Pravastatin Limits Endothelial Activation after Irradiation and Decreases the Resulting Inflammatory and Thrombotic Responses

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Endothelial dysfunction has been implicated in the pathogenesis of atherosclerosis, fibrosis and vascular occlusion after radiation therapy. Statins have been reported to improve endothelial function; however, this beneficial effect on endothelial cells has never been investigated after irradiation. Therefore, using human microvascular endothelial cells from lung that had been irradiated with 5 or 10 Gy, we assessed the effect of pravastatin on endothelial activation by ELISA, cell-ELISA and electrophoretic mobility shift assay and increased blood-endothelial cell interactions by a flow adhesion assay. Pravastatin inhibited the overproduction of monocyte chemoattractant protein 1, IL6 and IL8 and the enhanced expression of intercellular adhesion molecule 1 but had no effect on platelet-endothelial cell adhesion molecule 1 expression. Moreover, pravastatin down-regulated the radiation-induced activation of the transcription factor activator protein 1 but not of nuclear factor-κB. Finally, an inhibition by pravastatin of increased adhesion of leukocytes and platelets to irradiated endothelial cells was observed. The effect of pravastatin was maintained up to 14 days after irradiation and was reversed by mevalonate. Pravastatin exerts persistent anti-inflammatory and anti-thrombotic effects on irradiated endothelial cells. Statins may be considered in therapeutic strategies for the management of patients treated with radiation therapy. © 2005 by Radiation Research Society

INTRODUCTION

Vascular injury after radiation therapy for cancer or in-stent restenosis is believed to be a key factor in the pathogenesis of cardiovascular diseases such as atherosclerosis (*1*), fibrosis leading to organ dysfunction (*2–4*), and thrombosis with vascular occlusion (*5–7*). The high radiation sensitivity of the vasculature is mainly linked to endothelial

cells (EC), a critical target cell for radiation injury (*8–10*). Radiation induces endothelial activation, reflecting a specific state of endothelial dysfunction (*11*) characterized by activation of NF-κB (NFKB) (*12, 13*), alterations in adhesion molecule expression (*14, 15*), inflammatory and chemotactic cytokine production (*16*), release of von Willebrand factor (*17*), plasminogen activator and inhibitor secretion (*18*), and tissue factor (*19*) and thrombomodulin (*3*) activities. These activated EC are prone to atherogenesis (*1*) and have pro-inflammatory and thrombogenic properties, thereby promoting leukocyte- or platelet-endothelial cell adherence (*14, 20*), leukocyte infiltration into tissues (*15, 17, 21*), and thrombus formation (*22*). The response of EC to radiation occurs within hours of exposure and is sustained for several weeks (*2, 3, 14, 16, 17*). The maintenance of such a response could contribute to the development and progression of late vascular and tissue radiation damage (*3, 4, 7*). Therefore, improving endothelial cell function after radiation exposure with pharmacological agents may limit the crucial steps contributing to the pathogenesis of these late deleterious effects. Recently, statins have been reported to have beneficial vascular effects in addition to their cholesterol-lowering properties (*23*). In fact, statins exert anti-inflammatory action, especially on endothelial cells, by inhibiting endothelial activation, i.e. NF-κB activation (*24*), adhesion molecule-like intercellular adhesion molecule 1 (ICAM1) expression (*24–26*), cytokine (IL6) and chemokine [monocyte chemoattractant protein 1 (MCP1), IL8] production (*24, 27–30*), and by preventing leukocyte adherence to the endothelium (*24, 31–33*). To our knowledge, it has never been tested whether statins have this anti-inflammatory activity on irradiated EC. Statins also exert an anti-thrombotic effect through a direct action on platelets. Statins decrease platelet activation and activity (*34*) and affect thrombus formation by the reduction of platelet aggregation (*35*). However, no data are available on the role of statins in modulating the thrombogenic properties of the EC induced by radiation.

In the present study, we assessed the ability of pravastatin to affect EC activation induced by radiation and the resulting increased interactions with leukocytes and platelets. We also examined whether mevalonate could prevent

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the effect of pravastatin, since one of the effects of statins is to inhibit the conversion of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) to mevalonate by competitive inhibition of the rate-limiting enzyme HMG-CoA reductase (*23, 36*).

MATERIALS AND METHODS

Endothelial Cell Cultures

Primary human microvascular EC of lung origin (HMVEC-L) were obtained from Clonetics (Cambrex), seeded at density of 5000 cells/cm², and routinely cultured at 37°C in 95% air/5% CO_2 according to the vendor recommendations in the supplier's endothelial cell growth culture medium (EGM-2-MV), referred to here as complete medium. HMVEC-L cells were used between the fifth and seventh passage. Cells were allowed to reach confluence before irradiation.

