

Inhibitory effects of cyclosporin A on calcium mobilization-dependent interleukin-8 expression and invasive potential of human glioblastoma U251MG cells

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Interleukin (IL)-8 produced from glioblastoma is suggested to contribute to its own proliferation and progression. Since various external stimuli have been shown to increase intracellular Ca²⁺ in glioma cells, we investigated Ca²⁺ mobilization-dependent IL-8 expression and effect of cyclosporin A (CsA), an inhibitor of calcineurin (Cn), on the expression and invasive potential of human glioblastoma U251MG cells. Combined treatment with Ca²⁺-ionophore and phorbol-myristate-acetate (A23187/PMA) increased IL-8 mRNA and protein levels. This increase was suppressed by CsA and by another Cn inhibitor FK506. Luciferase reporter gene assay and electrophoretic mobility shift assay revealed that activation of p65-containing nuclear factor- κ B was essential for A23187/PMA-dependent activation of IL-8 promoter. CsA suppressed the promoter activity by attenuating I κ B- α degradation. U251MG cells expressed IL-8 receptors CXCR-1 and -2, and Matrigel invasion assay revealed that CsA attenuated A23187/PMA-dependent stimulation of invasive potential, probably by inhibiting IL-8 production. In addition, IL-8-dependent proliferation was also suppressed by CsA. Taken together, these results demonstrate the novel inhibitory effects of CsA on glioblastoma cell functions, suggesting CsA as a potential therapeutic adjuvant for glioma treatment.

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Introduction

Glioblastoma is an extremely aggressive neoplasm resistant to intensive therapies including surgical operation, irradiation and anticancer drugs, so that median

survival time of the affected patients is estimated to be less than 2 years (Shapiro *et al.*, 1989). Its histological features are characterized by abundant neovascularization and the paradoxical presence of necrosis, and infiltration of leukocytes (Daumas-Duport *et al.*, 1988). Predominant blood supply to rapidly growing tumor cells and/or thrombotic occlusion of vessels are believed to contribute to the development of necrosis in glioblastoma. Along with a variety of humoral and microenvironmental factors including growth factors, cytokines, acidosis and hypoxia, involvement of interleukin (IL)-8 in the development and progression of glioblastoma has been intensively investigated, because IL-8 can mediate recruitment of leukocytes and neovascularization by recruiting and activating endothelial cells, and because it can stimulate proliferation and motility of some cancer cells (Koch *et al.*, 1992; Schadendorf *et al.*, 1993; Smith *et al.*, 1994; Luca *et al.*, 1997).

IL-8, originally identified as a chemotactic factor for leukocytes, belongs to CXC chemotactic cytokine family, and has been demonstrated to play important roles in pathogenesis of autoimmune and inflammatory diseases (Smyth *et al.*, 1991; Koch *et al.*, 1992; Mukaida *et al.*, 1998). Its expression is therefore tightly controlled in cells. In normal noninflamed cells, its expression is extremely low or undetectable. However, inflammatory cytokines such as tumor necrosis factor (TNF)- α and IL-1 stimulate its expression in various cell types (Mukaida *et al.*, 1990). In glioblastoma-derived cell lines, IL-8 expression has also been shown to be inducible in response to TNF- α , IL-1, substance P, IL-17, Fas ligand, TNF-related apoptosis-inducing ligand (TRAIL), irradiation and hypoxia (Yamanaka *et al.*, 1993; Desbaillets *et al.*, 1997; Kasahara *et al.*, 1998; Palma and Manzini, 1998; Kehlen *et al.*, 1999; Choi *et al.*, 2001, 2002). Furthermore, IL-8 synthesis *in vivo* was demonstrated in sections prepared from glioblastoma by immunohistochemical analysis (Desbaillets *et al.*, 1997). However, intracellular signaling pathway leading to IL-8 expression has been poorly elucidated.

A minimal promoter region (–133 to –1) in the human IL-8 gene was identified, which is essential for its transcriptional regulation by inflammatory cytokines

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(Mukaida *et al.*, 1990). This region contains potential binding sites for activator protein-1 (AP-1), CCAAT/enhancer-binding protein β (C/EBP β) and nuclear factor- κ B (NF- κ B). The NF- κ B site is indispensable for the activation of the promoter in all cell types studied, whereas the potential AP-1 and C/EBP β sites are dispensable (Mahe *et al.*, 1991; Yasumoto *et al.*, 1992; Matsusaka *et al.*, 1993). It is thus suggested that the promoter is controlled in cell-type-specific and stimulus-dependent manners. NF- κ B is a transcription factor consisting of a dimer of the Rel family proteins such as p65 (RelA), p50, p52, c-Rel and RelB (Baldwin, 1996). In unstimulated cells, NF- κ B is sequestered in the cytoplasm bound to an inhibitory protein I κ B that includes several isoforms such as I κ B- α and I κ B- β . External stimuli such as TNF- α induce activation of NF- κ B by promoting I κ B kinase-dependent phosphorylation of I κ B. The phosphorylated I κ B is then ubiquitinated and degraded by proteasome. After release from I κ B, NF- κ B translocates into the nucleus, binds to a regulatory element of the target gene and controls its transcription.

