Induction of receptor for advanced glycation end products by EBV latent membrane protein 1 and its correlation with angiogenesis and cervical lymph node metastasis in nasopharyngeal carcinoma.

Title

Induction of Receptor for Advanced Glycation End Products by Epstein-Barr virus Latent Membrane Protein 1, and its Correlation with Angiogenesis and Cervical Lymph Node Metastasis in Nasopharyngeal Carcinoma

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Running Title

Induction of RAGE by EBV LMP1

Key Words

Epstein-Barr virus, latent membrane protein 1, receptor for advanced glycation end products, lymph node metastasis, angiogenesis, invasion.

Footnotes

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Abbreviations used are: AGE, advanced glycation end product; EBV, Epstein-Barr virus; LMP1, latent membrane protein 1; MMP, matrix metalloproteinase; NF-κB, nuclear factor-κB; NPC, nasopharyngeal carcinoma; RAGE, receptor for advanced glycation end products; VEGF, vascular endothelial growth factor.

ABSTRACT

Purpose: The EBV oncoprotein, latent membrane protein 1 (LMP1), contributes to the metastasis of nasopharyngeal carcinoma (NPC) by inducing factors to promote tumor invasion and angiogenesis. The receptor for advanced glycation endproducts (RAGE) is associated with abnormal angiogenesis in diabetic microangiopathies. Moreover, some papers have suggested the association of RAGE overexpression with tumor metastasis; thus, the associations of RAGE with LMP1 and angiogenesis in NPC were examined.

Experimental Design: Forty-two patients with NPC were evaluated for expressions of LMP1, RAGE, and S100 proteins, and for microvessel counts by immunohistochemistry. Then, the RAGE-induction by LMP1 was examined with Western blotting and luciferase reporter assay.

Results: The microvessel counts were significantly higher in patients with high LMP1 expression or high RAGE expression compared to cases with low expressions ($P = 0.0049$ and $P < 0.0001$), respectively. Patients with advanced N classification were also significantly increased in these groups ($P = 0.0484$ and $P = 0.0005$). The expressions of LMP1 and RAGE proteins were clearly correlated in NPC tissues ($P =$

0.0093). Transient transfection with LMP1 expression plasmid induced RAGE protein in Ad-AH cells. The expression of LMP1 transactivated the RAGE promoter as demonstrated by luciferase reporter assay. Mutation of the reporter at nuclear factor-κB (NF-κB) binding site (-671 to -663) abolished transactivation of the RAGE promoter by LMP1.

Conclusion: These results suggest that LMP1-induced RAGE enhances lymph node metastasis through the induction of angiogenesis in NPC. NF-κB binding site (-671 to -663) is essential for transactivation of the RAGE promoter by LMP1.

INTRODUCTION

Nasopharyngeal carcinoma (NPC) has invasive and metastatic features, and approximately 90 % of patients show cervical lymph node metastasis at the time of initial diagnosis (1). As the biological behavior of NPC depends on its nodal status, patients with advanced nodal disease have a higher tendency toward a poor outcome. Another distinctive character of NPC is the consistent association with Epstein-Barr virus (EBV) (2). EBV-encoded latent membrane protein 1 (LMP1) is capable of transforming rodent fibroblasts into tumorigenic cells *in vitro*. (3). Two domains in the cytoplasmic carboxyl tail of LMP1, C-terminal-activating regions (CTAR) 1 and CTAR2, are essential for the activation of multiple cellular signaling pathways, including nuclear factor (NF)-κB, p38/mitogen-activated protein kinase (MAPK), signal transducer activators of transcription (STAT), AP-1 and phosphatidylinositol 3 kinase (PI3-K) (4). Previously, we reported that LMP1-positive NPCs are more progressive with an increased tendency toward lymph node metastasis than LMP1-negative cases (5, 6). LMP1 is able to directly induce matrix metalloproteinase 9 (MMP9), which plays a critical role in invasion of the basement membrane (7). Recently, we reported that induction of Twist by LMP1 is directly associated with the metastatic nature of NPC (8).

LMP1 also promotes cell migration and invasive growth *via* Ets-1 expression (9). In addition, LMP1 induces and causes the release of fibroblast growth factor 2 (FGF2) into extracellular fluid (10). LMP1 also induces vascular endothelial growth factor (VEGF) through induction of cyclooxygenase 2 (COX2) and hypoxia-inducible factor 1α (HIF-1α) (11, 12). Thus, LMP1 induces a comprehensive set of cellular factors that mediate steps in the process of invasion and metastasis of tumor cells as well as angiogenesis.

