Induction of Interleukin-8 by Epstein-Barr Virus Latent Membrane Protein-1 and Its Correlation to Angiogenesis in Nasopharyngeal Carcinoma

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ABSTRACT

Purpose: The EBV latent membrane protein-1 (LMP-1) is a multifunctional protein. Recently, the contribution of LMP-1 to the metastasis of nasopharyngeal carcinoma (NPC) has been suggested. Angiogenesis is a key step for metastasis. Thus, the association of LMP-1 to neovascularization of NPC was examined in this study.

Experimental Design: The association of LMP-1 to angiogenesis in 39 patients with NPC was evaluated by immunohistochemical study, and then induction of angiogenic factors by LMP-1 was examined by ELISA and luciferase reporter assay.

Results: In an immunohistochemical study, the expression of LMP-1 was significantly correlated to microvessel counts (P = 0.0003), suggesting that LMP-1 may induce some angiogenic factors. Therefore, we studied the relationship between LMP-1 expression and interleukin-8 (IL-8), vascular endothelial growth factor (VEGF), and basic fibroblast growth factor (bFGF) expression by immunohistochemical analysis. IL-8, VEGF, and bFGF expression were correlated to microvessel counts, but only IL-8 expression was significantly correlated to LMP-1 expression (P < 0.0001). Transfection with LMP-1 expression plasmid induced IL-8 promoter protein expression in C33A cells. The expression of LMP-1 transactivated IL-8 promoter, as demonstrated by IL-8 promoter luciferase reporter assay. Mutation of the nuclear factor kB responsive element in the IL-8 promoter region completely abolished transactivation by LMP-1, whereas mutation of the activator protein responsive element did not affect promoter activity.

Conclusion: These results suggested that LMP-1 induces expression of IL-8 through the nuclear factor kB binding site, which may contribute in part to angiogenesis in NPC.

INTRODUCTION

Angiogenesis is a key step in tumor growth, invasion and metastasis. Massive formation of blood vessels at the tumor site increases the opportunity for tumor cells to enter the circulation. Thus, microvessel density is considered to influence tumor metastasis and consequently prognosis in various human cancers (1). VEGF, bFGF and IL-8 are prominent angiogenic molecules. These molecules have been demonstrated to influence microvessel synthesis in various tumors (2, 3).

EBV is a ubiquitous human γ herpes virus and is associated to several human malignant neoplasms such as endemic Burkitt lymphoma and NPC (4, 5). LMP-1 is the principal EBV oncoprotein. LMP-1 is essential for immortalization of B lymphocytes and transforms rodent fibroblasts and human keratinocytes (6, 7). In addition to such transforming properties, LMP-1 is associated to down-regulation of E-cadherin, induction of MMP9, and up-regulation of cell motility (8–10). Thus, LMP-1 has been suggested to contribute to invasion and metastasis. As angiogenesis is associated to tissue remodeling, which resembles tumor invasion, it is of interest to determine whether multifunctional LMP-1 protein could contribute to microvessel synthesis (11).

In this study, we examined the role of LMP-1 in angiogenesis of NPC.

MATERIALS AND METHODS

Tissue Samples. Thirty-eight specimens were obtained from patients with NPC who underwent biopsy at the National Taiwan University Hospital from 1996 to 1997. They were composed of three squamous cell carcinomas (WHO type I), 15 nonkeratinizing carcinomas (WHO type II), and 20 undifferentiated carcinomas (WHO type III). Twenty-nine patients were male, and nine patients were female (mean age, 54.9 years; 2 The abbreviations used are: VEGF, vascular endothelial growth factor; bFGF, basic fibroblast growth factor; IL-8, interleukin 8; NPC, nasopharyngeal carcinoma; MMP9, matrix metalloproteinase.

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range, 22–81 years). Biopsy specimens were embedded in OCT compound (Miles, Elkhart, IN), frozen immediately, and stored at −80°C.

**Immunohistochemical Analysis.** Immunohistochemical studies were performed using the avidin-biotin-complex method as described previously (12, 13). Frozen sections 5 μm thick were fixed with 100% ethanol. Endogenous peroxidase was blocked with Endo-blocker (Biomeda, Foster City, CA) and nonspecific reaction was blocked with normal sheep serum (DAKO, Copenhagen, Denmark), then incubated with primary antibodies at 4°C overnight. Primary antibodies against LMP-1 (CS1-4; DAKO), IL-8 (RS0002–08; R&D Systems, Inc., Minneapolis, MN), VEGF (A-20; Santa Cruz Biotechnology, Santa Cruz, CA), bFGF (SC-79G; Santa Cruz Biotechnology), and vWF (A0082; DAKO JAPAN, Kyoto, Japan) were used. The samples were incubated with a universal secondary antibody (Research Genetics, Huntsville, AL) containing biotinylated goat antimouse immunoglobulin and horseradish peroxidase-conjugated streptavidin and then 3,3′-diaminobenzidine tetrahydrochloride as a chromogen and hematoxylin as a nuclear counterstain. NPC sections that had been used in previous studies were used as positive controls for LMP-1, VEGF, and vWF staining (12, 14). Human placenta sections were used as positive controls for bFGF, and tonsil sections were used as