Ca²⁺-mobilizing reagents such as Ca²⁺-ionophore (A23187) and thapsigargin, and a protein kinase C (PKC) activator phorbol-myristate-acetate (PMA) are also shown to induce IL-8 expression in various cell types (Okamoto *et al.*, 1994; Kuhns *et al.*, 1998). PKC is shown to increase intracellular Ca²⁺ by phosphorylating inositol triphosphate receptor and allowing efflux of Ca²⁺ from the endoplasmic reticulum (Cameron *et al.*, 1995). Although a variety of external stimuli such as epidermal growth factor (EGF), IL-1 β , platelet-derived growth factor (PDGF), substance P, endothelin, nerve growth factor (NGF) and serotonin have been demonstrated to increase intracellular Ca²⁺ in glioma cells (Bando *et al.*, 1995; Kugaya *et al.*, 1995; De Bernardi *et al.*, 1996; Pita *et al.*, 1999; Saqr *et al.*, 1999), effect of Ca²⁺ mobilization on IL-8 expression in the cells has not been studied to date. On the other hand, IL-8 action is exerted through G protein-coupled membrane receptors, CXCR-1 and -2, whose expression is detected not only in leukocytes but also in various other cell types (Mukaida *et al.*, 1998). Although the IL-8 synthesis by glioblastoma has been established, its receptor expression and its autocrine and paracrine actions remain to be addressed.

A rise in intracellular Ca²⁺ leads to activation of calcineurin, a serine/threonine-specific and Ca²⁺-calmodulin-dependent protein phosphatase (Klee *et al.*, 1998; Hemenway and Heitman, 1999). Cyclosporin A (CsA) and FK506, powerful immunosuppressants widely used in autoimmune disorders and organ transplantation, are known as specific inhibitors for calcineurin (Rao *et al.*, 1997). Recently, it is reported that CsA induces apoptosis of rat C6 glioma cells, demonstrating the direct effect of CsA on glioma cell functions (Pyrzynska *et al.*, 2002).

Based on these findings, the present study was undertaken to evaluate the effects of Ca²⁺ mobilization on IL-8 expression in human glioblastoma U251MG cells by utilizing Ca²⁺-ionophore (A23187) and PMA,

and also effects of CsA on the expression. Furthermore, their effects on invasive potential and proliferation of the cells were investigated, since IL-8 possesses mitogenic and mitogenic actions as described above.

Results

CsA attenuates A23187/PMA-dependent IL-8 expression

Effects of A23187/PMA, TNF- α , CsA and FK506 on IL-8 mRNA expression in U251MG cells were examined by Northern blot analysis. As shown in Figure 1a, the basal expression was not detected. Treatment of the cells with A23187/PMA markedly increased IL-8 mRNA level. A similar degree of induction was observed by treatment with TNF- α . Interestingly, CsA suppressed the A23187/PMA-dependent increase in IL-8 mRNA, but not TNF- α -dependent increase. Another calcineurin inhibitor, FK506, also attenuated the A23187/PMA-dependent increase, but not the TNF- α -dependent one (Figure 1b).

Concentrations of IL-8 in culture media produced from U251MG cells are shown in Figure 2. Treatment

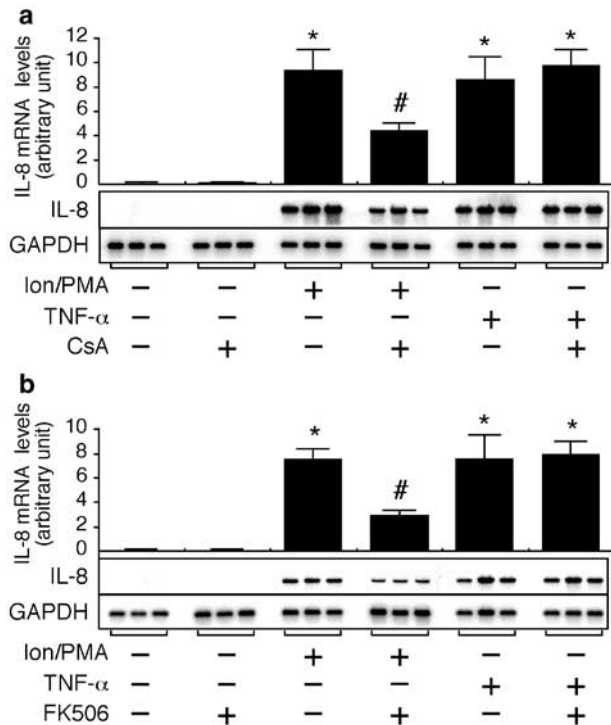


Figure 1 CsA attenuates A23187/PMA-dependent expression of IL-8 mRNA. U251MG cells were treated with A23187 (Ion)/PMA or with TNF- α in the presence or absence of CsA (panel a) or FK506 (panel b). After 6-h incubation, total RNA was extracted, and Northern blot analysis was performed using IL-8 and GAPDH cDNAs as probes. Representative images by BAS 2000 system are shown. Experiments were performed in triplicate. IL-8 mRNA levels were normalized by those of GAPDH mRNA. Data are shown as mean \pm s.d. ($n=3$). * $P<0.01$ vs unstimulated cells. ** $P<0.05$ vs Ion/PMA alone

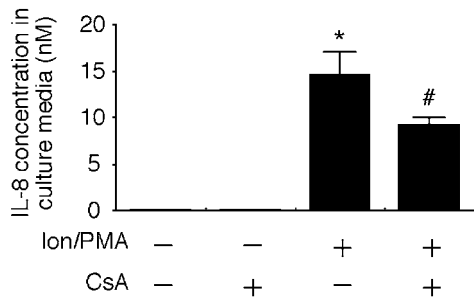


Figure 2 CsA attenuates A23187/PMA-dependent accumulation of IL-8 in culture media. U251MG cells were treated with A23187 (Ion)/PMA in the presence or absence of CsA. After 24-h incubation, amounts of IL-8 in culture media (3 ml) were determined by ELISA. Experiments were carried out in triplicate. Data are shown as mean \pm s.d. ($n=3$). * $P<0.01$ vs unstimulated cells. ** $P<0.05$ vs Ion/PMA alone

with CsA alone had no effect on IL-8 production, whereas A23187/PMA markedly increased IL-8 concentration in culture media. This increase was significantly suppressed by CsA. The IL-8 accumulations in culture media well reflected its mRNA levels.

