Aspirin Inhibits Tumor Cell Invasiveness Induced by Epstein-Barr Virus Latent Membrane Protein 1 through Suppression of Matrix Metalloproteinase-9 Expression

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ABSTRACT

Matrix metalloproteinases (MMPs) are thought to play crucial roles in tumor invasion and metastasis. Because we have shown that EBV latent membrane protein 1 (LMP1) enhances MMP-9 expression by activation of nuclear factor (NF)-κB and activator protein (AP)-1 (T. Yoshizaki, et al., Proc. Natl. Acad. Sci. USA, 95: 3621–3626, 1998), we therefore tested whether up-regulation of MMP-9 by LMP1 could be correlated with enhanced invasiveness of tumor cells in vitro. Whether aspirin and sodium salicylate could reduce invasiveness and whether LMP1 could enhance MMP-9 expression in tumors grown in nude mice were also tested. C33A cells stably expressing LMP1 had increased expression of MMP-9 and showed greater invasion through reconstituted basement membrane compared with vector-transfected C33A cells (P < 0.02). Treatment with aspirin or sodium salicylate inhibited invasiveness of the LMP1-expressing C33A cells (P < 0.03) and suppressed both the LMP1-induced MMP-9 expression in zymographic analyses and LMP1-induced MMP-9 promoter activity in CAT reporter assays (P < 0.01). Endogenous MMP-2 levels were unaffected by either drug. Both drugs repressed the CAT activity of the truncated MMP-9 promoter construct, which only contained a binding site for AP-1, to the basal level (P < 0.05). Moreover, EMSA indicated that the effects of the salicylates were through the inhibition of not only NF-κB but also AP-1 binding activity. Inhibitory effect of salicylates could be reversed by p50/p65 subunits of NF-κB or c-Jun overexpression. The inhibitory effect of aspirin on NF-κB activity was attributable to the inhibition of IκB kinase activity. Finally, tumors derived from C33A cells stably expressing LMP1 grown in nude mice showed enhanced MMP-9 levels compared with tumors derived from vector-transfected C33A cells. This enhancement was inhibited by treatment of the mice with aspirin. These results suggest that aspirin may be able to suppress invasion and metastasis of EBV-associated tumors that express LMP1 by suppression of MMP-9.

INTRODUCTION

MMPs play a crucial role in degradation of extracellular matrix associated not only with normal tissue remodeling but also with pathological conditions such as tumor invasion and metastasis. Invasion into surrounding tissues and metastasis to distant organs are characteristics of many malignant tumors. Degradation of the basement membrane is the critical step in the process of both invasion and metastasis. Among human MMPs reported previously, MMP-2 (gelatinase A/M, 72,000 type IV collagenase) and MMP-9 (gelatinase B/M, 92,000 type IV collagenase) are thought to be key enzymes for degrading type IV collagen, which is a major component of the basement membrane. Contributions of both enzymes to invasion and metastasis have been documented in numerous reports (1, 2). EBV is a ubiquitous human herpesvirus and the causative agent of infectious mononucleosis. EBV is also associated with several malignancies such as Burkitt’s lymphoma, Hodgkin’s disease (3), non-Hodgkin’s lymphoma in immunocompromised hosts (4), nasal T/natural killer cell lymphoma (5), and NPC (6, 7). In all of these tumors, EBV infection is predominantly latent. EBV latent infection is divided into three types by the pattern of expression of six EBNA (EBNA1, EBNA2, EBNA3A, EBNA3B, EBNA3C, and EBNA-LP) and three LMPs (LMP1, LMP2A, and LMP2B). Only EBNA1 is expressed in Burkitt’s lymphoma (type I latency). On the other hand, all six EBNA and three LMPs are expressed in non-Hodgkin’s lymphoma in immunocompromised hosts (type III latency). LMP1, LMP2A, and LMP2B are expressed in NPC, nasal T/natural killer cell lymphoma and Hodgkin’s disease (type II latency) in addition to EBNA1. Thus, expression of LMP1 is common to type II and type III latency. LMP1 is considered an oncoprotein because it can transform rodent fibroblasts (8). In addition to its oncogenic properties, LMP1 was shown recently to enhance expression of MMP-9 (9), which might contribute to the invasion and metastasis of EBV-associated tumors especially in type II and type III latency.

