

## **In vitro effect of cyclosporine A, mitomycin C and prednisolone on cell kinetics in cultured human umbilical vein endothelial cells.**

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**Abstract:** *Introduction:* Vascular endothelial cell damage plays an important role in microvascular thrombogenesis. In vivo administration of cyclosporin A or mitomycin C sometimes results in thrombotic microangiopathy in patients. *Materials and methods:* The effects of cyclosporin A, mitomycin C and/or prednisolone on the cell cycle in cultured human umbilical vein endothelial cells were investigated to evaluate drug-induced endothelial cell damage and the protective effect of prednisolone on endothelial cells against the damage by cyclosporin A or mitomycin C in vitro. *Results:* The addition of cyclosporin A to cultures caused proliferation arrest in the G1-phase in a dose-dependent manner, while mitomycin C inhibited DNA synthesis, which resulted in cell cycle arrest and inhibition of BrdUrd incorporation in the S-phase. The administration of prednisolone also caused cell cycle arrest in the G1 by itself, and protected the cells from the damage caused by mitomycin C. The inhibitory effects of cyclosporin A and prednisolone on the cell cycle were reversible, while mitomycin C was not. The highly phosphorylated retinoblastoma protein expressed in human umbilical vein endothelial cells decreased in the presence of mitomycin C. Soluble thrombomodulin levels in the culture supernatant were elevated by the addition of cyclosporin A. *Conclusion:* These effects of the drugs may cause the cell cycle arrest and the prolonged repair of damaged endothelial cells in patients. © 2004 Elsevier Ltd. All rights reserved.

**KEYWORDS:** Human umbilical vein endothelial cells; Endothelial cell damage; Cell cycle; Cyclosporin A; Mitomycin C; Prednisolone.

**Abbreviations:** CsA, cyclosporin A; GVHD, graft-versus-host disease; BMT, bone marrow transplantation; TTP, thrombotic thrombocytopenic purpura; HUS, hemolytic—uremic syndrome; TMA, thrombotic microangiopathy; MMC, mitomycin C; PSL, prednisolone; EC, endothelial cell(s); HUVEC, human umbilical vein endothelial cells; TM, soluble thrombomodulin; vWf:Ag, von Willebrand factor antigen; hrs., hours; min., minutes; PBS, phosphate-buffered saline; RT, room temperature; CDK4, cyclin-dependent kinase-4; Rb, retinoblastoma protein; TBS, Tris-buffered saline; LDH, lactate dehydrogenase; ELISA, enzyme-linked immunosorbent assay; ANOVA, analysis of variance; pRb, underphosphorylated Rb; ppRb, highly phosphorylated Rb.

### **Introduction**

Cyclosporin A (CsA) [1] is one of the immunosuppressive agents widely used to prevent graft-versus-host disease (GVHD) in patients treated with bone marrow transplantation (BMT). However, the administration of CsA is frequently accompanied by a series of adverse vascular effects in some patients, and the incidence is high at a therapeutic dose of CsA for GVHD [2]. The vascular effects were reported to be thrombotic thrombocytopenic purpura (TTP)/adult/ sporadic hemolytic—uremic syndrome (HUS)-like microangiopathy in patients treated with CsA. It

is also accompanied by acute GVHD [3]-associated or BMT-associated thrombotic microangiopathy (TMA) [4,5]. Subclinical and clinical TMA is detectable in 74% of patients receiving CsA prophylaxis [3]. The development of these syndromes shows high mortality, and hemorrhagic complications are sometimes encountered, such as diffuse alveolar hemorrhage [6,7].

Mitomycin C (MMC) is used for the treatment of neoplasms of the gastrointestinal tract and breast [8]. MMC is bioactivated by a number of enzyme systems to a potent, bifunctional alkylat-

ing species that is capable of cross-linking DNA [9,10]. TTP/HUS-like syndromes (microangiopathic hemolytic anemia, thrombocytopenia, and renal insufficiency) have been reported as toxic responses to MMC [11–13]. Patients who develop these syndromes also have high mortality [14], often related to the effects of uremia or hemorrhagic complications.

Prednisolone (PSL) is also widely used for severe GVHD in BMT, although its effects on vascular endothelial cells (EC) are still unclear.

It is well known that EC damage causes thrombogenesis. Recently, it has been reported that the plasma obtained from patients with TTP or HUS induces apoptosis in a restricted lineage of human microvascular endothelial cells in vitro [15]. This report indicates that EC damage may closely participate in these diseases.

