings suggest that EBV infection has a definite effect in the secretion of immunosuppressive cytokines such as IL-10 and transforming growth factor β1. This suggests that cytokines may play a pathogenic role in EBV-associated laryngeal carcinoma, by both suppressing anti-tumour responses and by promoting tumour growth.

- **Epstein-Barr virus (EBV) may have a role in the pathogenesis of laryngeal malignancies**
- **This study aimed to determine the serum levels of cytokines in EBV DNA positive patients with laryngeal carcinoma**
- **The cytokines interleukin 10 and transforming growth factor β1 may represent potential targets for molecular treatments of laryngeal carcinoma, and may also be useful to indicate disease prognosis**

Our study aimed to determine cytokine levels in patients with laryngeal carcinoma possibly associated with EBV. This was one of the first studies to investigate EBV DNA positive laryngeal carcinoma patients and tissue samples; thus, our findings have significance.

The significance of our findings may be limited by our small patient numbers, and by the fact that the association between laryngeal carcinoma and EBV infection has not been clearly determined.

Our future investigations will involve larger patient numbers and will analyse cytokine expression within laryngeal biopsy tissue, and the effect of treatment efficacy on this expression.

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