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Mycophenolic Acid Inhibits Syncytium Formation Accompanied by Reduction of gp120 Expression

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Abstract Mycophenolic acid (MPA) was identified as an inhibitor of syncytium formation during the screening of human immunodeficiency virus (HIV) entry inhibitors. MPA is a well-known inhibitor of inosine monophosphate dehydrogenase and anti-HIV activity has been reported *in vitro* and *in vivo*. MPA inhibited syncytium formation in T cell-tropic and macrophage-tropic systems with IC_{50} values of 0.1 and 0.5 μM, respectively. The reduction of HIV gp120 expression by MPA (1.0 μM) was observed by use of Western blot analysis. Furthermore, the addition of guanosine restored both syncytium formation and gp120 expression in the presence of MPA. These results suggest that MPA inhibits not only reverse transcription by depletion of a substrate, GTP, as has been reported, but also syncytium formation through a predominant reduction in the amount of gp120 that is vigorously expressed in the above transformed cells and may be in HIV-infected cells.

Keywords AIDS, HIV, fusion, gp120, mycophenolic acid, syncytium formation

Introduction

During the course of screening for new inhibitors against HIV entry to cells using a syncytium formation assay with HIV *env*-expressing cells and HIV receptor-expressing cells [1], a syncytium formation inhibitor isolated from a culture filtrate of *Penicillium* sp. FO-8017 was identified as mycophenolic acid (MPA, Fig. 1). MPA is well-known as an inosine monophosphate dehydrogenase (IMPDH) inhibitor that blocks the conversion of inosine monophosphate (IMP) to GMP. Mycophenolate mophetil, an ester derivative of MPA, is used clinically as an immunosuppressive agent in organ transplantation [2~4]. Its anti-HIV activity has also been reported in individual use [5, 6] and in combination with reverse-transcriptase (RTase) inhibitors [7~9]. In fact, the combination of abacavir and MPA exhibits profound and synergistic anti-HIV activity. Two mechanisms have been proposed for the effect of MPA on HIV/AIDS. One is that MPA enhances the inhibition of HIV RTase by abacavir through depletion of the substrate GTP by the inhibition of IMPDH. Another is that MPA induces a reduction in the pool of HIV target cells, $CD4^+$ T lymphocytes, which do

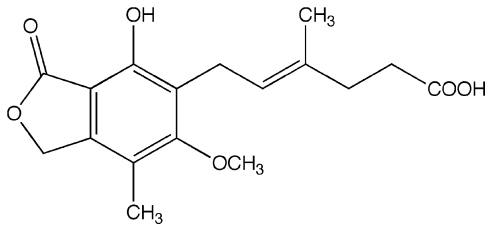

Fig. 1 Structure of mycophenolic acid.

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not have the salvage pathway for guanosine nucleotide and thus are sensitive to inhibition of IMPDH. However, neither mechanism can fully explain the inhibition of the syncytium formation assay system. In this article, we propose another mechanism for anti-HIV activity of MPA–that MPA inhibits highly enhanced synthesis of viral proteins such as gp120 through the inhibition of IMPDH to occur the inhibition of syncytium formation and HIV entry to cells.

Materials and Methods

Chemicals

Mycophenolic acid was purchased from Wako Chemicals, Osaka, Japan.

Cells

HeLa/T-*env*/Tat, HeLa/CD4/LacZ, HeLa/M-*env*/Tat, and HOS/CD4/CCR5/LacZ cells, established as described previously [1], were used. All the above cell lines were grown in Dulbecco's modified Eagle's medium (Gibco-BRL, Grand Island, NY) supplemented with 10% fetal bovine serum (Intergen, Purchase, NY) and 100 μg/ml kanamycin sulfate (Meiji Seika, Tokyo, Japan).

Reporter Gene Activation-mediated Syncytium Formation Assay

The assay was performed as described previously [1]. In the assay system, multinuclear giant cells (syncytia) were formed by fusion between *env*-expressing cells and receptor-expressing cells. Accompanied by the fusion, the *trans*-activation protein (Tat) expressed in the *env*-expressing cells enhances the expression of *Lac-Z* gene that is regulated with HIV-1 long-terminal repeat. HeLa/CD4/LacZ or HOS/CD4/CCR5/LacZ cells, a sample solution, and then HeLa/T-*env*/Tat or HeLa/M-*env*/Tat cells were put into each well of a 96-well microplate, respectively. They were cocultured under 5% CO_2 at 37°C overnight. After the culture medium were removed, the cells were lysed with 20 μl of 0.05% Tween 20. The lysate was mixed with 80 μl of a buffer (Na_2HPO_4 60 mM, NaH_2PO_4 40 mM, KCl 10 mM, $MgSO_4 \cdot 7H_2O$ 1 mM, and β-mercaptoethanol 50 mM) and 20 μl of 0.5 mg/ml *O*-nitrophenyl-β-D-galactopyranoside and then incubated for 80 minutes at 37°C. The reaction was stopped with 50 μl of 2.0 M Na_2CO_3, and the absorbance at 405 nm was measured with a microplate photometer (Bio-Tek Instruments, Power Wave X 340).