We and other researchers reporting the problem in a literature sometimes encounter patients with TMA like TTP/HUS, which is possibly induced by the pathological state of immunological activation or some other unknown cause (probably infection, chemotherapy, radiation, and immunosuppressants like CsA) after BMT or by MMC after cancer chemotherapy. However, the mechanism and effect of the drugs on vascular EC are not well understood. Our hypothesis is that the putative effects of the drugs on vascular EC are direct injury of the cell surface and an indirect effect of prolonged repair of the cells by cell cycle arrest. We then studied the effects of CsA, MMC and PSL on the cell kinetics in cultured human umbilical vein endothelial cells (HUVEC), as well as released soluble thrombomodulin (TM) and von Willebrand factor antigen (vWf:Ag) from the cells [16–19] which are often referred to as markers of EC damage. We also studied the additive effect of PSL on the adverse effects of CsA and MMC in the cells.

## Materials and methods

### *EC cultures*

HUVEC (Clonetics, Walkersville, MD) were grown on 90 mm diameter plastic dishes precoated with  $3.0 \times 10^{-3}\%$  of bovine type I collagen (Sumitomo, Osaka, Japan) in MCDB131 medium (Sigma, St. Louis, MO) containing 20 mM HEPES-NaOH (pH7.4), 5% fetal bovine serum (GIBCO, Grand Island, NY), 10 ng/ml recombinant human basic fibroblast growth factor (R&D Systems, Minneapolis, MN), 10 U/ml heparin and penicillin streptomycin mixture (Bio Whittaker, Walkersville, MD). All experiments

were performed using preconfluent stage cells prepared as described below. CsA (containing polyoxyethylene castor oil and ethanol, Novartis, Basel, Switzerland), MMC (Sigma) and prednisolone sodium succinate (PSL, Shionogi, Osaka, Japan) were added to the medium at the following four concentrations: CsA at 0.1, 1, 5 or 10  $\mu\text{g/ml}$ ; MMC at 0.05, 0.2, 1 or 3  $\mu\text{g/ml}$ ; and PSL at 30, 100, 300 or 600  $\mu\text{g/ml}$ .

### *EC preparation*

First, HUVEC were grown as described above and fourth passage confluent cell cultures were started in new, collagen-coated dishes with  $4$  to  $5 \times 10^5$  cells/ dish. Trypsin—EDTA mixture (0.05% and 0.02% in PBS, respectively, Sigma) was used to detach the cells from the dishes during the passages. Twelve hours (hrs.) after the passage, all media in the dishes were changed and CsA, MMC and/or PSL were immediately added to the media to obtain each concentration of the drugs. The same volume of physiological saline was added as control. Twenty-four hours after the addition of the drugs, the culture media were harvested and the dishes with attached cells were washed twice with Hanks' balanced salt solution (HBSS, Sigma). Then the cells were detached from the dishes using the trypsin—EDTA mixture. The cells were suspended in HBSS containing 10% FBS and used as samples after the cell count of each dish.

### *Flow cytometry*

#### *DNA/RNA quantification (7AAD/PY)*

The kinetics of the cultured cells was analyzed using 7-amino-actinomycin D (7AAD, Sigma) and pyronin Y (PY, Polysciences, Warrington, PA) as described previously [20]. In brief, after washing the single cells once in a suspension with IFA buffer (10 mM/l HEPES buffer in 0.15 M NaCl supplemented with 0.1%  $\text{NaN}_3$  and 4% FBS), up to about  $1 \times 10^6$  cells were suspended in 1 ml of nucleic acid staining solution (NASS: 0.15 M NaCl in 0.1 M phosphate/citrate buffer containing 5 mM sodium EDTA (Sigma) and 0.5% bovine serum albumin (Sigma; BSA, fraction V), pH 6.0) containing 0.2% saponin (from quillaja bark, Sigma), then 50  $\mu\text{l}$  of a 400  $\mu\text{M}$  7AAD solution (final 20  $\mu\text{M}$ ) was added and the cells were incubated at RT for 30 minutes (min.). After incubation with the DNA dye, the cells were cooled on ice for at least 5 min., then 50  $\mu\text{l}$  of a 100  $\mu\text{M}$  PY solution (final 5  $\mu\text{M}$ ) was added to stain RNA. The cell suspension was

incubated for an additional 10 min. on ice and analyzed.

Cell fluorescence was analyzed with a FACScan flow cytometer (Becton-Dickinson, Mountain View, CA). The cells were excited with a single, 488 nm argon laser, analyzed for simultaneous orange (FL2; 570 nm) and red (FL3; N650 nm) fluorescence emission, and 10,000 events were collected. The data were analyzed using Lysis II™ and CellFit™ software (Becton-Dickinson).

