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Review

Promotion of metastasis in nasopharyngeal carcinoma by Epstein-Barr virus latent membrane protein-1

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Summary. Nasopharyngeal carcinoma (NPC) is a malignant tumor associated with Epstein-Barr virus (EBV). Latent membrane protein-1 (LMP-1) is an EBV-encoded oncoprotein and is detected in approximately 50-70% of patients with NPC. LMP-1 is thought to play an essential role in tumorigenesis of NPC. In addition to its transforming properties, LMP-1 has been suggested to be associated with promotion of metastasis. Metastasis is a phenomenon composed of multiple sequential cascades. Reduction of tumor cell adhesion, degradation of extracellular matrix, basement membrane, enhancement of cell motility, and promotion of neovascularization are thought to be essential steps. LMP-1 down-regulates expression of E-cadherin, induces matrix metalloproteinase-9 and urokinase type-plasminogen activator through activation of NF-κB and AP-1, and enhances cell motility via ets-1 activation. LMP-1 also induces vascular endothelial growth factor through cyclooxygenase-2 activation and interleukin-8 through NF-κB activation. Clinical studies suggested the association of these factors with metastatic status of patients with NPC. In this review, the role of LMP-1 in the metastasis of NPC is discussed.

Key words: Epstein-Barr virus, Latent membrane protein-1, Matrix metalloproteinase, Metastasis, Nasopharyngeal carcinoma

Introduction

Metastatic spread of cancer cells is a result of a complex cascade of cellular events. The cascade is composed of multiple sequential steps such as down-regulation of intercellular adhesion, degradation of extracellular matrix (ECM) and up-regulation of cell motility. In addition, tumor size and likelihood of metastasis are thought to depend on increased vascularity in tumors (Forkman, 1971).

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Nasopharyngeal carcinoma (NPC) is an Epstein-Barr virus (EBV)-associated malignant tumor. A prominent clinical characteristic of NPC is frequent involvement of cervical lymph nodes and distant organs compared with other head and neck carcinomas (Ortiz-McWilliams, 1991). Latent membrane protein-1 (LMP-1) is an EBV oncoprotein that transforms rodent fibroblast (Fahraeus et al., 1990) and inhibits human keratinocyte differentiation (Dawson et al., 1990). These effects of LMP-1 on cell growth and proliferation are considered to be attributable to its tumor necrosis factor α receptor (TNFR) II and CD40 mimicking function (Mosialos et al., 1995; Hatzivassiliou et al., 1998). LMP-1 is composed of a short cytoplasmic N-terminus of 25 amino acids, a transmembrane domain with six membrane-spanning segments, and a long cytoplasmic C-terminus of 200 amino acids (Liebowitz et al., 1986). The C-terminus cytoplasmic domain of LMP-1 has two functional domains, C-terminal activation region (CTAR)-1 and CTAR-2. CTAR-1, is located in the proximal portion, interacts with tumor necrosis factor receptor-associated factors (TRAFs) and activates NF-κB (Izumi et al., 1997). CTAR-2, is located in the distal portion, interacts with tumor necrosis factor receptor-associated death domain protein and activates NF-κB and AP-1 (Kieser et al., 1997) (Fig. 1).

Recent studies have suggested the association of LMP-1 with metastasis of NPC. In this review, the mechanism of LMP-1-mediated promotion of metastasis is discussed.

Down-regulation of intercellular adhesion by LMP-1

One of the main adhesion molecules that plays a major role in epithelial cells is E-cadherin. E-cadherin is a homophylic connector that is localized in adherens junction, and its function is supported by ß-catenin that binds to the cytoplasmic portion of E-cadherin. Reduction of E-cadherin expression or functional insufficiency of E-cadherin, due to abnormal function of ß-catenin, are frequently seen features of various cancers, and have been reported to correlate with

metastatic properties of tumors (Paciucci et al., 1998; Groden, 2000; Jiang et al., 2000). Expression of LMP-1 in human epithelial and murine adenocarcinoma cells down-regulated expression of E-cadherin. Moreover, these LMP-1-expressing cells increased invasive capacity (Fahraeus et al., 1992; Farwell et al., 1999). These reports supported the suppressive effect of LMP-1 on the E-cadherin-mediated cell adhesion system in epithelial cells. Decreased expression of E-cadherin/ß-catenin has also been reported in NPC (Lou et al., 1999; Zheng et al., 1999). However, the downstream signal pathway from LMP-1 for down-regulation of E-cadherin/ß-catenin systems has not been clarified.