The receptor for advanced glycation end products (RAGE) is a multi-ligand receptor belonging to the immunoglobulin superfamily (13). It is implicated in homeostasis, the development of embryonic neurons, inflammation, and tissue damage in diabetes mellitus and Alzheimer's disease. In addition, RAGE is associated with abnormal neovascularization in diabetic retinopathy (14). Furthermore, it has been shown that overexpression of RAGE promotes cell survival through increased expression of the anti-apoptotic protein bcl-2 (15). Recently, RAGE has been regarded as a tumor invasion- and metastasis-related protein (16). Several reports on the relationship between RAGE expression and cancer have been published to date (17-21). RAGE expression has been reported to be significantly associated with lymph node

metastasis in gastric, colorectal, and prostate cancer (18, 20, 21). Major ligands of RAGE are S100/calgulanin, amphoterin, advanced glycation end products (AGEs), and amyloid-β peptide (22). Among them, S100 molecules are small (9-12 kDa) calcium-binding proteins that display 30% to 50% homology within the family. S100 proteins have recently become of major interest owing to their differential expression in a variety of tumors (23).

Here, we present a novel view on the mechanism of the high metastatic features of NPC: EBV-LMP1 contributes to the metastasis of NPC by induction of RAGE. To confirm our view on metastasis, we investigated the expressions of LMP1, RAGE, and S100 proteins by immunohistochemical analysis in NPC tissues to assess the correlations of these expressions with clinicopathologic parameters. Furthermore, we examined the induction of RAGE by LMP1 in nasopharyngeal epithelial cells *in vitro*. Finally, we confirmed the importance of NF-κB in transcriptional activation of RAGE promoter by LMP1.

MATERIALS AND METHODS

Tissue Samples. Forty-two specimens were obtained from patients with NPC who underwent biopsy at Kanazawa University Hospital or Toyama Prefectural Central Hospital from 1996 to 2006. All specimens were fixed in 10% neutral formalin and embedded in paraffin. After a review of all hematoxylin-eosin-stained slides of the specimens, they were classified histopathologically on the basis of the World Health Organization (WHO) system (24). Tumors were subdivided into three histopathological groups: WHO Type I, keratinizing squamous cell carcinoma; WHO Type II, differentiated non-keratinizing carcinoma; and WHO Type III, undifferentiated carcinoma. The clinical staging of NPC was evaluated based on the TNM classification of the Union Internationale Contre le Cancer (UICC) (25).

Immunohistochemical Analysis. Consecutive 4 μm sections were cut from each block. Immunohistochemical staining was performed as previously described (5, 6, 26). The following antibodies were used as primary antibodies: rabbit RAGE polyclonal antibody from Santa Cruz Biotechnology (Santa Cruz, CA); mouse LMP1 monoclonal antibody, rabbit S100 polyclonal antibody, and rabbit von Willebrand factor (vWF) antibody were from DAKO (Glostrup, Denmark). The sections were color-developed

with substrate/chromogen diaminobenzidine (DAKO, Copenhagen, Denmark). The sections were counterstained with methyl green. NPC specimens that had been used in previous studies, lingual carcinoma specimens, and malignant melanoma specimens were used as positive controls for LMP1 (6, 26), RAGE, and S100 proteins, respectively. The specificity of staining was confirmed using non-immune serum instead of the primary antibody as a negative control.

Evaluation of Immunohistochemical Staining for RAGE, LMP1, and S100 proteins. The stained sections were independently examined by two of the authors (A.T. and N.W.). In each case, two arbitrary separate microscopic fields (x 200) containing > 200 tumor cells were evaluated. 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.

The results for each protein were classified into three scores depending on the percentage of immunoreactive tumor cells: for RAGE protein, expression score 0, < 20%; expression score 1, 20 to 50%; expression score 2, > 50%; for LMP1 protein, expression score 0, < 10%; expression score 1, 10 to 50%; expression score 2, > 50%; and for S100 protein, expression score 0, < 30%; expression score 1, 30 to 60%; expression score 2, > 60%.

Also, the results for each protein were classified into two subgroups depending on the percentage of immunoreactive tumor cells: for RAGE protein, < 20% (score 0) and ≥20% (score 1,2) (18); for LMP1 protein, < 10% (score 0) and ≥10% (score 1,2) (27); and for S100 protein, < 30% (score 0) and ≥ 30% (score 1,2) (28); respectively.

