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Interleukin-10 levels in Epstein-Barr virus-associated nasopharyngeal carcinoma

Dyah R Budiani[1,3], Salomo Hutahaean[1], Sofia M Haryana[2,3], Marsetyawan HNE Soesatyo[2], Wihaskoro Sosroseno[4]

[1]Department of Biology, Faculty of Mathematics and Natural Science, Sumatera Utara University, Medan, Sumatera Utara; [2]Department of Histology and Cell Biology, School of Medicine; [3]IUC-Biotechology, Gadjah Mada University, Yogyakarta, Indonesia; and [4]Deparment of Oral Biology, School of Dental Sciences, Universiti Sains Malaysia, Kota Bharu, Malaysia

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The aim of this study was to determine the levels of interleukin-10 in patients with nasopharyngeal carcinoma. Both biopsies and sera were obtained from patients with nasopharyngeal carcinoma as well as Epstein-Barr virus-seronegative patients as a control. Nasopharyngeal carcinoma patients were classified using the World Health Organization pathological assessment and clinical staging of nasopharyngeal carcinoma. The numbers of interleukin-10 positive cells and the levels of serum interleukin-10 were assessed by immunohistochemical methods and enzyme-linked immunosorbent assay, respectively. The levels of serum interleukin-10 were determined by enzyme-linked immunosorbent assay. The results showed that the number of interleukin-10 positive cells and serum interleukin-10 levels were significantly increased in patients with nasopharyngeal carcinoma-World Health Organization type III and with clinical late stage ($p<0.05$), suggesting that interleukin-10 may have a crucial role in the progression of nasopharyngeal carcinoma.

Key words: Epstein-Barr virus, interleukin-10, nasopharyngeal carcinoma

Nasopharyngeal carcinoma (NPC) is a tumor of epidermoid origin and prevalent in several regions around the world. The association between Epstein-Barr virus (EBV) and NPC is well known as shown by the fact that the EBV genome was found in the NPC specimens [1]. However, the precise pathogenesis by which EBV induces the development of NPC remains to be further elucidated. Evidences have shown that cytotoxic T lymphocytes (CTL) specific to EBV antigens have a crucial role in the host defense against EBV-associated tumors [2,3]. Despite abundant infiltrating CTL in the stroma of NPC, the development of tumor is undisturbed, suggesting that the immune responses to the tumor may be downregulated [1-3]. One possible mechanism by which EBV-specific CTL functions are suppressed is via tumor-derived immunosuppressive cytokines. Indeed, EBV encodes a late lytic cycle protein called BCRF1 or viral interleukin-10 (vIL-10), which shares amino acid homology with human IL-10 [4]. Interleukin-10 is produced by human monocytes and both T and B cells and acts to inhibit MHC class II expression and variety of cytokines, such as interferon-gamma (IFN-γ), indicating that IL-10 may function as an immunosuppressive cytokine [4]. Previous studies have shown that increased IL-10 messenger RNA expression and IL-10-positive cells in biopsies of NPC could be observed [5,6], but other failed to demonstrate similar results [7]. The aim of this study was to determine the number of IL-10-positive cells and the levels of serum IL-10 in patients with NPC.

Materials and Methods

Nasopharyngeal carcinoma biopsies were obtained from 8 patients with nonkeratinizing carcinoma (World Health Organization [WHO] type II) and 8 patients with undifferentiated carcinoma (WHO type III). Serum samples were obtained from 7 patients with NPC-WHO type II and 8 patients with NPC-WHO type III. The clinical staging of NPC was examined according to tumor-node-metastasis (TNM) classification of the International Union Against Cancer rules for head and neck cancer [6]. These patients were then classified as early stage (stage I and II) and late stage (stage III and IV) [8]. Both biopsies and serum samples were collected from patients undergone observations and treatments

Corresponding author: Dr. Wihaskoro Sosroseno, Department of Oral Biology, School of Dental Sciences, Universiti Sains Malaysia, 16150, Kota Bharu, Malaysia. E-mail: wihaskoro@kb.usm.my

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at Dr. Sardjito's General Hospital, Yogyakarta, Indonesia. All patients were EBV-positive by serologic tests. The positive control of IL-10 staining was derived from the human tonsils. Sera from nondiagnostic-asymptomatic, EBV-negative patients were used a control. All patients and/or their relatives gave informed contents and this study was approved by the ethical committee of the Faculty of Medicine, Gadjah Mada University.

