

## Induction of Micronuclei in CHO Cells by Bleomycin but not by X-irradiation is Decreased by Treatment with HMG-CoA Reductase Inhibitors

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### Free radicals/HMG-CoA reductase inhibitor/Micronuclei/X-rays/Bleomycin.

We investigated the effect of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, pravastatin and fluvastatin, on the induction of micronuclei by ionizing radiation or bleomycin in Chinese hamster ovary cells in order to assess the radical-scavenging ability of these inhibitors. The results indicated that both pravastatin and fluvastatin had no effect on the induction of micronuclei by X-irradiation when they were applied for either pre-treatment or post-treatment. In contrast, both drugs effectively reduced the frequency of bleomycin-induced micronuclei when they were applied for simultaneous treatment or post-treatment, but not for pre-treatment. This indicates that the radical-scavenging ability of these two HMG-CoA reductase inhibitors differs according to the origins of the radicals - e.g., X-rays or bleomycin - even when the two drugs are compared at an equivalent cytotoxic dose. Our results suggest that both pravastatin and fluvastatin have the ability to scavenge certain types of radicals and to protect cells against oxidative stress.

### INTRODUCTION

Hyperlipidemia is one of the major risk factors for arteriosclerosis (along with smoking and hypertension) and is thought to involve an oxidative form of low-density lipoprotein (OxLDL).<sup>1–3)</sup> This implicates that oxidative stress *in vivo* might contribute to the development of arteriosclerosis. Conversely, it is expected that drugs that have anti-hyperlipidemic and anti-oxidant activities prevent the development of arteriosclerosis.

Statins are well known inhibitors for 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA), and are used to reduce serum cholesterol in patients with hyperlipidemia. In addition to the inhibitory effect on HMG-CoA, one of the statins,

fluvastatin, exhibits radical-scavenging activity.<sup>4–9)</sup> This implies that a certain type of statins might be expected to prevent the progression of atherosclerotic disease by dual actions—i.e., by reduction of both serum cholesterol and OxLDL. However, several studies have reported that two other statins, pravastatin and simvastatin, had no effect as radical scavengers.<sup>7–9)</sup>

In other studies, however, pravastatin suppressed the superoxide production of glomerular mesangial cells,<sup>10)</sup> and simvastatin displayed antioxidant activity *in vivo*.<sup>11)</sup> To determine the reason for these discrepancies, more information about the antioxidant activities of statins other than fluvastatin is needed.

Therefore, we investigated the radical-scavenging ability of fluvastatin and pravastatin by examining their protective effect against the induction of micronuclei (MN) by ionizing radiation and bleomycin in Chinese hamster ovary (CHO) cells. MN have been shown to be an effective marker for quantitating DNA damage, and correspond to the loss of an acentric fragment or a whole chromosome induced by ionizing radiation or chemical mutagens.<sup>12)</sup> Moreover, it is indicated that the induction of MN is responsible for the reduction of proliferative activity,<sup>13,14)</sup> implying that MN induction may be a good marker of cellular damage induced by radicals.

In the present study, we demonstrate that both pravastatin and fluvastatin scavenge radicals that are induced by bleo-

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mycin depending on the treatment conditions.

## MATERIALS AND METHODS

### Cell culture

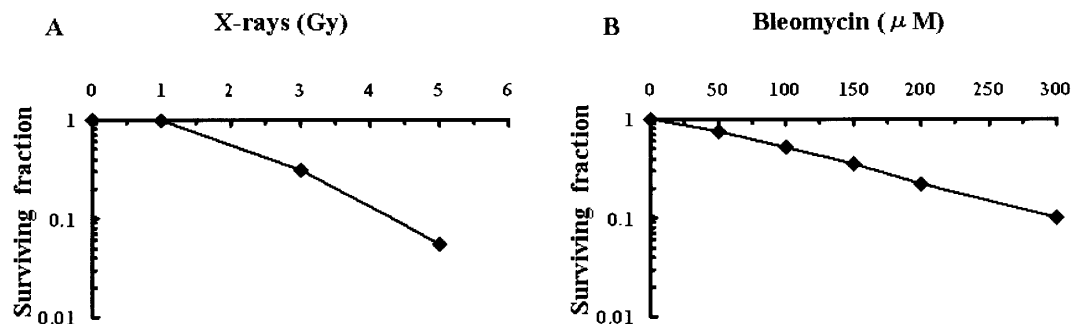
CHO-K1 cells were grown in Eagle's minimum essential medium (MEM) supplemented with 20 mM 2-4-(2-hydroxyethyl)-1-piperazinyl ethan-sulfonic acid (HEPES, Dojin Chemicals, Kumamoto), 0.2 mM serine, 0.2 mM aspartate, 1 mM pyruvate, and 10% fetal bovine serum (Trace Scientific, Melbourne), and maintained in plastic 75 cm<sup>2</sup> culture