Irradiation Protocol

Just before irradiation, the culture medium was replaced with complete medium containing pravastatin (1–1000 μ*M,* final concentration), kindly provided by Bristol-Myers Squibb, alone or in combination with mevalonate (400 μ*M*) (Sigma). HMVEC-L cells were uniformly irradiated with a total dose of 5 or 10 Gy of γ radiation delivered from a ^{137}Cs source (IBL 637, CISBio International, dose rate of approximately 0.7 Gy/min). Sham-irradiated control HMVEC-L cells were treated under the same conditions. Mevalonate and/or pravastatin were added each time the culture medium was changed after irradiation (days 3, 7, 10).

Cell-ELISA for Adhesion Molecule Expression

ICAM1 and PECAM1 expression was determined as described previously (*16*) using cell-ELISA on paraformaldehyde-fixed HMVEC-L cells in 24-well plates. The cells were first incubated with primary antibody (Beckman Coulter) for 1 h at 20°C and then with a peroxidase-conjugated secondary antibody (Jackson) for 1 h at 20°C. Absorbance was measured at 492 nm on a microplate reader (Bio-Tek Instruments). In parallel, for each time, the number of cells was determined after trypsinization of the monolayer.

Quantification of Cytokine Production

Quantitative determinations of IL6, IL8 and MCP1 in supernatants of HMVEC-L cells cultured in 24-well plates were performed using a sandwich ELISA according to the manufacturer's recommendations (R & D Systems). For each time, the number of cells was determined after trypsinization of the monolayer.

Electrophoretic Mobility Shift Assay

Nuclear protein extraction was performed as described previously (*37*). Protein concentrations were determined with the Coomassie Protein assay (Pierce). The oligonucleotides 5-AGTGAGGGGACTTTCCCAGGC-3′ (NF-κB consensus), 5′-CGCTTGATGAGTCAGCAGCCGGAA-3′ (AP1 consensus), 5′-CGCTTGAT*AGTG*CAGCAGCCGGAA-3′ (AP1 mutant), were ³²P-end-labeled by incubation for 30 min at 37°C with 10 U of T4 polynucleotide kinase (Promega) and 1.85 MBq [γ-³²P]ATP. The protein-DNA complexes were visualized with a Phosphoimager and quantified using Image Gauge software (FLA-3000, Fujifilm). For supershift experiments, 0.1 μg/μl (final concentration) of anti-RelA (p65) or anti-p50 antibodies (Santa Cruz Biotechnology) was used. Unlabeled competitor oligonucleotides were added in 10-fold excess.

Quantification of Platelet- and Leukocyte-Endothelial Cell Interactions

Confluent monolayers of HMVEC-L cells grown on glass slides were assembled in a parallel-plate flow chamber and mounted on the stage of
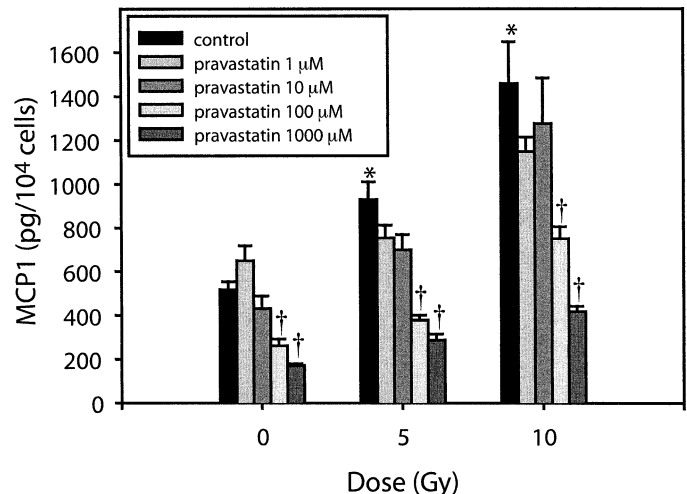

FIG. 1. Dose-dependent effect of pravastatin on MCP1 production by nonirradiated and irradiated HMVEC-L cells 1 day after exposure. Histograms represent the means ± SEM of one to four separate experiments performed in triplicate. *$P \leq 0.001$ compared with nonirradiated cells, †$P < 0.05$ compared with control cells.

an inverted fluorescence microscope (Eclipse TE 300, Nikon) as described previously (*20*). Whole blood fluorescently labeled with Rhodamine 6G was perfused through the flow chamber for 20 min at constant wall shear rates of 25 s⁻¹ and 500 s⁻¹ using an electric pump. The entire experiment was visualized in real time at 40× magnification with a color video camera and a 20-inch monitor and recorded with a digital videotape recorder. Leukocyte movements and surface area coverage by platelets were analyzed off-line using image analysis software (Histolab, Microvision Instruments). The number of rolling and firmly adherent leukocytes and the surface occupied by platelets and thrombi on endothelial cells were determined as described previously (*20*).

Statistical Analysis

All values are reported as means ± SEM unless indicated. Data were analyzed using the Student's *t* test or the Mann-Whitney rank-sum test (Sigmastat software, Jandel Scientific). Significance was set at $P < 0.05$.

