Lovastatin Induction of Cyclin-dependent Kinase Inhibitors in Human Breast Cells Occurs in a Cell Cycle-independent Fashion

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Abstract

Cyclin-dependent kinase inhibitors (CKIs) p21, p27, p16, and p15 are an essential and integral part of cell cycle regulation. Studies on the expression of these inhibitors in normal versus tumor human breast cancer cells revealed that although p27 and p16 are expressed at higher levels in tumor cells, p21 and p15 expression were higher in normal cells. Analysis on the expression pattern of these proteins throughout the cell cycle in synchronized cells demonstrated a substantial increase in p21 during the S-phase in normal cells and barely detectable expression of p21 in any phase of the tumor cell cycle. Levels of p15, p16, and p27 remained relatively constant throughout the cell cycle of normal and tumor cells. Synchronization of tumor cells by lovastatin, which arrests cells in G1, resulted in increased levels of p21 and p27 with a concomitant decrease in cyclin-dependent kinase 2-associated kinase activity. Synchronization of cells by double-thymidine block did not result in the induction of p21 or p27. These observations suggest that lovastatin causes a profound cell cycle-independent alteration of CK1 expression which is distinct from growth factor deprivation or thymidine block.

Introduction

Cell proliferation, the ability of cells to traverse the cell cycle, is intricately regulated in normal cells by the coordinate activity of both positive and negative regulating proteins. However, tumor cells have defective cell cycle control mechanisms, resulting in the uncontrolled growth and proliferation characteristic of all cancers. The cell cycle is driven forward by complexes of stable kinases termed cdks and unstable regulatory subunits called cyclins (1–4). An additional layer of cell cycle regulation has emerged with the discoveries of low molecular weight CKIs which represent a novel mode of negative regulation (5–7). There are two classes of CKIs: the CIP/KIP family including p21Cip1/Waf1 and p27Kip1 and the INK4 family including p15INK4b and p16INK4a reviewed elsewhere (8). Both classes of inhibitors function to block the activity of cdks. p21 and p27 inhibit cdk2–5 and cdk6, whereas p15 and p16 inhibit cdk4 and cdk6 (8). CKI levels have recently been examined during the cell cycle by synchronization of cells with the drug lovastatin (9, 10), which is routinely used to treat hypercholesterolemia. Lovastatin inhibits the activity of the 3-hydroxy-3-methylglutaryl CoA (HMG CoA) reductase enzyme, critical to the cholesterol biosynthetic pathway. Among other effects, inhibition of this pathway disrupts protein prenylation and, therefore, also disrupts the subcellular localization and function of proteins such as ras and protein glycosylation via inhibition of dolichol production. As a result the pleiotropic cellular effects of lovastatin are not thoroughly understood or characterized. Lovastatin has been used recently to arrest diverse cell types in both mitotic (11–13) and meiotic cell cycles (14). Hengst et al. (9) reported on the induction of p27 in cells synchronized via lovastatin treatment. More recently, it was demonstrated that this induction, rather than being transcriptionally mediated, is at least partially attributable to translational control of p27 (10). The induction of p27 by lovastatin was assumed to be a cell cycle effect rather than a drug-specific effect. Here, we report on the cell cycle expression of four CKIs of both the p21 and p16 families and demonstrate that their expression is modulated by lovastatin via a cell cycle-independent mechanism. Synchronization of human breast cells by means other than lovastatin treatment did not lead to the induction of any of the CKIs examined.