Cytotoxicity Assay

The number of viable cells was determined by the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) colorimetric assay [1, 10].

Microscopic Analysis for β-Galactosidase Expression Associated with Syncytium Formation

HeLa/CD4/LacZ cells, a sample solution, and HeLa/T-*env*/Tat cells were put into 6-cm dishes. They were cocultured under 5% CO_2 at 37°C overnight. After the cells were washed with PBS(−) three times, they were fixed with 1% formaldehyde for 5 minutes at room temperature. They were washed with PBS(−) again and were stained with 400 μg/ml of 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) (Takara, Otsu, Japan) at 37°C for 20 minutes. Stained syncytia were observed microscopically.

Western Blot Analysis

To detect β-galactosidase or gp120 expression, HeLa/T-*env*/Tat and HeLa/CD4/LacZ cells were cocultured in a 6-well culture dish for 18 hours. The cells were washed with PBS(−), collected with a scraper, and centrifuged at 1,000×*g* for 10 minutes at 4°C. The cells were resuspended in lysis buffer (1% Triton X-114, 150 mM NaCl, and 50 mM Tris-HCl [pH 8.0]) and placed in an ice bath for 30 minutes. The cell lysate was centrifuged at 10,000×*g* for 10 minutes at 4°C; then, supernatant fluid was subjected to SDS-polyacrylamide gel electrophoresis. Proteins in the gel were electrophoretically transfected to a polyvinylidene difluoride membrane (Biocraft, Tokyo, Japan) using a semidry blotting apparatus (Biocraft, Tokyo, Japan). The membrane was immersed with 1% blocking reagent (Roche, Penzberg, Germany) for 18 hours at 4°C and washed three times with PBS(−) that contained 0.5% Tween-20 (PBS-T). For β-galactosidase detection, the membrane was incubated with horseradish peroxidase-conjugated anti-β-galactosidase polyclonal antibody (Rockland, Gilbertsville, PA) diluted to 1 : 1000. To detect gp120, a goat HIV-1 gp120 polyclonal antibody (Virostat, Portland, ME) diluted to 1 : 1000, followed by horseradish peroxidase-conjugated rabbit anti-goat immunoglobulin G (Bethyl Laboratories, Montgomery, TX) diluted to 1 : 2000 were used to detect gp120. The membrane was washed with PBS-T three times and rinsed with horseradish peroxidase buffer that contained 667 μg of 3,3′-diaminobenzidine (Sigma-Aldrich, St. Louis, MO) per ml and 0.06% of H_2O_2.

Results

Identification of MPA as an Inhibitor of Syncytium Formation

In our screening program for new inhibitors against HIV entry to cells using a syncytium formation assay system, *Penicillium* sp. FO-8017, isolated from a soil sample at the Kitasato Institute, was found to produce an inhibitor of syncytium formation. The inhibitor was isolated from the cultured broth and then identified as MPA (Fig. 1) through mass and NMR analyses (data not shown).

Inhibition of Syncytium Formation by MPA and its Reversion by Guanosine

MPA inhibited the syncytium formation in a dose-dependent manner (Fig. 2). The IC_{50} values for syncytium formation were 0.1 and 0.5 μM for T-tropic and M-tropic systems, respectively, whereas MPA did not show cytotoxic activity up to 300 μM in the above system.

Because MPA is known to be an IMPDH inhibitor that blocks conversion of IMP to GMP, we examined the effect of guanosine on the inhibition of syncytium formation by MPA. As shown in Fig. 3, the addition of guanosine with MPA (3.1 μM) restored the syncytium formation in a dose-dependent manner. The inhibition was completely cancelled by the addition of 353 μM guanosine.

These results were confirmed by microscopic observation after X-gal staining of fixed cells treated with MPA (Figs. 4A and B)—syncytium formation was quantified with β-galactosidase activity of cell lysate in our assay system. The addition of 3.1 μM MPA reduced the blue product from X-gal by β-galactosidase.

