Statin-Induced Inhibition of 3-Hydroxy-3-Methyl Glutaryl Coenzyme A Reductase Sensitizes Human Osteosarcoma Cells to Anticancer Drugs

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ABSTRACT

Osteosarcoma is the most common primary bone tumor in children and young adults. Resistance to chemotherapeutic drugs is a major problem that is responsible for the failure of treatment. This points to the need for increasing the responsiveness to cytotoxic drugs. We previously showed that lipophilic statins induce apoptosis in human osteosarcoma cells. In this study, we investigated the effects of atorvastatin [(3R,5R)-7[2-(4-fluorophenyl)-5-(1-methylethyl)-3-phenyl-4-[phenylamino]carbonyl]-1H-pyrrol-1-yl]-3,5-dihydroxyheptanoic acid] in combination with chemotherapeutic drugs on human osteosarcoma cell apoptosis, invasion, and migration. We report here that atorvastatin enhances the reduced cell viability induced by the anticancer drugs doxorubicin (Adriamycin; (1S,3S)-amino-3 tridesoxy-2,3,6 α-L-lyxo-hexopyranoside glycolyl-3 trihydroxy-3,5,12 methoxy-10 dioxo-6,11 naphtacetyl-1) and cisplatin in human osteosarcoma cells. In particular, we found that atorvastatin enhances the induction of osteosarcoma cell apoptosis by anticancer drugs. In addition, we show that atorvastatin enhances the inhibitory effect of anticancer drugs on osteosarcoma cell migration. Moreover, atorvastatin and chemotherapeutic drugs had additive inhibitory effects on osteosarcoma cell invasion. In consistent tests, atorvastatin further augmented the reduction of matrix metalloprotease 2 activity induced by doxorubicin or cisplatin in osteosarcoma cells. The results show for the first time that atorvastatin sensitizes osteosarcoma cells to anticancer drugs, resulting in reduced cell viability, migration, and invasion, which suggest a strategy to improve the response to chemotherapy and reduce tumorigenesis in human osteosarcoma.

Osteosarcoma is the most common primary tumor of bone in children and young adults that affects proximal or distal long bones (Himelstein, 1998). These tumors are characterized by a highly malignant and metastatic potential (Bruland et al., 2005). Although chemotherapeutic strategies have been improved, the survival rate of patients who relapse on therapy has not improved during the last decade. Aberrant regulation of cell growth and deregulation of apoptosis occur commonly in cancer cells and may play an essential role in tumor development (Lowe and Lin, 2000; Ding and Fisher, 2002). Accordingly, currently anticancer agents act by inhibiting cell growth or inducing cell apoptosis (Johnstone et al., 2002; Stenner-Liewen and Reed, 2003; Waxman and Schwartz, 2003). In osteosarcoma, resistance to chemotherapeutic drugs is the major mechanism responsible for the failure of treatments (Johnstone et al., 2002; Waxman and Schwartz, 2003). This highlights the need for more effective treatments using proapoptotic factors that are able to increase the responsiveness of osteosarcoma cells to classic anticancer cytotoxic drugs.

The cholesterol-lowering agents statins act as HMG-CoA reductase inhibitors, resulting in alteration of prenylation and function of small G proteins that regulate cell cycle and survival (Wong et al., 2002; Graaf et al., 2004). One of these GTPase proteins, RhoA, is implicated in the control of cell growth and apoptosis (Aznar and Lacal, 2001). Accordingly, statins that inactivate RhoA were found to trigger apoptosis in several cancer cells (Aznar and Lacal, 2001; Li et al., 2006). In recent studies, we demonstrated that lipophilic statins induce caspase-dependent apoptosis in all human cancer cell lines. Moreover, we found that atorvastatin enhances the induction of osteosarcoma cell apoptosis by anticancer drugs. In addition, we show that atorvastatin enhances the inhibitory effect of anticancer drugs on osteosarcoma cell migration. Moreover, atorvastatin and chemotherapeutic drugs had additive inhibitory effects on osteosarcoma cell invasion. In consistent tests, atorvastatin further augmented the reduction of matrix metalloprotease 2 activity induced by doxorubicin or cisplatin in osteosarcoma cells. The results show for the first time that atorvastatin sensitizes osteosarcoma cells to anticancer drugs, resulting in reduced cell viability, migration, and invasion, which suggest a strategy to improve the response to chemotherapy and reduce tumorigenesis in human osteosarcoma.
osteosarcoma cells tested independently of their phenotypic characteristics (Fromigué et al., 2006). Notably, we showed that lipophilic statins, by inhibiting HMG-CoA reductase, induce apoptosis in human osteosarcoma cells by a RhoA-p42/p44 mitogen-activated protein kinase-Bcl-2 mediated mechanism without affecting cell replication (Fromigué et al., 2006). This raises the possibility that statins may enhance the cytotoxic effects of anticancer drugs in osteosarcoma cells. We therefore investigated the effect of statins in combination with commonly used anticancer agents such as doxorubicin (Adriamycin) and cisplatin on osteosarcoma cell death and tumorigenic capacity. In this study, we show that statins sensitize human osteosarcoma cells to these anticancer agents and further reduce tumor cell migration and invasion.

