



PROAPOPTOTIC EFFECT OF ATORVASTATIN ON STIMULATED RABBIT SMOOTH MUSCLE CELLS

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The *in vitro* and *in vivo* activity of atorvastatin and other 3-hydroxy-3-methylglutaryl coenzyme A (HMGCoA) reductase inhibitors (fluvastatin, pravastatin and simvastatin) has been investigated. Atorvastatin, fluvastatin, pravastatin and simvastatin caused a significant and dose-dependent (0.1–50 μM) reduction in cell multiplication of vascular smooth muscle cells (SMC) in cultures associated with the retardation of cycling cells in the G1 and G2/M compartments at 24 h, a phenomenon leading to apoptosis (programmed cell death) in several experimental *in vitro* models. The effects on the cell cycle resulted in a strong inhibition of cell proliferation at 48 h, followed by apoptosis when incubation was prolonged to 72 h as assessed by nuclei morphology and cytofluorimetric analysis of DNA. The apoptotic effect observed for the statins is completely prevented by the addition of exogenous mevalonate at a 100 μM concentration. *In vivo* SMC proliferation was stimulated by applying a silastic collar to the outside surface of carotid arteries in normocholesterolemic rabbits in the presence of an anatomically intact endothelium. The positioning of the collar promoted apoptosis in control vessels as assessed by Terminal Deoxynucleotidyl Transferase-dUTP-Biotin Nick-End Labeling (TUNEL) assay. The pre-treatment with 5 mg kg^{-1} per day of atorvastatin before collar insertion strongly increased the number of TUNEL-positive cells, suggesting a pro-apoptotic effect of HMGCoA reductase inhibitors also *in vivo*, even though cell DNA rearrangement still needs to be excluded. No apoptotic signal was detectable in sham operated arteries with no collar in either control or atorvastatin-treated rabbits. These data indicate that HMGCoA reductase inhibitors effect on the arterial wall may involve the modulation of both cell proliferation and programmed cell deaths supporting a possible role of statins in the prevention of early lesion and restenosis.

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KEY WORDS: apoptosis, smooth muscle cells, atherosclerosis, HMGCoA reductase inhibitors, atorvastatin.

INTRODUCTION

Dynamic and continued 'stress events' represented by either chemical or mechanical risk factors or alterations of the physiological balance in the arterial wall promote functional endothelium injury and thus migration and multiplication of smooth muscle cells (SMC) and apoptosis [1]. SMC proliferation and intimal hyperplasia is a prominent feature in human and experimental atherosclerosis [2].

Atherosclerosis occurs in the intima at sites of SMC proliferation and often in the presence of a continuous yet functionally and structurally altered endothelium. Smooth muscle cells contribute to the lesion by cell accumulation and extracellular matrix production and secretion [1]. The control of the proliferative process is presently considered a favourable component of potential antiatherosclerotic drugs [3]. The occurrence of apoptosis in normal and especially in rapidly proliferating cell populations suggests a potential role of this process in the maintenance of stable numbers of normal cells in tissues with various degrees of proliferative activity, including the arterial wall [4, 5]. Apoptosis is an important regulator of cell number in the vessel

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wall, balancing cell proliferation and thus helping to maintain vessel wall architecture [4]. Cell kinetic studies indicate a tight link between vascular SMC rate of formation and death in normal arteries [6, 7], a link that may be lost upon the development of atherosclerosis [4–6, 8, 9].

We have recently observed that several 3-hydroxy-3-methylglutaryl coenzyme A (HMGCoA) reductase inhibitors are able to affect smooth muscle cell proliferation in the carotid artery intima of normocholesterolemic rabbits who had a silastic collar placed on them [10], by mechanisms independent from serum lipoprotein lowering, possibly through the local inhibition of mevalonate biosynthesis [11]. We also reported that statin-treated C6 glioma cells show the inhibition of cell cycling in G1 and arrest/retardation in G2/M both *in vitro* and *in vivo*, a phenomenon often associated with apoptosis [12]. Furthermore, an apoptotic effect of lovastatin, an HMGCoA reductase inhibitor, has already been described in HL-60 [13], leukaemic [14] and human glioma cells [15].