Fig. 1  Immunohistochemical detection of LMP-1 (A), IL-8 (B), VEGF (C), bFGF (D), and microvessels (E) in NPC. Original magnification, ×200.
positive controls for IL-8. The specificity of the staining was confirmed using nonimmune serum instead of the primary antibodies as a negative control.

Microvessel Counting. Blood vessels were identified by anti-vWF antibody staining. The area of tumor that showed the most dense neovascularization was found by light microscopy at ×10. Then, microvessels were counted at ×400. These assessments were performed by two observers (T. Y. and T. H.). The highest number of microvessels identified within any single ×400 field was defined as the microvessel index of the tumor.

Evaluation of Immunohistochemical Staining for LMP-1, VEGF, bFGF, and IL-8. The stained specimens were examined independently by two of the authors (T. Y. and T. H.). In each case, two arbitrary separate microscopic fields (×200) containing >200 tumor cells were evaluated. These two arbitrary areas were selected from the area where microvessels were counted. After counting both immunoreactive cells and the total number of tumor cells, the average percentages of immunoreactive cells were calculated, without knowledge of the clinical data, and the results were reviewed by the other author. The average percentage of immunostained cells was defined as the expression score and was used for statistical analyses.

Statistical Analysis. The data were analyzed with a Macintosh personal computer (Apple Computer, Cupertino, CA) with Stat View software (Abacus Concepts, Berkeley, CA). Statistical analysis was performed by Spearman’s correlation coefficient by rank test and Pearson’s correlation coefficient analysis, with Ps of <0.05 considered to indicate statistical significance.

Plasmid, Cell Line, and Transient Transfection. The LMP-1 expression plasmid LMP1 was constructed as described previously (10). Using the primers 5′-TTAGATCTGTAGCATCAGTTGCGGCCTG-3′ and 5′-GCCTAGAAGCTTGTTAGCTCTCTGCTG-3′, the 169-bp IL-8 promoter fragment (−130 to +39 relative to the transcriptional initiation site) was amplified by PCR from human genomic DNA and inserted into the multiple cloning site of the pGL3-Basic Vector (Promega, Madison, WI) to generate a construct containing the firefly luciferase gene as a reporter (pGL-8-Luc; Ref. 15). Mutations in the multiple cloning site of the appropriate reporter and effector plasmids by the calcium phosphate method as described previously (9).

ELISA. LMP-1-transfected C33A cells were cultured with serum-free DMEM for 24 h. Then, the IL-8 content of the supernatants was measured using a specific ELISA assay kit (R&D Systems, Inc.) according to the manufacturer’s protocol.

Luciferase Reporter Assay. Luciferase reporter assays were performed with extracts of C33A cells after transient transfection. Cells were incubated for 48 h after transfection, then harvested. Luciferase activity was quantified with a chemoluminometer (EG&G Berthold, Bad Wildbad, Germany).

RESULTS

Immunohistochemical Features of LMP-1, Angiogenic Factors, and vWF. LMP-1, VEGF, bFGF, IL-8, and vWF expression were examined by immunohistochemical staining on biopsy specimens of NPC. LMP-1, VEGF, bFGF, and IL-8 were localized on the cell surface and in the cytoplasm of the tumor cells. However, their distribution patterns were protein-specific. LMP-1 and IL-8 proteins were scattered throughout the tumor cells and showed no specific tendency in its distribution pattern. VEGF and bFGF proteins were diffusely detected and were mainly localized to the periphery of the tumor cells. Microvessels, indicated by vWF staining, were detected as scattered in the tumor cell nests and showed similar distribution to LMP-1 and IL-8 in the area. They were also detected at the tumor stroma encircling tumor cell nests and showed a distribution pattern similar to VEGF and bFGF in the area (Fig. 1). The data of expression scores are shown in Table 1.

Relationship of LMP-1 to Microvessel Counts. To examine the relevance of LMP-1 to the neovascularization of NPC, the expression score of LMP-1 and microvessel counts were evaluated. A significant relationship was observed between LMP-1 expression score and microvessel counts (P = 0.0003; Table 2). Regression analysis also showed a significant correlation between these two factors (Fig. 2a). These results suggested that the expression of LMP-1 protein is closely associated with angiogenesis in NPC.

