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Cellular zinc status regulates cytomegalovirus major immediate-early promoter

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Abstract

Diethylenetriaminepenta-acetic acid (DTPA) inhibits human cytomegalovirus (CMV) replication in vitro, although the mechanism has remained unclear. The present study shows that DTPA inhibits CMV major immediate-early (MIE) promoter activity in a luciferase reporter assay, whereas its enhancer-less promoter was not affected. The inhibitory effect of DTPA on CMV MIE promoter activity was abrogated by stoichiometric amounts of cations in the following (decreasing) order, $Zn^{2+} > Co^{2+} > Ni^{2+} > Cu^{2+} > Fe^{3+} > Fe^{2+}$, but not by Mn^{2+}. These cations bind to DTPA and may limit the zinc-chelating capability. In the absence of DTPA, exogenous zinc activated CMV MIE promoter activity in a dose-dependent manner, but not its enhancer-less promoter. The intracellular metallothionein content of DTPA- and cation-treated cultures was significantly correlated with CMV MIE promoter activity. DTPA may inhibit CMV replication by regulating CMV MIE promoter activity through controlling the availability of cellular zinc. Since the CMV MIE promoter has no consensus sequence for a metal responsive element, a novel mechanism for metal-regulated transcription may be involved in this process.

Keywords: CMV promoter; Anti virus; Zinc; DTPA; Metallothionein

1. Introduction

The reactivation of latent human cytomegalovirus (CMV) is thought to be the main cause of severe active CMV disease in immunosuppressed patients, such as solid organ and bone marrow transplant recipients and those with AIDS. Death, mental retardation or sensorineural

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hearing loss often results from CMV infection of the fetus (Fowler et al., 1992). The disease may involve the retina, lung, gastrointestinal tract, liver, kidneys, or nervous system (Meyers et al., 1982; Rubin, 1990; Gallant et al., 1992). The anti-CMV compounds, ganciclovir (Laskin et al., 1987), phosphonoformate (Singer et al., 1985; Walmsley et al., 1988), cidofovir (De Clercq, 1993, 1996) and CMV-specific immunoglobulin (Meyers, 1988) have received approval for treatment of patients with CMV disease. However, prolonged use of ganciclovir and phosphonoformate may be associated with serious side effects such as anemia, neutropenia and nephrotoxicity (Felsenstein et al., 1985; Erice et al., 1987; Laskin et al., 1987; Hecht et al., 1988).

Cinatl et al. have demonstrated that CMV replication can be inhibited by the metal chelators, desferrioxamine (DFO), 2,2′-bipyridine (BPD) and diethylenetriaminepenta-acetic acid (DTPA) (Cinatl et al., 1996). Among these, only the hydrophobic and membrane-impermeable chelator, DTPA, can gain access to the extracellular space and bind iron as well as other ions from the extracellular pool (Aisen and Listowsky, 1980). In addition, DTPA completely inhibits infectious CMV replication in human foreskin fibroblast cells, an activity that is suppressed by Mn^{2+} and Zn^{2+}. However, the mechanism by which DTPA exerts its activity against CMV has remained unclear. Cinatl et al. proposed four scenarios (Cinatl et al., 1996), (1) DTPA inhibits virus replication through affecting several cellular and viral enzymes that require metal ions; (2) DTPA affects the plasma membrane, which is important for viral infection; (3) various ions are involved in signal transduction (similar to the requirements of growth factors and hormones), which is important for the initiation of viral DNA replication; and (4) DTPA directly interacts with membrane-associated proteins and lipids. The roles of metal ions in cellular functions, particularly that of zinc, have been studied using DTPA in vitro (Chesters et al., 1989; Petrie et al., 1991; Chattopadhyay and Freake, 1998; Lefebvre et al., 1998; Nakatani et al., 1998). Zinc is a constituent of many proteins and enzymes and is indispensable for catalysis, gene expression, and intracellular signaling. We surmised that reducing the cellular zinc content would influence CMV replication. In addition, DTPA is selective for CMV, and does not affect herpes simplex virus, adenovirus or poliovirus replication (Cinatl et al., 1994). We therefore focused on the effect of zinc on cellular signal transduction and CMV gene expression.