In the present study, SMC apoptosis in tissue culture and *in vivo* has been investigated. *In vivo* SMC proliferation was stimulated by the silastic collar method. The positioning of the collar promotes an early infiltration of the vessel wall by polymorphonuclear cells and monocytes, followed by smooth muscle cell replication in the media and gradual subendothelial smooth muscle cell accumulation, with deposition of extracellular matrix within the neointima [10]. The effect of statins (atorvastatin, fluvastatin, pravastatin and simvastatin) on *in vitro* and *in vivo* SMC apoptosis has been evaluated.

MATERIALS AND METHODS

Materials

Tissue culture plastics were from Corning (Corning, NY, USA). Atorvastatin, fluvastatin, pravastatin and simvastatin were from Parke-Davies, Novartis (Basel, Switzerland), Bristol Mayers Squibb and Merck, Sharp and Dohme (Rahway, NJ, USA). Mevalonic acid lactone and all materials for flow cytometry were from Sigma Chemical Co. (St. Louis, MO, USA). The TUNEL kit was supplied from Boehringer-Mannheim.

Cell culture

Smooth muscle cells were cultured according to Ross [16], from the intima-media layers of the common carotid of male New Zealand rabbits weighing 2–2.3 kg (Charles River, Calco, Italy) as previously described [17]. Cells were maintained in monolayer culture at 37°C in a humidified atmosphere containing 5% CO₂ in Dulbecco's minimum essential

medium (Gibco Laboratories, Grand Island, NY, USA) supplemented with 10% foetal calf serum, penicillin–streptomycin, sodium pyruvate (Gibco). Cells were maintained. At confluence, cultures were replated by enzymatic dissociation with trypsin/EDTA (Gibco). All experiments were performed within the 10th subculture.

Atorvastatin and fluvastatin were diluted in methanol (final concentration 0.1%) and added in required amounts to the culture media. Pravastatin was dissolved in PBS. Simvastatin was added to the cultures as the sodium salt, obtained from the lactone according to the procedure of Kita *et al.* [18]. In each experiment, proper control cell dishes incubated with methanol (0.1%) were included.

Cell viability was determined by the Trypan blue exclusion methods as previously described [19].

Cell cycle analysis

Approximately 10⁶ cells per experimental condition were harvested by trypsinization, washed with 0.1 M phosphate-buffered saline (PBS) and subsequently fixed in 70% cold ethanol. Propidium iodide staining and DNA analysis by flow cytometry were carried out as previously described [12].

Myocyte proliferation

Myocytes were seeded in triplicate in 35-mm tissue culture dishes at a density of about 150,000 cells per dish and after 24 h received drug treatment as described in the Results section. The cell number was determined using a Coulter counter (Coulter Instruments, Milan, Italy) following trypsinization.

Visualization of apoptotic nuclei

Smooth muscle cells were grown on coverslips and treated as described in the text. Cells were fixed with 70% cold ethanol and stained with the DNA intercalating dye Hoechst 33528, 0.5 µg ml⁻¹ in PBS containing 100 µg ml⁻¹ DNase-free RNase A (Sigma Chemical Co.) and 0.1% NP40. The coverslips were rinsed, air dried and mounted with Permafluor mounting medium (Immunon, Pittsburgh, PA, USA). Fluorescence was visualized using a Zeiss Axioskop microscope.

Neointimal hyperplasia

New Zealand rabbits (approx. 2 kg in weight) were used. The animals were randomly separated into two groups (three animals per group): (1) control animals; (2) atorvastatin, 5 mg kg⁻¹ per day continuous i.p. infusion for 7 days.

At day 5 a silastic collar of 1.5 cm in length was inserted on the left carotid artery, essentially as described [10]. The contralateral artery was identically manipulated but no collar was left on (sham). Animals were killed 3 days after collar positioning.

Terminal Deoxynucleotidyl Transferase-dUTP-Biotin Nick-End Labeling (TUNEL) assay

Paraffin sections were dewaxed, rehydrated, treated with proteinase K ($20 \mu\text{g ml}^{-1}$) and blocked for endogenous peroxidase activity with 3% H_2O_2 . After end-labelling for 1 h at 37°C according to the kit instructions, sections were washed in PBS and incubated with anti-fluorescein antibody conjugated with horseradish peroxidase complex, rinsed in PBS and stained with DAB substrate. Apoptotic nuclei were identified by the presence of fluorescent staining.