Enhanced expression of MMP-9 by LMP1 is through the activation of NF-κB and AP-1 (9). There are two regions that activate NF-κB in the COOH-terminal region of LMP1 (10), and both contribute to the enhanced expression of MMP-9 (11). NF-κB remains sequestered in the cytoplasm under unstimulated conditions by tightly bound inhibitory proteins, the IκBs (12). Many of the signals known to activate NF-κB result in phosphorylation of IκBα on serine residues 32 and 36 and subsequent degradation of IκBα, allowing NF-κB to translocate into the nucleus and activate target genes (13). Acetylsalicylic acid (aspirin) and sodium salicylate have been shown to suppress the NF-κB activation induced by TNF-α, lipopolysaccharide, and interferon-γ in certain systems (14). A recent report demonstrated that aspirin and sodium salicylate inhibited the activity of IκB kinase β, which is responsible for phosphorylation of IκBα (15). Additionally, aspirin was shown recently to inhibit JNK in several systems (16), which resulted in the inactivation of AP-1. Here we demonstrate that LMP1 increases invasiveness of tumor cells in vitro and that both aspirin and sodium salicylate inhibit this invasiveness by suppression of the induced constitutive MMP-9 expression. In addition, inhibition of NF-κB and AP-1 binding to the MMP-9 promoter is involved in the suppression of MMP-9 by both drugs. Furthermore, LMP1 induces expression of MMP-9 in tumors grown in nude mice, which is suppressed by aspirin. These results indicate that both aspirin and sodium salicylate may contribute to reduce invasion and metastasis in EBV-associated tumors.
MATERIALS AND METHODS

Cell Culture. C33A cells, derived from a human papillomavirus-negative cervical cancer cell line, were obtained from the American Type Culture Collection and cultured in DMEM supplemented with 10% FBS and penicillin and streptomycin.

Plasmids. LMP1 expression plasmid was described previously (9). The IκBα(S32/36AA) expression plasmid, the p50 subunit of NF-κB (referred to as p50) expression plasmid, and the p65 subunit of NF-κB (referred to as p65) expression plasmid were kindly provided by Dr. Albert Baldwin (University of North Carolina, Chapel Hill, NC). In the IκBα(S32/36AA) expression plasmid, both serines at position 32 and 36 were substituted by alanines, resulting in the prevention of degradation of IκBα and subsequent activation of NF-κB.

A series of 5′ flanking sequences of MMP-9 were inserted upstream of the CAT reporter gene as described previously (18). A C-Jun expression plasmid was described previously (19).

Transient and Stable Transfection. C33A cells were transfected with 5 μg of appropriate reporter and/or effector plasmids using LipofectAMINE reagent (Life Technologies, Inc., Grand Island, NY), following the manufacturer’s instructions. To establish either the vector-transfected C33A cells (referred to as C33A/pcDNA3 cells) or the C33A cells stably expressing LMP1 (referred to as C33A/LMP1 cells), 0.5 μg of pcDNA3 (Invitrogen, Carlsbad, CA) or LMP1 expression plasmid, respectively, was transfected into C33A cells and cultivated in the presence of geneticin (700 μg/ml Geneticin; Life Technologies).

Reagents. Aspirin, sodium salicylate, and MTT were purchased from Sigma Chemical Co. (St. Louis, MO).

MTT Assay. MTT assays were performed to evaluate the cytotoxicity of aspirin and sodium salicylate. One hundred μl of each cell type (1 × 10⁴ cells/ml) were plated and incubated for 24 h in a 96-well plate. Then various concentrations of each drug were added to the wells. After another 24 h incubation, 10 μl of sterile MTT dye were added, and the cells were incubated for 6 h at 37°C. Then 100 μl of 0.4% SDS in isopropanol were added and thoroughly mixed. Spectrometric absorbance at 595 nm (for formazan dye) was measured with the absorbance at 655 nm for reference.

Conditioned Media. Eight × 10⁵ cells were plated and cultured for 48 h in DMEM with 10% FBS. Then cells were incubated for 24 h in DMEM with neither FBS nor antibiotics with or without drug. Media were harvested and used for gelatin zymography.