Materials and Methods

Materials, Cell Lines, and Culture Conditions. [methyl-3H]thymidine (81 Ci/mmol) and [α-32P]dCTP (3000 Ci/mmol) were purchased from New England Nuclear (Boston, MA). Lovastatin was kindly provided by A. W. Alberts (Merck, Sharp and Dohme Research Pharmaceuticals, Rahway, NJ). Mevalonic acid lactone and serum were purchased from Sigma Chemical Co. (St. Louis, MO) and cell culture medium from Life Technologies, Inc. (Grand Island, NY). All other chemicals used were of reagent grade. The scintillation cocktail used was Budget-Solve from Research Products International (Mount Prospect, IL). Before addition to cultures, lovastatin and mevalonic acid were converted from their inactive lactone prodrug form to their active dihydroxyopen acid as described previously (12). The culture conditions for 70N, 81N, and 76N normal cell strains, MCF-10A cell line, and MCF-7, MDA-MB-157, MDA-MB-231, MDA-MB-436, T47D, BT-20, HBL100, Hu578T, SKBR3, and ZR75 tumor cell lines were described previously (15, 16). All cells were cultured and treated at 37°C in a humified incubator containing 6.5% CO2 and maintained free of Mycoplasma as determined by the MycoTect kit (Life Technologies, Inc.).

Synchronization and Flow Cytometry. Synchronization by lovastatin treatment or growth factor deprivation was performed as described previously (12). Briefly, medium was removed 36–48 h after the initial plating of MDA-MB-157 cells and replaced with fresh medium plus 40 μM lovastatin for 36 h. At time 0 h, cells were stimulated by replacing the medium with fresh medium containing 4 mm mevalonic acid. Cells were harvested at the indicated times, and DNA synthesis and cell density were measured as described previously (12). The synchronization of normal mammary epithelial 76N cells by growth factor deprivation is as follows: At 48 h following plating subconfluent 76N cells, medium was removed and cells were washed three times and incubated in DFCI-3 medium for 72 h. DFCI-3 medium is DFCI-1 medium without essential growth factors (17). At time 0 h, cells were stimulated by replacing the medium with fresh medium containing 4 mm mevalonic acid. Cells were harvested at the indicated times, and DNA synthesis and cell density were measured as described previously (12).
iodide in 38 mM sodium citrate. Cells were then incubated at room temperature in the dark for 30 min and filtered through a 75-μm Nitex mesh. DNA content was measured on a FACScan flow cytometer system (Becton Dickinson, San Jose, CA), and data were analyzed using the CELLFIT software system (Becton Dickinson).

RNA Isolation and Northern Blot Hybridization. Total cellular RNA was extracted from normal and tumor cells by guanidinium isothiocyanate and subjected to cesium chloride gradient purification. For Northern blot analysis, 20 μg of total RNA were fractionated under denaturing conditions on a 1.2% agarose/0.66 M formaldehyde gel and transferred to a Nytran filter (Schleicher & Schuell, Keene, NH) for subsequent hybridization. The DNA probes were prepared by random primed labeling (Boehringer Mannheim, Indianapolis, IN). Vector containing p21 was provided by S. Elledge and W. Harper, cDNA to p27 was provided by Joan Massague, cDNAs to p15 and p16 were provided by David Beach, and cDNAs to histone H4 and 36B4 as described previously (15). All cDNA inserts were labeled with [α-32P]dCTP to a specific activity of 1 × 106 dpm/μg DNA.

Western Blot and Immune Complex Kinase Analysis. Cell lysates and tissue homogenates were prepared and subjected to Western blot analysis as described previously (15, 20). Briefly, 50 μg of protein from each tissue sample or cell line were electrophoresed in each lane of either a 10% SDS-polyacrylamide gel (cyclin A), a 13% SDS-polyacrylamide gel (p21 and p27), a 15% SDS-polyacrylamide gel (p15 and p16), and transferred to Immobilon P. Primary antibodies used were pRb monoclonal antibody (PharMingen, San Diego, CA) at a dilution of 1:100, monoclonal antibody to p16 (a gift from J. W. Harper, Baylor College of Medicine, Houston, TX) at a dilution of 1:20,000, and monoclonal antibody to actin (Boehringer Mannheim) at 0.63 μg/ml Blotto.

Immunoprecipitations and H1 kinase assays were performed as described previously (16, 21). For immunoprecipitation followed by Western blot analysis, 250 μg of protein were used per immunoprecipitation with a polyclonal antibody to cdk2 in lysis buffer as described above. The Immunoprecipitates were then electrophoresed on a 13% SDS-polyacrylamide gel, transferred to Immobilon P, blocked, and incubated with either monoclonal antibody to p21 (Oncogene Science, Cambridge, MA) or p27 (Transduction Laboratories, Lexington, KY) at a dilution of 1:100 and analyzed as described above.