RESULTS

Statin-induced apoptosis: cell cycle distribution analysis

The DNA histograms obtained from smooth muscle cells treated for 24 h with atorvastatin ($1\text{--}10 \mu\text{M}$) showed a dose-dependent drug effect on the cell cycle distribution (Fig. 1, upper panel). The entry of cells into the S phase progressively decreased. At a concentration above $1 \mu\text{M}$ the G₀/G₁ block was

accompanied by an increase of the proportion of cells in G₂/M, as indicated by the decline of the S:G₂/M ratio. When incubation with atorvastatin was prolonged up to 72 h the G₂/M peak declined coincident with the appearance of a dose-dependent sub-diploid peak (sub G₁), typical of apoptotic cells, starting from a concentration of $5 \mu\text{M}$ (Fig. 1, lower panel). The addition of mevalonate to cultured cells completely abolished the effects of atorvastatin on cell cycle distribution and DNA content (Fig. 1). Simvastatin, pravastatin and fluvastatin showed a similar pattern on the cell cycle.

Antiproliferative effect of statins on SMC

Smooth muscle cells were treated in culture with increasing concentrations of either atorvastatin, fluvastatin, pravastatin or simvastatin and the cell number was determined after 48 h. Exposure to either statin resulted in a dose-dependent inhibition of cell growth rates as shown in Fig. 2. A similar pattern, somehow more effective for simvastatin, was observed for all statins. The addition of mevalonate ($100 \mu\text{M}$) to cells exposed to the statins completely abolished the antiproliferative effect observed (Fig. 2).