Degradation of ECM by LMP-1

Destruction of ECM is an essential step for tumor metastasis. The basement membrane is an important barrier, and thus, the degree of basement membrane destruction is one of the major predictors of tumor metastatic ability. Type IV collagen is a major component of the basement membrane, and expression of two type IV collagenases, matrix metalloproteinase (MMP)-2 and -9, is closely associated with metastatic status of the tumor (Stetler-Stevenson, 2001).

Expression of MMP-9 protein is markedly correlated with that of LMP-1 protein in various Burkitt's lymphoma cell lines (Fig. 2). Moreover, transfection of LMP-1-expression vector to the human epithelial cell line C33A induced expression of MMP-9. Electrophoretic mobility shift assay revealed that cellular transcription factors bound to NF-κB and AP-1 binding DNA sequences in the MMP-9 promoter. Furthermore, mutation of either NF-κB or AP-1 binding region in the MMP-9 promoter dramatically decreased transactivation of the MMP-9 promoter by LMP-1 (Yoshizaki et al., 1998). Thus, LMP-1 transactivates MMP-9 production through the activation of NF-κB and AP-1.

LMP-1 mutants lacking either CTAR-1 or CTAR-2 could transactivate the MMP-9 promoter and induce MMP-9 independently and additively, although both had weaker MMP-9 induction ability than wild-type LMP-1 (Fig. 3). Moreover, both CTAR-1 and CTAR-2 could activate both AP-1 and NF-κB, which was suggested to be mediated by TRAF2 (Takeshita et al., 2001).

LMP-1 and MMP-9 protein expression were studied immunohistochemically in NPC tissue, and a significant correlation was observed. Furthermore, expression of MMP-9 was significantly correlated with cervical lymph node metastasis. NPC patients with LMP-1 expression showed a tendency for cervical lymph node metastasis, although it was not statistically significant. These observations suggested a close association of MMP-9 with spread of cervical lymph node metastasis (Horikawa et al., 2000).

Urokinase-type plasminogen activator (u-PA) is a serine proteinase that also degrades ECM. LMP-1 also induces u-PA (Kim et al., 2000).

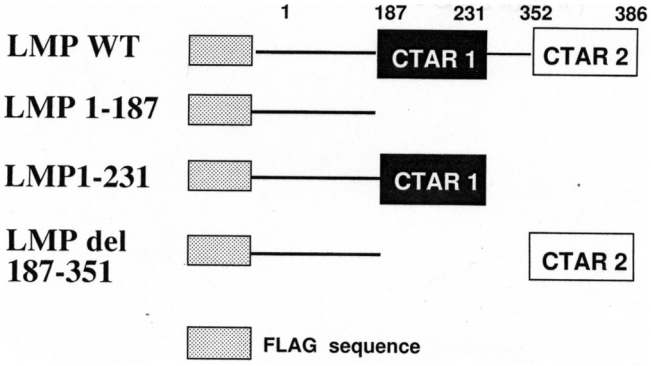

Fig. 1. Schematic representation of LMP-1 mutants. WT, wild-type. CTAR, COOH-terminal activation region.

Upregulation of cell motility by LMP-1

Those tumor cells that do not migrate lose the chance of entering the lymphatic system and microvessels. Thus, motility is also an important factor that influences invasion and metastaic ability of tumor cells. The correlations of lymphatic and hematogenous metastasis with expression of cell motility up-regulating factors such as ets-1, autocrine motility factor receptor, and c-met have been reported in various cancers (Nakayama et al., 1996; Nakada et al., 1999; Nakajima et al., 1999).

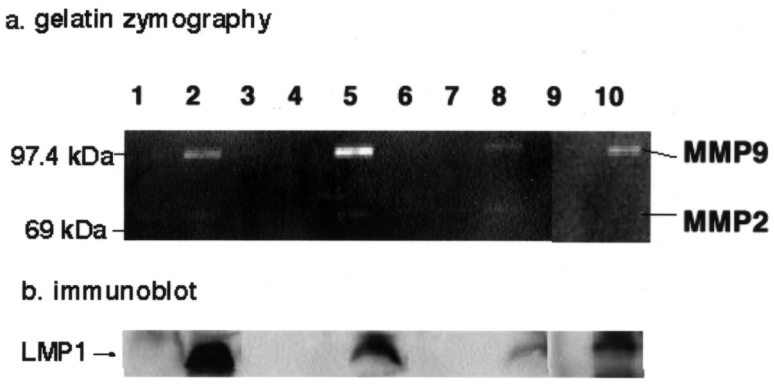

Fig. 2. Expression of MMP2, MMP9 and LMP-1 in Burkitts' lymphoma cell lines. **a**) MMP2 and MMP9 expression were determined by gelatin zymography, **b)** LMP-1 expression was determined by immunoblot. 1: SAV I; 2: SAV III; 3: BL30; 4: BL30-P3HR-1; 5: BL30-B95-8; 6: BL41; 7: BL41-P3HR-1; 8: BL41-B95-8; 9: P3HR-1; 10, Jijoye.