Results and Discussion

CKIs Are Differentially Expressed in Normal versus Tumor-derived Exponentially Growing Cells. To determine the relative levels of CKIs in normal versus tumor-derived breast epithelial cells, we initiated our studies by investigating the expression of four CKIs (p21, p27, p15, and p16) in three normal cell strains, one immortalized mammary epithelial tumor cell lines (Fig. 1). The three normal cell strains (70N, 81N, and 76N) were established from reduction mammoioplasties obtained from three different individuals (17). The immortalized MCF-10A cell line is a subline of a normal breast epithelial cell strain, MCF-10, derived from human fibrocystic mammary tissue which was immortalized after extended cultivation in medium containing low concentrations of calcium (22). The mammary epithelial cell types, estrogen receptor, p53, pRb (retinoblastoma), and cyclin E status of these normal and the nine established tumor cell lines used were described previously (15, 23). Total RNA and protein were prepared from exponentially growing cells and subjected to Northern or Western blot analyses, respectively. Loading controls were performed for both Northern and Western blot analysis using an invariant mRNA (i.e., 36B4 cDNA) and protein (i.e., actin antibody) as probes. As demonstrated, p21 mRNA is expressed at higher levels in normal versus tumor cells, whereas p27 and p16 mRNAs are more highly expressed in some tumor cells (Fig. 1A). The pattern of p21 expression is interestingly different at the protein level with some tumor cell lines as demonstrated by Western blot analysis (Fig. 1B). For example, MCF-7, T47D, BT-20T, and ZR75T cells, all

Fig. 1. Altered expression of CKIs in exponentially growing normal versus tumor cells. Northern blot (A) and Western blot (B) analyses of CKI expression in normal versus tumor breast epithelial cells. RNA was analyzed on Northern blots (20 μg of RNA/lane). The list of normal cells (Lanes 1–4) and tumor cell lines (Lanes 5–13) is presented below. Blots were hybridized with the indicated probes or 36B4 (15) used for equal loading. B, Western blot analysis of CKIs from cell extracts obtained from the same cell lines used in A. Fifty μg of total cell extract were run on SDS-polyacrylamide gels. Proteins were transferred to Immobilon P and blots were incubated with the indicated anti-CKI antisera, and immunoreactive proteins were detected with the enhanced chemiluminescence reagent (Amersham). Lane assignments: 1-70N; 2-81N; 3-76N; 4-MCF-10A; 5-MCF-7; 6-MDA-MB-157; 7-MDA-MB-231; 8-MDA-MB-436; 9-T47D; 10-BT-20; 11-HBL-100; 12-HS578T; and 13-ZR75T. Blots were hybridized with the indicated probes or actin used for equal loading.
estrogen-receptor positive cell lines, express relatively high levels of p21 protein, whereas the levels of p21 mRNA in these cells were very low. In addition, in all of the tumor cell lines that show increased p27 protein levels, the p21 levels are not as low as is typical in tumor cells, but instead are similar in levels to those of normal cells. Although the levels of mRNA for each of the CKIs generally correspond to their protein levels, some particular discrepancies are noted with respect to p15 expression. In the normal cell strains, p15 protein is expressed at high levels, whereas mRNA levels are only increased in the 81N cells (Fig. 1, Lane 2). Additionally, the tumor cell line Hs-578T (Fig. 1, Lane 12) expresses very high levels of p15 mRNA and undetectable levels of p15 protein. These observations suggest that p15 levels may be regulated by translational or posttranslational mechanisms.

Although the differences in CKI expression observed between normal breast cells and breast cancer cells could be a result of tissue specificity, it is more likely due to changes which occur during tumorigenesis. In most cases where a difference in expression was noted between normal versus tumor cells, all four normal cells (i.e., p21) or all three normal cell strains (i.e., p15) consistently exhibited a clearly detectable expression of these two proteins compared to tumor cells, even though the normal cells were derived from different individuals.