#### *Analysis of DNA synthesis (BrdUrd incorporation analysis)*

Simultaneous analysis of DNA quantification and incorporated BrdUrd was utilized to estimate the actual DNA synthesis using 7AAD and anti-BrdUrd antibody after minor modification as described previously [21]. Sixty minutes before the harvest of the cells, 5% (v/v) of 0.4 mM bromodeoxyuridine (BrdUrd, Sigma, final 20  $\mu$ M) and 5% (v/v) of 0.04 mM FdUrd (Sigma, final 2  $\mu$ M, control culture did not contain BrdUrd) were added and incubated for 60 min., then the cells were harvested. After washing the cells with working buffer (WB: phosphate-buffered saline (PBS) containing 0.1% sodium azide, 1% human serum albumin, and 0.15% sodium citrate), they were resuspended in 100  $\mu$ l of natural saline on ice. The cells were fixed with 5 ml of ice-cold 70% ethanol in a 15 ml tube, incubated on ice for 30 min., pelleted, and washed once with PBS. The cell pellet was suspended in 1 ml of 4N HCl, and incubated for 30 min. at room temperature (RT). The cells were pelleted and resuspended in 1 ml of 0.1 M  $\text{Na}_2\text{B}_4\text{O}_7$  (pH 8.5), incubated for 5 min. at RT, pelleted, and resuspended in 1 ml of 0.5% Triton X100 (Sigma) in PBS, and incubated for 3 min. at RT. After washing once with 5 ml of WB, the fixed cells were stained with FITC-conjugated anti-BrdUrd antibody (Becton-Dickinson) or control IgG1 in the dark for 30 min. at RT. They were then washed with PBS and resuspended in 1 ml of PBS, 7AAD (final 20  $\mu$ M) and 0.1 ml RNase A (Sigma, final 1 mg/ml) were added, and then incubated in the dark for 30 min. at RT, followed by analysis with a flow cytometer. The cells were analyzed for simultaneous green (FL1) and red (FL3) fluorescence emission, and 10,000 events were collected.

#### *Western blotting analysis for cyclin-dependent kinase (CDK) 4 and retinoblastoma protein (Rb)*

To understand the molecular events in the G1-checkpoint, the expressions of cyclin-dependent kinase-4 (CDK4) and retinoblastoma protein (Rb) were analyzed by Western blotting. Rabbit anti-human CDK4 antibody and mouse anti-human retinoblastoma protein (Rb) monoclonal antibody (clone G3-245, IgG1) were purchased from PharMingen (San Diego, CA).

After removal of the conditioned medium, the cells were washed twice with pre-warmed PBS, and then harvested. Cell lysates from HUVEC were made from the cells ( $2.0 - 3.4 \times 10^6$  cells) by incubation with 400  $\mu$ l lysis buffer [50 mM Tris—HCl (Sigma), 0.15 M NaCl, 0.5% Triton X-100, and 1 mM Benzamidine (Sigma)] for 1 hr. at 4°C. The lysate was collected and cell debris was removed by centrifugation at 12,000 x g for 20 min. The supernatant was used for the following analysis.

Protein assay protocol was done using a DC protein assay kit (Bio-Rad, Hercules, CA). Equivalent volumes of the lysates and 2 x sample buffer containing 2-mercaptoethanol were mixed, and used as samples. SDS-PAGE was performed by the method of Laemmli [22] using a 7.5% polyacrylamide gel. Western blotting was performed using a modification of the method of Beardsley et al. [23]. The blots were electrophoretically transferred to poly-vinylidene difluoride (PVDF, Immobilon-P, Millipore, Bedford, MA) blotting membranes. Nonspecific binding was blocked with 5% low-fat milk powder in Trisbuffered saline (TBS: Tris 20 mM, NaCl 137 mM, pH 7.5) (blocking buffer). After extensive washing with TBS, the immunoblots were incubated overnight with the antibodies (1:500 dilution) in the blocking buffer at 4°C, then washed a further three times with TBS-T (0.05% Triton X-100 in TBS). Antibody binding was detected using horseradish-peroxidase-conjugated sheep anti-mouse IgG or goat anti-rabbit IgG (Amersham, Little Chalfont, UK) and visualized with ECL chemiluminescence reaction reagents and ECL-hyperfilm (Amersham).

#### *Assays for procoagulant activities*

Besides the cell cycle analysis, TM and vWf:Ag levels in the culture supernatant were measured to estimate the functional cellular damage of HUVEC induced by these drugs in vitro. Lactate dehydrogenase (LDH) level in the culture supernatant arisen from dead cells was also measured to estimate the direct cytotoxic

effect of the drugs [24]. The harvested culture media were centrifuged at 3000 rpm for 15 min. and stored at  $-80^{\circ}\text{C}$  to serve as culture supernatants. TM was measured by an enzyme-linked immunosorbent assay (ELISA) (Mitsubishi Gas Chemical, Tokyo, Japan) [25]. vWf:Ag was measured by ELISA (Diagnostica Stago, France). LDH was measured by an ultraviolet method. Each antigen level in the culture supernatant was calculated as percent of antigen compared with the control.

