BRCA1 overexpression sensitizes cancer cells to lovastatin via regulation of cyclin D1-CDK4-p21WAF1/CIP1 pathway: Analyses using a breast cancer cell line and tumoral xenograft model

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Abstract. It is well established that statins display potent anticancer activity in several types of proliferating tumor cells. However, how to promote the sensitivity of statins to mammary cancer is yet to be completely deciphered. The purpose of this study was to investigate whether breast cancer susceptibility gene 1 (BRCA1) overexpression sensitizes mammary cancer cells to statins. MCF-7 cells, which have only one wild-type BRCA1 allele, were transfected with pcDNA3-beta-HA-hsBRCA1 plasmids via liposomes to reconstitute BRCA1 overexpression human breast cancer cell line, and tumoral xenografts with BRCA1 overexpression were subsequently established in BALB/c nude mice. Then, the inhibitory activity of lovastatin on cancer cells and tumoral xenografts, and the underlying mechanism involving in cell-cycle regulatory proteins were analyzed. The proliferative ability of MCF-7 cells treated with lovastatin was reduced compared to normal, and further decreased in the presence of excess BRCA1, detected by methyl thiazolyl tetrazolium and flow cytometry techniques in vitro or by 5-bromodeoxyuridine incorporation in vivo. Additionally, the mRNA and protein expression of cyclin D1, cyclin-dependent kinase 4 (CDK4) and retinoblastoma protein (pRb), was further down-regulated under exposure to lovastatin in condition of BRCA1 overexpression, but the expression of p21WAF1/CIP1, a cyclin-dependent kinase inhibitor (CDKI), was further up-regulated, both in vitro and in vivo detected with quantitative real-time polymerase chain reaction (qRT-PCR) and Western blot analysis. Moreover, we found the further reduced volume of tumoral xenografts treated with lovastatin in the presence of BRCA1 overexpression. Our results suggest that BRCA1 overexpression sensitizes cancer cells to lovastatin via regulation of cyclin D1-CDK4-p21WAF1/CIP1 pathway, which will provide an innovative experimental framework to study control of breast cancer cell proliferation.

Introduction

Breast cancer is the most common malignancy in women, accounting for 23% of cases in developed or developing countries. However, its pathogenesis and perfect therapeutic measures are not fully demonstrated so far (1,2). Similar to normal cell cycle, the progression of cancer cell cycle is positively regulated by cyclins and cyclin-dependent kinases (CDKs) which associate to form heterodimeric complexes (cyclins/CDKs) (3-5). The activated cyclins/CDK complexes sequentially phosphorylate substrates, such as the retinoblastoma protein (pRb), throughout the cell cycle. Furthermore, the restriction point through the cell cycle is also negatively regulated by the association with CDK inhibitors (CDKIs), such as p21WAF1/CIP1, which is the first mammalian CDKI to be identified (3-5).

The BRCA1 gene is associated with inherited susceptibility to breast and ovarian cancer (6-9), and its expression product is a multifunctional protein that has been implicated in many normal cellular functions such as DNA repair, transcriptional regulation, cell cycle check-point control, and ubiquitination (6,10). Recent data have suggested that women who carry a germline mutation in BRCA1 have a cumulative lifetime risk of 50-85% of developing breast cancer (11). BRCA1 expression is down-regulated by 30% in sporadic breast cancers and by 70% in ovarian cancer cases, respectively (12). However, whether or not BRCA1 gene overexpression inhibits breast cancer proliferation and the detailed mechanism involving in cell-cycle regulatory proteins remains unknown.

Lovastatin, like other statins, an inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG CoA), the key...
regulatory enzyme in the mevalonate pathway of cholesterol synthesis, belongs to fungal antibiotic firstly used in treatment of hypercholesterolemia (13-15). Recently, lovastatin has been paid more attention for its wide-range effects on human cancer cells (16,17). Previous studies have demonstrated that lovastatin up-regulates p21WAF1/CIP1 expression and down-regulates activity of cyclin/CDK2 or cyclin D/CDK4 complexes in breast cancer cells by modulating the ubiquitin-proteasome pathway, independent of the HMG-CoA reductase enzyme. These findings show that lovastatin has the activity of inhibiting tumor cell proliferation, or inducing tumor cell differentiation, maturity or apoptosis (18-20). Therefore, it is possible that lovastatin could be used as an antitumor agent with potentially useful clinical applications in breast cancer. Nevertheless, malignant cells have the capacity to develop mechanisms to resist or escape the cytotoxic effects of statin-mediated chemotherapy (21). Recent results from our laboratory also demonstrated the antitumor activity of lovastatin against breast cancer is not satisfactory (18). Hence, how to sensitize breast cancer to lovastatin is a major question unsolved still for clinical therapy.