Gelatin Zymography. MMP-2 and MMP-9 enzymatic activity were assayed by gelatinolysis by means of gelatin zymography as reported previously (9, 19). For mouse tissues, equal amounts of each tissue were homogenized in RIPA buffer [25 mM Tris-HCl, 150 mM NaCl, 2 mM EDTA, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 0.2 mM sodium orthovanadate, 100 mM NaF, 10 μg/ml aprotinin, 5 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride]; then extracts were obtained as reported (21), and the concentration of protein was calculated as described previously (20), then mixed with 2× SDS sample buffer containing reducing agent. For tumors grown in nude mice, equal amounts of each tumor were homogenized in RIPA buffer [25 mM Tris-Cl (pH 7.5), 150 mM NaCl, 2 mM EDTA, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 0.2 mM sodium orthovanadate, 100 mM NaF, 10 μg/ml aprotinin, 5 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride]; then extracts were obtained as reported (21), and the concentration of protein was calculated. After SDS/PAGE, immunoblotting was performed as described previously (9). Antimouse immunoglobulin κ chain antibody (1:2000 dilution; Southern Biotechnology Associates, Birmingham, AL) was used for the secondary antibody in analyses of tumors grown in nude mice.

CAT Reporter Assay. CAT assays were performed as described previously (9). Construction of the MMP-9 promoter reporter has been described (Ref. 17; Fig. 1C). Cells were incubated 48 h after transfection with or without adding either drug 24 h after transfection and then harvested. Acetylated [1-1⁴C]chlorophenicol was quantified with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Transfection efficiency was monitored by cotransfection with β-galactosidase reporter construct.

EMSA. Nuclear extracts were prepared from C33A/pcDNA3 cells or C33A/LMP1 cells with or without treatment of either drug as reported previously with some modifications (22). Synthetic oligonucleotides used for probes were identical to the NF-κB or AP-1 binding sequences in the promoter region of MMP-9 (9). Oligonucleotides were annealed with the use of Klenow DNA polymerase and then labeled with [γ-³²P]ATP with the use of T4 polynucleotide kinase. The unlabeled oligonucleotides were used for competition. Five μg of nuclear extracts were incubated with 50,000 cpm labeled probe and analyzed by 1.5% PAGE; then autoradiography was performed as described (9).

IκB Kinase Assay. Assays were performed after transient transfection as described previously (23). Briefly, lysates were obtained from C33A cells transfected with pcDNA3 or with the LMP1 expression construct with or without treatment with 5 mM aspirin; then supernatants were collected, and protein concentration was calculated. Five hundred μg of protein were incubated with 15 μg of IKK2 CT antibody (kindly provided by Drs. Albert Baldwin and Minnie Holmes-McNary [University of North Carolina, Chapel Hill, NC]). Incubation was continued with protein A beads (50 μl). The immunoprecipitates were then vigorously washed and subjected to an in vitro kinase assay. Kinase assays were done with 2 μCi of [γ-³²P]ATP in the presence of 2 μg of GST-IκBα or GST-IκBα(S32/36TT) (kindly provided by Drs. Albert Baldwin and Minnie Holmes-McNary) for substrates, followed by SDS-PAGE analysis and autoradiography.

In Vitro Invasion Assay. Assays were performed with the use of Biocoat Matrigel Invasion Chambers (Becton Dickinson Labware, Bedford, MA) as described elsewhere. Biocoat Cell Culture Inserts were used for uncoated assays. We established two indices to evaluate in vitro invasiveness: percent invasion (% invasion) and invasion index. Both indices were calculated as follows: % invasion = number of cells invading through Matrigel-coated membrane/number of cells migrating through uncoated control insert membrane. Invasion index = % invasion of tested cells/ % invasion of C33A/pcDNA3 cells.

Injection of Cells into Athymic Mice. Either C33A/pcDNA3 cells or C33A/LMP1 cells (2 × 10⁴ cells) in a volume of 0.1 ml PBS were s.c. injected into athymic nude mice. Four weeks after injection, PBS alone or 9 mg/day aspirin were given s.c. at a distance from the tumor for 3 days, and then mice were sacrificed. Equal amounts of each tumor were homogenized in SDS sample buffer without reducing agent or RIPA buffer for gelatin zymography or Western blot analysis, respectively.

Statistical Analysis. Significant differences were determined by the paired t test. P < 0.05 was considered to be statistically significant.