#### *Statistical analysis*

Two-way analysis of variance (ANOVA) was used for testing two by two categories of two combined drugs. One-way ANOVA and post hoc Dunnett test was used to estimate the differences between various concentrations of MMC and control. Differences between the groups were considered significant at a P value of  $<0.05$ .

### **Results**

#### *Cell kinetics analysis by 7AAD/PY*

Representative RNA/DNA dot plots are shown in Fig. 1, and a summary is shown in Figs. 2—5. CsA and PSL induced G1-arrest especially in a high concentration medium, i.e. G1 (%) increased and S (%) decreased. Combined effect of CsA and PSL also showed increased G1-arrest (not significant). (Figs. 1B,E, 2 and 3). MMC functions as a DNA polymerase-inhibitor. Lower concentrations of MMC inhibited DNA synthesis at the late S-phase, while higher concentrations of MMC inhibited DNA synthesis at the early S-phase (Fig. 1C and D). G1 (%) decreased and S (%) increased as a result of S-arrest by MMC (Figs. 2 and 3). The level of the measured G1 (%) in the presence of the higher concentrations of MMC seemed to be higher than that in the presence of the lower concentrations of MMC as a result of the possible admixture of the very early S-phase cells into G1 calculated by a fit program of cell cycle analysis (Fig. 3). Measured RNA fluorescence of the S-phase cells by PY increased in the presence of lower concentrations of MMC and decreased in the presence of higher concentrations of the drug as a result of later and earlier S-arrest, respectively (data not shown).

PSL protected HUVEC from the toxic effect of MMC by inhibiting the progression of the cells into the S-phase, while CsA did not (Figs. 1F and 4).

In two-way ANOVA, MMC significantly decreased G1, and increased S. PSL significantly

increased G1, and decreased S. The interaction between the effects of MMC and PSL was significant in the S phase (Tables 1 and 2). Accordingly, the simultaneous use of MMC and PSL diminished the effect of MMC in the S phase and reinforced the effect of PSL. Concerning MMC adding to CsA or PSL, both MMC and CsA/PSL had significant effect on each phase (Fig. 4, Table 3).

To estimate the reversibility of the drug effects, the culture media were completely changed after the 24-hrs. in the presence of the drugs, and an additional 48-hr. culture was carried out in the absence of the drugs (Fig. 5). The effects of CsA or PSL were basically reversible (compare with Fig. 3). The cells released from the effect of higher concentrations of CsA showed decreased G1 (%) and increased S (%) as a result of emancipation from cell cycle synchronization in the G1. The cells released from the effect of higher concentrations of PSL also showed decreased G1 (%). In contrast, the effect of MMC was basically irreversible. The S-arrested cells by MMC were still captured in the S-phase even after a 48-hr. washout of the drug (Fig. 5 compared with Fig. 3).

#### *BrdUrd incorporation in MMC-treated HUVEC*

To estimate the actual DNA synthesis, a BrdUrd incorporation assay was carried out three times. The incorporated BrdUrd-fluorescence intensity of the cells in the S-phase decreased with the addition of MMC in a dose-dependent manner (Fig. 6). The measured percents of BrdUrd-positive cells were as follows: control culture,  $31.1 \pm 5.3\%$ ;  $0.05 \mu\text{g/ml}$  MMC,  $52.5 \pm 8.0\%$ ;  $0.2 \mu\text{g/ml}$  MMC,  $48.5 \pm 15.2\%$ ;  $1.0 \mu\text{g/ml}$  MMC,  $22.5 \pm 9.0\%$ , and  $3.0 \mu\text{g/ml}$  MMC,  $5.8 \pm 0.8\%$ . BrdUrd (%) increased in the presence of lower concentrations of MMC as a result of S-arrest, and the amount of incorporated BrdUrd in each cell (BrdUrd fluorescence intensity) decreased as a direct effect of MMC as a DNA polymerase inhibitor. In contrast, BrdUrd (%) decreased and the BrdUrd fluorescence almost disappeared in the presence of higher concentrations of MMC; therefore, the apparent S-phase cells did not synthesize DNA.

#### *Western blotting*

No remarkable alterations of the CDK4 expression were observed in each culture condition (Fig. 7). The monoclonal antibody against human Rb protein used in this study stains

underphosphorylated Rb (pRb) and highly phosphorylated Rb (ppRb) as different bands as shown in Fig. 7. The expression of ppRb was reduced by the addition of CsA. Significant reduction of Rb expression was observed only at a highest concentration of PSL, while a notable reduction of ppRb was observed in the presence of 600 µg/ml of PSL. The expression of ppRb was decreased by the addition of MMC in a dose-dependent manner.