We conducted this study to investigate whether BRCA1 overexpression sensitizes cancer cells to lovastatin and to further explore possible mechanisms. To address this hypothesis, we reconstituted MCF-7 cells with BRCA1 overexpression, and established a tumoral xenografts model in BALB/c nude mice. Our results suggest that BRCA1 gene overexpression sensitizes cancer cells toLovastatin via regulation of cyclin D1-CDK4- p21WAF1/CIP1 pathway both in vitro and in vivo.

Materials and methods

Cell culture. The human breast cell line MCF-7 was purchased from Shanghai Cell Biology Institute of Chinese Academy of Sciences and cultured in Dulbecco’s modified Eagle’s medium (Invitrogen), supplemented with 10% fetal bovine serum (HyClone), and 100 U/ml penicillin and 100 mg/ml streptomycin (Invitrogen) in a humidified atmosphere of 5% CO2 at 37°C.

BRCA1 gene transfection and identification. BRCA1 gene expression vector, pcDNA3-beta-HA-hsBRCA1 plasmids, was kindly provided by Dr Ashok Venkitaraman (Cambridge University, UK). After the plasmids were identified by electrophoresis on 1% agarose gels. Then BARCA1 gene transfection and identification by RT-PCR, immunocytochemistry or Western blot techniques with mouse anti-human BRCA1 antibody. The primer sequences used for RT-PCR are as follows: for BRCA1 gene, forward 5'-TGCTCGGAGGAAATGGTTGATT-3', reverse 5'-TTGCGATGGAAGTGAGCTGAAG-3', which afforded a 120-bp fragment. The MCF-7 cells stably transfected with BRCA1 gene were named as MCF-7/BRCA1 cells.

Proliferative ability assay with MTT. 200 μl of 5x10^5/ml MCF-7 and MCF-7/BRCA1 cells were grown in microtiter plates and subjected to the experimental culture conditions and treated with lovastatin at dose of 8 μmol/l as previously described for 24, 48 or 72 h (22). After removal of culture medium, 20 μl of 5 mg/ml methyl thiazolyl tetrazolium (MTT) (Invitrogen) in phosphate-buffered solution (PBS) were added to each well and the cultures were incubated for an additional 4 h at 37°C. The supernatant was aspirated and formazan crystals were dissolved in 150 μl dimethyl sulfoxide. Absorbance at 490 nm was read on an automated plate reader. The proliferation inhibitory rate = (1-ODexperimental group/ODcontrol group) x 100%.

Cell cycle phase distribution analyzed with flow cytometry. After treatment with or without the tested lovastatin for 26-72 h, both MCF-7 and MCF-7/BRCA1 cells were collected to centrifugal tubes, centrifuged at 2000 r/min for 5 min, and washed twice with PBS. The distribution of cell cycle phases was assessed by CycleTest™ Plus DNA Kit (BD Biosciences Pharmingen) with a BD FACScan (Macintosh operation platform, BD CellQuest1.0 software analysis).