LMP-1-transfected Madin-Darby canine kidney (MDCK) cells lost cell-to-cell contacts and showed morphological changes. Enhanced motility of LMP-1 transformants was also observed by scrape-wound migration assay (Fig. 4). Differential display analysis of mRNA between control MDCK cells and LMP-1-transformed MDCK cells identified up-regulation of ets-1 (Fig. 5). Expression of dominant negative form of ets-1 (ets-DN) markedly reduced motility of LMP-1-transfected MDCK cells, but they still showed reduced cell-to-cell adhesion (Kim et al., 2000). These results suggested an important role of ets-1 in LMP-1-downstream signal cascades for motility enhancement.

As described previously, LMP-1 has two cytoplasmic domains (CTAR-1 and CTAR-2), which activate NF-κB. The next issue was which domain is responsible for up-regulation of cell motility. Expression of LMP-1 mutant lacking the CTAR-1 domain failed to transform MDCK cells and thereby lost its motility up-regulating effect. However, MDCK cells with LMP-1 mutant lacking CTAR-2 exhibited morphological changes and high motility in the scrape-wound migration assay (Kim et al., 2000). Therefore, CTAR-1 was suggested to be the domain responsible for up-regulation of cell motility, although both CTAR-1 and CTAR-2 could induce MMP-9.

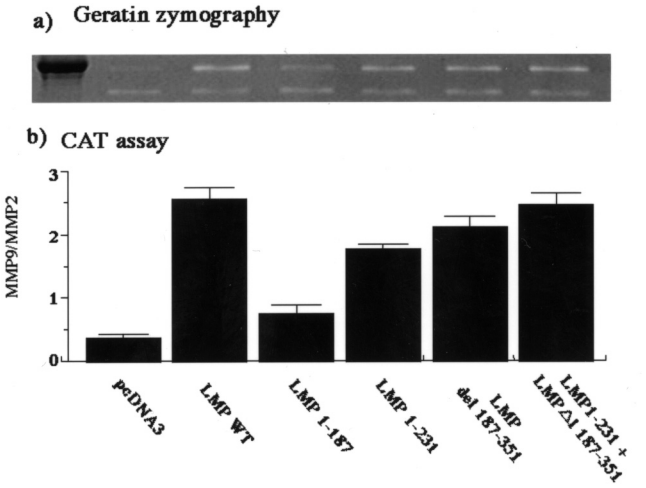

Fig. 3. Induction of MMP2 and MMP9 by LMP-1 mutants. **a.** Gelatin zymography, **b.** The ratio of MMP9/MMP2 was determined by assessing the densities of MMP2 and MMP9 bands observed in **a**.

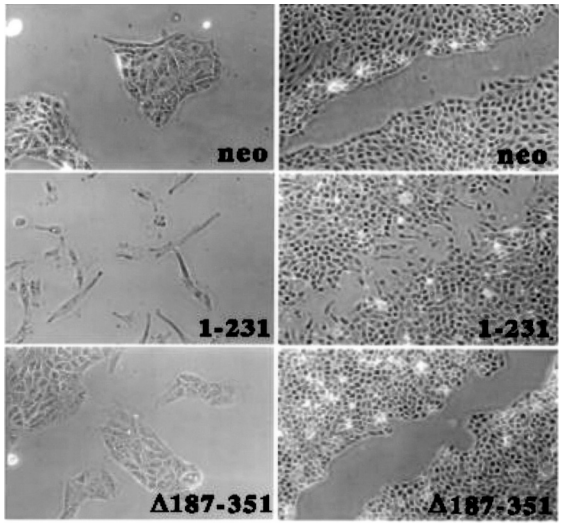

Fig. 4, Cell motility of LMP mutants was studied by wound-scrape assay. The figures on the left represent morphological status of LMP-1 mutant-transfected MDCK cells. The figures on the right show cell migration of LMP-1 mutant transfectants. The numbers in each figure represent the LMP-1 amino acid residue from the COOH terminus. Results are from 5 hr after wounding.