After primary infection, CMV, like other herpesviruses, establishes lifelong persistence in the host. Three classes of viral genes are expressed by CMV, i.e. immediate-early (IE), early, and late genes (Wathen and Stinski, 1982). The CMV MIE genes are transcribed prior to viral protein synthesis and the direct production of both viral and cellular genes. CMV early genes direct viral DNA synthesis, whereas CMV late genes direct the production of structural nucleocapsid proteins (Wathen and Stinski, 1982). The transcription of CMV MIE genes is regulated by a large and complex promoter/enhancer, which is critical for the expression of all viral gene products (Meier and Stinski, 1996). In practice, the CMV MIE promoter is thought to be constitutively active and unregulated (Schmidt et al., 1990; Coleman et al., 1991). However, we found that cellular zinc status regulates the expression of transgenes that are regulated by the CMV MIE promoter. If CMV MIE promoter can be regulated by zinc, then novel anti-CMV agents and vectors of therapeutic genes can be developed.

2. Materials and methods

2.1. Reagents

Restriction enzymes and DNA modification enzymes were purchased from TaKaRa (Shiga, Japan) or TOYOBO (Osaka, Japan). Other chemicals were purchased from Nakalai Tesque (Kyoto, Japan).

2.2. Construction of plasmids

Plasmids were derived from a luciferase vector bearing the CMV MIE gene promoter, pRL-CMV (Promega, WI, USA). Deletion mutated

fragments derived from the CMV MIE gene promoter were generated using PCR (Saiki et al., 1988). All plasmids were amplified and purified by CsCl-EtBr and CsCl density gradient centrifugation. All constructs were verified by sequencing the relevant portions.

2.3. Cells and cell culture

L929 cells were obtained from Dr H. Ochiai (Toyama Medical and Pharmaceutical University) and cultured in Dulbecco's modified eagle's (DME) medium supplemented with 5% fetal bovine serum (FBS) at 37°C in 5% CO_2/95% air. Human embryonic lung (HEL) fibroblast cells were prepared from 4-month-old female embryo and cultured with DME medium supplemented with 10% FBS.

2.4. Transfection and luciferase reporter assay

Calcium phosphate transfection was performed in six-well culture plates by Chen and Okayama (1988). After 48 h, the cells were lysed and luciferase activity in the lysate was measured using a Luminometer (EG&G Berthold, Bad Wildbad, Germany). All transfection experiments were repeated at least three times using two or three different DNA preparations. Luciferase activities of the cells were normalized using the luciferase vector, PGV-P2, bearing a minimal SV40 promoter, (Toyo Ink, Tokyo, Japan). The luciferase activities from the cells transfected with PGV-P2 were not affected significantly by DTPA (0–50 μM) and zinc (0–50 μM). The CMV MIE promoter response (fold induction) was defined as the ratio of luciferase activity in the stimulated cells to that in the unstimulated cells.

2.5. Treatment

Cells were incubated with DTPA (0–50 μM) with or without cations for 48 h. When exposed to zinc (0–50 μM in medium containing 2.5% of zinc-free FCS), the cells were first incubated in serum-free medium for 24 h after transfection. For zinc depletion, FCS was treated with Chelex 100 (BioRad, CA) as described by Tate et al. (1995).

2.6. Miscellaneous

Zinc content in the HNO_3-solubilized cells was determined by atomic absorption spectrophotometry and the metallothionein concentration was determined by the ^{109}Cd-hemoglobin assay, as described by Eaton and Toal (1982).