Both AP-1 and NF- κ B sites in IL-8 promoter are necessary for A23187/PMA-dependent IL-8 expression and its inhibition by CsA

To identify the promoter region responsible for A23187/PMA-dependent induction of IL-8 expression and its inhibition by CsA, U251MG cells were transfected with luciferase reporter plasmids driven by a series of 5'-deletion fragments of human IL-8 promoter. As shown in Figure 3a, when the cells transfected with -1460, -651 or -133 plasmid were treated with A23187/PMA, the luciferase activity was markedly increased. This increase was significantly inhibited by CsA. In contrast, the A23187/PMA-dependent promoter activation was not observed in the cells transfected with -60 plasmid, demonstrating that the -133 to -60 promoter region is responsible for the regulation by A23187/PMA and CsA.

Since earlier studies demonstrated the important roles of the AP-1 site (-126 to -120) and the NF- κ B site (-81 to -72) in transcriptional activation of human IL-8 promoter (Yasumoto *et al.*, 1992), -133 reporter plasmids with a mutated AP-1 or NF- κ B site were constructed, and luciferase reporter assay was carried out. As shown in Figure 3b, disruption of either AP-1 or NF- κ B site resulted in a marked attenuation of the A23187/PMA-dependent promoter activation, indicating that both sites are necessary for full promoter activation by A23187/PMA and for its inhibition by CsA.

CsA attenuates A23187/PMA-dependent NF- κ B activation

To elucidate proteins interacting the AP-1 and NF- κ B sites, nuclear extracts were prepared from U251MG cells cultured with A23187/PMA and/or CsA for 1 h,

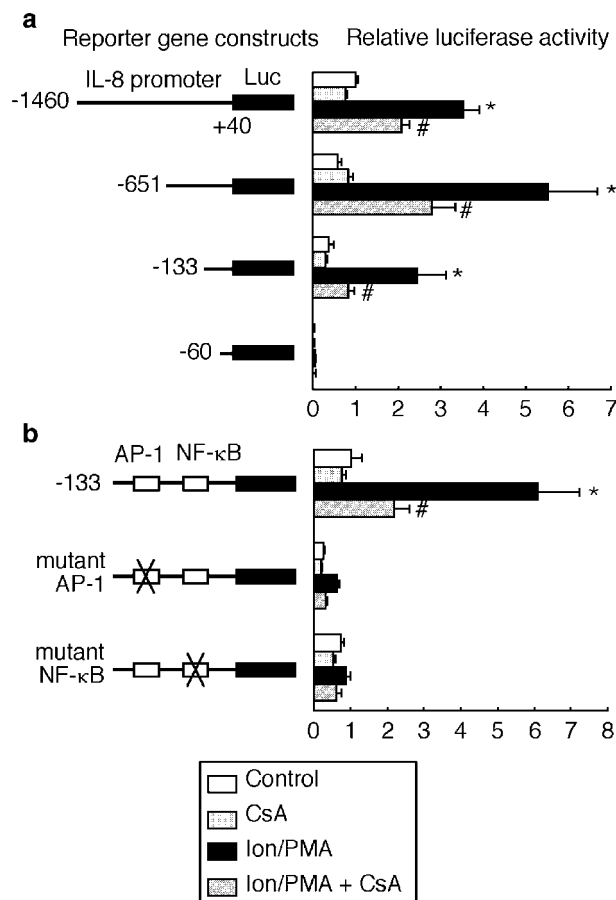


Figure 3 Mutation of NF- κ B or AP-1 site abolishes A23187/PMA-dependent activation of IL-8 promoter and its inhibition by CsA. Panel a: U251MG cells were transfected with luciferase reporter plasmids driven by various lengths of human IL-8 promoter (-1460, -650, -133 and -60), together with pSV- β -gal plasmid. Panel b: The cells were transfected with the luciferase reporter plasmid driven by -133 to +40 of human IL-8 promoter (p-133), p-133 containing mutated κ B-like site or p-133 containing mutated AP-1 site, together with pSV- β -gal plasmid. After incubation with DNA-liposome mixture for 10 h, the cells were cultured in growth media for 12 h, and then treated with A23187 (Ion)/PMA and/or CsA for 24 h. The cells were harvested, and the luciferase and β -gal activities were measured. Promoter activity is expressed as luciferase activity normalized by β -gal activity (mean \pm s.d., $n=3$). * $P<0.01$ vs control. ** $P<0.05$ vs Ion/PMA alone

and EMSA was performed using hIL8-AP1 oligo and hIL8- κ B oligo as probes. As shown in Figure 4a, when hIL8-AP1 oligo was used, a single protein/DNA complex was detected in the unstimulated cells (lane 1). Density of the complex was not affected by CsA (lane 2), by A23187/PMA (lane 3), nor by the combined treatment (lane 4). The complex was supershifted by anti-c-Fos antibody (lane 5). When anti-c-Jun antibody was used for supershift analysis, density of the complex was reduced, and a faint supershifted complex appeared (lane 6). The complex was displaced with 50-fold molar excess of cold canonical AP-1 oligo (lane 8). These results indicated that the protein/DNA complex includes c-Fos/c-Jun heterodimer AP-1. However, the