(T-75) flasks at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub> as previously described.<sup>15)</sup>

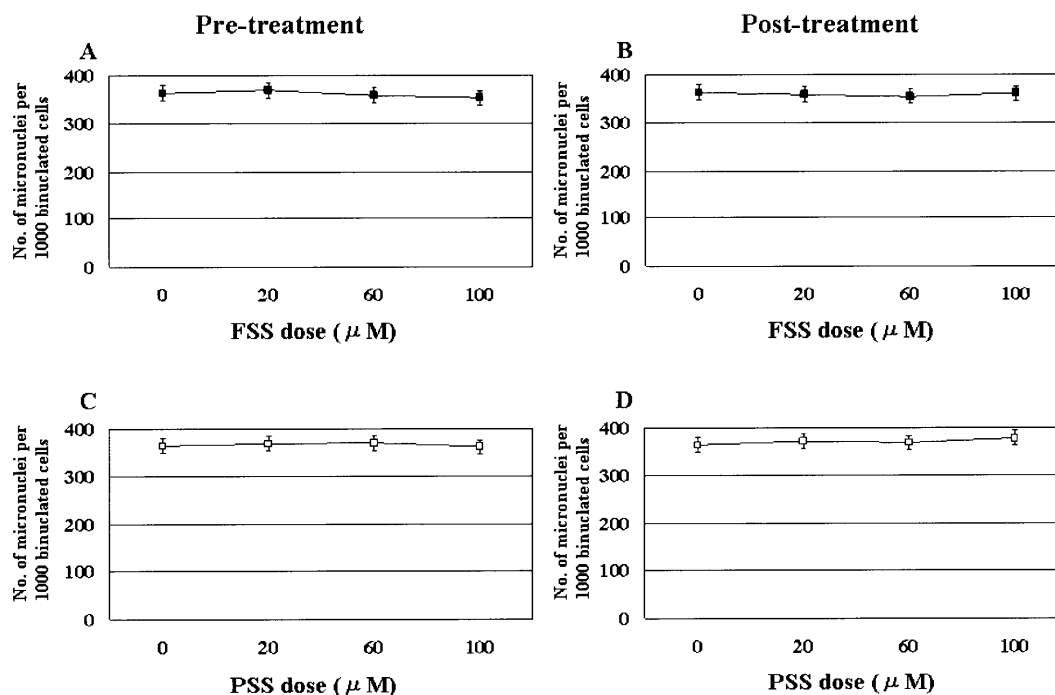
### X-irradiation and bleomycin treatment

Cells were irradiated with X-rays using an M-150WE X-ray machine (Softex, Osaka) operated at 150 kV and 5 mA with a 1.0 mm Cu filter at room temperature at a dose rate of 0.43 Gy/min.

Bleomycin was dissolved in serum-free medium, and the cells were exposed to the complete medium containing graded doses of bleomycin for 2 h as the same treatment period



**Fig. 1.** Survivals of CHO-K1 cells after X-irradiation and bleomycin treatment. A, Clonogenic survival by X-irradiation; B, Clonogenic survival by bleomycin treatment for 2 h. The equivalent doses for 30% survival of X-irradiation and bleomycin treatment were 3 Gy and 150 μM, respectively.



**Fig. 2.** Effect of HMG-CoA reductase inhibitors on the induction of micronuclei (MN) by X-irradiation. A, Induction of MN by X-irradiation combined with pre-treatment with fluvastatin (FSS); B, Induction of MN by X-irradiation combined with post-treatment with fluvastatin (FSS); C, Induction of MN by X-irradiation combined with pre-treatment with pravastatin (PSS); D, Induction of MN by X-irradiation combined with post-treatment with pravastatin (PSS). The induction of MN was represented by the number of binucleated cells with MN per 1000 binucleated cells.

as those of scavengers.

### Cell-killing effects by X-irradiation and bleomycin treatment

Exponentially growing cells ( $2-3 \times 10^6$  cells in a T-75 flask) were irradiated with X-rays or treated with bleomycin (Nippon Kayaku, Tokyo) for 2 h. Immediately after X-irradiation or bleomycin treatment, the cells were trypsinized, plated in 100-mm dishes and incubated for 14 days. Surviving colonies were fixed with ethanol and stained with 3% Giemsa solution. Colonies consisting of more than 50 cells were scored as survivors.