Quantitative real-time PCR-based gene expression analysis. Oligonucleotide primers and TaqMan probes were designed by using Primer Express software 2.0 (PE Biosystems) and were synthesized by Takara Biotechnology Inc. Sequences of probes and primers used were as follows: for cyclin D1 gene, forward 5'-GTGCTAACGTCTGGAAGTGGACCTCT-3', reverse 5'-TTGGCATTCTTGAGAAAGGATGTC-3'; and probe 5'-FAM-ATTAACAAATCAGAGGACCAGAGGACCAAAGATG-3'. For CDK4 gene, forward 5'-CTACCTCTGCATACTGGCCACCTG-3', reverse 5'-CTCACGATGCTGATCTCTGTA-3'; and probe 5'-FAM-TGCCTGACCTCCTGCTG-3'. For pRb gene, forward 5'-ATAAACATCAAAAGAGGACCAGGAAATGTTCTCA-3', reverse 5'-AGCGATGATAGGAGAATACGCTG-3'; and probe 5'-FAM-CTTCGACCTACCTGCCTC-3'. For p21WAF1/CIP1 gene, forward 5'-GGAGCACCAAAATGGATTGATG-3', reverse 5'-GGGTTGATGAGGAGGAAAT-3'; and probe 5'-FAM-CTGCTGACCTGAACT-3'. For p53 gene, forward 5'-CCCAATGAGTATCTGATTG-3', reverse 5'-TAGCAGCTCCTCCATGTAG-3'; and probe 5'-FAM-GCGCTCGATGAACTGA-3'. For GAPDH gene, forward 5'-CTCGAGGATGTATCTCCTG-3', reverse 5'-CTGATGGAAGGTTAAAATG-3'; and probe 5'-FAM-MTGGTGGCTGAGGAGAATCC-3'. Total RNA was extracted from the cultured cells using TRIzol reagent according to the protocol provided by the manufacturer (Invitrogen). Real-time quantitative TaqMan PCR analysis was used to measure the levels of cyclin D1, CDK4, pRb and p21WAF1/CIP1 mRNA expression according to our previous method (23). The thermal cycling conditions included 2 min at 93°C, 1 min at 93°C, and 1 min at 55°C. Thermal cycling proceeded with 40 cycles. Levels of different mRNAs were subsequently normalized to GAPDH mRNA levels.
Protein expression of cell cycle regulatory proteins analyzed with Western blot analysis. To analyze the protein expression of cyclin D1, CDK4, pRb and p21WAF1/CIP1, protein extracts of MCF-7 and MCF-7/BRCA1 cells incubated without or with 8 μmol/l lovastatin for each experimental condition were made by homogenization in 5 Vol of ice-cold Tris-buffered saline (0.15 mol/l of sodium chloride and 20 mmol/l of Tris-HCl, pH 7.0) containing 1% Triton X-100, 1 mmol/l phenylmethylsulfonylfluoride and 1 mg/l aprotinin, and were measured with Bradford method. Extracts containing 40 μg of total protein were loaded onto 12% SDS-PAGE using a protein assay (Bio-Rad Laboratories, Hercules, CA), and the separated proteins were electrophoretically transferred to a polyvinylidene fluoride membrane (PVDF). The membrane was blocked in Tris-buffered saline with 0.1% Tween-20/5% non-fat milk and probed with 1:1,000 diluted antibody (anti-cyclin D1, CDK4, pRb, p21WAF1/CIP1 or GAPDH, Santa Cruz Biotechnology, CA) overnight, followed by a horse-radish peroxidase linked secondary antibody (1:1,000 dilution). Specific protein bands were revealed by enhanced chemiluminescence and visualized by immediate exposure to autoradiographic film and scanned by gel imaging analytical system (Bio-Rad Laboratories, Hercules, CA).

Tumoral xenografts establishment. The animal experimental procedures were approved by the Animal Care and Use Committees of the The Third Military Medical University. A total of 20 nude mice (nu/nu mutants on a BALB/c background, n=20) were purchased from Experimental Animal Center of The Third Military Medical University at 8-week of age and 18±2 g weight. All animal were housed in sterile laminar flow room and allowed to acclimatize to the facility, personnel, and daily handling for 1 week. During this acclimatization period, mice were housed under standard conditions of light (12-h light, 12-h dark cycle, lights on at 07:00 h and temperature 24˚C), with free access to standard ground, n=20) were purchased from Experimental Animal Committees of the The Third Military Medical University. A total of 20 nude mice (nu/nu mutants on a BALB/c background, n=20) were purchased from Experimental Animal Center of The Third Military Medical University at 8-week of age and 18±2 g weight. All animal were housed in sterile laminar flow room and allowed to acclimatize to the facility, personnel, and daily handling for 1 week. During this acclimatization period, mice were housed under standard conditions of light (12-h light, 12-h dark cycle, lights on at 07:00 h and temperature 24˚C), with free access to standard rodent chow AIN-93 formulation (24) and tap water. After that, the 20 mice were randomized into two groups (n=10, each group respectively) to receive injections of 200 μl of 1×10^7/ml MCF-7 or MCF-7/BRCA1 cells under the lateral dorsal skin respectively as previous method (25). Four weeks after inoculation of tumor cells, when tumors were visible, each group was randomly subdivided into two groups (n=5, each group respectively) that received subsequent s.c. shoulder injections (at sites away from the growing tumors): placebo (0.9% sodium chloride) or lovastatin (10 mg/kg body weight, at a level corresponding to the human dosage of 1-2 mg/kg/day). Injections were continued every other day for 2 weeks. During this period, the growth of the tumors was observed. At the end of this period, the mice were sacrificed by decapitation under diethyl ether anesthesia between 10 a.m. and noon, and tumors were carefully dissected and studied. Many of the in vitro molecular studies mentioned previously were carried out on mRNA and protein extracts of the extirpated tumoral xenografts.