Interleukin-10 positive cells in paraffin-embedded blocks were determined using the avidin-biotin-peroxidase complex. Following cut and deparaffinization, the sections were blocked in 3% H_2O_2 dissolved in absolute methanol and then mounted with protein blocking agent (Lipshaw, Pittsburgh, PA, US). Biotinylated mouse anti-human IL-10 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, US) were applied on all sections, which were then incubated for 30 min at room temperature. After washing, all sections were reacted with the streptavidin-peroxidase (Lipshaw) for 30 min, visualized using a 3-3'-diaminobenzidine tetrahydrochloride solution (DAB; Lipshaw) for 10 to 20 min and subsequently counterstained with hematoxylin. Cells with positive staining per mm^2 were microscopically counted.

The levels of serum IL-10 were determined using the Biotrak human IL-10 enzyme-linked immunosorbent assay kit as described by the manufacturer (Amersham Pharmacia Biotech, Buckinghamshire, UK).

The data was statistically determined by one-way analysis of variance followed by Fisher's least squared differences using a statistical package (SPSS version 9.0; SPSS Inc., Chicago, IL, US). The data on the levels of IL-10 in the clinical stages of NPC was calculated by an independent *t* test.

Results and Discussion

The positive staining for IL-10 seen in the cytoplasm of cells could be observed in tissues from patients with NPC (data not shown). The numbers of IL-10-positive cells in NPC-WHO type III were significantly higher than those in NPC-WHO type II ($p<0.05$) (Fig. 1). Furthermore, no significant difference between the levels of serum IL-10 in patients with NPC-WHO type II and those in the control was seen ($p>0.05$) (Fig. 2). In contrast, the serum IL-10 levels in patients with NPC-WHO type III were significantly elevated as compared with those in patients with NPC-WHO type II ($p<0.05$) (Fig. 2). The numbers of IL-10+ cells and the levels of serum IL-10 in patients with NPC late stage were significantly higher than those in the patients with NPC early stage ($p<0.05$) (Table 1).

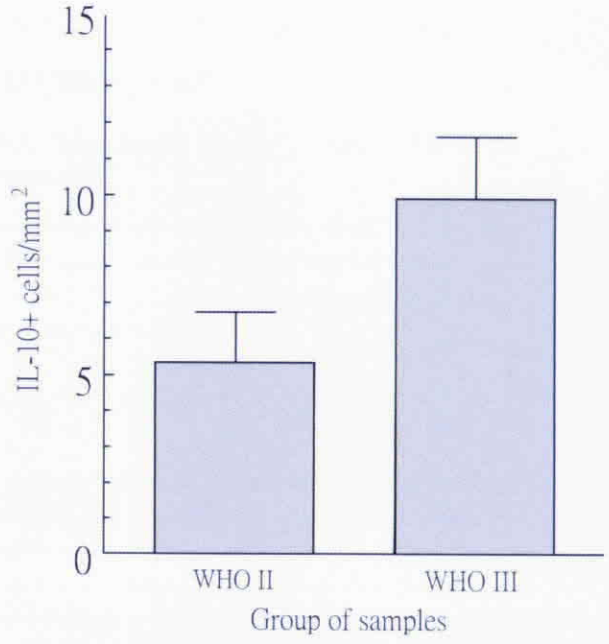

Fig. 1. Mean and standard deviation of the numbers of IL-10 positive cells in tissues of NPC patients. Tissues were obtained from patients with NPC-WHO type II and type III and were stained with monoclonal anti-human IL-10 antibodies. The numbers of IL-10-positive cells /mm^2 were determined microscopically.