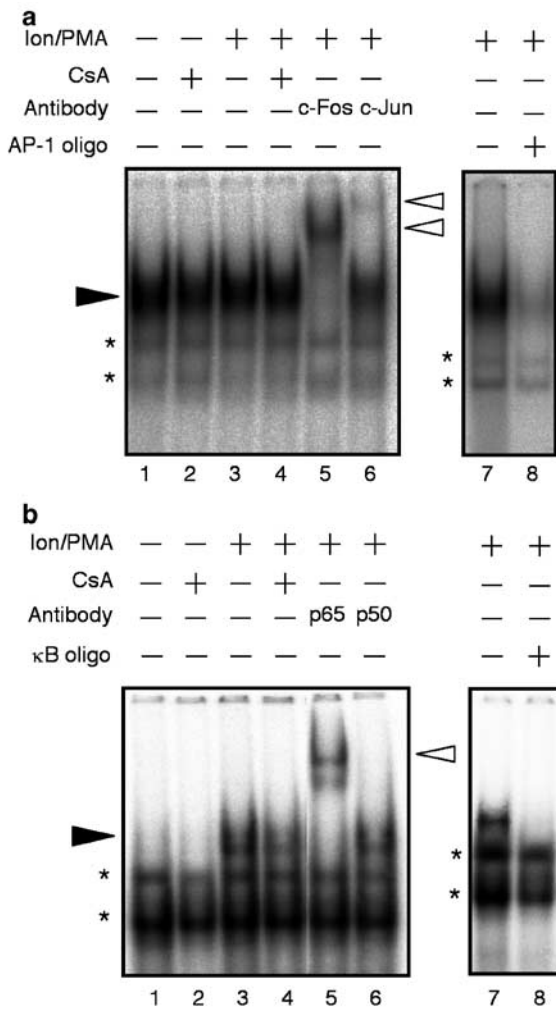


Figure 4 CsA attenuates A23187/PMA-dependent NF- κ B activation. Nuclear extracts prepared from U251MG cells cultured with A23187 (Ion)/PMA in the presence or absence of CsA for 1 h were subjected to EMSA using hIL8-AP1 oligo (panel a) and hIL8- κ B oligo (panel b) as probes. Representative images by BAS 2000 system are shown. Supershift analysis was performed using antibodies directed against c-Fos, c-Jun (panel a), p65 and p50 (panel b). Competition assay was performed using canonical AP-1 oligo (panel a) and canonical κ B oligo (panel b). The closed and open arrowheads represent specific protein/DNA complexes and supershifted complexes, respectively. The asterisks indicate non-specific binding

DNA-binding activity of AP-1 was not altered by A23187/PMA, nor by CsA. The faint, fast-migrating complexes indicated by asterisks were not affected by anti-c-Fos and c-Jun antibodies, nor by cold AP-1 oligo.

In contrast, when hIL8- κ B oligo was used as a probe (Figure 4b), treatment of the cells with A23187/PMA induced a single protein/DNA complex (lane 3), which was absent in unstimulated (lane 1) and CsA-treated (lane 2) cells. This complex was supershifted by anti-p65 antibody (lane 5), but not by anti-p50 antibody (lane 6), and was displaced with 50-fold molar excess of cold canonical κ B oligo (lane 8), indicating that this complex includes p65-containing NF- κ B. The A23187/PMA-

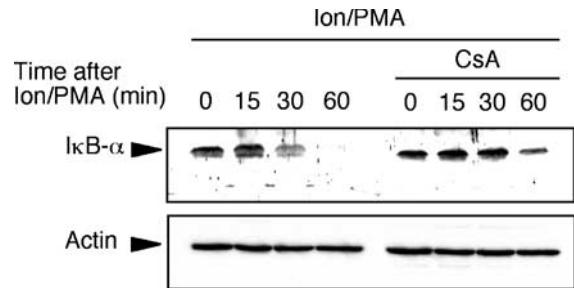


Figure 5 CsA attenuates A23187/PMA-dependent degradation of I κ B- α . Cytosolic extracts prepared from U251MG cells cultured with A23187 (Ion)/PMA in the presence or absence of CsA for the indicated time were subjected to Western blot analysis using anti-I κ B- α or anti-actin antibodies

dependent activation of p65-containing NF- κ B was suppressed by treatment with CsA (lane 4).

Constitutive DNA-binding activities of C/EBP β and no alteration in its activities by A23187/PMA nor by CsA were observed in EMSA using hIL8-C/EBP oligo (data not shown).

CsA suppresses A23187/PMA-dependent degradation of I κ B- α

To explore the mechanism for CsA-dependent suppression of NF- κ B activation, degradation of I κ B- α was investigated by Western blot analysis (Figure 5). In accordance with EMSA study, I κ B- α was rapidly degraded in response to A23187/PMA. However, CsA markedly delayed the degradation by A23187/PMA. Actin contents were altered neither by A23187/PMA nor by CsA. I κ B- β degradation was not observed in response to either A23187/PMA or CsA (data not shown).