3. Results

3.1. Inhibition of CMV major immediate-early promoter activity by DTPA

Since human CMV replication is inhibited by DTPA in vitro (Cinatl et al., 1996), we determined whether or not the CMV MIE promoter was inhibited by this metal chelator using a plasmid in which luciferase expression is determined by the CMV MIE promoter ($-735/+62$). HEL and L929 cells were transiently transfected with the luciferase reporter plasmid. Thereafter, the cells were incubated with 50 μM DTPA for 48 h. Fig. 1 shows that DTPA decreased luciferase activity by almost 40% in transfected HEL and L929 cells. To further investigate the effect of DTPA on the CMV MIE promoter, the DTPA-response of a deletion construct containing the enhancer-less CMV MIE promoter ($-117/+62$) was examined. Further analyses were performed using L929 cells, because they are more suited to transient transfection assays than HEL cells. The activity of the enhancer-less CMV MIE promoter ($-117/+62$) was not suppressed by DTPA (Fig. 1).

3.2. Effects of various cations on DTPA inhibition of CMV MIE promoter activity

To investigate whether exogenous metals influence the inhibitory effect of DTPA, L929 cells transfected with CMV MIE promoter ($-735/+62$) plus reporter were incubated with 50 μM DTPA for 48 h in the presence or absence of seven divalent cations (Co^{2+}, Cu^{2+}, Ni^{2+}, Mg^{2+}, Fe^{2+}, Fe^{3+} and Zn^{2+}). The inhibitory effect of DTPA on CMV MIE promoter activity was completely abrogated by stoichiometric amounts of Cu^{2+}, Co^{2+}, Ni^{2+} or Zn^{2+} (Fig. 2). The DTPA

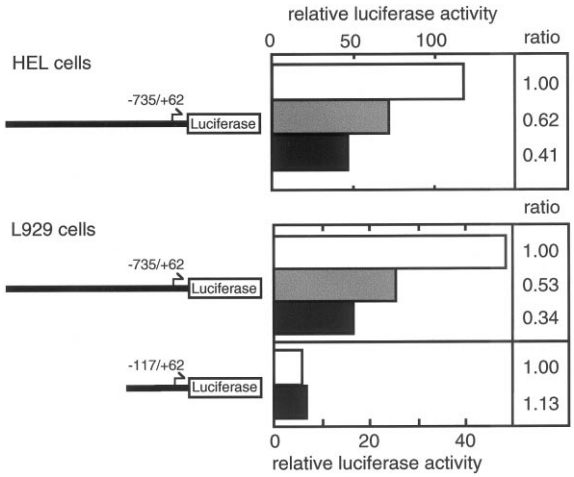

Fig. 1. Inhibition of CMV MIE promoter activity by DTPA. HEL and L929 cells were transiently transfected with 1.5 μg of plasmid containing −735 or −117 bp of the human CMV MIE promoter/firefly luciferase reporter and 0.5 μg of internal control plasmid containing sea pansy luciferase gene. Cells were incubated with 0 (open column), 25 (hatched column) and 50 (closed column) μM DTPA in DME medium containing 5% FCS. Luciferase activities were examined in cell lysates 48 h later. Data are presented as relative values of luciferase activities against activities in untreated cells. Values were normalized against internal controls. This experiment was independently repeated three times and similar results were obtained. Results of one representative experiment are shown.

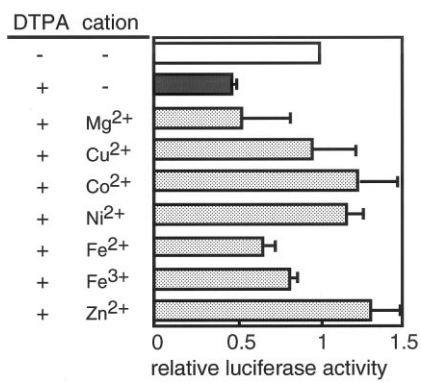

Fig. 2. Effects of various cations on DTPA inhibition of CMV MIE promoter activity. L929 cells were transiently transfected with luciferase reporter plasmid containing CMV MIE promoter and thereupon incubated for 48 h with DTPA (50 μM) and various cations (50 μM) in medium containing 5% FCS. Other experimental conditions were same as those described in the legend to Fig. 1. This experiment was independently repeated three times and similar results were obtained. Results of one representative experiment are shown.