#### *Levels of TM, vWf:Ag and LDH in the culture supernatant*

The levels of TM and LDH in the culture supernatant are shown in Fig. 8. The TM antigen level in the supernatant was elevated in the presence of CsA in a dose-dependent manner ( $p < 0.05$  at 10 µg/ml of CsA), while no elevations of the TM level were observed in the presence of MMC or PSL. None of the tested drugs influenced the vWf:Ag level in this study (data not shown). A high dose of MMC at 3 µg/ml caused an increased level of LDH (not significant), while PSL administration decreased the LDH levels at 100 and 600 µg/ml (not significant).

#### **Discussion**

The study presented in this article showed the mechanism of endothelial damage by CsA, MMC and high doses of PSL in part, i.e., the functional damage, but not cytotoxic effect, of the endothelial cells by release of the TM antigen from the cell surface, the inhibition of DNA synthesis, and the delayed recovery of the endothelial cells, even after the preventive effect from damage of the cells by chemotherapy, respectively.

In terms of the G1-checkpoint of the cell cycle, dephosphorylated Rb proteins combined with E2F in dormant cells are phosphorylated by the G1-cyclin/CDK complex and release E2F by growth stimulation, and the free E2F induces growth progression into the S-phase [26]. As one of the checkpoints in the S, the inhibition of DNA polymerization causes down-modulation of G1-cyclin through p53 and p21, and the growth of the cells is suppressed as a result of dephosphorylation of Rb and capture of E2F by the Rb [26,27]. CsA, a growth inhibitor in the G1 [28], caused G1-arrest in HUVEC with a reduction of ppRb, and the effects were reversible. CsA also induced TM release from the cell surface of HUVEC without any remarkable cellular destruction, therefore, functionally modulated endothelial

cells by CsA may cause hypercoagulative conditions on the vascular endothelial surface through a decrement of expressed TM. High dose administration of PSL at 600 µg/ml also caused G1-arrest through down-modulation of ppRb, and the effect on the G1-checkpoint was reversible as well. PSL did not increase the TM level in the supernatant, but decreased the LDH level. PSL prevented HUVEC from the dramatic effects of MMC in the cell cycle. Therefore, the cytostatic effects of PSL may protect endothelial cells from cellular damage by chemotherapy. However, long-term administration of a high dose of PSL may delay the intact EC recovery from vascular damage once EC damage has occurred through the G1-arrest effect.

MMC, one of the DNA polymerase-inhibitors, caused an apparent increment of the S-fraction as an accumulation effect by DNA synthesis inhibition. The amount of incorporated BrdUrd [29—31] in the cells in S decreased by the direct effect of MMC. ppRb also decreased in the presence of MMC in a dose-dependent manner. This phenomenon may have been caused by MMC through p53, p21 and G1-cyclin, and the increment of G1 and decrement of S, G1-arrest, in the presence of a high dose of MMC may have been an indirect effect of MMC through down-modulation of ppRb in addition to a direct inhibition of DNA synthesis (Fig. 1F). The effects of MMC on the cell cycle were basically irreversible. MMC at 3 µg/ml tended to increase the LDH level in the medium, therefore, a high dose administration of MMC may also cause cellular destruction of the vascular endothelium. The importance of the interactions among these cell cycle modulators on cellular biological events, p53, p21, cyclins, CDK, Rb and E2F, is still being elucidated step by step [32—35].

TM is a receptor for thrombin on the vascular endothelium, and plays an important role in the thrombin-catalyzed activation of protein C [36—40]. An elevated level of soluble TM in the serum is inferred as the result of endothelial cell damage [41]. Because no remarkable alterations of the vWf:Ag level were observed in each condition in this study contrary to the former reports [16—19], TM might have been a quick and sensitive marker of EC damage in vitro, and other stimulations like cytokines after BMT might be necessary to elevate the vWf:Ag level in vivo. Otherwise, increase in vWf:Ag or soluble intercellular adhesion molecule-1 (sICAM-1) might indicate EC activation [18], which are not simulated by

our study, rather than EC damage. A low grade elevation of the LDH level in the supernatant was observed in the presence of 3 µg/ml of MMC and 10 µg/ml of CsA (not significant). Therefore, no direct cytotoxic effect of the drugs was evident in vitro in these conditions.