CsA suppresses A23187/PMA-dependent invasive potential of U251MG cells

IL-8 belongs to the CXC chemokine family, and its action is mediated by membrane receptors, CXCR-1 and -2. Expression of these receptors in U251MG cells was examined by RT-PCR (Figure 6a). When RT products prepared from human aortic smooth muscle cells (SMCs) were used for PCR, a single band was amplified by CXCR-1 primers and by CXCR-2 primers. The sizes of the bands were the expected lengths: 363 bp for CXCR-1 and 385 bp for CXCR-2, demonstrating the expression of both receptors in SMCs. A high level of CXCR-1 expression was also detected in U251MG cells, whereas CXCR-2 expression appeared to be much lower than that of CXCR-1. No bands were amplified from the samples in which reverse transcriptase was omitted from reverse transcription reaction (data not shown), indicating that contamination of our RNA samples with genomic DNA was, if any, below the detectable level.

To address whether CXCR-1 and -2 expressed in U251MG cells are functional or not, we first assessed the invasive potential of U251MG cells utilizing Matrigel invasion chambers. The cells were seeded on

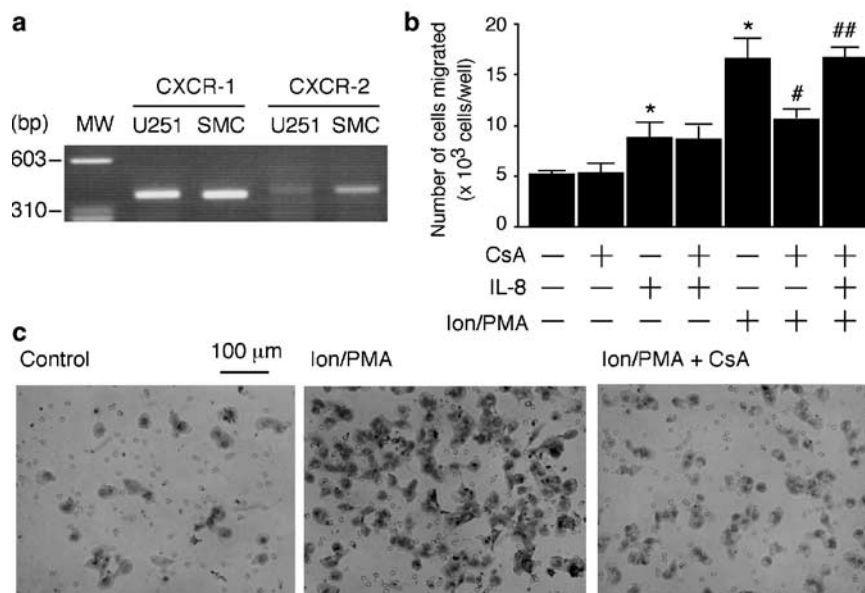


Figure 6 Effects of IL-8, A23187/PMA and CsA on the invasive potential of U251MG cells. Panel a: Expression of IL-8 receptors, CXCR-1 and -2, in U251MG cells and human aortic SMCs (positive control) was determined by RT-PCR. The expected sizes of the amplified cDNAs for CXCR-1 and -2 are 363 and 385 bp, respectively. MW: molecular weight marker. Panel b: U251MG cells (2×10^4 cells/well) were seeded on the filter of the upper compartment of Matrigel invasion chambers in the presence or absence of IL-8, A23187 (Ion)/PMA, and CsA. After 24-h incubation, the cells on the lower surface of the filter were stained, and the number of the cells was counted. Total numbers of the migrated cells are shown as mean \pm s.d. ($n = 3$). * $P < 0.05$ vs unstimulated cells. ** $P < 0.05$ vs Ion/PMA alone. *** $P < 0.05$ vs Ion/PMA and CsA. Panel c: Representative microscopic photographs for the migrated cells are shown. Multiple small circles indicate 8- μ m pores of the filter

the filter of the upper chamber in the presence or absence of IL-8, A23187/PMA and CsA. After 24-h incubation, the cells that migrated to the lower side of the filter by passing through the basement membrane matrix and 8- μ m pores of the filter were counted (Figure 6b). Even in unstimulated conditions, approximately 25% cells migrated to the lower side of the filter. CsA alone had no effect on the invasive potential. Treatment of the cells with 10 nM IL-8 resulted in a significant increase in the potential, which was, however, not inhibited by CsA. When the cells were treated with A23187/PMA, the number of migrated cells was markedly increased by threefold above the unstimulated conditions. This increase was significantly suppressed by CsA. A23187/PMA-dependent suppression of invasive potential was reverted by 10 nM IL-8. Representative microscopic photographs for the migrated cells are shown in Figure 6c.

CsA suppresses IL-8-dependent proliferation of U251MG cells

We next examined the effects of A23187/PMA and IL-8 on proliferation of U251MG cells by WST-1 assay. As shown in Figure 7, treatment of the cells with 10 nM IL-8 significantly increased the number of cells. This increase was significantly attenuated by treatment with CsA. In contrast, CsA alone had no effect on the growth. Unexpectedly, A23187/PMA markedly decreased, rather than increased, the cell number at Day 4.