**Materials and Methods**

**Cell Cultures.** Human cancer cells derived from different osteosarcoma tumors were used in this study: p53-deficient SaOS2 osteosarcoma cells (Rodan et al., 1987), obtained from American Type Culture Collection (Manassas, VA); p53 mutant and ARF-deficient MG63 cells, obtained from Dr. N. Rochet (Nice, France); and ARF mutant U2OS cells and CAL72 cells (Rochet et al., 1999), which display chromosomal abnormalities and a distinct cytokine expression profile compared with MG63 and SaOS2 cells (Rochet et al., 1999). All cells were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen, Paisley, UK) in the presence of 10% heat inactivated FCS, 1% L-glutamine, and penicillin/streptomycin (10,000 U/ml and 10,000 µg/ml, respectively), with medium change every 2 to 3 days. All experiments were performed in serum-supporting conditions, i.e., in the presence of 10% FCS.

**Reagents.** Atorvastatin (provided by Dr. Feyen, Bristol-Myers Squibb Co., Stamford, CT) was solubilized in dimethyl sulfoxide and used at 10 µM as described previously (Fromigué et al., 2006). Doxorubicin (Sigma-Aldrich, Lyon, France) and cisplatin (cis-diaminedichloroplatinum; Sigma-Aldrich) were solubilized in H2O at concentrations of 10 µg/µl and 50 µM, respectively.

**Cell Viability Assay.** Cells were seeded in 96-well plates at a density of 10^4 cells/well and incubated for 24 h. Indicated compounds were added in medium containing 10% FCS for 24 h. Cell viability was evaluated by the MTT test as described previously (Fromigué et al., 2006). Doxorubicin and cisplatin (20 µM) are used at 100 ng/ml, or cisplatin (20 µM) alone reduced cell viability by 30 to 50% in the four human osteosarcoma cell lines (Fig. 2A). The combination of atorvastatin with doxorubicin or cisplatin further reduced cell viability in all osteosarcoma cell lines (Fig. 2A).

**Apoptosis Assay.** Cells were seeded in 48-well plates at a density of 3 x 10^4 cells/well for 24 h. The compounds or combination of treatments were then added in medium containing 10% FCS and incubation was carried out for 24 h. Caspase-3 like activity was determined using a colorimetric assay as described previously (Fromigué et al., 2006). In brief, cellars extracts were incubated with 0.2 mM acetyl-Asp-Glu-Val-Asp-para nitro aniline (Alexis Laboratories, San Diego, CA) as substrate for various times at 37°C. The specific activity (nanomoles of pNA per minute per milligram of protein) was expressed as treated over control ratios.

**Cell Migration and Invasion Assays.** In vitro cell migration was measured in the modified Boyden chamber assay, using cell culture inserts with a polycarbonate filter (∼8-µm pore; BD Biosciences, Le Pont de Claix, France). Cells (50,000 cells/insert) were preincubated 2 h with or without the indicated drug before seeding in inserts and incubated for a further 22 h at 37°C. Cells that did not migrate through the filter were removed by wiping out with a cotton swab. The cells migrated to the underside were fixed in 3.7% paraformaldehyde in phosphate-buffered saline at 4°C, washed in phosphate-buffered saline, and stained with toluidine blue. Membranes were then cut from the insert and observed under the microscope. Three fields were randomly selected and counted for each assay. Triplicates were used for each assay, performed in duplicate. For in vitro cell invasion assay, the same experiments were performed using cell culture inserts coated with basement membrane Matrigel (BD Biosciences). A negative control was performed in serum-free medium supplemented with 0.1% bovine serum albumin.