A direct effect of CsA on the endothelial cells has been reported [42,43], and TTP-like microangiopathy is caused by CsA administration in patients [44,45]. Serum levels of CsA in patients are 0.2 to 0.8 µg/ml, and the levels of this hydrophobic chemical in the organs are supposed to be higher. CsA at 1.2 to 60 µg/ml was used in vitro in a previous paper [42], and 0.1 to 10 µg/ml of CsA was used in this article. Even the highest concentration of CsA used in this study (10 µg/ml) might have been higher than in a clinical situation. MMC at 5 to 30 mg is administered intravenously to treat solid tumors, and the maximum serum level of MMC is believed to be 0.5 to 5 µg/ml. PSL at 50 to 1000 mg is used for immunosuppressive treatment, and the maximum serum level in patients is thought to be 10 to 200 µg/ml. CsA is also used for treating TTP [46,47], and the administration of CsA may protect EC against some pathological factors, such as activation of the immune system in vivo, that lead to vascular injury. However, once EC injury develops after BMT with conditioning, infections, venoocclusive disease, GVHD and other drugs, restoration of damaged EC is required through the proliferation of intact EC, and a possible delay of endothelial repair by CsA and PSL through cell cycle arrest may lead to prolongation or progression of the vascular damage which is thought to lead to TMA.

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Table 1. Basic effects of cyclosporine A and prednisolone for cell cycle.

	G1 (%)	S (%)
Natural saline	59.6 + 7.7	25.0 + 3.4
Natural saline + CsA	63.9 + 5.1	21.9 + 3.3
PSL + Natural saline	73.8 + 6.7	11.7 + 5.1
PSL + CsA	75.0 + 7.5	11.0 + 5.7
<i>Main effect</i>		
CsA	ns	ns
PSL	*	**
Interaction	ns	ns

Mean±S.D., n=3. \* $p<0.05$ , \*\*  $p<0.01$  by two-way ANOVA.



Table 2. Basic effects of mitomycin C and prednisolone for cell cycle.

	G1 (%)	S (%)
Natural saline	59.6 + 7.7	25.0 + 3.4
Natural saline + MMC	20.9 + 2.3	60.2 + 8.4
PSL + Natural saline	73.8 + 6.7	11.7 + 5.1
PSL + MMC	57.8 + 11.0	20.6 + 6.3

*Main effect*

MMC	**	**
PSL	**	**
Interaction	ns	*

Mean±S.D., n=3. \* $p<0.05$ , \*\*  $p<0.01$  by two-way ANOVA.

Table 3. Influence of cyclosporine A or prednisolone on the effect of mitomycin C.

(µg/ml)	G1 (%)	P with MMC 0	S (%)	P with MMC 0
CsA + MMC 0	68.6 + 3.4		15.1 + 2.7	
CsA + MMC 0.05	41.1 + 1.7	###	21.7 + 3.1	
CsA + MMC 0.2	30.5 + 6.0	###	40.3 + 1.5	###
CsA + MMC 1	35.8 + 8.7	###	43.8 + 5.4	###
CsA + MMC 3	59.1 + 11.0		23.2 + 11.0	
P with groups	**(0.0002)		**(0.0004)	
PSL + MMC 0	74.0 + 2.5		11.4 + 1.9	
PSL + MMC 0.05	59.9 + 4.7		13.2 + 0.7	
PSL + MMC 0.2	52.0 + 9.1	#	23.5 + 6.9	
PSL + MMC 1	57.2 + 6.5		23.2 + 7.7	
PSL + MMC 3	64.2 + 4.3		17.6 + 5.2	
P with groups	*(0.00999)		(0.0545)	

Mean±S.D., n=3. \*\* $p<0.01$ , \*\*\*  $p<0.001$  by two-way ANOVA.

# $p<0.05$ , ### $p<0.001$ , compared with MMC 0 (Control).

CsA: cyclosporine A, 5 µg/ml, PSL: prednisolone, 600 µg/ml.

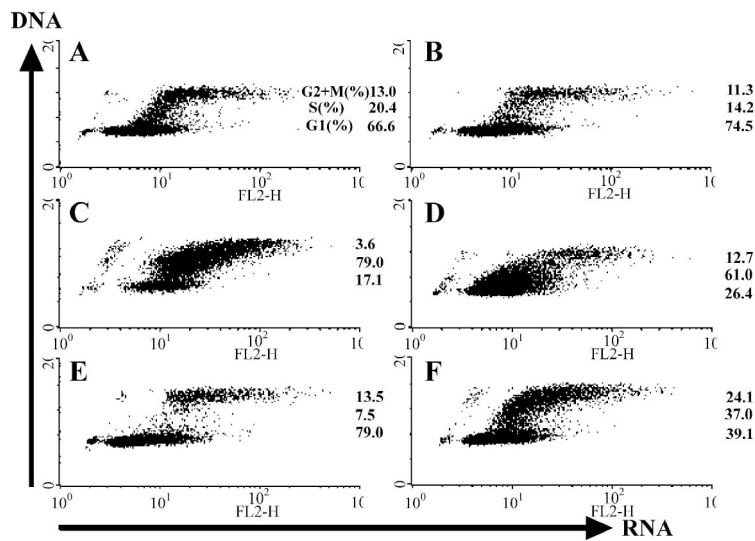


Figure 1. 7AAD/PY dot plots. (A) Control culture; (B) cyclosporine A (5 µg/ml); (C) mitomycin C (0.2 µg/ml); (D) mitomycin C (1.0 µg/ml); (E) prednisolone (600 µg/ml) and (F) prednisolone (600 µg/ml) plus mitomycin C (0.2 µg/ml).