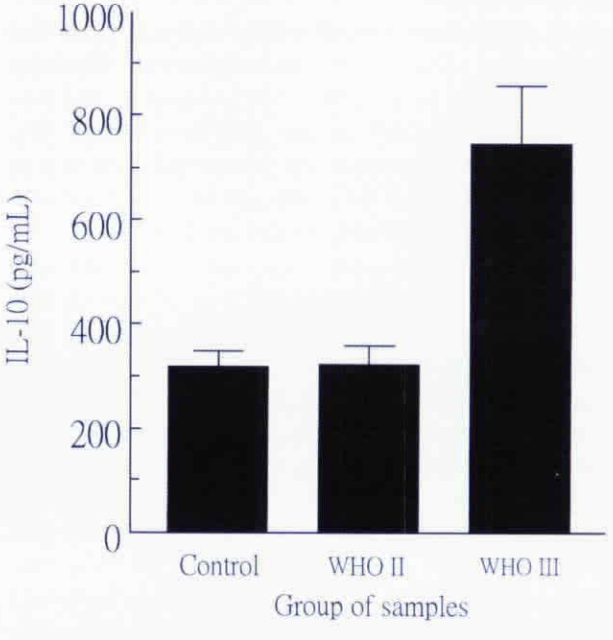

Fig. 2. Mean and standard deviation of serum IL-10 levels in NPC patients. Sera were obtained from patients with NPC-WHO type II and type III as well as the EBV-seronegative patients (the control group). The serum IL-10 levels were determined by a commercial enzyme-linked immunosorbent assay kit.

Table 1. The mean and standard deviation of IL-10 levels in different clinical stages of NPC

Clinical stage[a]	IL-10+ (cells /mm^2)	*p*	Serum IL-10 (pg/mL)	*p*
Early	4.067 (0.987)	0.004	372.250 (26.986)	0.006
Late	9.633 (2.170)		595.167 (116.625)	

Abbreviations: IL-10 = interleukin-10; NPC = nasopharyngeal carcinoma

[a]Early = stages 1 and II; Late = stages III and IV. An independent *t* test with 95% confident levels was used to determine the statistical differences between the levels of IL-10 in early and late stage of NPC.

The results of this study showed that, based upon both pathologic classification and clinical staging of NPC, the numbers of IL-10-positive cells and the levels of serum IL-10 increased as the NPC progressed. These results support previous studies that demonstrate an increased IL-10 gene and protein levels in NPC tissues [5,6]. However, the exact mechanism by which EBV infection leading to the development of NPC induces increased IL-10 levels *in vivo* as seen in this study remains unclear. The previous studies indicated that EBV-encoded RNAs (EBERs) are responsible for a direct stimulation of IL-10 production by EBV-transformed cells and that only IL-10 acts as an autocrine growth factor for EBV-related tumor [9,10]. Indeed, tumor cells of NPC produce IL-10 *in situ* [5,6]. Therefore, one may assume that *in vivo* IL-10 production is directly induced by EBV and subsequently, used as the autocrine growth factor for autonomous growth of EBV-associated tumors. Not surprisingly, increased EBV-induced tumor growth as seen in the present and previous studies was paralleled with increased production of IL-10 [5,6].

The extrapolation of the results of this study in the pathogenesis of NPC remains speculative. Interleukin-10 is known as an immunosuppressive growth factor for T cells and cytokine production [4]. Indeed, Muller and colleagues [11] demonstrated that vIL-10 may directly induce T-cell tolerance by inhibiting the costimulatory signals mediated via B7 receptors, such as CD28 or CTLA-4. It seems plausible, therefore, that decreased numbers of cytotoxic T cells in NPC tissues expressing high levels of IL-10 *in situ*, as seen in the previous study carried out by Yao *et al* [5], may be due to the fact that IL-10 produced by EBV-induced tumor cells directly suppresses the function of this T-cell population by downregulating signal transduction activated via B7 receptors, thereby allowing tumor cells to escape from the immune surveillance. This notion remains to be further clarified.

Although further studies remains to be carried out, the results of this, to some respects, indicated that the detection of IL-10 may be used as one of the diagnostic tools to determine the progression of NPC. A support of this notion can be drawn from the fact that the serum IL-10 levels may be a good predictor of the progression of gastrointestinal carcinoma, since the serum levels of this cytokine in patients with metastatic stage were higher than those in patients with undisseminated one [12]. Of interest, this study showed that increased clinical staging of NPC was paralleled with increased levels of IL-10 as seen in a previous study carried by Fujieda *et al* [6]. Their study indicated that the survival rate of NPC patients with IL-10 positive cancer cells was much lower than that with IL-10 negative cells, suggesting that increased levels of IL-10 may represent a poor prognosis [6]. Whether or not increased levels of IL-10 in NPC patients with either pathologic feature of WHO type III or clinical late stage seen in this study may reflect a poor prognosis remains to be investigated further.

In conclusion, this study showed that both IL-10 positive cells and serum IL-10 levels are elevated in patients with NPC-WHO type III and late clinical NPC stage, suggesting that IL-10 may have a crucial role in the development of NPC.

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