Histological studies. To analyze the impact of BRCA1 overexpression on the lovastatin-mediated anti-proliferative ability of breast cancer cells in vivo, 5-bromodeoxyuridine (BrdU) incorporation was used in tumoral xenografts (26). Before the sacrifice of nude mice, for 2 h, 50 mg/kg BrdU was applied with intraperitoneal injection, then tumoral xenografts were carefully dissected, fixed with 10% paraformaldehyde overnight at room temperature and sectioned to 8 μm of thickness. The sections were washed in PBS, and blocked with 1% hydrogen peroxide solution (Sigma) in PBS for 10 min. Sections were then further blocked and permeabilized with permeabilization buffer for 60 min. Immediately prior to staining, primary antibody (mouse anti-human BrdU antibody, 1:100 dilution) was added and then incubated overnight at 4˚C. Secondary antibody (anti-mouse IgM-biotin, 1:100 dilution) and streptavidin-HRP (1:200 dilution) were sequentially incubated for 60 min at room temperature. Color was developed using 3,3’-diaminobenzidine (DAB) and slides were dehydrated in ethanol, cleared in xylene and mounted in Histomount. The percentage of positive staining cells with BrdU was determined with the help of a digital imaging system, which used a Leitz Dialux microscope linked to a Vidicon camera, an IBM PC with PC Vision digitizer, and Microscience software. The evaluation of extirpated tumor volume was performed as previous described (25). The maximum and minimum diameters of the xenograft tumor were measured, and the tumor volume was calculated using the formula V=1/2(LxD), in which V stands for the volume of the tumor, L for the largest diameter, and D for the smallest diameter of extirpated tumor.

Data analysis. Results are expressed as means and standard deviation. Multiple group means of detected targets between MCF-7 group and MCF-7/BRCA1 group treated with or without lovastatin both in vitro and in vivo were compared by single-factor analysis of variance (ANOVA) and post hoc Student’s t-tests. Difference was considered significant at P<0.05. SPSS version 10.0 was used for all statistical analysis.

Results

The reconstitution of MCF-7 cells with BRCA1 overexpression. We reconstituted MCF-7 cells with BRCA1 gene overexpression via liposomes for further study. After the pcDNA3-beta-HA-hsBRCA1 plasmids (11683 bp) enzymolysised by MluI and KpnI restriction enzymes, two bands of 3667 and 8016 bp were observed under UV-lamp on 1% agarose gels (Fig. 1A), which is consistent with the theoretical values. The expression of BRCA1 mRNA in MCF-7 cells transfected with pcDNA3-beta-HA-hsBRCA1 plasmids was higher than that in MCF-7 cells transfected with pcDNA3 plasmids detected with RT-PCR (Fig. 1B), a similar profile of which was observed by Western blotting (Fig. 1C) and immunocytochemical technique (Fig. 1D and E). Above results show that we have successfully established the reconstituted MCF-7 cells with BRCA1 gene overexpression (named as MCF-7/BRCA1 cells).