(50 μM)-induced suppression of CMV MIE promoter activity was prevented by zinc (1–100 μM) in a dose-dependent manner (data not shown). Stoichiometric amounts of Fe^{2+} and Fe^{3+} partially prevented the effect of DTPA, and Mg^{2+} did not significantly affect DTPA-induced suppression. Although Mn^{2+} completely prevented the DTPA inhibition of viral replication in human foreskin fibroblasts (Cinatl et al., 1996), Mn^{2+} was cytotoxic under our experimental conditions (data not shown).

3.3. Effects of various cations on DTPA-induced reduction of zinc concentration

Zinc is a trace element that is essential for normal cell growth and metabolism. The DTPA-induced suppression of CMV MIE promoter activity was abrogated by stoichiometric amounts of zinc (Fig. 2). Zinc deficiency is one potential mechanism by which DTPA inhibits CMV MIE promoter activity. In fact, exposing cells to 50-μM DTPA for 24 h promoted a decrease in cellular zinc content of 50% of the control value, and the normal level of cellular zinc was recovered by adding stoichiometric amounts of cations (Fig. 3).

3.4. Activation of CMV MIE enhancer activity by zinc

The results from Figs. 2 and 3 imply that cellular zinc status regulates the activity of the CMV MIE promoter. L929 cells transfected with a reporter plasmid containing the CMV MIE promoter (−735/+62) or its enhancer-less promoter (−117/+62) were incubated with 0–50 μM zinc for 24 h in DME medium containing 2.5% of zinc-free FCS. Fig. 4 shows that the CMV MIE promoter (−735/+62) was up-regulated by zinc in a dose-dependent manner, whereas the enhancer-less CMV promoter was not stimulated.

3.5. Effects of various cations on DTPA-induced reduction of metallothionein concentration

Metallothionein (MT) is a low molecular weight, metal binding protein with a high cysteine

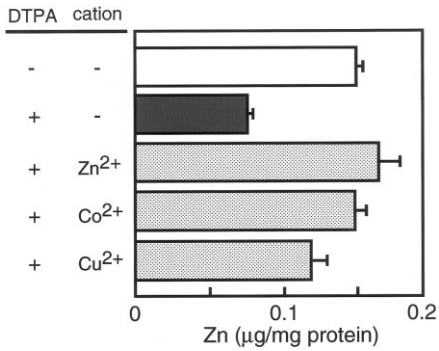

Fig. 3. Effects of various cations on DTPA-induced reduction of zinc concentration. L929 cells were incubated with DTPA (50 μM) and various cations (50 μM) in medium containing 5% FCS for 24 h. Zinc contents in nitric acid-extracts from the cells were assayed by atomic absorption spectrophotometry. Values are expressed as mean ± S.D. of three cultures.

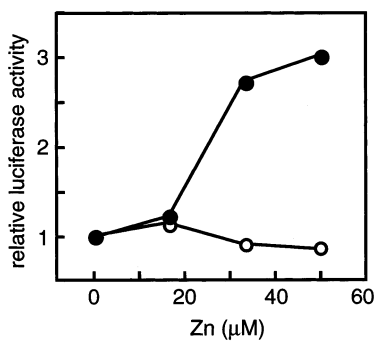

Fig. 4. Activation of CMV MIE promoter activity by zinc. L929 cells were transiently transfected with luciferase reporter plasmid containing CMV MIE promoter, −735/+62 (●) or −117/−62 (○), and then stimulated with zinc (0–50 μM) in medium containing 2.5% zinc-free FCS. Other experimental conditions were the same as those described in the legend to Fig. 1. This experiment was independently repeated three times and similar results were obtained. Results of one representative experiment are shown.