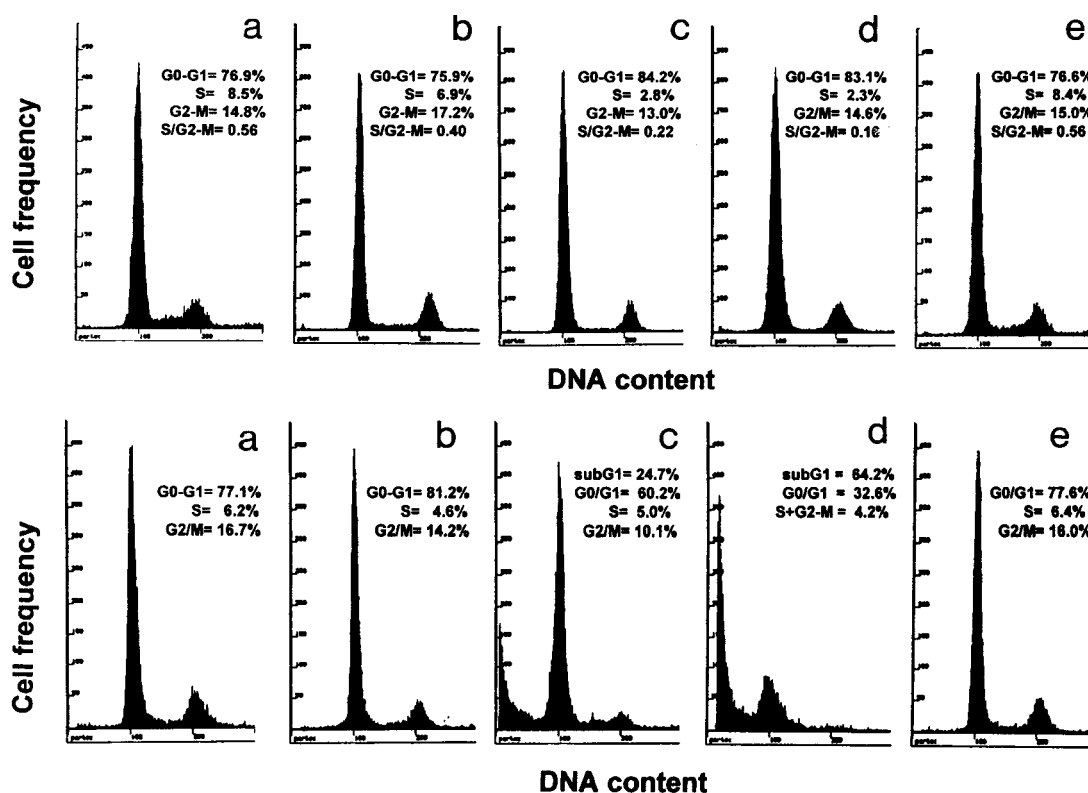


Fig. 1. *In vitro* effect of atorvastatin on DNA frequency distribution histograms of rabbit SMC. Exponentially growing cells were incubated for 24 h (upper panel) or 72 h (lower panel) in the presence of various concentrations of atorvastatin. The DNA content was analyzed by flow cytometry after propidium iodide staining. a, controls; b, $1 \mu\text{M}$ atorvastatin; c, $5 \mu\text{M}$ atorvastatin; d, $10 \mu\text{M}$ atorvastatin; e, $10 \mu\text{M}$ atorvastatin plus $100 \mu\text{M}$ MVA. Percentages of cells in different phases of the cell cycle are given in each panel. Similar results were obtained in six independent experiments. The sub-diploid peak in DNA frequency distribution histograms from 72 h treated cells indicates the presence of apoptotic nuclei.

Morphological analysis and effect on cell viability in statin-treated cells

The induction of apoptosis by atorvastatin was also detected in smooth muscle cells by micronucleation after staining with the DNA intercalating dye Hoechst 33528 (Fig. 3). Cell viability, determined by the Trypan blue exclusion assay, was always greater than 80% under all experimental conditions (data not shown).

Statin-induced apoptosis: *in vivo* effect

The neointimal hyperplastic lesion composed of vascular smooth muscle cells was generated by applying a silastic collar to the outside of normocholesterolemic rabbit carotid arteries. Rabbits were killed 24 and 72 h after collar surgery. The positioning of the collar promoted an early infiltration of the vessel wall by polymorphonuclear cells and monocytes (24 h after collar placement), followed by smooth muscle cell replication in the media and a gradual subendothelial smooth muscle cell accumulation, with deposition of extracellular matrix within the neointima. Apoptotic cells were detectable starting 24 h after collar positioning. Figure 4 (A) is an overview at low magnification illustrating the general distribution of TUNEL labelling of nuclei for internucleosomally degraded DNA indicating apoptosis at day 3 in control collared arteries. Labeling

was prominent in the outer media. Carotid arteries obtained from rabbits treated with either atorvastatin or fluvastatin (5 mg kg⁻¹ per day) starting 5 days prior to collar insertion revealed a stronger TUNEL labelling [Fig. 4 (B)], compared to controls, which was especially apparent in the outer media but common also elsewhere. No apoptosis signal was detectable in arteries with no collar from either control or statin-treated rabbits.

DISCUSSION

We have recently reported an *in vitro* and *in vivo* inhibition of vascular SMC proliferation by HMG-CoA reductase inhibitors independent of their hypocholesterolemic properties [10]. The present study indicates programmed cell death as an additional possible mechanism for the modulation of the cellularity of the arterial wall in proliferative atherosclerotic or restenotic lesions where the SMC component is predominant. These results and previous *in vitro* and *in vivo* observations [10, 11] demonstrate a direct effect by statins on the arterial wall which merits clinical investigation.

HMGCoA reductase inhibitors have received increasing attention as pharmacological tools for con-