Microvessel Counting. All microvessels were highlighted by staining endothelial cells with anti-vWF antibody. The area of most intense neovascularization was found by light microscopy in x 10 field followed by counting in x 400 field. Any endothelial cell or endothelial cluster staining positive for vWF that was clearly separate from adjacent microvessels was considered to be a single, countable vessel. Results were expressed as the highest number of microvessels identified within any single x 400 fields.

Plasmids, Cell Line, and Transient Transfection. A pcDNA3-based LMP1 expression plasmid (pcLMP1) has been described previously (7). RAGE promoter luciferase chimeras (pGL-1, -5, -6, -7, and -5 NF-κB2m) were a kind gift from Dr. Hiroshi Yamamoto (Department of Biochemistry, Kanazawa University School of Medicine, Ishikawa, Japan) (29). Ad-AH cells, kindly provided by Dr. Erik K. Flemington (Tulane University, LA), are an EBV-negative human nasopharyngeal epithelial cell line (30). Ad-AH cells were maintained in Dulbecco's modified Eagle's

medium (DMEM) with 10% FBS and penicillin and streptomycin. For Western blot analysis, cells were transfected with 1 μg of the appropriate plasmids using the Effectene transfection kit (QIAGEN, Tokyo, Japan) in accordance with the manufacturer's instructions. For luciferase reporter assay, cells were transfected with 0.5 μg of the appropriate plasmids with or without 0.001 μg of pcLMP1. Forty-eight hours after transfection, Ad-AH cells were held for 24 h in DMEM with neither FBS nor antibiotics and with or without treatment with 50 ng/ml phorbol 12-myristate 13-acetate (PMA) (Sigma, MO).

Western Blot Analysis for RAGE, LMP1, and S100 proteins. Whole cell lysates were extracted in 100 μl of RIPA buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 2 mM EDTA, 1% Nonidet P-40, 0.5% Na deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 25 mM dithiothreitol, 0.2 mM Na orthovanadate, 100 mM NaF, 1 mM phenylmethylsulfonyl fluoride], and protein concentration was determined by a Bio-Rad protein assay. Each cell lysate (100 μg) was solubilized for 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and transferred to nitrocellulose membranes (Amersham, Buckinghamshire, UK) by ATTO semidry transfer apparatus. The membranes were incubated with an antibody to RAGE, LMP1, or S100 protein at 4□

overnight. Primary antibodies for each protein were the same as for immunohistochemistry. After incubation with horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, CA) for 60 min, reactive bands were visualized using the Super Signal West Pico Chemiluminescent Substrate (PIERCE, IL). To verify equal protein loading, the α-tubulin level in each lane was examined with the antibody to α-tubulin (Santa Cruz Biotechnology, CA).

Luciferase Reporter Assay. Luciferase reporter assays were performed with extracts of Ad-AH cells after transient transfection. Cells were incubated for 48 h after transfection and then harvested. Transfection efficiency was monitored by cotransfection with β-galactosidase reporter plasmid. Luciferase activities were measured using a Dual-Luciferase^{TM} reporter assay system (Promega, Tokyo, Japan) according to the manufacturer's protocol with a luminometer (Labsystems, Helsinki, Finland).

Statistical Analysis. Data were analyzed using SPSS for Windows software version 11.0.1. The microvessel counts and the expressions of RAGE, LMP1, and S100 proteins in relation to the clinicopathological data were analyzed using the Mann-Whitney U test or Kruskal-Wallis rank test. Correlations among RAGE, LMP1,

and S100 proteins were analyzed with the chi-square test. The relationship between microvessel counts and each protein expression was analyzed with the Mann-Whitney U test. *P* values of < 0.05 were considered significant.

RESULTS

Expressions of RAGE, LMP1, and S100 proteins in NPC tissues. RAGE protein was immunolocalized on the cell membrane and cytoplasm of tumor cells (Fig. 1A), consistent with other tumors (18, 20, 21). LMP1 protein was also detected on the membrane and cytoplasm of tumor cells (Fig. 1B). The expression of RAGE and LMP1 was usually high or low in parallel in the same case. Case I (Fig. 1A, B) shows a representative result in which both RAGE and LMP1 are highly expressed (score 2) in tumor cells. Surrounding normal cells were negative for both LMP1 and RAGE. In contrast, case II (Fig. 1C, D) is a representative result when RAGE and LMP1 expressions were low (score 0). It was previously reported that 60 % of the primary site and 100 % of metastatic NPC showed S100 protein-positive cells (28), which suggests the constitutive existence of S100 protein as a ligand for RAGE in NPC. Thus, we performed immunohistochemical study for S100 protein in NPC tissues. S100 protein was observed mainly in the cytoplasm, and also occasionally in the nucleus of tumor cells with diffuse distribution in tumor cell nests (Fig. 1E). Most microvessels highlighted by staining vWF were localized at the tumor stroma. In WHO Type II,