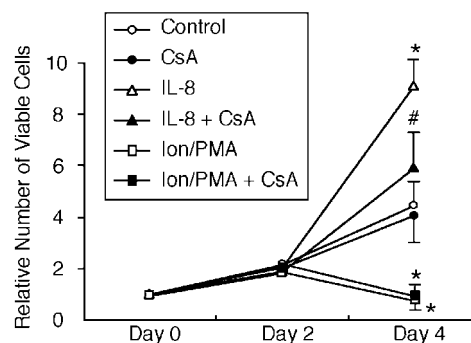


Figure 7 Effects of IL-8 and CsA on proliferation of U251MG cells. U251MG cells were seeded in 96-well plates and cultured overnight in α -MEM supplemented with 1% FBS, followed by treatment with IL-8, A23187 (Ion)/PMA and/or CsA (day 0). Cell number was determined by WST-1 assay on days 0, 2, and 4. Values are expressed as mean \pm s.d. ($n = 8$). * $P < 0.01$ vs control. ** $P < 0.05$ vs IL-8 alone

Discussion

The present study demonstrated that two different stimuli, A23187/PMA and TNF- α , increase IL-8 mRNA levels in human glioblastoma U251MG cells. A23187/PMA-dependent increase is, at least in part, due to transcriptional activation of IL-8 gene, because A23187/PMA was capable of increasing the activity of luciferase

reporter gene driven by IL-8 promoter. Subsequent transfection analysis using 5'-deleted promoters revealed that the -133 to -60 region is responsible for the transcriptional activation. Mutation of either AP-1 or NF- κ B site in this region resulted in a marked reduction in the promoter inducibility by A23187/PMA. However, EMSA study revealed that only NF- κ B binding is inducible in response to A23187/PMA, whereas AP-1 is constitutively activated. These results thus suggest that AP-1 contributes to the basal promoter activity, and that NF- κ B, cooperatively with AP-1, mediates A23187/PMA-dependent IL-8 induction. Similar results were reported by Yasumoto *et al.* (1992). They showed that mutation of either site for NF- κ B or AP-1 abolished IL-8 promoter responsiveness to TNF- α and interferon- γ in human gastric cancer cells.

Interestingly, although A23187/PMA and TNF- α both increased IL-8 expression, CsA and FK506 attenuated only A23187/PMA-dependent expression. This inhibitory effect can be attributed to the decrease in transcription of IL-8 gene, because luciferase reporter gene assay showed that CsA also attenuates A23187/PMA-dependent activation of IL-8 promoter. Subsequent EMSA analysis demonstrated that the decrease in NF- κ B binding to its cognate site in the promoter is responsible for the inhibitory action of CsA. Indeed, it was also demonstrated that CsA markedly delays the A23187/PMA-dependent I κ B- α degradation.

CsA was reported to inhibit chymotrypsin-like activity of 20S proteasome *in vitro* and that it prevented lipopolysaccharide-induced I κ B- α degradation in RAW 264.6 murine macrophage cell line (Meyer *et al.*, 1997). This is, however, not the case in our study, because CsA prevented only A23187/PMA-dependent NF- κ B activation. If CsA inhibits proteasome activity in U251MG cells, both TNF- α - and A23187/PMA-dependent NF- κ B activation should be prevented. It is thus speculated that CsA might affect a certain step upstream of I κ B- α phosphorylation in A23187/PMA-elicited intracellular signaling pathway. As it is established that CsA and FK506 are specific inhibitors for calcineurin (Cn), it is likely that Cn mediates A23187/PMA-dependent I κ B- α degradation and NF- κ B activation in U251MG cells. Indeed, involvement of Cn in NF- κ B activation was demonstrated in Jurkat T cells (Frantz *et al.*, 1994). Expression of the constitutively active form of Cn was shown to increase NF- κ B binding by enhancing degradation of I κ B- α . It was recently shown that Cn and PKC synergize to activate I κ B kinase and NF- κ B in human T lymphocytes (Trushin *et al.*, 1999). Our present result is compatible with these findings, and suggests that Cn/PKC-mediated degradation of I κ B- α is not restricted to T cells. However, how Cn/PKC activates I κ B kinase is still unknown. As I κ B kinase was shown to be phosphorylated and activated by other kinases such as NF- κ B-inducing kinase (NIK) and NF- κ B-activating kinase (NAK), it is unlikely that Cn directly activates I κ B kinase. Since some isoforms of PKC were shown to activate I κ B kinase (Vertegaal *et al.*, 2000; Saijo *et al.*, 2002), Cn may function upstream of I κ B kinase by potentiating the activity of such PKC or

by modifying other effectors for I κ B kinase. Although a precise pathway is unknown, the present study strongly suggests the presence of Ca²⁺-Cn-NF- κ B signaling pathway, leading to IL-8 expression in U251MG cells.

IL-8 has been shown to stimulate proliferation of a variety of cancer cells such as melanoma, pancreatic cancer and colon cancer cells (Schadendorf *et al.*, 1993; Miyamoto *et al.*, 1998; Brew *et al.*, 2000). However, the effect of IL-8 on glioma cell growth seems to be elusive. It was reported that IL-8 can increase growth of human glioma NP-1 cells, and this increase is inhibited by anti-IL-8 antiserum and retroviral expression of antisense IL-8 in the cells (Yamanaka *et al.*, 1995), suggesting that IL-8 may serve as an autocrine growth factor. It was, however, reported by using *in situ* hybridization that CXCR-1 and -2 expression is not detected in glioblastoma sections prepared from patients, but detected in infiltrating leukocytes (Desbaillets *et al.*, 1997). Recently, it was shown by RNase protection assay that five out of 16 glioma cell lines express substantial amounts of CXCR-1 and -2 mRNA (Zhou *et al.*, 2002). In this study, it was demonstrated by RT-PCR that both CXCR-1 and -2 are expressed in U251MG cells. These receptors may be functional, because IL-8 was capable of stimulating proliferation of the cell. Higher expression of CXCR-1 than -2 in U251MG cells is relevant to the recent report by Flynn *et al.* (2003), demonstrating a high level of CXCR-1 expression in primary human astrocytes isolated from adult temporal lobe, with a low level of CXCR-2. The discrepancy for CXCR expression among glioma cell lines and glioblastoma sections might be due to their variable expression levels and/or culturing conditions.