Expression of CKIs in Synchronized Normal and Tumor Cells. The CKIs have been proposed to establish a threshold of inhibition that must be exceeded if cell cycle progression is to occur (24). Therefore, a disruption in the levels of CKIs or cyclin-cdk5 could offset the threshold balance or result in the displacement of particular regulatory proteins. The net result of such an imbalance suggests an aberrant cell cycle progression. This hypothesis led us to analyze the pattern of expression and relative levels of the CKIs, p21, p16, p21, and p27 throughout the cell cycle in synchronized populations of both normal cell strains and tumor cell lines (Figs. 2–4).

Initially, we assessed the effect of growth factors on CKI expression by arresting normal 76N cells in G0 (Fig. 2; i.e., growth factor deprivation-induced quiescence). This allowed us to determine whether a particular phase of the cell cycle might enrich for the expression of these CKIs. Briefly, cells were cultured in growth factor-deficient medium for 72 h and then stimulated to reenter the cell cycle with the addition of growth factors. Reentry into the cell cycle and S-phase was monitored by [3H]thymidine incorporation (Fig. 2B). At the indicated times after readdition of growth factors, cells were harvested and extracted proteins were analyzed on Western blots with antibodies to p21, p27, p15, p16, and cyclin A (Fig. 2A). In normal 76N cells, the pattern of expression of cyclin A protein is consistent with that seen for other normal cell types with levels rising dramatically at the S-phase and disappearing by the end of G2–M. The pattern of expression of the four CKIs examined following growth factor stimulation indicates that p21 levels increase substantially (10-fold) during the S-phase, whereas p27, p15, and p16 levels remain relatively unchanged throughout the cell cycle (Fig. 2A). These observations corroborate our hypothesis that these CKIs may each function differently during the cell cycle, with p21 clearly having a pronounced role during the S-phase of cycling cells and p27 and p15 being important in quiescent cells as recently documented in fibroblasts (25).

Induction of p21 and p27 by Lovastatin in Tumor Cells. Unlike normal cells, it is difficult to growth factor deprive tumor cells or arrest them in G0 since most tumor cells have lost their growth factor requirement. As such a pharmacological method has to be used to synchronize tumor cells in early G1. For that purpose we used lovastatin, an inhibitor of the cholesterol biosynthetic pathway, to synchronize MDA-MB-157 breast cancer cells in G1 (Fig. 3). We have previously reported on the use of lovastatin as an agent to synchronize cells in early G1 (12, 13). Cells were cultured in lovastatin for 36 h, at which time lovastatin media were removed and replaced with media containing mevalonate (the end product of the cholesterol biosynthetic pathway), which is routinely used to stimulate cells to reenter the cell cycle and advance to the S-phase. Synchrony of tumor cells at various times after release from the lovastatin block was monitored by histone H4 mRNA expression and [3H]thymidine incorporation (Fig. 3, A and B). Immediately following lovastatin treatment there is inhibition of DNA synthesis followed by a dramatic increase in incorporation of [3H]thymidine as well as histone H4 expression. This increase is indicative of the cells being arrested in G0.

Total RNA and protein were extracted from cells harvested at various times after release from lovastatin and analyzed on Northern and Western blots using p21[CP1]NAP1, p27[CP1], histone H4, and 36B4 cDNAs and p21 and p27 antisera as probes (Fig. 3, A and B). Unexpectedly, we found that lovastatin treatment dramatically induced the expression of p21 and p27 in MDA-MB-157. These two CKIs are barely detectable in exponentially growing tumor cells (Fig. 4).
Cencereagent. C, histone H1 kinase assay: 500 μg of extracts were immunoprecipitated and assayed for kinase activity using cdk2-containing complexes prepared from extracts of synchronized cells (Fig. 3D). These results indicate that the induced levels of p21 and p27 are accompanied by a dramatic decrease in cdk2-associated kinase activity which then increases upon disappearance of p21 and p27 proteins. To determine whether p21 and/or p27 can directly associate with cdk2 and inhibit its activity, we performed a two-step immunoprecipitation/Western blot analysis. We immunoprecipitated lovastatin-treated cell extracts with anti-cdk2 antisera followed by immunoblotting with p21 or p27 antisera (Fig. 3D). Anti-p21 immunoblot analysis of cdk2-containing complexes demonstrates that p21 is directly associated with cdk2 and that the relative amount of p21 associated with cdk2 is inversely correlated with kinase activity of cdk2.