The reduction of the blue product by the addition of MPA was also reversed to control levels by the addition of 353 μM guanosine (Fig. 4C). On the other hand, MPA did not have any effect on β-galactosidase activity up to 100 μM (data not shown). MPA, therefore, is considered to

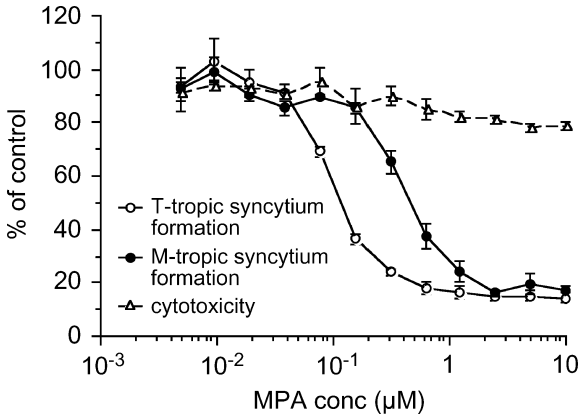

Fig. 2 Effect of MPA of syncytium formation and cell viability. Inhibition of syncytium formation is displayed as percent against β-galactosidase activity of drug-free control cells. Cytotoxicity is also shown in the same. Error bars indicate±S.D. (n=3).

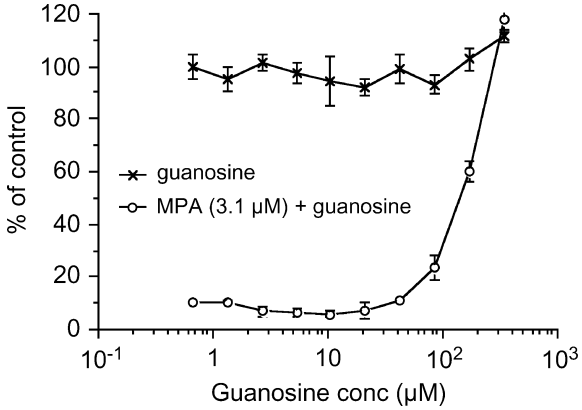

Fig. 3 Reversal of the inhibition of syncytium formation by MPA. Indicated guanosine was added in the presence (3.1 μM) or absence of MPA. Inhibition of syncytium formation is displayed as percent against β-galactosidase activity of drug-free control cells. Error bars indicate±S.D. (n=3).

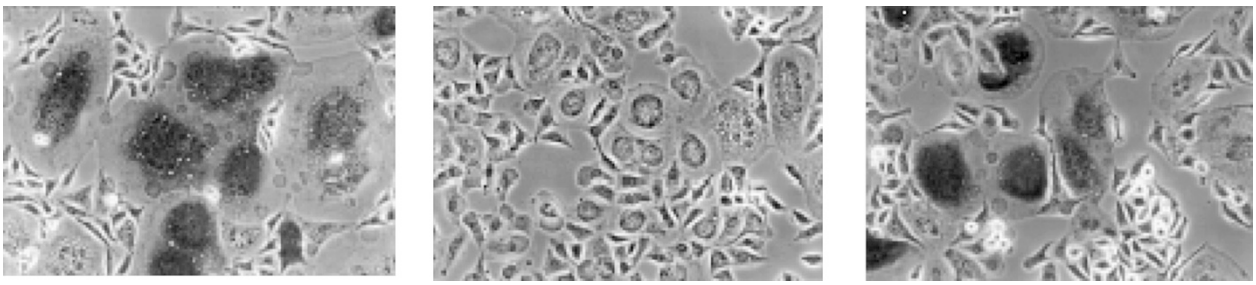

Fig. 4 Effects of MPA and guanosine on the syncytium formation in microscopic analysis. Cocultured HeLa/T-*env*/Tat cells and HeLa/CD4/LacZ cells were stained with X-gal. Beta-galactosidase expressing cells that formed syncytium were stained in blue.

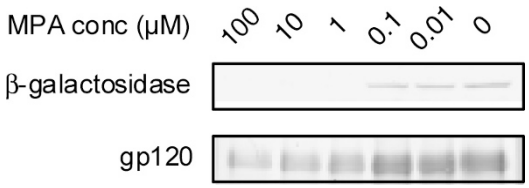

Fig. 5 Inhibition of the expression of β-galactosidase and gp120 by MPA. Cell lysate was prepared after co-cultivation of HeLa/T-*env*/Tat cells and HeLa/CD4/LacZ cells in the indicated concentrations of MPA. Expression of β-galactosidase and gp120 were verified with Western blot analysis.