BRCA1 overexpression enhanced the anti-proliferative ability of lovastatin both in vitro and in vivo. A previous study (27) suggested the possible inhibitory role of BRCA1 gene expression in breast cancer, which led us to hypothesize that BRCA1 overexpression might enhance anti-proliferative...
Figure 1. BRCA1 gene transfection and identification of BRCA1 overexpression. The products of pcDNA3-beta-HA-hsBRCA1 plasmids, BRCA1 gene expression vector, zymohydrolysed with restrictive endonuclease MluI and KpnI, were identified by electrophoresis (A). Then, MCF-7 cells transfected with pcDNA3-beta-HA-hsBRCA1 plasmids (named MCF-7/BRCA1 cells) were characterized of BRCA1 overexpression by RT-PCR (B), Western blotting (C) or immunocytochemical staining without (D) or with mouse anti-human BRCA1 antibody (E) as described in Materials and methods. Bar, 50 μm.

Figure 2. Enhanced anti-proliferative ability of lovastatin in the presence of BRCA1 overexpression in MCF-7 cells measured with MTT (A) as well as in tumoral xenografts analyzed with 5-bromodeoxyuridine (BrdU) incorporation (B) (bar, 200 μm), as described in Materials and methods. Values are means for six determinations for each time-point, with standard deviations represented by vertical bars. *P<0.05, **P<0.01 versus corresponding group without lovastatin at the same time-point, respectively; ^P<0.05, "P<0.01 versus MCF-7 group at the same time-point, respectively (Student's t-test).
activity of lovastatin. To address this possibility, the proliferative ability of 8 μmol/l lovastatin was compared between MCF-7 and MCF-7/BRCA1 cells with MTT method. As shown in Fig. 2A, the proliferative ability of MCF-7/BRCA1 cells in normal culture was decreased compared with MCF-7 cells after 48 h (P<0.05). And lovastatin reduced the growth of MCF-7 cells by 56.11% (P=0.013) whereas lovastatin reduced the growth of MCF-7/BRCA1 cells by 84.52% (P<0.001) at 72 h.

Next, we analyzed the BrdU incorporation in tumoral xenografts treated with lovastatin, to test whether BRCA1 overexpression also enhance anti-proliferative ability of MCF-7 cells by 56.11% (P=0.013) whereas lovastatin reduced the growth of MCF-7/BRCA1 cells by 84.52% (P<0.001) at 72 h.

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lovastatin in vivo. As shown in Fig. 2B, the brown-granules of BrdU antibody staining were numerous in MCF-7 and MCF-7/BRCA1 xenografts without lovastatin exposure, and the ratio of positive cells was 65.3±7.8% in MCF-7 xenografts and 53.8±8.3% in MCF-7/BRCA1 xenografts, respectively. In both MCF-7 and MCF-7/BRCA1 xenografts, lovastatin reduced the number of brown granules (P<0.001), but lovastatin reduced the percentage ratio of positive staining of MCF-7 cells by 18.5% (P=0.018) whereas lovastatin reduced the percentage ratio of positive staining of MCF-7/BRCA1 cells by 32.1% (P=0.006).

BRCA1 overexpression enhanced the lovastatin-induced accumulation of cells in G0/G1 phase. The results from our previous study (28) and other investigations (16,17,19) have demonstrated G0/G1 phase is the cell cycle phase most affected by lovastatin in MCF-7 cells. Here, we analyzed the distribution alterations of cell phase in both lovastatin-loaded MCF-7 and MCF-7/BRCA1 cells, which was completed with flow cytometry. The percentage of G0/G1 phase was increased from 52.0±8±1.2 to 68.0±9±7.84% in MCF-7 cells and from 61.0±7.12 to 84.5±9±7.84% in MCF-7/BRCA1 cells, when lovastatin-loaded 24-72 h. Significant difference was observed in the percentage of G0/G1 phase between MCF-7 and MCF-7/BRCA1 cells, especially at lovastatin-loaded 72 h. The percentage of G2/M phase was decreased from 31.8±9±5.71% to 16.3±4±2.8% in MCF-7 cells (P=0.025) and from 28.4±3±5.71 to 13.7±0±4.26% in MCF-7/BRCA1 cells (P=0.019), respectively.