RESULTS

LMP1 Enhances Invasion of C33A Cells in Vitro. A Matrigel Invasion Chamber system was used to assay tumor cell invasiveness as described in “Materials and Methods.” This system was used to evaluate the contribution of LMP1 to invasiveness. C33A/LMP1 cells were about twice as invasive in this assay compared with C33A/pcDNA3 cells (P < 0.02; Fig. 1A), suggesting that LMP1 is a possible invasiveness-inducing factor.

NF-κB and AP-1 Are Involved in Expression of MMP-9 Induced by LMP1. We showed previously that transient transfection of LMP1 into C33A cells enhanced expression of MMP-9 (9). To investigate the contribution of this type IV collagenase to the LMP1-associated invasion in vitro, we first carried out gelatin zymography to determine MMP-9 expression. The zymographic analysis showed that C33A/LMP1 cells constitutively enhanced the expression of MMP-9 compared with C33A/pcDNA3 cells (Fig. 1B). As before (9), MMP-2 gelatinolytic activity was not affected by LMP1 expression. This result suggests that MMP-9 associated with LMP1 expression may be involved in cell invasiveness in vitro. Analysis of the effect of LMP1 expression on MMP-9 promoter constructs (Fig. 1C) was then carried out. MMP-9 promoter activity was increased in C33A/LMP1 cells, as shown by CAT reporter assays with the −670 CAT construct (P < 0.01; Fig. 1D, Lanes 1 and 2). C33A/LMP1 cells also increased activity of the −90 CAT construct, which only contains the AP-1 binding site and TATA box, compared with the −73 CAT construct in which the AP-1 binding site was deleted (P < 0.01; Fig. 1D, Lanes 3–6). These results are compatible with previous reports that LMP1...
activates JNK (24) because AP-1 is one of the downstream pathways of JNK. EMSA showed increases in nuclear factor binding not only to NF-κB but also to AP-1 binding sequence in the MMP-9 promoter in C33A/LMP1 cells (Fig. 1).

Influence of Aspirin on Cell Viability and LMP1 Expression. Aspirin and sodium salicylate are cytotoxic in high concentrations; therefore, we investigated the effect of both drugs on cell viability by MTT assay. The effects of 1, 2.5, 5, and 10 mM aspirin or sodium salicylate were tested. Absorbance was not reduced, nor was there any visible cytotoxic effect by any of the concentrations tested except with 10 mM aspirin (data not shown). Therefore, 5 mM aspirin and 10 mM sodium salicylate were used in the experiments. Neither drug affected the expression of LMP1 (Fig. 2, Lanes 3–5).

Aspirin Inhibits LMP1-induced Tumor Cell Invasiveness in Vitro. The effect of salicylates on cell invasiveness was tested. LMP1-induced invasiveness was clearly inhibited by aspirin \( (P < 0.03) \) and sodium salicylate \( (P < 0.03; \) Fig. 3, Lanes 5 and 6). Neither drug affected invasiveness of the C33A/pcDNA3 cells (Fig. 3, Lanes 2 and 3). These results suggest that both drugs can reduce LMP1-related invasiveness.

Aspirin Suppresses LMP1-induced MMP-9 Expression through Inhibition of NF-κB and AP-1. To clarify whether expression of MMP-9 is involved in inhibition of LMP1-induced in vitro invasion by salicylates, we first evaluated the effect of both drugs on LMP1-induced MMP-9 expression with the use of gelatin zymography. Zymographic analysis demonstrated that aspirin and sodium salicylate clearly abolished the LMP1-induced MMP-9 enhancement (Fig. 4, Lanes 3 and 4). MMP-2 gelatinolytic activity was affected by neither aspirin nor sodium salicylate. Direct exposure of collected conditioned medium of C33A/LMP1 cells to aspirin or sodium salicylate had no effect on MMP-9 and MMP-2 gelatinolytic activity (Fig. 4, Lanes 5 and 6), indicating that inhibition of LMP1-induced MMP-9 was not attributable to a direct effect of the drugs on secreted MMP-9 itself.