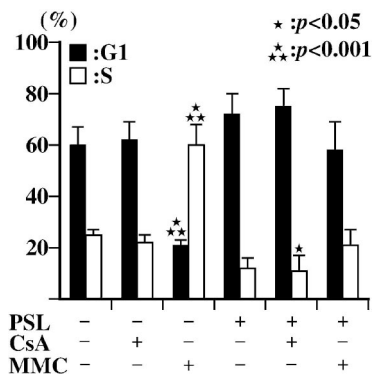


Figure 2 Basic effects of cyclosporin A, mitomycin C and prednisolone. Mean±S.D., n=3,  $p<0.05$  and  $p<0.001$ , compared with control (-, -, -), CsA, cyclosporine A (1.0 µg/ml); MMC, mitomycin C (0.2 µg/ml); PSL, prednisolone (600 µg/ml).

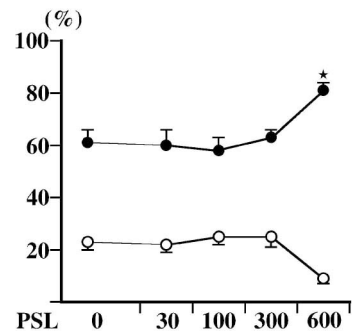
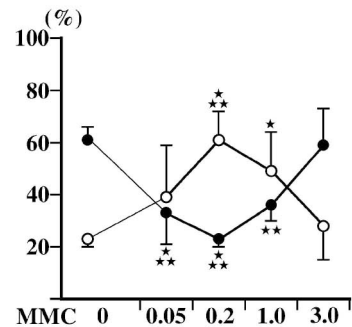
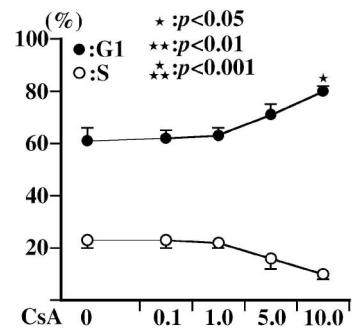


Figure 3 Dose-dependency of the drugs. Mean±S.D., n=4,  $p<0.05$ ,  $p<0.01$  and  $p<0.001$ , compared with control, CsA, cyclosporin A (µg/ml); MMC, mitomycin C (µg/ml) and PSL, prednisolone (µg/ml).

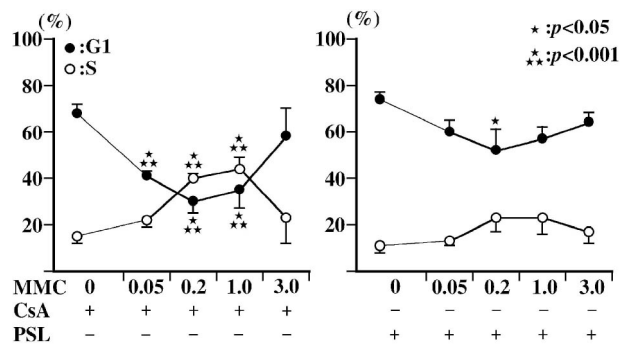


Figure 4 Influence of cyclosporin A or prednisolone on the effect of mitomycin C. Mean±S.D., n=3,  $p<0.05$ ,  $p<0.01$  and  $p<0.001$ , compared with control (no MMC), MMC, mitomycin C (µg/ml); CsA, cyclosporin A (5 µg/ml); PSL, prednisolone (600 µg/ml).