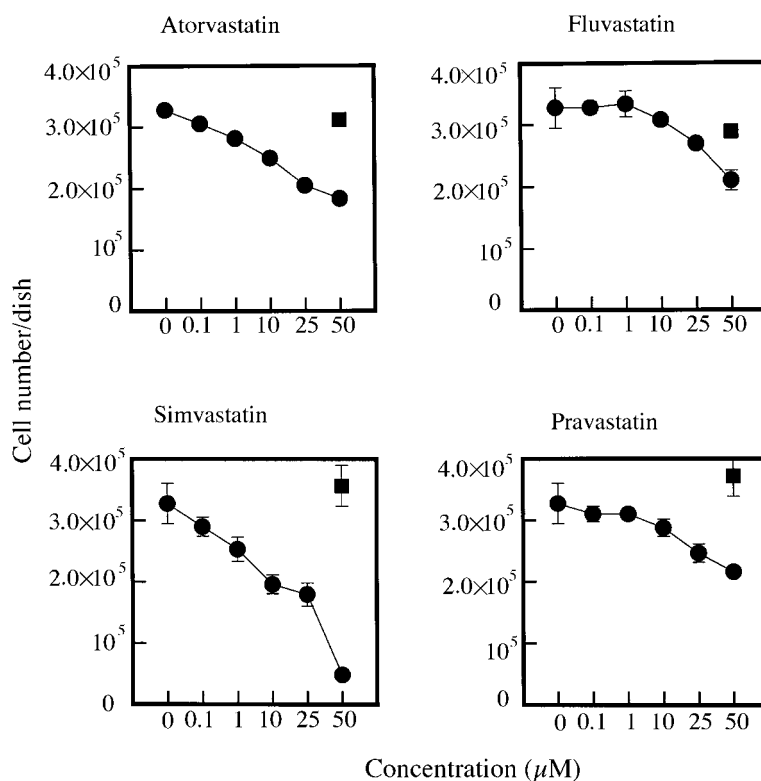


Fig. 2. Dose-dependent effect of different statins on SMC proliferation at 48 h. Exponentially growing cells were incubated for 48 h with increasing concentrations of statins in the presence (■) or absence (●) of 100 μM mevalonate.

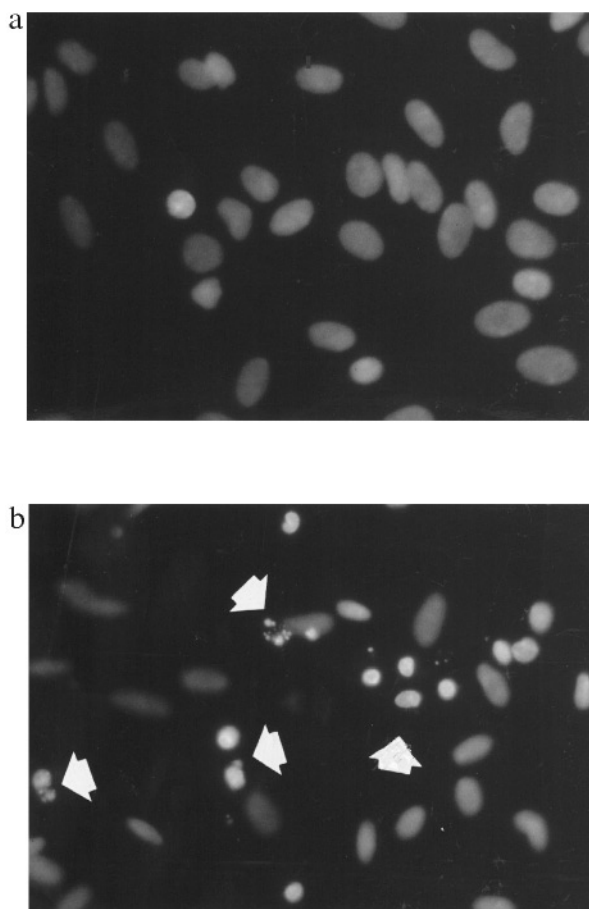


Fig. 3. Induction of apoptosis by atorvastatin: morphological analysis of Hoechst 33528-stained nuclei. SMC were treated for 72 h with 5 μM atorvastatin. Cells were fixed with 70% ice-cold ethanol and stained with Hoechst 33528. Fluorescence was visualized using a Zeiss Axioskop microscope (Objective PlanNeofluar 40X). a, untreated SMC; b, treated SMC. Apoptotic cells are indicated by arrows.

trolling abnormal cell growth, such as myocyte proliferation in atherogenesis [3, 10] or tumor development [12, 20].

Several *in vitro* and pre-clinical evidence support the hypothesis of a direct effect of statins on the arterial wall [3]. Recent *in vitro* studies demonstrate that statins dose-dependently decrease SMC migration and proliferation [17]. Pre-clinical data show that HMGCoA reductase inhibitors, albeit with different potencies, decrease neointimal proliferation in both hypercholesterolemic [21–24] and normocholesterolemic rabbits without affecting, in this case, plasma cholesterol levels [10]. The latter study suggests that these drugs can directly affect early alteration of wall responses irrespective of plasma lipid changes. Indeed *in vivo* data demonstrate that the mechanism underlying the antiproliferative effect of statins is related to the local inhibition of MVA synthesis within cells of the vascular wall, since local delivery of MVA fully prevented the inhibitory ef-