microvessels were localized at the tumor stroma encircling each tumor cell nest. In WHO Type III, microvessels were detected as scattered in tumor cell nests (Fig. 1F).

Microvessel counts and the expression of each protein in relation to the progression of N factor were analyzed using the Mann-Whitney U test (Table 1). The N classification was subdivided into two categories (N0 and N1-3), according to the methods used in our previous studies (5). Detailed results showing the expressions of each protein and microvessel counts in relation to the clinicopathological data are summarized in supplementary Table 1' online. In 33 cases of the lymph node metastasis-positive category (N1-3), the mean RAGE expression score was significantly higher than in nine cases of the lymph node metastasis-negative category (N0) (P = 0.0005). In addition, in cases of positive lymph node metastasis (N1-3), the mean LMP1expression score was significantly higher than in the lymph node metastasis-negative category (N0) (P = 0.0484). There was no statistical difference in the mean expression score of S100 protein between the lymph node metastasis-positive category and the lymph node metastasis-negative category. These results suggest that both RAGE and LMP1 proteins are closely associated with lymph node metastasis in NPC, but S100 protein is not. In addition to each protein expression, we analyzed the

relationship between the results of microvessel counting and the progression of N factor. In cases of positive lymph node metastasis, microvessel counts were significantly higher than in the lymph node metastasis-negative category, compatible with our previous reports (6, 26).

Associations between LMP1, RAGE, and S100 protein expressions are summarized in Table 2. The expression of RAGE protein is significantly associated with the expression of LMP1 protein ($P = 0.0093$); however, there is no significant association between LMP1 and S100 proteins, or RAGE and S100 proteins.

The relationships between each protein expression and microvessel count are summarized in Table 3. As shown in our previous report, cases of LMP1-immunoreactivetumor cells ≥ 10% showed a significantly higher number of microvessel counts compared to cases of < 10% ($P < 0.0001$), which reconfirmed the importance of LMP1 in tumor neovascularization in NPC (6). Cases of RAGE-immunoreactive tumor cells ≥ 20% had significantly higher microvessel counts than cases with < 20% ($P = 0.0020$), which suggests the important role of RAGE protein in angiogenesis in NPC.

Induction of RAGE by LMP1. The data obtained from immunohistochemical studies showed that all cases of high LMP1-immunoreactivity (≥ 10%) were also high in RAGE immunoreactivity (≥ 20%), in contrast to the 13 high RAGE immunoreactivity of 31 cases with low LMP1 immunoreactivity (< 10%) (Table 2), which suggests the expression of RAGE protein is significantly associated with the expression of LMP1 protein ($P = 0.0093$); therefore, we investigated whether LMP1 protein could induce RAGE protein production *in vitro*. Ad-AH cells, an EBV-negative nasopharyngeal epithelial cell line, were transiently transfected with pcLMP1. RAGE protein was detected by Western blotting of cell extracts. As demonstrated in Figure 2, trace amounts of RAGE protein were detected in cells transfected with pcDNA3, a control plasmid; however, LMP1 induced the expression of RAGE protein, depending on the amount of transfected LMP1 expression plasmid. RAGE protein was also upregulated in cells incubated with PMA, which was used as a positive control. On the other hand, trace amounts of S100 protein were not affected either by transfection of LMP1 or incubation with PMA. To verify the equal protein loading, the α-tubulin level in each lane was examined. As expected from immunohistochemical study, results from Western blotting indicate that LMP1 induces the expression of RAGE protein, but not S100 protein, in Ad-AH cells.