Next, the effects of the drugs on promoter activity were investigated. CAT reporter assays also showed that MMP-9 promoter activity was dramatically suppressed by aspirin \( (P < 0.01) \) and sodium salicylate \( (P < 0.01; \) Fig. 5A, Lanes 3 and 4). Cotransfection with IκBa(SS2/36AA) also sharply repressed CAT activity \( (P < 0.01; \) Fig. 5A, Lane 5). The residual activity might be attributable to the...
Overexpression of NF-κB p65/p50 and c-Jun Overcomes the Inhibitory Effect of Aspirin on LMP1-induced MMP-9 Expression. Overexpression of p50 and p65 can overcome NF-κB inhibition by IκB(SS32/36AA), confirming that it is NF-κB that is blocked by aspirin (Ref. 25). Therefore, transient transfection assays were performed to investigate whether the inhibitory effect of aspirin on LMP1-induced MMP-9 expression could be reversed by overexpression of NF-κB or c-Jun. Zymographic analysis showed that overexpression of p50 and p65 restored MMP-9 expression inhibited by aspirin (Fig. 7A, Lane 4). Overexpression of c-Jun also restored MMP-9 expression (Fig. 7A, Lane 5). CAT reporter assays with the −670 CAT construct also showed that overexpression of p50 and p65 restored MMP-9 promoter activity inhibited by aspirin (Fig. 7B, Lane 4). Overexpression of c-Jun also restored MMP-9 promoter activity (Fig. 7B, Lane 5). These results suggest that both NF-κB and c-Jun are involved in the inhibitory effect of aspirin on LMP1-induced MMP-9 expression.

Aspirin Inhibits LMP1-induced IKK Activity. IKK is responsible for phosphorylation of IκB on serine residues 32 and 36, which is a critical step in NF-κB activation. We tested whether LMP1 activates NF-κB through IKK, then tested whether aspirin had an inhibitory effect on IKK activity with the use of the LMP1 (9). Therefore, we investigated the effect of the drugs on AP-1 activity with the use of the −90 CAT construct and −73 CAT constructs. The activity of the −90 CAT construct was repressed to the basal level by both aspirin (P < 0.05) and sodium salicylate (P < 0.05; Fig. 5B, Lanes 4 and 5), suggesting that LMP1-induced AP-1 activity was significantly repressed by both drugs. However, residual activity compared with the −73 CAT construct was still observed. Because C33A/pcDNA3 cells had more activity with the −90 CAT than with the −73 CAT construct (Fig. 5B, Lanes 1 and 6), this residual activity seems to be independent of LMP1. Finally, binding activity of nuclear factors not only to NF-κB but also to the AP-1 binding sequence in the MMP-9 promoter was reduced by aspirin and sodium salicylate (Fig. 6A, Lanes 3, 5, and 7; Fig. 6B, Lanes 4, 6, and 8). Therefore, inhibition of LMP1-induced MMP-9 by aspirin and sodium salicylate is probably through the inactivation of both NF-κB and AP-1.

Fig. 2. Immunoblots for LMP1. Lysates were harvested after mock treatment or treatment with either 5 mM aspirin (Asp) or 10 mM sodium salicylate (NaSal) for 24 h. Fifty μg protein of each sample except for SAV III (20 μg) were loaded on a 10% polyacrylamide gel. SAV III is an EBV-positive Burkitt’s lymphoma line exhibiting type III latency.

Fig. 3. Association of in vitro invasiveness with LMP1 and its inhibition by aspirin and sodium salicylate. In vitro invasion assays were carried out as described in Fig. 1. Asp, treatment with 5 mM aspirin; NaSal, treatment with 10 mM sodium salicylate; pcDNA3, C33A cells stably transfected with pcDNA3; LMP1, C33A cells stably expressing LMP1. Bars, SD.

Fig. 4. MMP-2 and MMP-9 gelatinolytic activities were analyzed with gelatin zymography. Conditioned media were collected from C33A cells stably transfected with pcDNA3 (pcDNA3) and C33A cells stably expressing LMP1 (LMP1) with or without treatment with either 5 mM aspirin (Asp) or 10 mM sodium salicylate (NaSal). In some experiments, collected conditioned media of mock-treated C33A cells stably expressing LMP1 were incubated with either 5 mM aspirin or 10 mM sodium salicylate in vitro (direct) prior to incubation for 30 min at 37°C.