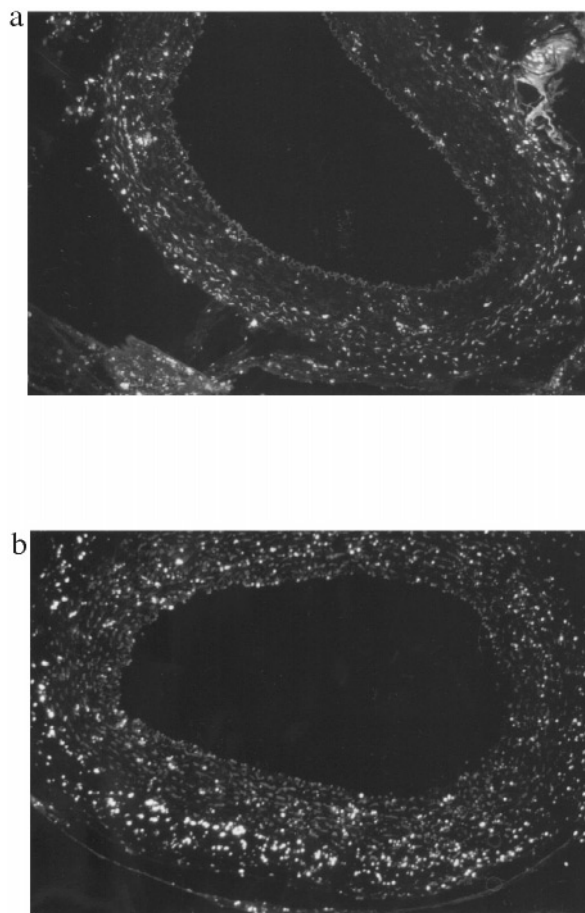


Fig. 4. Detection of apoptosis in collared arteries from normocholesterolemic rabbits 3 days after perivascular manipulation. Staining with TUNEL demonstrates the presence of fragmented nuclei. Fluorescence was visualized using a Zeiss Axioskop microscope (Objective PlanNeofluar 20X). a, carotid section from the control rabbit; b, carotid section from a rabbit treated with 5 mg kg⁻¹ day of atorvastatin starting 7 days before collar positioning.

fect of systemic fluvastatin [11]. It is well known that mevalonate is the precursor of a complex branched pathway the major product of which is cholesterol. However, mevalonate is also the precursor of a variety of essential non-sterol isoprenoids including: the polyisoprenoids side chains of heme A and ubiquinone, involved in oxidative respiration; dolichol, required for glycoprotein synthesis; and isopentenyl-adenine, present in some transfer RNAs. Furthermore, mevalonate is the precursor of two isoprenoids intermediates, farnesyl-pyrophosphate (C15) and geranylgeranyl-pyrophosphate (C20), substrates for a post-translational modification in which the lipid moiety is covalently transferred to cellular proteins fundamental for cell function secretion, migration and proliferation and proliferation [20].

In the present study statins were used in rabbit smooth muscle cell cultures; however, similar results were obtained with human SMC (unpublished data).

Statin concentrations utilized in both rabbit and human SMC were comparable to the peak plasma concentrations (0.1–1 μM) transiently achieved in the plasma of hypercholesterolemic patients treated with 160 mg per day of simvastatin [25]. Previous *in vitro* results demonstrated that the inhibition of cell growth correlates with intracellular HMGCoA reductase activity and takes place as long as mevalonate biosynthesis remains inhibited by at least 60–70% [27]. These observations suggest the use of HMGCoA reductase inhibitors either with very high *in vivo* efficacy or at very high doses and favour an uninterrupted administration to maintain biological activity by sustained mevalonate pathway inhibition. This could translate into a more significant prevention of cardiovascular disease [3].

The results of the present study indicate that statins play an important role on cell cycle progression and apoptosis in SMC. In fact, the exposure of SMC to statins induces at 24 h a dose-dependent decrease in S phase and an accumulation of the cells in G2/M, particularly at the highest concentrations. This results in an inhibitory effect on cell proliferation at 48 h, followed by apoptosis when incubation is prolonged to 72 h [Figs. 1 and 2]. Atorvastatin and fluvastatin systemic pre-treatment increased the number of TUNEL positive cells suggesting a pro-apoptotic effect of HMGCoA reductase also *in vivo*, at least under our experimental conditions, even though cell DNA rearrangement cannot be completely excluded.

In conclusion these data, together with previous results, suggest that the HMGCoA reductase inhibitors effect on the arterial wall may involve modulation of both cell proliferation and apoptotic cell death. Thus, statins merit further investigation as preventive approaches to early lesion development and restenosis.

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