RESULTS

Pravastatin Inhibits the Overproduction of MCP1, IL6 and IL8 by Irradiated HMVEC-L Cells in a Dose-Dependent Manner

Radiation induced an increased production of MCP1 by HMVEC-L cells 1 day after exposure ($P \leq 0.001$) (Fig. 1). Treatment of the cells with pravastatin inhibited this augmented production. The inhibitory effect of pravastatin was dependent on dose and was significant at concentrations of 100 and 1000 μ*M* ($P < 0.05$). Increased production of IL6 (Fig. 2A) and IL8 (Fig. 2B) by irradiated cells was also observed ($P < 0.005$), as described previously for EC from other tissue (*16, 38*), and the same inhibitory effect of pravastatin was obtained ($P < 0.05$) (Figs. 2A, B). However, the higher concentration (1000 μ*M*) did produce cytotoxicity after 3 days in culture. Therefore, the concentration of 100 μ*M* of pravastatin was used in subsequent experiments, as in previous studies (*39, 40*).
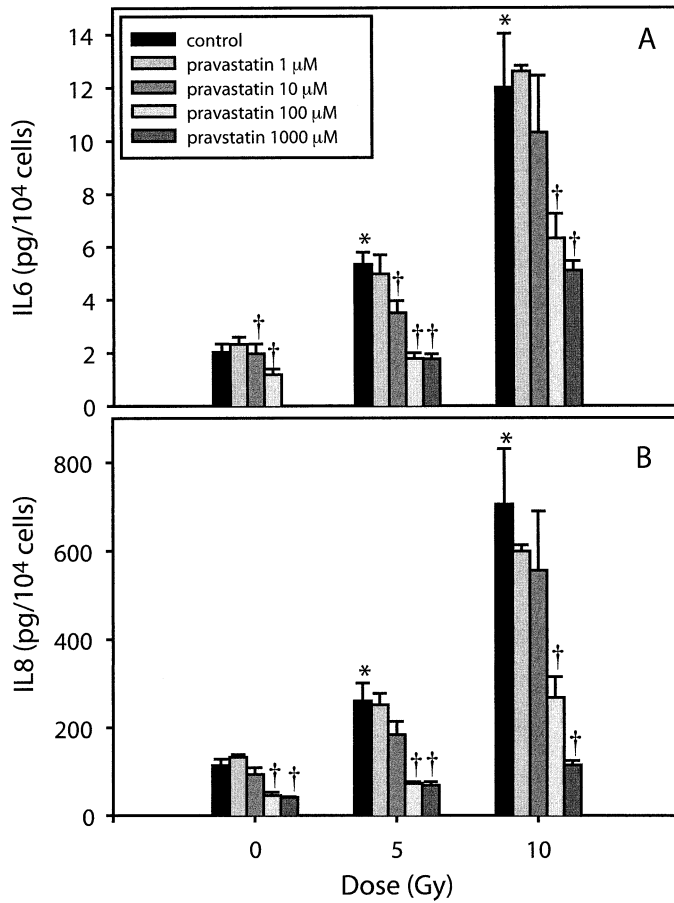
FIG. 2. Dose-dependent effect of pravastatin on IL6 (panel A) and IL8 (panel B) production by nonirradiated and irradiated HMVEC-L cells 1 day after exposure. Histograms represent the means ± SEM of one to four separate experiments performed in triplicate. $*P < 0.005$ compared with nonirradiated cells, $\dagger P < 0.05$ compared with control cells.

The Inhibitory Effect of Pravastatin on MCP1, IL6 and IL8 Production is Persistent

Figure 3 shows that the inhibitory effect of pravastatin on the production of MCP1, IL6 and IL8 by HMVEC-L cells irradiated with 10 Gy was maintained at least up to 14 days after irradiation, since the 1.7–2.2-fold reduced production of MCP1, IL6 and IL8 obtained 1 day after exposure was still observed 14 days after exposure. Pravastatin also had a persistent inhibitory effect on cells irradiated with 5 Gy (Table 1). Furthermore, production of MCP1, IL6 and IL8 by irradiated cells was inhibited by pravastatin either partially or completely when production returned to the basal levels in nonirradiated cells (Table 2).