The present study further demonstrated that CsA inhibits the IL-8-dependent growth of U251MG cells. Since CsA alone had no effect on growth of the control cells, CsA might exert a specific, inhibitory action on the IL-8-dependent intracellular signaling pathway, leading to cell proliferation. On the other hand, it was reported that CsA induces apoptosis of rat C6 glioma cells (Pyrzynska *et al.*, 2002). However, in our experiments, morphological changes of U251MG cells indicative of apoptosis were not detected by microscopic observation. This may be due to the difference in doses of CsA used. More than 40 μ M CsA is required for inducing apoptosis of C6 glioma cells, which is much higher than the dose we used in this study (1 μ M).

The finding that treatment of U251MG cells with A23187/PMA decreased, rather than increased, the cell number at day 4 was unexpected, because such treatment for 24 h results in more than 10 nM of IL-8 accumulation in culture media (Figure 2), seemingly sufficient to stimulate the proliferation. However, it is well known that long-lasting treatment of cells with PMA causes downregulation or exhaustion of PKC (Couldwell *et al.*, 1990). Thus, the growth inhibition by extended treatment with A23187/PMA may indicate that the proliferation and viability of U251MG cells largely depend on PKC activity.

IL-8 has also been shown to stimulate migration of cancer cells such as melanoma, colon cancer and

prostate cancer cells (Wang *et al.*, 1990; Reiland *et al.*, 1999; Wilson *et al.*, 1999), which may be relevant to tumor invasion and metastasis. However, its effect on glioblastoma cells has not been studied. In the present study, invasive potential of U251MG cells was assessed by using Matrigel invasion chambers. Treatment of the cells with IL-8 significantly increased the invasive potential, but this increase was not inhibited by CsA. In contrast, A23187/PMA-dependent increase was significantly suppressed by CsA. This CsA-dependent suppression was reversed by treatment with IL-8. These results strongly suggest that the newly synthesized IL-8 in response to A23187/PMA contributes, at least in part, to the increased invasive potential in an autocrine and paracrine fashion. Note that the IL-8-dependent migration was insensitive to CsA, but its proliferation was sensitive to CsA, indicating the presence of distinct intracellular signaling pathways leading to migration and to growth.

Recently, clinical application of CsA and FK506 to treatment of stroke and spinal cord injury has been expected, because these drugs have been proven to possess neuroprotective effects in many experimental models, although the underlying mechanism is unknown (Kelly and Sharkey, 2001). In the present study, we demonstrate a novel aspect of biological effects of CsA on glioblastoma U251MG cells. Since it is demonstrated that CsA exerts multiple inhibitory actions on glioblastoma cell functions such as IL-8 production, invasive potential and proliferation, CsA can also be expected as a potential therapeutic adjuvant for glioma treatment.

Materials and methods

Cell culture

U251MG cells, a human cell line derived from glioblastoma, were obtained from the Memorial Sloan Kettering Cancer Center (NY, USA), and were cultured in minimum essential medium alpha medium (α -MEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics (penicillin and streptomycin). The cells, cultured to subconfluence, were treated, alone or in combination, with 1 μ M Ca^{2+} -ionophore (A23187, Wako Pure Chemical, Japan), 50 nM phorbol-12-myristate-13-acetate (PMA, Sigma, USA), 100 U/ml human recombinant TNF- α (Asahi Chemical, Japan), 1 μ M CsA (Wako), 1 μ M FK506 (Fujisawa Pharmaceutical, Japan) and 10 nM human recombinant IL-8 (Sigma).

Northern blot analysis

Total RNA was extracted by the acid guanidinium thiocyanate-phenol-chloroform method (Chomczynski and Sacchi, 1987). Northern blot analysis was performed as described in our previous report (Kambe *et al.*, 1988). Briefly, 12 μ g of total RNA was electrophoresed and blotted onto a nylon membrane, which was then hybridized with [32 P]dCTP-labeled human IL-8 and human glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) cDNAs (Sato *et al.*, 1996). After wash, the membrane was subjected to quantitative analysis using BAS 2000 bioimage analyzer (Fuji Photo Film, Japan).

Electrophoretic mobility shift assay (EMSA)