**Matrix Metalloproteinase 2 Activity Assay.** Matrix metalloproteinase (MMP) 2 activity was evaluated by a colorimetric assay using Ac-Pro-Leu-Gly-[2-mercapto-4-methyl-pentanoyl]-Leu-Gly-OC2H5 thiopeptide (BIOMOL Research Laboratories, Plymouth Meeting, PA) as substrate in 500 mM HEPES, 100 mM CaCl2, 10 mM ZnCl2, 0.5% Brij35, and 10 mM DTNB buffer according to the manufacturer’s recommendations and corrected for total protein content.

**Data Analysis.** The results are representative of two to four independent experiments and are expressed as means ± S.E.M. of four to six replicates for each condition. The data were analyzed by two-factor analysis of variance followed by Kruskal-Wallis nonparametric test. A minimal level of p < 0.05 was considered significant.
and anticancer drugs on the migration potential of the four osteosarcoma cell lines. Cells were preincubated 2 h with atorvastatin and/or doxorubicin or cisplatin and then seeded in modified Boyden chambers with the drugs and 10% FCS-containing medium for a further 22 h. As shown in Fig. 3A, atorvastatin reduced cell migration by 30 to 50% in all osteosarcoma cells. Treatment with doxorubicin or cisplatin alone also reduced cell migration in the four cell lines. However, the efficacy of the drugs varied with the cell line. The two drugs reduced cell migration by 30 to 50% in SaOS2 and CAL72 cells, by more than 60% in MG 63 cells, and by up to 77% in U2OS cells (Fig. 3A). Nevertheless, the combination of atorvastatin with the cytotoxic drugs further inhibited cell migration in most cell lines. Cell migration was even reduced by up to 97% in U2OS cells (Fig. 3A). These results indicate that atorvastatin enhances the inhibition of osteosarcoma cell migration induced by chemotherapeutic drugs.

Combined Atorvastatin and Anticancer Drugs Reduce Osteosarcoma Cell Invasion. We next evaluated the effects of statins and chemotherapeutic drugs on the invasive potential of human osteosarcoma cell lines, using basement membrane Matrigel-coated Transwell filters. In these assays, SaOS2, U2OS, and CAL72 cells showed invasive capacity when cultured in 10% FCS-containing medium. U2OS cells were the most invading cells. In contrast, MG63 cells showed no invasive capacity (Fig. 3B). Treatment with atorvastatin markedly reduced osteosarcoma cell invasion by 60 to 80% in Matrigel. Treatment with doxorubicin or cisplatin alone also reduced cell invasion, with a maximal inhibitory effect in U2OS cells (90%, \( p < 0.05 \) versus untreated cells).
Fig. 3B). The combination of atorvastatin with either doxorubicin or cisplatin further inhibited cell invasion capacity of SaOS2 or U2OS cells (up to approximately 97% inhibition of cell invasion in U2OS cells). These results indicate that atorvastatin enhances the inhibitory effect of chemotherapeutic drugs on human osteosarcoma cell invasion ability.

Combined Atorvastatin and Anticancer Drugs Reduce MMP2 Activity. Finally, we investigated the possible mechanisms underlying the positive interaction of atorvastatin and anticancer agents on osteosarcoma cell invasion. MMPs are known to regulate many processes involved in early stages of tumor development. It is noteworthy that MMP2 (gelatinase A) has been implicated in invasion and metastasis in several cancers (Coussens et al., 2002; Egeblad and Werb, 2002). Based on our finding that atorvastatin and anticancer drugs decrease osteosarcoma cell invasion (Fig. 3), we hypothesized that this effect may be mediated through reduction of MMP2 activity. The measurement of MMP2 activity in invading osteosarcoma cells showed that the different cell lines exhibit different basal level of MMP2 activity.