Fig. 5. Stimulation of MMP-9 promoter constructs by LMP1 and repression by aspirin and sodium salicylate. A, stimulation of MMP-9 promoter activity by LMP1 and its repression by aspirin, sodium salicylate, and IκB. The relative CAT activities from four experiments are shown; bars, SD. B, stimulation of deleted MMP-9 promoter activity (−90 CAT) by LMP1 and its repression by aspirin and sodium salicylate. The relative CAT activities from four experiments are shown; bars, SD. Asp, treatment with 5 mM aspirin; NaSal, treatment with 10 mM sodium salicylate; pcDNA3, C33A cells stably transfected with pcDNA3; LMP1, C33A cells stably expressing LMP1.
effect on phosphorylation of Ikβ by IKK. In vitro kinase assays showed phosphorylation of Ikβ in LMP1-transfected cells in contrast to vector-transfected cells (Fig. 8, Lanes 2 and 3), suggesting that IKK is involved in LMP1-induced NF-κB activation. No phosphorylation of Ikβ by LMP1 was detected when GST-Iκβα(SS32/36TT) was used as substrate (Fig. 8, Lane 7), indicating specificity of this assay. Furthermore, treatment with aspirin abolished phosphorylation of Ikβ (Fig. 8, Lane 4). This result suggests that aspirin inhibits LMP1-induced NF-κB activation through inhibition of IKK activity.

Aspirin Inhibits LMP1-induced MMP-9 Expression in Vivo.

Finally, whether LMP1 induces MMP-9 in vivo was investigated. Both C33A/pcDNA3 cells and C33A/LMP1 cells are tumorigenic in nude mice. LMP1 was detectable in only C33A/LMP1-derived tumors (Fig. 9A). Gelatin zymography showed that there was enhanced expression of MMP-9 in C33A/LMP1-derived tumors compared with C33A/pcDNA3-derived tumor (Fig. 9B, Lanes 1 and 2), indicating that LMP1 induces MMP-9 expression in vivo. Next the effect of aspirin on expression of MMP-9 in vivo was investigated. Treatment with aspirin dramatically decreased expression of MMP-9 in C33A/LMP1-derived tumor (Fig. 9B, Lane 3) in contrast to expression of LMP1, which was not affected (Fig. 9A, Lane 3). Expression of MMP-2 was not affected by aspirin. These results suggest that aspirin may be an inhibitor of LMP1-induced MMP-9 expression in vivo.

DISCUSSION

LMP1 expression is not only essential for B-cell immortalization by EBV, but it is the only EBV protein that transforms nonlymphoid cells such as rodent fibroblasts (8), human epithelial cells (26), and human keratinocytes (27). Consequently, the oncogenic potential of LMP1 has been the focus of many investigations. A different aspect of LMP1 function, i.e., its ability to induce MMP-9, which might contribute to invasion and metastasis, is addressed here. LMP1 is expressed in at least 70% of NPCs and in all EBV-infected preinvasive NPC lesions (28). NPC, which exhibits type II latency, shows clinically aggressive metastasis not only into regional lymph nodes but distant organs such as lung and vertebrae, although the primary tumor is still small and beyond carcinoma in situ. Additionally, NPC often shows aggressive intracranial invasion. Although levels of MPPs including MMP-9 in NPC have not been reported yet, a clinical study showed that LMP1-positive NPCs tended to invade more frequently outside the nasopharynx and were more progressive and prone to invade the lymph nodes than LMP1-negative tumors (29).

The COOH-terminal region of LMP1 contains two functional domains named CTAR1 and CTAR2. CTAR1 is needed for binding the TRAF-1, TRAF-2, and TRAF-3 proteins (30, 31), and CTAR2 has been shown to bind the TNFR-associated death domain protein (32), suggesting that LMP1 might act as a TNFR family-like molecule. Indeed, downstream signaling of LMP1 is similar to that of CD40, which is one of the TNFR family members (33). Although LMP1 is not homologous to any of the known TNFR family members and its activity does not appear to depend on the binding of a specific ligand, LMP1 activates NF-κB through a pathway including IKK, as shown here and previously (34). Both CTAR1 and CTAR2 contribute to NF-κB activation. In addition, LMP1 also activates the JNK pathway through CTAR2, which results in the activation of AP-1 (24).