Mevalonate Reverses the Pravastatin-Mediated Inhibition of Production of IL6 and IL8 by Irradiated HMVEC-L Cells

Figure 3B and C shows that co-treatment with mevalonate reversed the effect of pravastatin on IL6 and IL8 production in HMVEC-L cells irradiated with 10 Gy. This reversion was complete except at 7 days after exposure. In
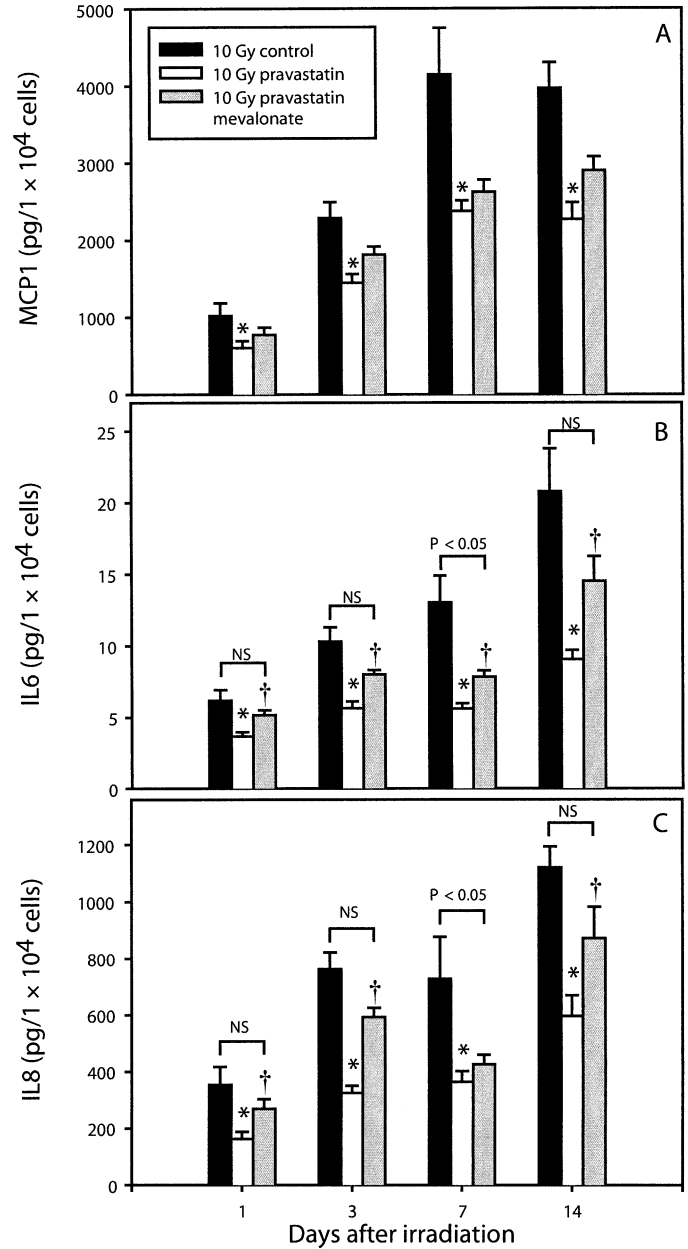

FIG. 3. Effect of mevalonate (400 μM) and/or pravastatin (100 μM) on MCP1 (panel A), IL6 (panel B) and IL8 (panel C) production by HMVEC-L cells irradiated with 10 Gy. Histograms represent the means ± SEM of two to six separate experiments performed in triplicate. $*P < 0.05$ compared with control cells, $\dagger P < 0.05$ compared with pravastatin-treated cells. NS: nonsignificant.

contrast, mevalonate did not reverse the effect of pravastatin on production of MCP1 (Fig. 3A). Co-treatment with mevalonate also reversed the effect of pravastatin on production of IL6 and IL8 in cells irradiated with 5 Gy (Table 1).

Effect of Mevalonate and/or Pravastatin on Adhesion Molecule Expression

As described previously for EC from other tissue (*16*), radiation up-regulated ICAM1 and PECAM1 on the surface

TABLE 1
Comparison of the Production of IL6 and IL8 by HMVEC-L Cells Irradiated with
5 Gy and Treated or not with Mevalonate (400 μ*M*) and/or Pravastatin (100 μ*M*)

Days after irradiation	Treatment	IL6 (pg/liter $\times 10^4$ cells)	IL8 (pg/liter $\times 10^4$ cells)
3	5 Gy, control	6.7 ± 0.5	512 ± 60
	5 Gy, pravastatin	4.4 ± 0.3*	246 ± 22*
	5 Gy, pravastatin, mevalonate	6.4 ± 0.4†, NS	420 ± 43†, NS
7	5 Gy, control	9.6 ± 0.9	513 ± 43
	5 Gy, pravastatin	6.4 ± 0.8*	393 ± 52*
	5 Gy, pravastatin, mevalonate	10.5 ± 1.2†, NS	562 ± 68†, NS

Notes. Values are the mean $\pm$ SEM of 3–5 separate experiments performed in triplicate. * $P < 0.05$ compared with control HMVEC-L cells, † $P < 0.05$ compared with pravastatin-treated HMVEC-L cells, NS: non-significant compared with control HMVEC-L cells.

of HMVEC-L cells ($P \leq 0.001$) (Fig. 4A, B). The radiation-induced up-regulation of ICAM1 was inhibited by pravastatin but only partially ($P = 0.023$ and $P \leq 0.001$, 0 Gy compared respectively with 5 or 10 Gy-pravastatin). Co-treatment with mevalonate completely reversed the effect of pravastatin on ICAM1 expression in cells irradiated with 5 Gy (Fig. 4A). In contrast, no effect of pravastatin was observed for PECAM1 (Fig. 4B). The same results were obtained 3 days after irradiation (data not shown).