BRCA1 overexpression enhanced the lovastatin-induced reduction of tumoral xenografts. To investigate whether BRCA1 overexpression exerts fortified suppressive effects of lovastatin on breast carcinoma progression, we measured the volume of MCF-7 and MCF-7/BRCA1 tumoral xenografts in nude mice treated with lovastatin. The tumorigenic ratio of MCF-7 and MCF-7/BRCA1 cells was 100% in the 20 BALB/c mice, and the time of visible tumor was 6±1 days. The volume of xenograft was 1.75±0.22 cm³ and 1.14±0.13 cm³ in MCF-7 and MCF-7/BRCA1 cells respectively (P=0.017). Lovastatin administration reduced the volume by 72.57% in MCF-7 xenografts (0.48±0.01 cm³) (P<0.001) and by 87.80% in MCF-7/BRCA1 xenografts (0.21±0.01 cm³) (P<0.001), compared to corresponding placebo group.

Effects of BRCA1 overexpression on alteration of mRNA expression of cell cycle regulatory proteins by lovastatin in cells and tumoral xenografts. As stated above (3-5), the progression of cancer cell cycle is positively regulated by cyclins and CDKs, and negatively regulated by CDKIs. Therefore, we measured the relative mRNA expression of cyclin D1, CDK4, p21WAF1/CIP1, and pRb in lovastatin-loaded MCF-7 and MCF-7/BRCA1 cells with qRT-PCR, to explore the underlying molecular mechanisms. For relative expression of cyclin D1 mRNA, lovastatin reduced the cyclin D1 mRNA expression of MCF-7 cells by 0.46-1.1-fold (P<0.05) whereas lovastatin reduced the cyclin D1 mRNA expression of MCF-7/BRCA1 cells by 0.9-5.7-fold (P<0.05) in incubation of 24-72 h (Fig. 3A). Similar fortified inhibitory effects of lovastatin were observed for CDK4 mRNA (Fig. 3B) and for pRb mRNA (Fig. 3C). In contrast, lovastatin increased the p21WAF1/CIP1 mRNA expression of MCF-7 cells by 0.3-0.5-fold (P<0.05) whereas lovastatin increased the p21WAF1/CIP1 mRNA expression of MCF-7/BRCA1 cells by 0.6-0.9-fold (P<0.05) in incubation of 24-72 h (Fig. 3D).

Furthermore, we measured the relative mRNA expression of cell cycle regulatory proteins mentioned above in MCF-7 xenografts and MCF-7/BRCA1 xenografts treated with

![Figure 4. Effect of BRCA1 overexpression on the protein expression of cyclin D1, CDK4, p21WAF1/CIP1 and pRb, in cells (A) as well as in tumoral xenografts (B) treated with or without lovastatin. Glyceralde-3-phosphate dehydrogenase (GAPDH) served as control. Expression alterations of the targets were obtained by Western blotting as described in Materials and methods.](image-url)
lovasitatin. Lovastatin treatment for 2 weeks reduced the relative expression of cyclin D1 by 31±5% in MCF-7 xenografts and by 52±8% in MCF-7/BRCA1 xenografts, compared to corresponding placebo group (Fig. 3E). The fortified inhibitory rate of lovastatin was observed for CDK4 mRNA (Fig. 3F) and for pRb mRNA (Fig. 3G). On the other hand, lovastatin treatment caused a marked rise in relative expression of p21WAF1/CIP1 mRNA (Fig. 3H), which clearly exceeded those detected in untreated mice (P<0.001).

BRCA1 overexpression reinforced the alteration of protein expression of cell cycle regulatory proteins by lovastatin in cells and tumoral xenografts. Finally, we measured the expression of cyclin D1, CDK4, p21WAF1/CIP1 and pRb proteins in lovastatin-loaded MCF-7 and MCF-7/BRCA1 cells. As shown in Fig. 4A, the expression of cyclin D1, CDK4 and pRb proteins was decreased, but the expression of p21WAF1/CIP1 protein was increased in MCF-7/BRCA1 cells, compared to MCF-7 cells. Lovastatin application gradually decreased the expression of cyclin D1, CDK4 and pRb proteins, and progressively increased the expression of p21WAF1/CIP1 protein in MCF-7 and MCF-7/BRCA1 cells, especially at incubation 72 h. Moreover, the regulatory effects of lovastatin on expression of D1, CDK4, p21WAF1/CIP1 and pRb proteins were more notable in MCF-7/BRCA1 cells than that of MCF-7 cells.

