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Pro-apoptotic effect of fluvastatin on human smooth muscle cells

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Abstract

The antiatherosclerotic effect of statins has been attributed to their hypocholesterolemic action. We therefore evaluated the effect, in vitro, of the addition of the serum of patients taking fluvastatin on human smooth muscle cells in order to ascertain the effect of the drug on cell proliferation and apoptosis. We found that the addition of serum from patients treated with fluvastatin for 6 days caused a significant reduction in cell proliferation, increased cell apoptosis and reduced the B cell leukemia-2 (bcl-2) concentration. It is concluded that the induction of apoptosis by statins could be a supplementary mechanism in the prevention of atherosclerotic lesions in humans.

Keywords: Fluvastatin; Apoptosis; Atherosclerosis; B cell leukemia-2

1. Introduction

Corsini et al. (Corsini et al., 1996) recently demonstrated that there is a marked reduction in cell proliferation following the addition of the serum of fluvastatin-treated patients to smooth muscle cells. This suggests that the antiatherosclerotic effect of statins might be attributed to their antiproliferative effect on atherosclerotic plaques as well as to their hypocholesterolemic effect. Increased cell replication is, however, only one of the mechanisms underlying the progression of atherosclerotic lesions. Various experimental models have stressed the importance of programmed cell death, or apoptosis, in the atherosclerotic thickening of the vascular wall and in the onset of atherosclerotic disease (Bjorkerud et al., 1994). We therefore added the serum of patients treated with fluvastatin to human vascular myocytes in order to evaluate its effects on cell proliferation and apoptosis.

2. Materials and methods

The study was conducted with 12 patients (6 males–6 females; mean age 25 ± 4 years), without hypercholesterolemia, who had been on 40 mg of fluvastatin a day for the 6 days prior to the study. Blood samples were collected before the start of the protocol, and 0, 0.5, 1 and 6 h after the administration of the last dose of fluvastatin. We then evaluated the effects of the addition of whole-blood serum on both the proliferation and the appearance of apoptosis in human vascular myocytes. Human vascular myocytes (A617 from the human femoral artery) were cultured according to Ross (Ross, 1991). Cells were grown in single layer at 37°C in a humidified atmosphere of 5% CO_2 in minimum essential medium Eagle (MEM), supplemented with 10% (v/v) fetal calf serum, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 20 mM tricine buffer and 1% (v/v) non-essential amino acid solution (Sun et al., 1992). The medium was changed every third day. Cells were used between the 4th and 10th passages. Smooth muscle cells were identified by their growth behaviour and morphology and by using monoclonal antibody specific for alpha smooth muscle actin, the actin isoform typical of smooth muscle cells (Skalli et al., 1986). The cells grew out of explants after 12–16 days, formed layers after confluency had been reached and contained numerous myofilaments and dense bodies, as observed by transmission electron microscopy. The cultures were incubated for 72 h. Cells (3×10^5 cells/well) were plated out in 1 ml of culture medium onto glass coverslips in 24-well culture plates.

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The percentage of apoptotic cells was evaluated using different techniques. Morphological analysis was performed following staining with acridine orange or Hoechst chromatin dyes (Sigma). Over 600 cells, including those showing typical apoptotic characteristics, were counted by using a Biomed fluorescence microscope (Leitz, Wetziar, Germany). The percentage of apoptotic cells was calculated:

$$\%\,\text{apoptosis} = \frac{\text{Total no. of cells with apoptotic nuclei}}{\text{Total no. of cells counted}} \times 100.$$

Flow cytometry analysis of isolated nuclei was performed. Briefly, cells were collected from culture wells and washed twice in CM in 12×75 mm polypropylene tubes (Falcon, Becton Dickinson Labware, Lincoln Park, NJ). The cell pellet was gently resuspended at room temperature in 1 ml of hypotonic solution consisting of propidium iodine 50 μg/ml (Becton Dickinson, Mountain View, CA) 0.1% sodium citrate (Merck, Darnstadt, Germany) and 1% Triton X-100 (Sigma) in distilled water. The tubes were placed at 4°C in the dark overnight. Flow cytometry analysis was subsequently performed using a FACscan flow cytometer (Becton Dickinson). The correct threshold value was experimentally selected in order to exclude the majority of cell debris. Data collection was gated by using adequate values of forward- and side-angle scatter to exclude remaining cell debris and large aggregates of nuclei and to include nuclei from apoptotic, necrotic and living cells. For each sample, 5000 events were acquired. Analysis of DNA fragmentation was carried out at single cell level using the terminal transferase uridyl nick end labelling (TUNEL) assay or at culture level by gel electrophoresis. Labeling of DNA strand breaks with fluorescein–dUTP by terminal deoxynucleotidyl transferase was performed using a commercial kit ('in situ cell death detection kit fluorescein', Boehringer Mannheim, Mannheim, Germany) according to the manufacturer's instructions. The positive control consisted of cells treated with DNase 1 (1 mg/ml) for 10 min at room temperature. Analysis of labeled cells was performed both by fluorescence microscopy and flow cytometry. Flow cytometry was performed as described above. For analysis of DNA fragmentation by gel electrophoresis, cells were washed with phosphate buffered saline (PBS) and then lysed in 0.5 ml of lysis buffer (10 mM Tris, pH 7.4, 1 mM EDTA, pH 8.0, 0.2% Triton X-100) containing proteinase K (100 μg/ml) for 1 h at room temperature. Samples were then centrifuged at 13 000 r.p.m. for 15 min. DNA in the supernatants was precipitated with 5 N NaCl in 1 volume of isopropanol for 1 h at −20°C. The DNA precipitates were recovered by centrifugation at 13 000 r.p.m., air dried for 15 min at room temperature, resuspended in TE buffer (10 mM Tris, pH 7.4, 1 mM EDTA, pH 8.0) containing RNase (100 μg/ml) and incubated for 1 h at 37°C. Before electrophoresis, loading buffer (2% lauryl sulfate sodium salt (SDS), 15 mM EDTA, 0.25% w/v bromophenol blue, 50% v/v glycerol) was added to each sample at a ratio of 1:5.