Expression of ets-1 and c-Met in NPC

In vitro analyses suggested that ets-1 plays an important role in LMP-1-mediated up-regulation of cell motility. Meanwhile, there has been increasing evidence that expression of c-Met, an HGF receptor, is highly associated with motility (Nakajima et al., 1999; Cortesina et al., 2000; Comoglio and Boccacio, 2001). Immunohistochemical analysis of expression of LMP-1, ets-1 and c-Met revealed close associations between them in patients with NPC. Moreover, both ets-1 and c-Met expression were shown to be significantly correlated with cervical lymph node metastasis status. Finally, ets-1-mediated c-Met induction by LMP-1 in MDCK cells was also demonstrated (Horikawa et al., 2001). c-Met is localized at the cell membrane, and binding of HGF with c-Met induces autophosphorylation of the intracellular portion of c-Met, which triggers activation of various cellular signal transduction factors

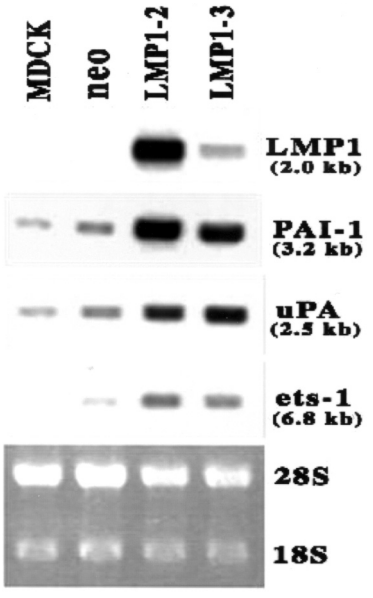

Fig. 5. Northern blotting analysis of gene expression induced by LMP-1 transfection in MDCK cells. Neo, MDCK cells with control plasmid; LMP 1-2 and LMP 1-3, two clones of LMP-1-transformed MDCK cells.

such as Rho, and as a result, enhances cell motility (Bardelli et al., 1997; Comoglio and Boccacio, 2001). Although enhancement of motility by LMP-1 is HGF-independent, induction of c-Met by LMP-1 may have additional roles in up-regulation of cell motility in the presence of HGF.

Role of LMP-1 in angiogenesis

Angiogenesis is an essential phenomenon to obtain nutrients for tumor cells. Increased numbers of lymph and microvessels increases the opportunity for tumor cells to enter the circulatory system, which will result in an increase in metastasis. Association of metastatic properties with microvessel density as well as expression of angiogenic factors such as VEGF, bFGF, TGF-α and IL-8 have been reported (Liotta et al., 1991). In NPC, expression of VEGF is correlated with invasive and metastatic features (Wakisaka et al., 1999). The main trigger of VEGF induction is thought to be hypoxia. However, Murono et al. (2001) reported that transfection of LMP-1-expression vector induced cyclooxygenase-2 (COX-2) in several epithelial cell lines, which resulted in VEGF production. In this system, either CTAR-1 or CTAR-2 induced COX-2 but to a lesser extent than wild-type LMP-1, and activation of NF-κB by CTAR-1 and CTAR-2 played a crucial role in induction of COX-2 (Murono et al., 2001). Activation of the epidermal growth factor receptor (EGFR) signal pathway induced COX-2 (Coffey et al., 1997). As LMP-1 constitutively activated EGFR, LMP-1-induced EGFR overexpression could also have some role in COX-2 induction (Miller et al., 1995).

Another angiogenic factor, IL-8, was also induced by LMP-1 via activation of p38 mitogen-activated protein kinase and NF-κB (Eliopoulos et al., 1999; Yoshizaki et al., 2001). Expression of IL-8 was significantly correlated with LMP-1 expression in NPC. Thus, LMP-1 was suggested to promote neovascularization, which is mainly attributable to activation of NFκB.

Transcription of IL-8 promoter was mainly activated by CTAR-2 (Fig. 6). Thus, the CTAR-1 and CTAR-2 contribute to promotion of each step of the metastatic cascade in a different manner (Fig. 7).

Role of LMP-1 in the progression of NPC

Generally, the acquirement of metastatic ability is thought to be a rather late event in the progression of

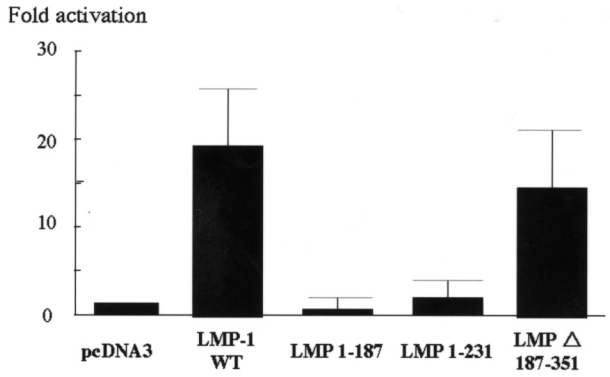

Fig. 6. Activation of IL-8 promoter by LMP-1 mutants. The IL-8 promoter sequence (-130 to +39) was subcloned into the pGl3-Basic vector to generate a construct containing the firefly luciferase gene as a reporter.