Fig. 4. Combined atorvastatin and anticancer drugs further reduces MMP2 activity. A, subconfluent human osteosarcoma cells were incubated for 24 h, and basal MMP2 activity was assessed by a colorimetric assay. B, migrating cells were incubated in the presence or absence of atorvastatin (10 μM) and/or chemotherapeutic drugs (100 ng/ml doxorubicin, 20 μM cisplatin), and specific MMP2 activity was evaluated. Results are expressed as treated over control ratio (mean ± S.E.M.). a, p < 0.05 versus untreated cells; b, p < 0.05 versus atorvastatin alone; c, p < 0.05 versus either chemotherapeutic drug alone.
may reduce osteosarcoma cell invasion and migration. Our determine whether statins combined with cytotoxic drugs 2003) cancer cells. One important issue was therefore to renal (Horiguchi et al., 2004) or melanoma (Collisson et al., Woodhouse et al., 1997; Van Noorden, 1998). Recent data invade and migrate, resulting in dissemination of cancer cells. An essential mechanism is the capacity of cancer cells to cated in the development of metastasis from primary tumors. ARF expression or alteration, indicating that the combined treatment decreased cell viability in a panel of osteosarcoma cell lines, independently of Rb, p53, or this study, we show that atorvastatin enhanced the inhibitory effect of chemotherapeutic drugs on osteosarcoma cell viability. It is noteworthy that atorvastatin enhanced the induction of osteosarcoma cell apoptosis by anticancer drugs. This effect was not specific to atorvastatin because we found similar effects with simvastatin (data not shown), another lipophilic statin with proapoptotic effects in osteosarcoma cells (Fromiguet et al., 2006). Strikingly, we found that the combined treatment decreased cell viability in a panel of human osteosarcoma cell lines, independently of Rb, p53, or ARF expression or alteration, indicating that the combined treatment can induce osteosarcoma cell apoptosis independently of the genotype. These results suggest that the combination of statins with anticancer drugs is more effective to induce osteosarcoma cell death than single therapies. Based on the amplitude of effects we obtained on cell viability, apoptosis, or cell invasion, the two classes of compounds seem to have additive effects.

The development of metastasis is an important problem in drug-resistant osteosarcoma. Several mechanisms are implicated in the development of metastasis from primary tumors. An essential mechanism is the capacity of cancer cells to invade and migrate, resulting in dissemination of cancer cells (Woodhouse et al., 1997; Van Noorden, 1998). Recent data indicate that statins can reduce migration and invasion in renal (Horiguchi et al., 2004) or melanoma (Collisson et al., 2003) cancer cells. One important issue was therefore to determine whether statins combined with cytotoxic drugs may reduce osteosarcoma cell invasion and migration. Our finding that atorvastatin markedly enhanced the inhibitory effect of anticancer drugs on cell migration and invasion in vitro indicates that atorvastatin combined to cytotoxic drugs can reduce osteosarcoma cell invasion potential. This effect is not specific to atorvastatin because similar results were obtained using simvastatin (data not shown). These results suggest that lipophilic statins combined with cytotoxic drugs may effectively reduce tumorigenesis in osteosarcoma cells. Whether the combination of cytotoxic drugs and statins at a dose that does not affect angiogenesis or induce myotoxicity may reduce tumorigenesis in an experimental model of osteosarcoma in vivo warrants further investigation.

MMPs are known to play a key role in cell growth, invasion, and migration, which are important events in the pathogenesis of metastasis (Sternlicht and Werb, 2001). Consistently, up-regulation of MMPs is associated with invasiveness in several cancers (Guo et al., 2005; Blavier et al., 2006). This raises the hypothesis that inhibition of MMP2 activity may have therapeutic applications to prevent metastasis. In the present study, we show that MMP2 activity is weak in noninvasive MG63 cells, suggesting that MMP2 activity may be involved in osteosarcoma cell invasion. We consistently show that atorvastatin and anticancer drugs reduced MMP2 activity in osteosarcoma cells that express high MMP2 activity and that the combined treatment was more effective to reduce MMP2 activity than single agents. These results suggest that targeting the mevalonate pathway by statins may improve the efficacy of anticancer drugs on osteosarcoma cell invasion through reduction of MMP2 activity. This is highly relevant to osteosarcoma because increased expression of MMP2 was found to correlate with poor prognosis in human osteosarcomas (Uchibori et al., 2006). Thus, the combination of HMG-CoA reductase inhibitors and conventional anticancer drugs may not only be used to trigger cancer cell apoptosis (Wong et al., 2002; Fromiguet et al., 2006) but also to inhibit the invasion of cancer cells. This is supported by recent data showing that inactivation of Rho GTPases can reduce human cancer cell invasion and migration in vivo (Kusama et al., 2006). The possibility that combined statin and anticancer agents may reduce osteosarcoma cell invasiveness and tumor burden in vivo warrants further investigation. Although the statin concentration required to reduce osteosarcoma cell invasion and migration in vitro may not be therapeutically achieved in vivo (Thibault et al., 1996), the use of lower doses for longer period of time may augment the anticancer efficacy of conventional cytotoxic drugs to reduce the risk of cell progression and metastasis (Wong et al., 2002).

In summary, the results of this study show for the first time that statin-induced inhibition of HMG-CoA reductase sensitizes human osteosarcoma cells to anticancer drugs, which leads to increased efficacy not only to trigger apoptosis but also to inhibit osteosarcoma cell invasion and migration. This suggests a strategy using the combined treatment to improve the response to chemotherapy and reduce tumorigenesis in human osteosarcoma.

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References


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