### Treatment with radical scavengers

Two inhibitors of HMG-CoA reductase, pravastatin sodium (PSS; Sankyo, Tokyo) and fluvastatin sodium (FSS; Ciba-Geigy, Hyogo), were used as radical scavengers. These scavengers were dissolved in serum-free medium, adjusted to pH 7.2, and sterilized by a filter (0.22  $\mu$ m). For the treatment with the scavengers, the cells were exposed to the complete medium containing the scavenger for 2 h.

### Micronuclei test

$10^6$  cells were plated in a 100 mm dish, incubated for 24 h, and then irradiated with 3 Gy of X-rays or treated with 150 mM bleomycin for 2 h. Radical scavengers were administered as a pre- or post-treatment for 2 h. In case of the pre-treatment with the scavenger followed by X-irradiation, the cells were exposed to the complete medium containing the scavenger for 2 h and irradiated with X-rays (3 Gy) during the last 7 min in the treatment time of the scavenger. In case of the post-treatment, the cells were exposed to the complete medium containing the scavenger for 2 h after X-irradiation was completed. For the treatment with bleomycin, a simultaneous treatment of bleomycin and the scavengers was also examined in addition to the pre- and post-treatment. After combined treatment with the DNA-damaging agents and the radical scavengers, the cells were treated with cytocharasin B at a final concentration of 2  $\mu$ g/ml, incubated for another 24 h to inhibit cytokinesis, and then harvested. The collected cells were fixed with Carnoy's fixative (methanol:acetic acid, 3:1) twice and the cell suspensions were dropped onto a clear slide. Air-dried slides were stained with 2% Giemsa. A thousand binucleated cells were scored for dose, and the induction of MN was represented by the number of binucleated cells with MN per 1000 binucleated cells.

### Statistical analysis

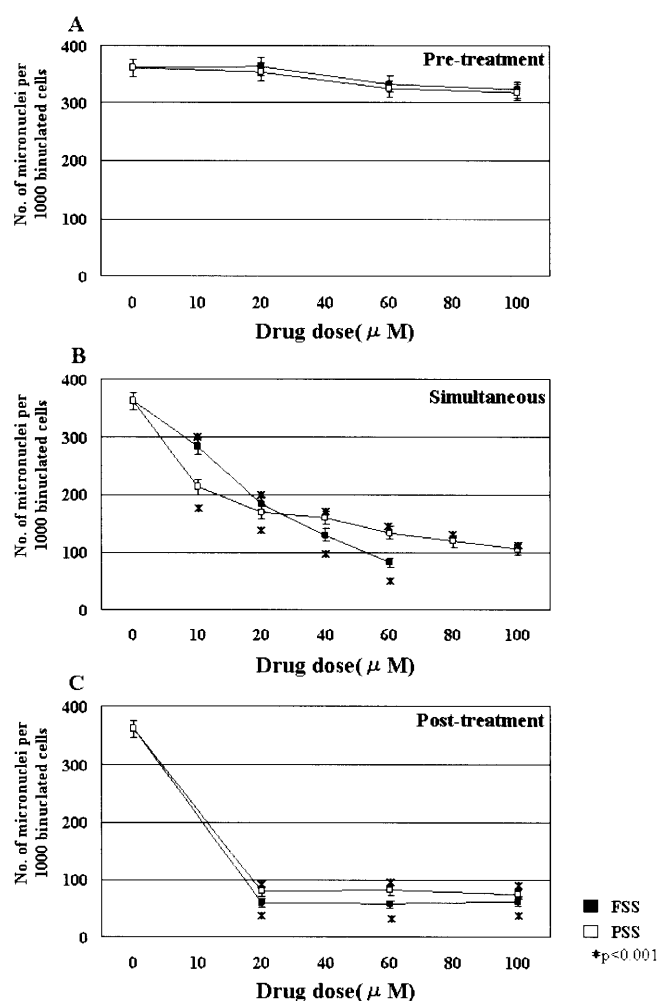
Statistical analysis was performed using a Chi-square for independence test.

## RESULTS

To determine an equivalent survival dose of X-rays and

bleomycin, we determined cell survivals after X-irradiation and bleomycin treatment of CHO-K1 cells as shown in Fig. 1.  $D_0$  values of X-rays and 2-h treatment of bleomycin were 1.16 Gy and 119.6 mM, respectively. Based on these results, we determined that the equivalent doses for 30% survival of X-irradiation and bleomycin treatment were 3 Gy and 150  $\mu$ M, respectively.