content. The metal-regulated transcription of the MT gene has been thoroughly characterized (Heuchel et al., 1994). The MT promoter is exquisitely controlled by the intracellular zinc concentration (Searle, 1990). L929 cells were treated with 50 μM-DTPA for 24 h, whereupon intracellular MT levels were assayed. The MT content was decreased to 20% of the control level by DTPA (Fig. 5). The DTPA-induced suppression of cellular MT content was recovered by adding stoichiometric amounts of various cations. We examined the relationship between DTPA-induced suppression of CMV MIE promoter activity (Fig. 2) and the cellular MT content (Fig. 5). The MT content was significantly correlated to the CMV MIE promoter activity ($P < 0.001$; $r^2 = 0.914$, determined by Pearson's analysis).

4. Discussion

After primary infection, CMV expresses MIE genes in host cells. The CMV MIE genes are transcribed prior to viral protein synthesis and the products from the MIE genes engender production of both viral and cellular proteins. The transcription of CMV MIE genes is regulated by a large promoter/enhancer complex, which is critical for the expression of all viral gene products (Meier and Stinski, 1996). Therefore, we initially examined the effect of DTPA on the CMV MIE promoter. The results of the present study showed that reducing the zinc availability by chelation with DTPA affected expression of the CMV MIE promoter in fibroblast cultures. Therefore, the DTPA-induced inhibition of CMV replication described by Cinatl et al. (1996) is due to DTPA-induced inhibition of CMV MIE promoter activity. Cells contain about 1 mM Zn^{2+}, most of which is bound and not readily exchangeable. However, a

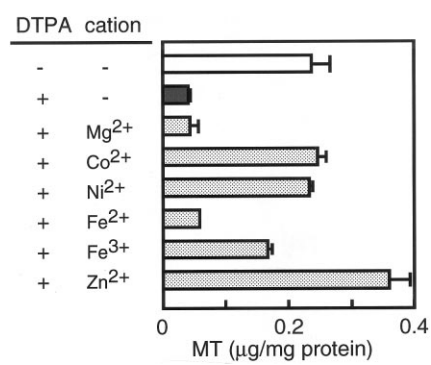

Fig. 5. Effect of cations on DTPA-induced reduction of MT concentration. L929 cells were incubated with DTPA (50 μM) and various cations (50 μM) in medium containing 5% FCS for 48 h, whereupon cellular MT levels were assayed. Other experimental conditions were as shown in the legend to Fig. 3. Values are expressed as mean ± S.D. of three cultures.

pool of less tightly bound Zn^{2+} has been implicated in metabolic regulation (Bettger and O'Dell, 1981) and is increased during signal transduction in G_0 phase fibroblasts, largely by enhanced uptake from the medium (Grummt et al., 1986). Hence zinc is essential for normal growth and metabolism, and an DTPA-induced zinc deficiency leads to the inhibition of CMV MIE promoter activity. Deletion constructs containing enhancer-less CMV MIE promoter ($-117/+62$) were not inhibited by DTPA (Fig. 1), and enhancer-less SV40 promoter (pGV-P2) was also not inhibited by DTPA (data not shown).

Faia et al. have shown that zinc deficiency of bovine pulmonary artery endothelial cells (BPAEC) induced with 25 μM DTPA decreases the amount of MT by 40% compared with the control level (Faia et al., 1996). Our results showed that cellular MT contents are correlated to CMV MIE promoter activity in the presence of DTPA and cations (Figs. 2 and 5). Zinc induces MT transcription (Heuchel et al., 1994) and the molecular mechanism thereof has been documented (Searle, 1990). The MT promoter has metal responsive elements (MREs) that are activated by metal-regulatory transcription factor 1 (MTF-1) bound to zinc (Searle, 1990). In addition to the MT genes, γ-glutamylcysteine synthetase and zinc transporter (ZnT-1) promoters possess MREs (Gunes et al., 1998). The CMV MIE promoter, like the MT gene, is probably controlled under the MRE–MTF-1 system. We did not identify an MRE consensus sequence in the CMV MIE promoter sequence. Therefore, another signaling mechanism (classical MRE-independent regulation of transcriptional activation, such as an unknown MRE- or another responsive element-mediated mechanism) may be involved in this process.