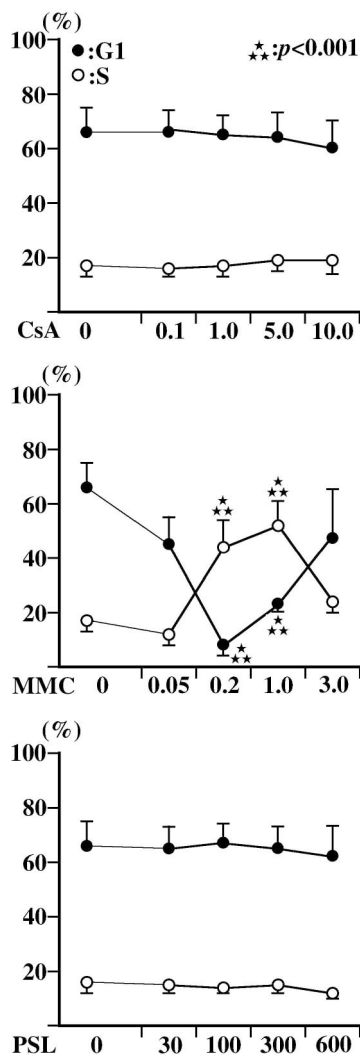


Figure 5 Reversibility of the effects of the drugs after washout (48 hrs.). Mean±S.D., n = 3,  $p<0.001$ , compared with control (no drugs). CsA, cyclosporin A (µg/ml); MMC, mitomycin C (µg/ml) and PSL, prednisolone (µg/ml).

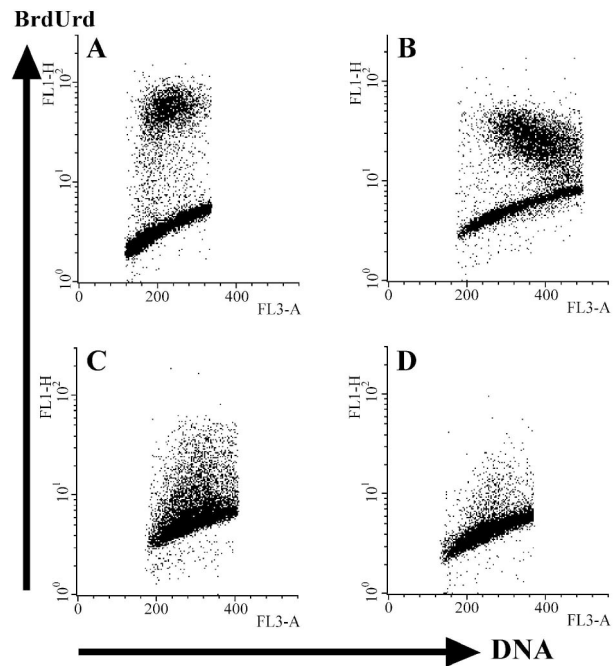


Figure 6 BrdUrd incorporation of HUVEC treated with mitomycin C. HUVEC were incubated in the presence of each concentration of mitomycin C, and simultaneously stained with 7AAD (DNA) and anti-BrdUrd (FITC). (A) Control culture; (B, C and D) 0.05, 1 and 3 µg/ml of mitomycin C, respectively.

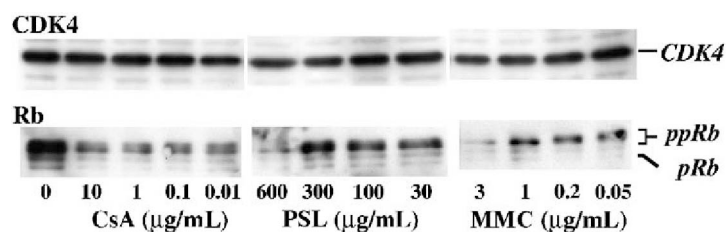


Figure 7 Western blotting analysis of CDK4 and retinoblastoma protein (Rb). HUVEC were incubated in the presence or absence of cyclosporine A (CsA), prednisolone (PSL) and mitomycin C (MMC). CDK4, between 31 and 43 kDa; Rb, between 110 and 116 kDa. pRb, underphosphorylated Rb; ppRb, phosphorylated and highly phosphorylated Rb.

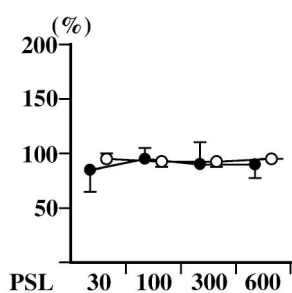
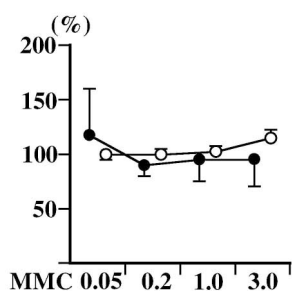
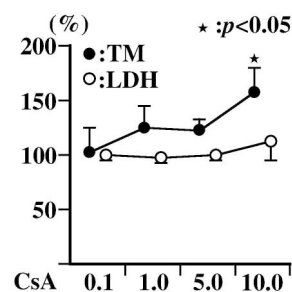


Figure 8 TM and LDH concentration in the culture supernatant. Mean±S.D.,  $n=4$ ,  $p<0.05$ , compared with control (no drugs). CsA, cyclosporin A (μg/ml); MMC, mitomycin C (μg/ml) and PSL, prednisolone (μg/ml). %TM and %LDH, relative concentration (%) of TM and LDH to the control, respectively.