	MMP9	Cell motility	IL-8
CTAR-1	↑	↑	→
CTAR-2	↑	→	↑

Fig. 7. Roles of CTAR-1 and CTAR-2 in promotion of invasion and metastasis.

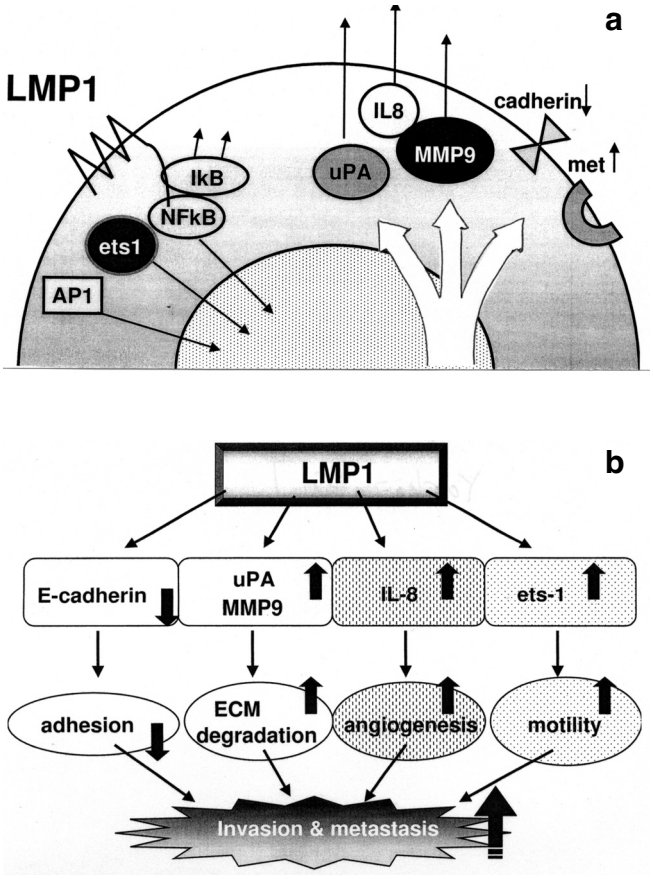

Fig. 8. a. Signal transduction and gene expression in LMP-1-expressing cell. **b.** Alteration of gene expression by LMP-1 and effects of such alterations on the metastasis cascade.

malignant tumors. Although LMP-1 is detected in approximately 70% of NPC tissues, all preinvasive NPC tumor cells already expressed LMP-1 (Pathmanathan et al., 1995). Therefore, LMP-1 might contribute not only to transformation of pharyngeal epithelial cells but also to frequent development of metastasis even in the early period of NPC. Hsu et al. reported that the occurrence of distant metastasis was more frequent in LMP-1-positive NPC than LMP-1-negative patients. Nevertheless, LMP-1-positive NPC patients showed better survival than negative patients. This could be partly explained by the status of LMP-1 as a target of cytotoxic T lymphocytes and would be eliminated in hosts with a normal immune system. However, LMP-1 induces bcl-2 in B lymphocytes and A-20 in epithelial cells, which protect EBV-infected cells from apoptosis (Henderson et al., 1991; Laherty et al., 1992). Moreover, LMP-1 induces IL-10, which helps EBV-infected cells evade immune surveillance (Nakagomi et al., 1994). Thus, EBV also defends against immune attack. It is likely that the interaction of the host immune system with EBV-infected cells is a key point in the development of NPC. In patients who develop NPC, LMP-1 seems to play dual roles, i.e. promotion of metastasis and transformation of epithelial cells.

Some MMP inhibitors and anti-angiogenic drugs are currently undergoing clinical studies (Nemunaitiset al., 1998; Zucker et al., 2000). In addition, aspirin suppresses the induction of MMP-9 by LMP-1 (Murono et al., 2000). LMP-1 is thought to promote metastasis by influencing each step of the metastatic cascade (Fig. 8). Inhibition of any step in the sequential metastatic cascade markedly reduces metastatic ability of the tumor. Thus, prophylactic administration of MMP inhibitors and/or anti-angiogenic drugs for LMP-1-positive NPC patients may improve prognosis of NPC.

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