Transactivation of RAGE Promoter by LMP1. To confirm whether LMP1 induces RAGE gene transcription and, if so, to delimit the regions involved in such transcriptional activations, a series of chimeric 5'-deletion promoter-luciferase reporter constructs were prepared (Fig. 3A). Ad-AH cells were transiently transfected with the constructs, and the effects of co-transfection of pcLMP1 on luciferase activity were determined. When pGL-1 or pGL-5-transfected cells were co-transfected with pcLMP1, promoter activities increased significantly (more than 5-fold) compared with those without pcLMP1. These results suggest that Sp-1 (-1628), AP-1 (-1542), NF-κB (-1542), Ets-1 (-1183) binding sites are not essential for the transcriptional activation of RAGE promoter; however, when cells were cotransfected with pGL-6 or pGL-7, luciferase activities were abolished (Fig. 3B). These results suggest that the element including the NF-κB binding site (-671) in the RAGE promoter is essential for the response to stimulation by LMP1. To determine the role of the NF-κB binding site (−671) in LMP1 activation of the RAGE promoter, site-directed mutagenesis was performed at that site (pGL-5 NF-κB2m). When luciferase activities were assayed in cells transfected with the mutant, the inducibility by pcLMP1 was totally abolished (Fig. 3C). These results suggest that LMP1 induces RAGE promoter in an NF-κB-binding site (-671)-dependent manner.

DISCUSSION

In addition to its central role in transformation of cells by EBV, LMP1 is able to directly induce a comprehensive set of factors that promote tumor invasion and angiogenesis, which results in the increased metastatic potential of EBV-related malignancies including NPC (31). In the present study, the expression of LMP1 was significantly associated with microvessel counts and the progression of N factor in NPC, concordant with our previous report (6). In addition, we showed that the expression of LMP1 was correlated with the expression of RAGE protein. Although the number of NPC samples examined in this study was not sufficient to allow definite conclusions, this result prompted us to investigate whether LMP1 induces RAGE protein in nasopharyngeal epithelial cells. First, transient transfection revealed that LMP1 induced RAGE protein in Ad-AH cells. A luciferase reporter assay using various chimeric luciferase plasmids then revealed that the NF-κB-binding site (-671) in the RAGE promoter was essential to transactivate the RAGE promoter by LMP1. We didn't confirm that LMP1-induced NF-κB could bind to the putative NF-κB site in the RAGE promoter. However, these results of luciferase reporter assay indicate that LMP1 directly induces the expression of RAGE through transcriptional activation of the

RAGE promoter. Sp-1 (-1628), AP-1 (-1542), NF-κB (-1519), and Ets-1 (-1183) binding sites in pGL-1 luciferase reporter plasmid have only a limited effect on the transactivation of RAGE promoter, because truncation of these elements only partially affects the luciferase reporter activity induced by LMP1. As previously reported, LMP1 transactivates MMP9 production through the activation of NF-κB and AP-1 signaling pathways (7). We have also reported that LMP1 promotes cell migration and invasive growth *via* Ets-1 expression in human epithelial cells (9). These results suggest that the essential binding sites for transactivation of the promoter by LMP1 are, at least in part, gene-specific in epithelial cells.

A close relationship between angiogenesis and lymph node metastasis has been demonstrated in malignant tumors (32, 33). As we previously reported, angiogenesis is strongly correlated with lymph node metastasis also in NPC (6, 26). Although the angiogenic role of RAGE remains somewhat controversial, several studies report that activated RAGE can promote aspects of angiogenic processes *in vitro*, including stimulation of endothelial cell proliferation and tube formation (34). To examine the role of RAGE protein in angiogenesis in NPC, we analyzed the correlation between the level of RAGE protein expression and microvessel counts in NPC tissues. As expected, the expression of RAGE was clearly correlated with the microvessel counts and

significantly associated with the progression of N factor. Furthermore, microvessel counts were correlated with the progression of N factor. These results suggest that LMP1-induced RAGE stimulates lymph node metastasis through the induction of angiogenesis in NPC, although the mechanism of RAGE-associated angiogenesis is unknown. VEGF plays an important role in lymph node metastasis through the induction of angiogenesis in NPC (6, 26). LMP1 directly induces VEGF transcriptionally through the activation of HIF-1 (12). LMP1 is involved in VEGF production indirectly through the induction of COX2 (11). It has been reported that RAGE can elicit angiogenesis through the induction of VEGF, thereby playing an active part in the development and progression of diabetic microangiopathies (14, 34), in which activated RAGE induces VEGF expression transcriptionally through the activation of NF-κB, AP-1, and HIF-1 (35). Although we did not examine the expression of VEGF in this study, these reports support the hypothesis that LMP1-induced RAGE induces angiogenesis, at least in part, through the induction of VEGF in NPC. Thus, LMP1-induced RAGE can be a novel key regulator to stimulate VEGF expression in NPC.