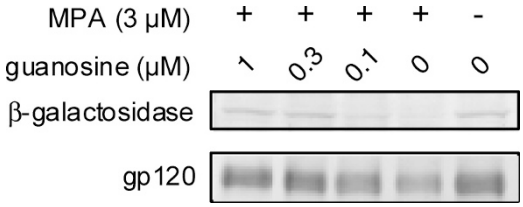

Fig. 6 Reversal by guanosine of the inhibition of β-galactosidase and gp120 expression with MPA. Cell lysate was prepared after co-cultivation of HeLa/T-*env*/Tat cells and HeLa/CD4/LacZ cells in the indicated conditions. Expression of β-galactosidase and gp120 were verified with Western blot analysis.

inhibit the expression of β-galactosidase that is caused by the fusion between *env*-expressing and receptor-expressing cells.

Inhibition of the Expression of gp120 and β-Galactosidase by MPA and Its Reversion by Guanosine

The expression of gp120 and β-galactosidase in syncytia formed from HeLa/T-*env*/Tat and HeLa/CD4/LacZ cells was checked by Western blot analysis. MPA repressed the expression of both proteins in a dose-dependent manner (Fig. 5), and their repression was reversed by addition of guanosine (Fig. 6). On the other hand, no remarkable changes in the amounts of other proteins were observed in the gel staining with Coomassie Brilliant Blue or in the protein quantification of cell lysates even at 10 μM MPA (data not shown).

Discussion

As our results indicate, MPA caused the inhibition of syncytium formation associated with β-galactosidase expression through the inhibition of gp120 expression. It is quite obvious that all of these phenomena derive from the blockade of IMPDH by MPA, given that all of the effects caused by MPA were reversed in the presence of guanosine.

On the other hand, it has been reported that MPA suppresses HIV replication *in vitro* and *in vivo* [5, 6]. The anti-HIV activity of MPA is thought to occur by two mechanisms. One is blocking of RTase activity by depleting its essential substrate, guanosine nucleotides. The depletion of intracellular deoxyguanosine triphosphate (dGTP) enhances anti-HIV-1 activities of nucleoside-type RTase inhibitors [7～9]. Another is a reduction in the pool of activated $CD4^+$ T lymphocytes [6]. The inhibition of IMPDH by MPA limits the rate of *de novo* synthesis of guanosine nucleotides in lymphocytes, which lack salvage metabolism of nucleotides. Such reduction in T cells, which are one of the major cellular targets of HIV, causes the decrease in productive HIV infection.

However, our syncytium formation system, which was inhibited by MPA (IC_{50}: 0.1 and 0.5 μM in T cell-tropic and macrophage-tropic systems, respectively), is not associated with RTase and lymphocyte growth. MPA has also been reported to be an inhibitor of additional reactions of the glycoside chain to proteins [11～13]. But the amounts of almost other proteins were not changed remarkably by MPA (10 μM) in our syncytium formation assay system (data not shown). MPA is a specific inhibitor of IMPDH, and the inhibition of IMPDH causes a depletion of guanosine nucleotides, which is followed by the inhibition of RNA and DNA syntheses requiring GTP or dGTP as a substrate, protein synthesis requiring GTP as an activating factor, and saccharide chain synthesis requiring GDP as a component of intermediates. As a result, MPA induces various effects such as immunosuppressive [2～4], antiviral [5～9], and antitumor [14～16] activities.

In the syncytium formation assay system, multinuclear giant cells (syncytia) were formed by fusion between *env*-expressing cells (HeLa/T-*env*/Tat, and HeLa/M-*env*/Tat) and receptor-expressing cells. The *env*-expression is enhanced by the cytomegalovirus promoter. Beta-Galactosidase expression followed by fusion is also enhanced by Tat from the *env*-expressing cells and the HIV-1 long-terminal repeat from the receptor-expressing cells. The expression of both gp120 and β-galactosidase is highly enhanced in the system. As a consequence, the enhanced expression is predominantly inhibited in the presence of a relatively low concentration of MPA, but expression of the other proteins is not reduced, as was described above. This could be because transcription, protein synthesis, and sugar chain synthesis are highly enhanced in the infection and proliferation of HIV, so they would be predominantly inhibited. The inhibition seems to contribute to anti-HIV

activity of MPA in addition to the above-mentioned two mechanisms that have been reported.

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