Pravastatin Down-regulates Activation of AP1 but not NF-κB after Radiation Exposure

NF-κB was activated 2 and 6 h after irradiation (2.6 ± 0.2- and 2.4 ± 0.2-fold, respectively) (Fig. 5A). The p65 subunit (Fig. 5C) but not the p50 subunit (data not shown) was present in this activity. Pravastatin did not modify NF-κB activation induced by radiation (Fig. 5C). AP1 was activated 6 h after exposure (2.5 ± 0.2-fold) (Fig. 5B). Pravastatin down-regulated this activation (1.6 ± 0.1-fold, $P = 0.006$, $n = 3$), and mevalonate partially reversed the effect of pravastatin (1446 ± 269 arbitrary units pravastatin com-

pared to 1852 ± 412 arbitrary units pravastatin/mevalonate) (Fig. 5D).

Pravastatin Inhibits Radiation-Induced Increased Blood-Endothelial Cell Interactions: Role of Mevalonate

As shown previously under physiological flow (*20*), irradiated HMVEC-L cells promoted platelet adhesion to a greater extent than nonirradiated cells ($P < 0.005$) regardless of time after exposure and at both wall shear rates tested (Fig. 6A, B). This radiation-induced increase in platelet adhesion was completely inhibited by pravastatin except at day 3 at a wall shear rate of 500 s^{-1} (Fig. 6B). Mevalonate completely reversed the effect of pravastatin on platelet adhesion on irradiated cells at both wall shear rates (Table 3). For the leukocyte-endothelial cell interactions at a wall shear rate of 25 s^{-1}, treatment with pravastatin did not modify the number of rolling leukocytes on irradiated cells (144 ± 5, $n = 5$) compared to control irradiated cells (145 ± 11, $n = 5$) whatever the time postexposure. In contrast, pravastatin inhibited the number of firmly adherent leukocytes on irradiated cells from $9.7 \pm$

TABLE 2
Comparison between the Production of MCP1, IL6 and IL8 by Nonirradiated HMVEC-L
Cells and Pravastatin-Treated Irradiated HMVEC-L Cells

Days after irradiation	Treatment	MCP1 (pg/liter $\times 10^4$ cells)	IL6 (pg/liter $\times 10^4$ cells)	IL8 (pg/liter $\times 10^4$ cells)
1	0 Gy, control	502 ± 50	2.1 ± 0.2	108 ± 12
	5 Gy, pravastatin	481 ± 59	1.6 ± 0.2	83 ± 7
	10 Gy, pravastatin	675 ± 53*	5.0 ± 0.6*	215 ± 29*
3	0 Gy, control	1250 ± 184	4.6 ± 0.7	277 ± 39
	5 Gy, pravastatin	1253 ± 121	4.0 ± 0.3	200 ± 17
	10 Gy, pravastatin	1565 ± 102*	6.3 ± 0.5*	365 ± 34*
7	0 Gy, control	1327 ± 104	4.3 ± 0.4	238 ± 28
	5 Gy, pravastatin	1950 ± 151*	4.9 ± 0.8	294 ± 52
	10 Gy, pravastatin	2178 ± 79*	5.1 ± 0.3	339 ± 34*
14	0 Gy, control	1150 ± 136	3.7 ± 0.5	291 ± 31
	5 Gy, pravastatin	1111 ± 59	2.3 ± 0.1	163 ± 3
	10 Gy, pravastatin	2052 ± 180*	5.3 ± 1.0	503 ± 67*