Procedures for preparation of nuclear extracts and EMSA were described in our previous report (Nagaya and Jameson, 1993). The nuclear extract (10 μ g) was incubated with 32 P-labeled EMSA probe, and electrophoresed with polyacrylamide gel. The gel was dried and subjected to BAS 2000 analyzer. Nucleotide sequences for EMSA probes are as follows: hIL8- κ B oligo, 5'-GATCGTGGAAATTCCTCTC-3', contains an NF- κ B-binding site (-81 to -72, underlined) in the promoter of human IL-8 gene (transcription start site is numbered as +1, DDBJ Accession Number M28130). hIL8-AP1 oligo, 5'-GTGTGATGACTCAGGTTTGC-3', contains an AP-1-binding site (-126 to -120, underlined). hIL8-C/EBP oligo, 5'-GCCATCAGTTGCAAATCGTGGC-3', contains a C/EBP β -binding site (-93 to -84, underlined). They were labeled with [32 P]dCTP by Klenow enzyme. To identify the binding proteins, supershift analysis was performed using antibodies directed against p50, p65, c-Rel, RelB, c-Fos, c-Jun and C/EBP β (Santa Cruz, USA). Competition assay was performed using 50-fold molar excess of cold oligonucleotide as follows. Canonical AP-1 oligo, 5'-TCGACTGTCTGACTCATGCTCTCGA-3', contains an AP-1 site in human collagenase promoter (DDBJ Accession No. AF007878 (Sarkar *et al.*, 2000). Canonical κ B oligo, 5'-TCGAGCAGAGGGGACTTTCGAGAGTCGA-3', contains a κ B site in mouse Ig κ enhancer (Baeuerle and Henkel, 1994).

Luciferase reporter gene assay

A DNA fragment of human IL-8 promoter spanning from -1460 to +40 bp (DDBJ: M28130) was amplified by PCR and subcloned into pGEM-T easy plasmid (Promega, USA). After sequence verification, the fragment was inserted upstream of a luciferase reporter gene in pGL3-basic (Promega). A series of 5'-deletion fragments of the promoter (-651 to +40, -133 to +40 and -60 to +40) were also amplified and inserted into pGL3-basic. The reporter plasmid driven by -133 to +40 promoter is named p-133. Site-directed mutagenesis of the AP-1 and κ B sites in p-133 was performed by PCR, which converted the AP-1 site (TGACTCA) to AGATCTA, and the κ B site (TGGAATTTCC) to TTAAC TTCC. Procedures for transfection and for luciferase and β -galactosidase (β -gal) assays were described in our previous report (Kikumori *et al.*, 2001). In brief, U251MG cells (6×10^4 cells/well), plated in 12-well plates and cultured for 1 day, were transfected with 0.8 μ g/well of reporter plasmid and 0.06 μ g/well of β -gal-expressing plasmid (pSV- β -gal, Promega) by using lipofectamine reagent (Gibco BRL). After 10-h incubation with the liposome-DNA solution, the cells were cultured in growth media for 12 h, and then treated with A23187, PMA and/or CsA for another 24 h. The cells were then harvested and the luciferase and β -gal activities in the cell lysates were determined. Promoter activity was expressed as luciferase activity normalized by β -gal activity.

Western blot analysis

Procedures for preparation of cytosolic extracts and Western blot analysis were described in our previous report (Kikumori *et al.*, 1998). In brief, cytosolic extracts (50 μ g) were subjected to polyacrylamide gel electrophoresis and blotted onto a membrane (Hybond-C super, Amersham, USA). The membrane was incubated with anti-I κ B- α or anti-I κ B- β antibodies (Santa Cruz) or anti-actin antibody (Sigma) and then incubated with anti-rabbit-IgG goat IgG conjugated with alkaline phosphatase (Zemed, USA). After wash, it was

subjected to color development reaction using NBT/BCIP tablet (Boehringer Mannheim, Germany).

Measurement of IL-8 concentrations in culture media

U251MG cells, grown to near confluence in a 60-mm dish, were treated for 24 h with A23187, PMA and CsA, alone or in combination in 3-ml fresh media. IL-8 concentration in the media was measured by enzyme-linked immunosorbent assay (ELISA, human IL-8 AN'ALYZA immunoassay kit, Genzyme Techne, USA).

Reverse transcription-polymerase chain reaction (RT-PCR)

Expressions of CXCR-1 and -2 were examined in U251MG cells and in human aortic SMCs (Iseki *et al.*, 2000b) by RT-PCR. The primers for CXCR-1 and -2 were designed according to Hayes *et al.* (1998); CXCR-1: 5'-GGGGCCACAC CAACCTTC-3' and 5'-AGTGCCTGCCCTCAATGTCTCC-3', CXCR-2: 5'-CCGGGCGTGGTGGTGAG-3' and 5'-TCTGC CTTTTGGGTCTTGTGAATA-3'.

Cell proliferation assay

U251MG cells (3×10^3 cells/well) were seeded in 96-well plates and cultured in α -MEM supplemented with 1% FBS. After overnight culture, the cells were exposed to A23187, PMA, IL-8 and CsA, alone or in combination, and cell proliferation was assessed by the water-soluble tetrazolium (WST)-1 method (Cell counting kit, Dojindo, Japan) as described in our previous report (Iseki *et al.*, 2000a).

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Matrigel invasion assay

Invasive potential of U251MG cells was assessed by using a Matrigel invasion chamber (BD Biosciences, USA). One chamber includes a cell insert and its well. The bottom of the cell insert consists of a filter with multiple 8- μ m pores, which is coated with a basement membrane matrix (Matrigel). Approximately 2×10^4 cells in 500 μ l of serum-free α -MEM containing 0.1% bovine serum albumin were seeded in the cell insert and cultured with IL-8, A23187/PMA and/or CsA. The well was filled with 500 μ l of α -MEM supplemented with 10% FBS. After 24-h incubation, the noninvasive cells present on the upper surface of the filter were wiped out with a cotton swab. The invasive cells migrated onto the lower surface of the filter were fixed and stained with Diff-Quick (American Scientific Products, USA), and the number of the cells were counted.

Statistical analysis

Statistical analysis was carried out by one-way fractional ANOVA (analysis of variance), followed by Fisher's protected least significant difference (PLSD) analysis.

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