Additionally, we analyzed the expression of these cell-cycle regulatory proteins in MCF-7 and MCF-7/BRCA1 cells xenografts treated with lovastatin. As shown in Fig. 4B, BRCA1 overexpression enhanced the down-regulation of expression of cyclin D1, CDK4, pRb proteins, and enhanced the up-regulation of expression of p21WAF1/CIP1 protein, by lovastatin treatment for 2 weeks. Hence, the results of protein expression of cell cycle regulatory proteins are coincident with the levels of respective mRNA expression (Fig. 3) both in vitro and in vivo.

Discussion

Recently, numerous studies have demonstrated the extensive antitumor activities of lovastatin against malignant tumor cells, such as suppressing proliferation by arresting cell cycle in G0/G1, inducing apoptosis and promoting differentiation (16,17). Lovastatin also shows low risk of adverse effects and no influence on normal cells (28). However, how to improve the sensitization of breast cancer to lovastatin is a question unsolved still for clinical therapy. The BRCA1 gene encodes a complex protein that appears to be involved in some aspects of DNA repair, transcription, or cell cycle regulation (12). Clinically, much is known about the risks associated with mutations in this gene, but less is certain with regards to its impact on treatment. In the present study, our findings have provided evidence that BRCA1 gene overexpression further decreases the down-regulation of the mRNA and protein expression of cyclin D1, CDK4 and pRb, and further increases the up-regulation of the expression of p21WAF1/CIP1 by lovastatin (Figs. 3 and 4), so as to sensitize the breast cancer cells and tumoral xenografts to lovastatin (Fig. 2). Therefore, the novel findings of our experiment is that BRCA1 overexpression reinforces the antitumor activity of lovastatin via regulation of cyclin D1-CDK4-p21WAF1/CIP1 pathway.

Previously, we have reported that lovastatin induces alterations of cellular hyperpolarization and intracellular Ca 2+ distribution, and an increase of gap junctional intercellular communication function, resulting in changes in mitogen-activated protein kinase (MAPK) downstream signal cascade, which leads to inhibit the growth of MCF-7 cells (18). Nevertheless, the detailed molecular mechanism involved in anti-proliferation of lovastatin against MCF-7 cells is yet to be completely deciphered. The cessation of cell proliferation, differentiation or even cell death is due to the reduction of cyclins/CDKs complex or induction of CDKIs in most cells, including tumor cells (5). Hence, if the cell cycle regulatory proteins could be induced consistently in tumor cells, and their induction causes to G1 arrest, the goal of controlling the proliferation of cancer cells could be achieved. Rao et al (29) have shown that treatment of breast cancer cells with lovastatin induces a cascade of events leading to inhibition of cell proliferation through the redistribution of the CDKIs from cyclin D/CDK4 complexes to cyclin E/CDK2 complexes, which like other growth arresting agents such as TGF-β use the switching of CDKI from CDK4 complex to CDK2 as a method to initiate growth arrest (30). As shown in this study, we have observed that lovastatin ceases cell proliferation arrest at G0/G1 phase through not only the reduction of mRNA and protein expression of cyclin D1, CDK4 and pRb, but also the increase of p21WAF1/CIP1 expression, in both MCF-7 cells and tumoral xenografts (Figs. 3 and 4). We also found that lovastatin treatment reduces the size of tumoral xenografts by 72.57%. Rb is a tumor suppressor protein and has inhibitory effects on cell cycle progression, down-regulation of Rb by lovastatin suggest that lovastatin may have potent chemo-preventative properties by inducing the inhibitory activity of the negative regulators of the cell cycle. Furthermore, the data from large clinical trial of patients suffering from hypercholesterolemia have suggested that lovastatin produces the unexpected finding of a decrease in cancer incidence (31). Additionally, lovastatin has also been shown to inhibit metastasis of highly metastatic B16F10 mouse melanoma in nude mice (32). From above observations, it can be deduced that lovastatin may have properties increasing cyclins/CDK expression, or decreasing CDKI expression, or mediating CDKI redistribution to cyclin/CDK complexes to negative regulate the cell cycle, which make it a very attractive agent for use as a potential chemo-preventative agent.