Notes. Values are the means $\pm$ SEM of one to eight separate experiments performed in triplicate. * $P < 0.05$ compared with nonirradiated cells.
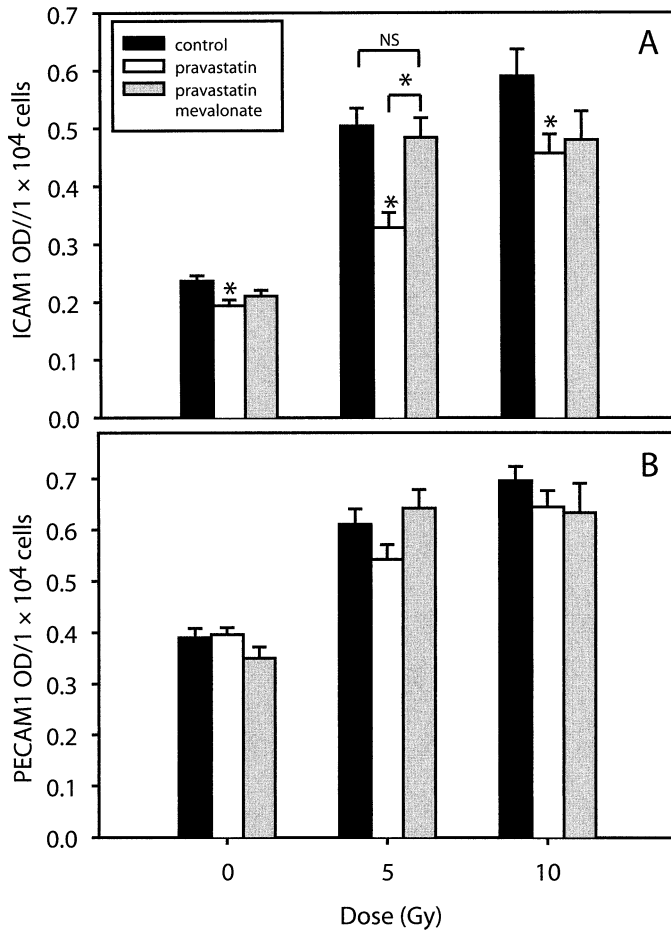
FIG. 4. Effect of mevalonate (400 μM) and/or pravastatin (100 μM) on ICAM1 (panel A) and PECAM1 (panel B) expression by nonirradiated and irradiated HMVEC-L cells 7 days after exposure. Histograms represent the means $\pm$ SEM of three to five separate experiments performed in triplicate. Statistical significance was determined by comparing the pravastatin-treated cells to the control cells. *$P < 0.05$.

3.0 to 4.2 $\pm$ 1.5 ($P < 0.05$, $n = 7$) 3 days after irradiation. The effect of pravastatin on leukocyte adhesion was limited by simultaneous treatment with mevalonate (5.7 $\pm$ 2.0).

DISCUSSION

EC injury is believed to be a key factor in the pathogenesis of radiation atherosclerosis, fibrosis and thrombosis, but only recently have studies proposed a link between the persistence of endothelial dysfunction and radiation pathologies (2–4, 7). We have shown previously that radiation induced an early and sustained activation of EC (14, 16, 41), resulting in maintenance of the functional pro-inflammatory and thrombogenic properties of these cells (14, 20). Because it has been proposed that there may be indications for statins in conditions with endothelial dysfunction (23, 36), we hypothesized that statins may improve endothelial function after radiation exposure. In the present study, we demonstrated for the first time that pravastatin inhibited the radiation-induced increased production of IL6, IL8 and

MCP1 and expression of ICAM1 by EC, thus providing additional evidence for the anti-inflammatory properties of statin on EC. We also showed that this inhibitory effect of pravastatin was persistent. Since the maintenance of increased inflammatory mediators or adhesion molecules has been reported to play a key role in the pathogenesis of radiation-induced tissue injury (2, 4, 42, 43), our results may support the future use of statins as a possible therapeutic intervention after radiation exposure. In fact, a recent study has demonstrated, in a rat model of chronic cyclosporin-induced nephropathy, that pravastatin suppresses interstitial inflammation and fibrosis through a mechanism involving persistent decreased production of chemokines and profibrogenic cytokines (44). Therefore, a sustained inhibitory effect of pravastatin after radiation exposure may effectively abrogate the progression of delayed radiation injury leading to impairment of organ function.

One of the mechanisms of action of statins is to block the conversion of HMG-CoA to mevalonate and the synthesis of isoprenoid intermediates of the mevalonate pathway by competitive inhibition of the rate-limiting enzyme HMG-CoA reductase (23, 36). The data reported here showed that mevalonate reversed the effect of pravastatin on IL6 and IL8 production and ICAM1 expression but had no effect on MCP1 production. Thus the anti-inflammatory effect of pravastatin on irradiated EC is due to its inhibition of mevalonate-derived isoprenoid synthesis. However, the absence of effect of mevalonate on MCP1 production is more difficult to explain. This is not consistent with an effect of pravastatin through isoprenoid intermediates and is not in agreement with the study of Romano *et al.* (29). Therefore, another mechanism of action of pravastatin on production of MCP1 that is unrelated to statin-mediated inhibition of HMG-CoA reductase could be implicated and could contribute to the anti-inflammatory potential of statins, as has recently been reported (45).