Burker's counting cell was used to evaluate cell proliferation. Apoptosis and cell proliferation were evaluated 24, 48, and 72 h after the addition of 1.5% 15% of serum.

We also measured the concentration of B cell leukemia-2 (Bcl-2) (Bcl-2 ELISA Oncogene Research Products, Cambridge, MA, USA), a proto-oncogene that can reduce apoptosis (Hokenberry et al., 1992) in the medium in the wells. Each experiment was repeated at least 3 times. Results were expressed as means ± S.E.M. The statistical analysis was made using a one-way analysis of variance (ANOVA) test, followed by Tukey's test.

3. Results

In agreement with Corsini et al. (1996), we found that the addition of serum from patients treated with fluvastatin caused a significant reduction in cell proliferation. The maximum effect was achieved with serum drawn 0.5 h after the last dose and when a 15% serum concentration was used (Fig. 1).

Moreover using flow cytometry, we found that the addition of the serum from treated subjects caused a marked increase in cell apoptosis, which reached its maximum at the 72nd h of incubation (Fig. 1). Morphological analysis by light microscopy and acridine orange staining confirmed the results obtained by flow cytometry (data not shown). Interestingly, the effects of fluvastatin on cell proliferation and on apoptosis appeared to be independent

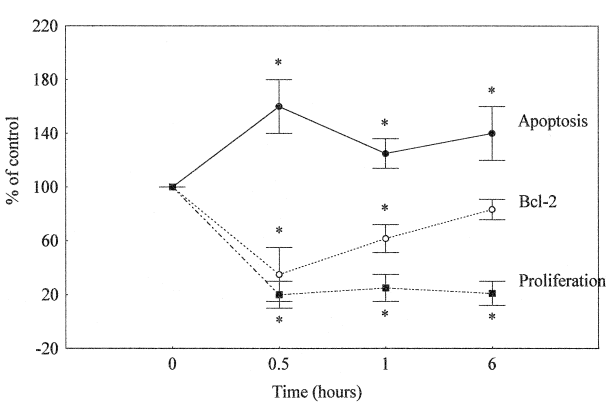

Fig. 1. Effect of sera from fluvastatin treated people on proliferation, apoptosis of human smooth cells and bcl-2 concentrations. Target cells were incubated for 72 h in a medium containing 15% whole blood sera from fluvastatin treated patients. Abscissa is time of sera collection after last dose. Means and S.E.M. of 12 experiments each run in triplicate, with different sera. The mean value of control (100%) for cell number, after subtracting $t = 0$, was 238×105 cells plate and 3.5% for apoptosis. $^{*}p < 0.05$ vs. $t = 0$.

of each other: the antiproliferative effect was also observed after the addition of 1.5% serum, while the pro-apoptotic action was observed with only 15% serum. After correction of bcl-2 concentrations, for the number of cells present, we found a significant reduction in the proto-oncogene concentrations, with the peak reduction corresponding to the peak of apoptosis (Fig. 1).

4. Discussion

Our findings confirm that fluvastatin has an antiproliferative action, as has already been observed by other authors (Corsini et al., 1996). To our knowledge, however, ours are the first findings to demonstrate that fluvastatin has a pro-apoptotic effect in humans. Our data, moreover, appear to confirm the preliminary results recently obtained in vitro for smooth muscle cells after the addition of substances such as lovastatin (Sindermann et al., 1996) and other 3-hydroxy-3-methyl-glutharil coenzyme A reductase inhibitors (Baetta et al., 1997; Guijarro et al., 1998). The capacity of fluvastatin to increase apoptotic phenomena may be mediated by the reduction in bcl-2 concentrations observed by us (Kroemer, 1997), although this effect on the system regulating apoptosis can only be clarified by making an in-depth study of the complex network of antagonist (bcl-2), and agonist (bcl-Xl, bcl-w, Mcl-1, A1, Bax, Bak, Bcl-Xs, Bad, Bid) factors that influence cell death (Yang and Korsmeyer, 1996). Although it has been suggested that a disordered apoptotis causes instability in atherosclerotic lesions (Crisby et al., 1997), a reduction in apoptotis, especially in the initial stages of the atherosclerotic process (Desmouliere et al., 1995) may occur secondary to the effect of protective factors such as insulin growth factor-1 and platelet derived growth factor (Raff et al., 1993). This reduced rate of apoptosis combined with cellular proliferation could lead to the accumulation of cells and intimal thickening, thereby contributing to the progression of atherosclerotic disease. In conclusion, our findings suggest that, in the prevention of atherosclerosis, the induction of apoptosis by statins may be a mechanism ‘supplementary’ to their hypocholesterolemic and antiproliferative actions. Other factors may be involved in the complex mechanism underlying the antiatherosclerotic effect of statins: it is well known that statins interfere with the production of the tissue factor (Colli et al., 1997). Further studies are therefore required in order to clarify the impact of fluvastatin on the progression of atherosclerosis and on the remodelling of atherosclerotic plaques (Cai et al., 1997; Mallat et al., 1997).

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