Relationship of LMP-1 to Angiogenic Molecules. To investigate the role of LMP-1 in the angiogenesis of NPC, the
relationships of LMP-1 to IL-8, VEGF, and bFGF were then analyzed by immunostaining. The expression score of IL-8 was significantly correlated to that of LMP-1 ($P < 0.0001$; Table 2; Fig. 2b). The expression scores of both VEGF and bFGF were related to that of LMP-1, but their correlation was not statistically significant ($P = 0.104$ and $P = 0.118$, respectively). These results suggested the close association of LMP-1 and IL-8 protein expression in NPC.

**Relationship of Angiogenic Molecules to Microvessel Counts.** The relevance of angiogenic factors to the angiogenesis of NPC was evaluated by studying the relationships of IL-8, VEGF, and bFGF expression scores to microvessel counts. The expression score of IL-8 was significantly correlated to microvessel counts ($P < 0.0428$; Table 2). Regression analysis also indicated a significant relationship between IL-8 and microvessel counts (Fig. 2c). Not only IL-8 but also the expression scores of both VEGF and bFGF showed significant correlation with microvessel counts ($P = 0.0468$ and $P = 0.0309$, respectively; Table 2). These results suggest that IL-8, VEGF, and bFGF contribute to microvessel development in NPC.

**DISCUSSION**

The associations of neovascularization to both angiogenic and lymphatic metastases have been examined in many malignant tumors, and the contributions of angiogenic molecules such as VEGF, bFGF, and IL-8 to the metastatic potential of tumors have been reported (2, 3).

The present study suggested associations of VEGF, bFGF, and IL-8 to neovascularization in NPC. It is noteworthy that expression of LMP-1 was significantly correlated to both the expression of IL-8 and the microvessel count but not to the expression of VEGF nor bFGF. Although the number of NPC samples examined in this study was not sufficient to allow us to make definite conclusions, these results suggested that LMP-1 may contribute to angiogenesis in NPC through the induction of IL-8.
LMP-1 has an activated-tumor necrosis factor receptor-mimicking function, which induces activation of NFκB and AP-1 (16, 17). Recently, we reported induction of MMP9 by LMP-1, which was mediated by both NFκB and AP-1 and blocked by aspirin or IκB (10, 18, 19). Both binding sites for AP-1 and NFκB in the promoter of MMP-9 were indispensable for transactivation of the promoter by LMP-1. In contrast, the data presented here indicated that the NFκB binding site in the IL-8 promoter was essential for the response to LMP-1, and that the AP-1 binding site played only a partial role.

Many molecules, including cell-surface markers such as CD23, CD40, intercellular adhesion molecule-1, and the anti-apoptotic protein bcl-2, are induced by LMP-1 in B lymphocytes (20–22). However, LMP-1 does not always have a similar effect on cellular signal transduction in epithelial cells. Functional NFκB complexes are combinations of the various NFκB family proteins, and different NFκB family proteins have varied affects on different NFκB-responsive promoters. In epithelial cells, LMP-1 activates a different subset of NFκB family proteins from that in lymphocytes (23). LMP-1 does not induce bcl-2 in epithelial cells but induces A-20 for preventing LMP-1-expressing cells from experiencing programmed cell death (24). Thus, we should be aware of the cell type-specific function of LMP-1.

Eliopoulos et al. (25) reported that activation of p38 mitogen-activated protein kinase, as well as NFκB, by LMP-1, contributed to IL-8 production in epithelial cells. Recently, we also reported the up-regulation of cell motility by LMP-1 via ets-1 activation (9). The multiple functions of LMP-1 could be largely attributable to the activation of NFκB and AP-1. However, all molecules modulated by LMP-1 have not yet been elucidated, and additional studies of the molecular mechanism of LMP-1-mediated transformation and tumor progression are required.

Despite much evidence which suggests the relationship of LMP1 to tumor initiation and progression, the relationship of LMP-1 to the clinical features of EBV-associated tumors have been a matter of issue. In this study, there was no statistically significant correlation between LMP-1 expression score and cervical lymph-node status (P = 0.07; data not shown). Presumably, LMP-1 could play some role in both tumorigenesis and metastasis. However, so many factors, such as hypoxia and personal immune system, would also influence the behavior of the tumor.

The results reported here add weight to the growing body of evidence suggesting a positive role of LMP-1 in invasion and metastasis. Metastatic potential of NPC might be reduced by the inhibition of the LMP-1-mediated signaling pathways.

REFERENCES

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