Recruitment of inflammatory cells and thrombus formation are viewed as important events in the pathogenesis of radiation damage (4, 7, 46). Therefore, in the present study, we tested the effect of pravastatin on adhesion of leukocytes and platelets to irradiated EC. We demonstrated a persistent inhibition by pravastatin of leukocyte and platelet adhesion to irradiated EC under physiological flow. This effect was abrogated by simultaneous treatment with mevalonate. Our data were the first to show that statin could modulate the functional thrombogenic properties of EC after irradiation (47), and that this effect was mediated through isoprenoid intermediates. Our data also showed for the first time the functional anti-inflammatory effect of statin after radiation exposure. Since ICAM1 mediates adhesion of leukocytes and platelets to endothelial cells (48, 49), the mechanism by which pravastatin inhibits blood cell adhesion could involve, at least in part, the inhibition of ICAM1 expression on irradiated HMVEC-L cells. Interestingly, statin treatment did not significantly change the fraction of rolling leukocytes under flow, confirming previous
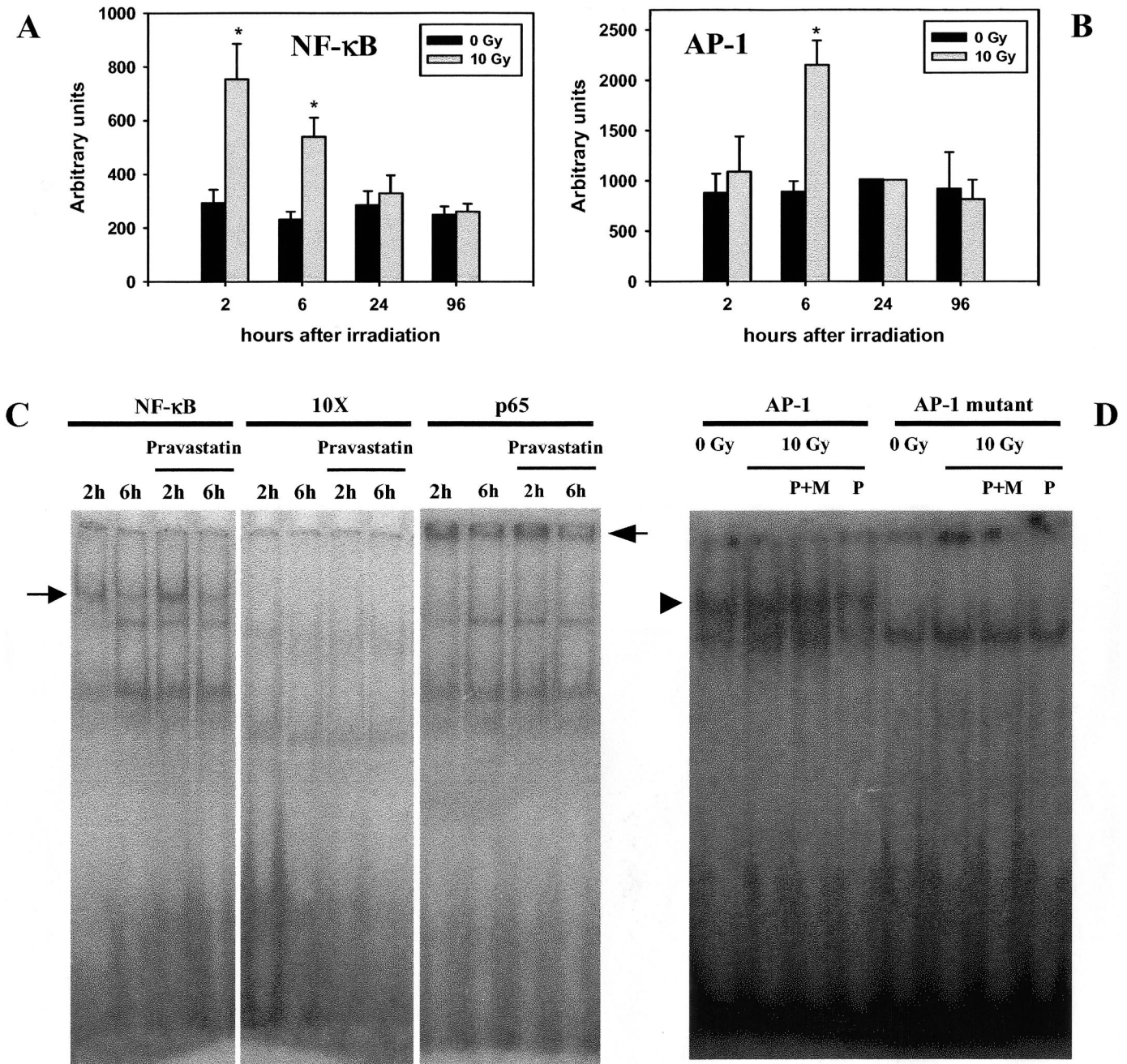
FIG. 5. NF-κB and AP1 activity analyzed by EMSA in nuclear protein extracts from HMVEC-L. Cells Quantification of the NF-κB (panel A) and AP1 (panel B) activity after 10 Gy irradiation. Data are means ± SEM of two to four independent experiments. *$P < 0.01$ compared with nonirradiated cells. Panel C: Gel-shift image showing the absence of effect of pravastatin on NF-κB activation in irradiated HMVEC-L cells. The 10X lanes were incubated in the presence of 10-fold excess of unlabeled NF-κB probe. The small arrow indicates the position of the NF-κB-specific complex. The p65 lanes present the supershifted NF-κB complex position indicated by the large arrow. Panel D: Gel-shift image showing the inhibiting effect of pravastatin on AP1 activation in irradiated HMVEC-L cells and its reversion with mevalonate. The arrowhead indicates position of the AP1-specific band.

observations on IL1B-activated EC (*33*). This suggests that the primary target of statin action is the firm adhesion step that is essential for leukocyte transmigration and that by preventing leukocyte recruitment could limit radiation pathologies.