To assess the radical-scavenging ability of FSS and PSS, we investigated the effect of these chemicals on the induction of micronuclei (MN) by 3 Gy of X-rays. The spontaneous level of MN in CHO-K1 cells was  $14 \pm 2.1$  MN per 1000 binucleated cells. As shown in Figs. 2, 3 Gy-irradiation



**Fig. 3.** Effect of HMG-CoA reductase inhibitors on the induction of micronuclei (MN) by the treatment with bleomycin. A, Induction of MN by bleomycin combined with pre-treatments with pravastatin or fluvastatin; B, Induction of MN by bleomycin combined with simultaneous treatments with pravastatin or fluvastatin; C, Induction of MN by bleomycin combined with post-treatment with pravastatin or fluvastatin. The induction of MN was represented by the number of binucleated cells with MN per 1000 binucleated cells. Statistical analysis was performed using the Chi-square for independence test (\* $p < 0.001$ ).

increased the level of MN, resulting in  $364 \pm 15.2$  MN per 1000 binucleated cells. The result revealed that the pre- and post-treatments with FSS had no effect on X-ray-induced MN in CHO-K1 cells. Similarly, neither pre- nor post-treatment with PSS changed the frequency of MN by X-irradiation. These results indicate that neither FSS nor PSS can scavenge X-ray-induced radicals involved in the induction of MN.

Then, we examined the effect of FSS and PSS treatments on the frequency of MN induction by pre-, simultaneous, and post-treatments with 150  $\mu$ M bleomycin as shown in Fig. 3. The results demonstrated that neither pre-treatment with FSS nor that with PSS had an effect on the induction of MN by bleomycin. In contrast, simultaneous treatment with bleomycin and either FSS or PSS decreased the induction of MN by bleomycin in a dose-dependent manner. In post-treatment with FSS and PSS, a low dose (20  $\mu$ M) treatment was sufficient to reduce the induction of MN by bleomycin to a basal level. These results indicate that both FSS and PSS have the ability to scavenge bleomycin-induced radicals when applied either concomitantly with bleomycin or as a post-treatment.

## DISCUSSION

In the present study, we demonstrated that fluvastatin and pravastatin, which are clinically used for anti-hyperlipidemia, reduced the frequency of bleomycin-induced micronuclei when they were applied concomitantly with bleomycin or as a post-treatment, but not when they were used as a pre-treatment (Fig. 3). Because the anti-cancer action of bleomycin is mainly due to the induction of DNA double-strand breaks (DSBs) mediated by production of radicals,<sup>16)</sup> the protective effect of these drugs against radical-induced DSBs indicates that they have the ability to scavenge radicals induced by bleomycin. However, the result that the pre-treatment of both drugs showed no protective effect on the MN frequency implies that neither drug protects the cells from radicals induced by ionizing radiation even though they are present in the culture medium during irradiation (Fig. 2, A and C).

This difference in the radical-scavenging ability of those drugs might be a reflection of a difference between acute (X-rays) and chronic (bleomycin) exposure to radicals. This is the first report showing a difference in the protective effects of fluvastatin and pravastatin between acute and chronic exposure to radicals. It is noteworthy that both drugs appeared to show the radical-scavenging activity under the chronic exposure condition, because chronic exposure to radicals is the most common condition for living cells *in vivo*. This means that these drugs have the potential to reduce oxidative stress in addition to anti-hyperlipidemia.

A former report demonstrated that bleomycin-induced chromosome aberrations in human lymphocytes were

reduced by simultaneous treatment with vitamin C but not with vitamin E.<sup>17)</sup> In contrast, we designed the present study to examine the protective ability of the HMG-CoA reductase inhibitors by pre- and post-treatments in addition to simultaneous treatment to clarify the characteristics of these drugs as antioxidant agents. Our results indicated that fluvastatin and pravastatin efficiently reduced the frequency of MN by post-treatment as well as when administered concomitantly with bleomycin, suggesting that bleomycin may produce oxygen radicals chronically. In contrast, the drugs were not effective in ameliorating the induction of MN by ionizing radiation, indicating that they are not effective scavengers of acutely produced radicals.

It was suggested that the active site for the anti-oxidative activity of fluvastatin is the allylic carbon conjugated with the indole ring.<sup>6)</sup> A study by Suzumura *et al.* also suggested that the fluorophenyl indole moiety of fluvastatin is important for the radical-scavenging activity.<sup>5)</sup> However, the present study demonstrated that pravastatin displayed the anti-oxidative activity, even though it may not have these active sites. This suggests that a common mechanism that is not mediated by the indole ring might be responsible for the radical-scavenging activity of these two drugs.

In conclusion, the present study indicates that both fluvastatin and pravastatin exert dual actions as anti-oxidant and anti-hyperlipidemia, suggesting that these drugs would slow the development of arteriosclerosis.

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