BRCA1 gene encodes a large protein thought to contribute to a variety of cellular processes (6-10). Wild-type BRCA1 protein binds to a number of cellular proteins, including DNA repair protein Rad51, tumor suppressor p53, RNA polymerase II holoenzyme, RNA helicase A, CtBP-interacting protein, c-myc, BRCA1-associated RING domain protein (BARD1), and BRCA2 protein (33,34). These proteins likely mediate the regulation of cell proliferation, participation in DNA repair/recombination processes related to the maintenance of genomic integrity, induction of apoptosis in damaged cells and regulation of transcription (35-37). Given that BRCA1 is required for cell proliferation, its chemo-preventive activity is believed to modify BRCA1 phenotypes.
and contribute to the etiology of BRCA1-deficient tumors. Herein, we have successfully stably transfected BRCA1 gene into MCF-7 cells (Fig. 1), which originated from sporadic breast cancers and has only one wild-type allele. We have found BRCA1 overexpression further inhibits the decreased proliferative ability of breast cancer by lovastatin through down-regulating expression of cyclin D1, CDK4, pRB, and up-regulating expression of p21WAF1/CIP1 (Figs. 2-4). The further down-regulation of Rb by lovastatin in the presence of excess BRCA1 hints that the sensitized effect by BRCA1 overexpression is relative to the reduction of tumor suppression protein. Our results suggest a BRCA1-cyclin D1-CDK4-p21WAF1/CIP1 pathway that coordinately functions in cell growth and tumor progression pathways, which are partly in agreement with the findings by Fan et al (38) in DU-145, a human prostate cancer cell line with low endogenous expression of BRCA1. In his investigation, wild-type BRCA1 clones exhibit a slightly decreased proliferation rate, reduced repair of single-strand DNA strand breaks, and alterations in expression of key cellular regulatory proteins (including BRCA2, p300, Mdm-2, p21WAF1/CIP1, Bcl-2 and Bax) (38). In other studies on BRCA1 overexpression for tumor treatment, Marot et al (39) found intratumor administration of wild-type BRCA1 also significantly inhibits growth of lung and colon steroid hormone-independent tumors, and Hoshino et al (40) demonstrated that BRCA1 overexpression in the murine mammary gland provides protection against carcino-nogen-induced tumors in transgenic mice. Coincidentally, Niwa and his colleagues (41) have shown the inhibition of BRCA1 expression by methylation greatly influences the grade of malignancy of sporadic breast cancers. Above mentioned findings suggest that overexpression of BRCA1 may affect cell cycle regulation to exert antitumor activity (42).

In the present study, the most significant finding is that BRCA1 gene overexpression and lovastatin can act jointly to regulate the expression of cell cycle regulatory proteins in arresting at G1/G0 phase. The expression of cyclin D1, CDK4 and pRB is further decreased, but the expression of p21WAF1/CIP1 is further increased by lovastatin in the presence of BRCA1 excess, and a similar profile is found in tumoral xenografts (Figs. 2 and 4). The results are in accordance with the findings of Fan et al (38) who have demonstrated that BRCA1 overexpression causes a 3-6-fold increase in sensitivity to chemotherapy drugs (adriamycin, camptothecin, and taxol) and an increased susceptibility to drug-induced apoptosis in DU-145 tumor cells. The fortified anticancer effect of lovastatin by BRCA1 overexpression may be explained by:

i) lovastatin inhibits the cholesterol biosynthesis pathway, and tumor cells have an increased level of cholesterol synthesis (29,43); ii) lovastatin might combine with BRCA1 gene, in which BRCA1 cooperates with other anti-oncogenes, such as p53 (44), to up-regulate p21WAF1/CIP1 expression and induce pRB dephosphorylation or phosphorylation altering the activity of cyclin D1/CDK4 complex. Hence, it can be extrapolated that tumors with higher levels of BRCA1 might be good candidates for co-therapy with lovastatin.

Chemosuppression of lovastatin to reduce the risk of breast cancer has been operated clinically, however its curative effect is not satisfactory. In this study, we have provided evidence that the antitumor activity of lovastatin is reinforced in the presence of BRCA1 overexpression via regulation of cyclin D1-CDK4-p21WAF1/CIP1 pathway. The focus of our research is on these genetic alterations in breast cancer that affect the response to therapy. More investigations are needed to further define the detailed molecular mechanisms by which BRCA1 gene overexpression sensitizes tumoral cell to lovastatin, which will provide an innovative experimental framework to study control of breast cancer cell proliferation.

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References