Aspirin is used as a nonsteroidal anti-inflammatory drug. The range of concentrations for the drug is correlated with the amounts in plasma (1–5 mM) for optimal anti-inflammatory effects in patients with rheumatoid arthritis. Moreover, both epidemiological and clinical studies have indicated that aspirin and related compounds have considerable potential as chemopreventive agents for cancers. It has been reported that patients with regular aspirin use had a reduced incidence of colon, lung, and breast cancers (35). Aspirin suppresses NF-κB activity or JNK activity induced by certain stimuli such as tumor necrosis factor-α, interleukin 1, and UVB in certain systems (14, 16, 36). It should be emphasized with respect to activation of NF-κB and JNK that LMP1 is considered to be a constitutively activated TNFR-like molecule (37). Here we show that aspirin inhibited constitutively active NF-κB and AP-1 and that inhibition could be reversed by p50/p65 or c-Jun overexpression. Inhibition of LMP1-induced NF-κB activation through inhibition of IKK is direct evidence of the inhibitory effect of aspirin on constitutively active NF-κB. Suppression of LMP1-inducible signaling pathways may be a potential therapeutic tool for the type II and type III EBV-associated diseases.
MMP-9 has been shown to have a direct role in tumor metastasis in certain systems (38). Here we demonstrate that LMP1-induced in vitro invasiveness is correlated with induction of MMP-9. The possibility that suppression of MMP-9 could prevent invasion and metastasis has been explored with the use of anti-MMP-9 ribozyme (39) or ursolic acid (40). Here we show that aspirin can be a possible candidate for repression of MMP-9 and inhibition of invasiveness. Aspirin completely suppressed MMP-9 promoter activity through its bifunctional effect on NF-κB and AP-1. We showed previously that MMP-9 promoter activity induced by LMP1 was abolished when either the NF-κB site or the proximal AP-1 site was mutated (9). On the basis of this observation, inhibition of either NF-κB or AP-1 binding by aspirin might be causal for MMP-9 down-regulation. However, the data in Fig. 5 suggest that the proximal AP-1 site may be responsible for mediating down-regulation, although the NF-κB site is essential for full induction, even in the presence of the proximal AP-1 site. A preferential inhibitory effect of aspirin on binding of the upper NF-κB band on EMSA, the one known to contain p65, may be beneficial because p65 has more transactivation capacity than p50 (41). Overexpression of either p50/p65 or c-Jun restored MMP-9 expression and promoter activity, suggesting that either one of them may be operative. This possibility may be explained by the previous reports showing the cross-coupling of NF-κB p65 and c-Jun (41, 42). Those reports showed that p65 activates AP-1 and c-Jun activates NF-κB (41), and that NF-κB inhibitor blocks AP-1 and AP-1 inhibitor blocks NF-κB (42). Accordingly, we showed previously that LMP1 CTAR1 induces AP-1 binding activity (11).

Thus, both aspirin and sodium salicylate significantly suppressed LMP1-induced in vitro invasion. Furthermore, aspirin inhibited LMP1-induced MMP-9 expression in tumors grown in nude mice. The present study is the first report showing that aspirin and sodium salicylate suppress not only the expression of MMP-9 in cell culture but also in vitro invasiveness. It is also the first to show induction of MMP-9 by LMP1 in tumors grown in nude mice and inhibition of that induction by treatment of the tumor-bearing mice with aspirin. Thus, hypothetically, aspirin or an aspirin-like effect may have potential to prevent invasion and metastasis, in addition to its anti-inflammatory and chemopreventive properties. Finally, this study is the first to show

![Fig. 7. Restoration of MMP-9 expression and promoter activity.](image)

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![Fig. 8. In vitro kinase assay of IKK.](image)

![Fig. 9. Induction of MMP-9 by LMP1 in tumors grown in nude mice and its suppression by aspirin.](image)
suppression of downstream signals of LMP1 by aspirin and sodium salicylate. Aspirin significantly, but not completely, inhibited LMP1-induced tumor cell invasion in vitro. Unfortunately, tumors caused by s.c. injection into nude mice are generally noninvasive because of the ectopic site. However, in vitro invasiveness predicts in vivo metastasis in certain systems (43). Therefore, we are now establishing an LMP1-induced tumor invasion and metastasis model in nude mice so as to evaluate the effect of aspirin on invasion and metastasis in vivo.

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ASPIRIN INHIBITS LMP1-INDUCED INVASION AND MMP-9
Aspirin Inhibits Tumor Cell Invasiveness Induced by Epstein-Barr Virus Latent Membrane Protein 1 through Suppression of Matrix Metalloproteinase-9 Expression

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