NF-κB and AP1 regulate of a variety of genes that participate in the atherosclerotic, inflammatory and thrombotic processes (*50, 51*). We found that NF-κB was activated after irradiation, confirming previous results on EC from other tissue (*12*), and that pravastatin, while inhibiting inflammatory molecule expression and blood cell adhesion to irradiated HMVEC-L cells, did not modify the radiation-induced activation of NF-κB. These observations have also been described for other anti-inflammatory molecules such
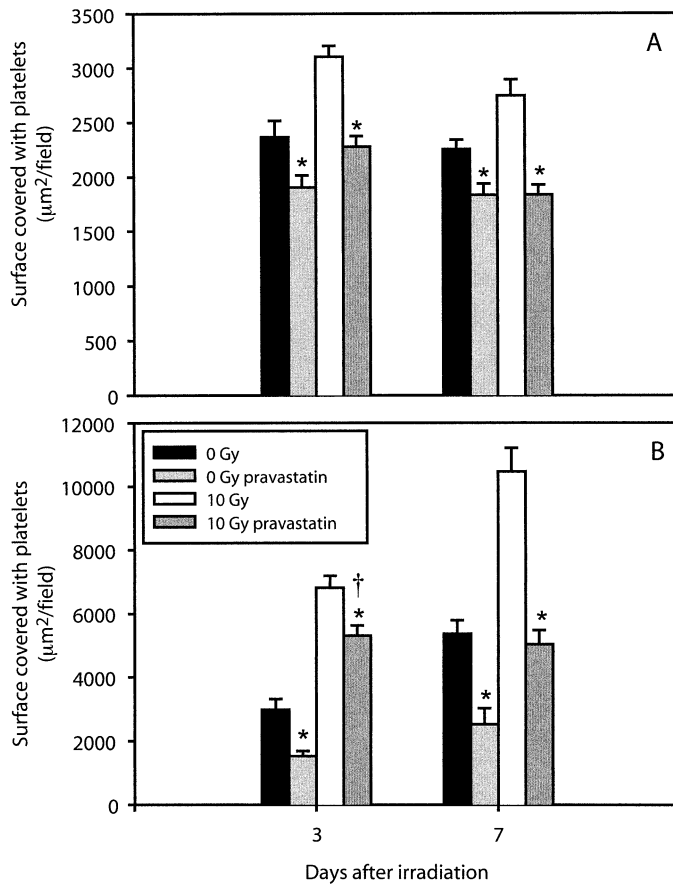
FIG. 6. Effect of pravastatin on the surface covered by single platelets and thrombi on nonirradiated and irradiated HMVEC-L cells at wall shear rates of 25 s^{-1} (panel A) and 500 s^{-1} (panel B). Histograms represent the means ± SEM of 20–50 fields randomly chosen at the end of whole blood perfusion from one to three separate experiments. *$P < 0.05$ compared with control cells, †$P \leq 0.001$ compared with nonirradiated cells.

as tepoxalin, which effectively prevented leukocyte adhesion to irradiated EC but had no effect on NF-κB activation after irradiation (13). This absence of effect of pravastatin on NF-κB could be due to the statin used (52) or to the involvement of transcription factors other than NF-κB. This study is the first to show that radiation activated AP1 in EC and that pravastatin down-regulated this activation. Therefore, AP1 rather than NF-κB is implicated in the anti-inflammatory effect of pravastatin after radiation exposure.

TABLE 3
Effect of Mevalonate on Platelet Adhesion on Irradiated HMVEC-L Cells

Wall shear rate (s^{-1})	Control (μm²/field)	Pravastatin (μm²/field)	Pravastatin and mevalonate (μm²/field)
25	2664 ± 83	2018 ± 107*	2563 ± 139†, NS
500	6418 ± 544	3428 ± 348*	5219 ± 296†, NS

Notes. Values are the means ± SEM of 40 fields randomly chosen at the end of the perfusion from two separate experiments. * $P \leq 0.001$ compared with control cells, † $P < 0.005$ compared with pravastatin-treated cells. NS: nonsignificant compared with control cells.

In summary, the data presented in this study suggested that inhibiting endothelial dysfunction with statins may limit the initiation and the progression of radiation atherosclerosis, fibrosis and thrombosis. Statins have also been described to have anticancer activity by acting as radiosensitizers (53, 54). Therefore, statins may be proposed as a therapeutic strategy not only to improve radiotherapy but also for reducing the risk of radiation pathologies.

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