The importance of lymphangiogenesis induced by tumor-secreted cytokines has been demonstrated recently (36). VEGF-C and VEGF-D expression in tumor cells has

been linked to lymphangiogenesis associated with tumor invasion into lymphatic vessels, and lymph node metastasis. Sasahira *et al.* examined the role of RAGE for lymphangiogenesis in human oral squamous cell carcinoma (SCC) (19). They showed that RAGE protein concentration in tumor tissues was not correlated with VEGF-C concentration, lymph vessel counts or nodal metastasis in oral SCC. Although the induction of VEGF-C by LMP1-induced RAGE may be controversial in NPC as well as in oral SCC, the direct induction of VEGF-C by LMP1 should be examined, because VEGF-C induction is a result of NF-κB or p38-MAPK, both of which are involved in the downstream signaling pathway of LMP1 (37).

Recently, Taguchi et al. reported that RAGE was closely associated with cell growth and invasion through MAPK activation, and matrix metalloproteinase (MMP) 2/9 induction in glioma cells (16). In pancreatic cancer cells, RAGE and MMP9 are expressed concordant with the metastatic ability of human pancreatic cancer cells both at protein and mRNA levels (38). Kuniyasu et al. reported that RAGE antisense *S*-oligodeoxynucleotide treatment suppressed the invasive activity of RAGE-positive gastric carcinoma cells by inhibiting the ability to degrade type IV collagen, as estimated by *in vitro* invasion assay (18). These reports suggest that RAGE induces the invasiveness of tumor cells, especially through the upregulation of type IV collagenase

activity. Although the importance of RAGE-induced MMP9 in the invasiveness of cancer cells is well established, MMP9-derived invasiveness indirectly stimulated by LMP1-induced RAGE should be limited or only partial, because LMP1 is a strong direct transcriptional inducer of MMP9 (7).

It has already been reported that S100 protein is constitutively expressed and detected in NPC tumor cells (28). Thus, we tested only the expression of S100 protein as a ligand of RAGE in this study. In the future, the present study should be expanded to examine the role of enhanced interaction of LMP1-induced RAGE with its other ligands, AGE or EN-RAGE, members of the S100/calgranulin family, which also affects RAGE activation.

Finally, we reported here that EBV-LMP1 induces the expression of RAGE protein, which is suggested to play a key role in angiogenesis in NPC, in addition to factors such as COX2 and HIF-1. This report shows additional evidence that inhibition of LMP1 signaling can be an effective therapy to suppress the metastatic potential of NPC.

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FIGURE LEGENDS

Fig. 1. Immunohistochemical detection of RAGE (A and C), LMP1 (B and D), S100 (E), and von Willebrand factor (F) in NPC biopsy tissues. Case I (A and B) shows representative results with high RAGE and LMP1 expression (score 2) in consecutive sections. Case II (C and D) shows the representative result with low expressions (score 0) of RAGE and LMP1. Original magnification, x 400.

Fig. 2. LMP1 induces the expression of RAGE in Ad-AH cells. Lysates of Ad-AH cells transfected with pcDNA3 with or without pcLMP1 are loaded on to corresponding lanes, and examined by Western blotting with antibodies to RAGE, S100, LMP1, or α-tubulin proteins, as described in Materials and Methods. Two different amounts of LMP1 expression plasmid were transiently transfected. Ad-AH cells treated with PMA (50 ng/ml) were used as a positive control for RAGE induction. α-tubulin was used as a loading control. PMA, phorbol 12-myristate 13-acetate.

Fig. 3. (A) Schematic constructions of RAGE promoter luciferase chimeras. The RAGE promoter has four Sp-1 binding sites, one Ap-1 binding site, three NF-κB binding sites, and one Ets-1 binding site. pGL-5 NF-κB2m contained the region -751 to +43, but with

the mutation in an NF-κB site (-671). (B) In cells transfected with pGL-1 and pGL-5, promoter activities increased significantly by LMP1. On the other hand, in cells transfected with pGL-6 and pGL-7, promoter activities were the same as controls. The results of triplicate experiments are shown. (C) Determination of responsive elements in the promoter of RAGE. pGL-5 or pGL-5 NF-κB2m was transiently cotransfected with pcLMP1 into Ad-AH cells. pcDNA3 was used as a control vector. Luciferase activities transfected wih the mutant are significantly lower than those with pGL-5. The results of triplicate experiments are shown.