On the other hand, this transactivation is not only via direct signal transduction through an MRE, but may also be mediated by protein synthesis for transactivation. For example, MT appears to play important roles in cellular Zn^{2+} homeostasis (Vallee, 1995). Apometallothionein in cell-free systems can remove zinc atoms from the zinc-finger transcription factor, Sp1, thus inhibiting binding to DNA and its transcription activator function (Zeng et al., 1991). Conversely, MT can also donate zinc to nuclear proteins (Maret et al., 1997). Chattopadhyay and Hedley (1998) reported that T3-induced gene expression is affected by DTPA and that MT may be involved in this effect, but no precise basis for this speculation was described. There are some consensus sequences for transcription factors such as Sp1, YY1 and Gfi-1 that have zinc finger domains (Meier and Stinski, 1996). Sp1 is a stimulator, Gfi-1 is a repressor and YY1 is both (Meier and Stinski, 1996). Sp1 binding site was within the enhancer-less CMV MIE promoter, which was not affected by DTPA and zinc. Therefore, the notion that these zinc finger-containing transcription factors are mainly involved in activation of the CMV MIE promoter by zinc remains tentative. Another transactivation mechanism by MT has been proposed — MT may directly interact with NF-κB (Abdel-Mageed and Agrawal, 1998). The aforementioned examples indicate that modulation of a transcription factor is mediated by MT (Zeng et al., 1991; Maret et al., 1997; Chattopadhyay and Freake, 1998). Therefore, further studies are necessary to define the relationship between the effect of zinc on the CMV MIE promoter and MT.

The CMV MIE promoter is widely recognized as being constitutively active and unregulated. However, variation in transcriptional activity of the CMV MIE promoter depending on the cell type and developmental age has been reported (Baskar et al., 1996). In addition, the CMV MIE promoter may be up-regulated under specific conditions. NF-κB plays a central role in the activation of the CMV MIE promoter when cells are stimulated with phorbol esters and CMV gene products (Sambucetti et al., 1989). Moreover, DTPA was reported to inhibit the function of NF-κB (Scholz et al., 1997). Activation of mitogen-activated protein kinases by sodium arsenite would activate the CMV MIE promoter (Boom et al., 1988; Bruening et al., 1998). Changing the cellular zinc content by various protocols may be difficult to interpret due to regulation of the CMV MIE promoter as well as the stress response (Bruening et al., 1998). The CMV MIE promoter is up-regulated by cAMP in lymphoid cell lines

(Stamminger et al., 1990), a process that is mediated through a cAMP response element within the enhancer.

Ganciclovir, phosphonoformate and cidofovir have been used to treat CMV infections (Singer et al., 1985; Laskin et al., 1987; Walmsley et al., 1988; De Clercq, 1993, 1996). However, prolonged use of ganciclovir and phosphonoformate may be associated with serious side effects. To overcome these effects, other drugs with a different mechanism of action, such as the metal chelator, DTPA, or derivatives thereof may be candidates for this purpose (Cinatl et al., 1996). It has been reported that DTPA and DFO exhibit a significant antiviral effect in vitro, but in vivo use of these metal chelators for the treatment of acute CMV infections has not been pursued (Kloover et al., 1999).

To the best of our knowledge, it has not been described that metals regulate virus promoters. The results of the present study indicate a novel approach to develop new anti-viral drugs. Our findings are indicative for the role of zinc in regulating CMV replication and CMV promoter-driven expression. The CMV MIE promoter is widely applied as a regulatory element in somatic gene therapy and as an experimental tool in biological sciences. Further studies are necessary to elucidate the role of MT under conditions of metal depletion and repletion to better understand the role of zinc in CMV promoter activity in vitro.

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