p27 is also similarly associated with cdk2, although in relatively less abundance than p21. Together these analyses indicate that not only are p21 and p27 induced during lovastatin synchronization in MDA-MB-157 tumor cells but they also functionally complex with cdk2. Furthermore, the expression of p21 and p27 is inversely correlated with cdk2 kinase activity, suggesting that these CKIs directly inhibit cdk2.

Induction of p21 and p27 in Tumor Cells by Lovastatin Is Cell Cycle Independent. We synchronized both normal and cell types in the G1-S-phase boundary by double-thymidine block ([16] and Fig. 4) to determine whether the lovastatin-mediated induction of p21 and p27 is cell cycle dependent or due to a direct (as of yet unknown) effect ofLovastatin. This synchronization trial was also used to compare the cell cycle pattern of expression of the four CKIs (Fig. 4C). Cells were harvested at various times after release from treatment, and extracted proteins were analyzed on Western blots with antisera to p21, p27, p16, p15 (Fig. 4), and cyclin A (16). In both normal and tumor cells, the expression pattern of cyclin A protein is consistent with that seen for other cell types, the levels are tightly regulated such that peak expression occurs during the S-phase and early M as documented previously ([16]).

The pattern of expression of p21 in normal cells revealed cell cycle regulation consistent with the growth factor deprivation results shown in Fig. 2. In normal cells, with both methods of synchronization, p21 is cell cycle regulated with peak levels coinciding with the peak S-phase and early M (Fig. 4A, left panels). In tumor cells, however, p21 levels remain low and virtually undetectable during all phases of the cell cycle. Thus, we were not able to enrich for p21 expression at G1 of tumor cells synchronized by double-thymidine block ([16] and Fig. 4B, right panels). These observations suggest that the induction of p21 during lovastatin synchronization (Fig. 3) is not cell cycle dependent but is rather a lovastatin- or mevalonate-specific effect.

The level of p27 remained unchanged in both normal and tumor with anti-cdk2 polyclonal antiserum and complexes reconstituted with the ability to phosphorylate histone H1. The H1-labeling reaction complexes were analyzed by SDS-PAGE and autoradiography. D, immunoprecipitation/Western blot analyses: 500 μg of protein extracts were immunoprecipitated with anti-cdk2 polyclonal antisera and precipitated complexes were analyzed using Western analysis with anti-p21 or anti-p27 monoclonal antisera. E, DNA synthesis rates were monitored by [3H]thymidine incorporation.
Fig. 4. Expression of CKIs in synchronized normal 76N and tumor MDA-MB-157 breast cells. Both cell lines were synchronized using the double-thymidine block procedure: 76N cells were incubated in 2 mM thymidine for 24 h, washed, and incubated in regular medium for 12 h and incubated in 2 mM thymidine for an additional 24 h. MDA-MB-157 cells were treated similarly, except incubation in thymidine was for 36 h and thymidine-free media for 24 h, as described previously (16). At the indicated times following release from double-thymidine block, cell lysates were prepared and subjected to Western blot (A), histone H1 kinase (B), and flow cytometry (C) analyses. Fifty µg of extracts of cells were subjected to Western blot analysis with antisera directed against the indicated CKI or cyclin A at indicated times after double-thymidine block. Note: p21 and p15 +ve C are from 76N cell extracts and are used as a positive control. Blots were probed with actin used for equal loading (data not shown), and the pattern was identical to that of p15 in normal cells and p16 in tumor cells whose levels do not change during the cell cycle. For histone H1 kinase activity, equal amount of proteins (500 µg) from cell lysates prepared from each cell line at the indicated times were immunoprecipitated with anti-cdk2 (polyclonal) coupled to protein A beads using histone H1 as substrate. B, quantification of the histone H1-associated cdk2 kinase activities by scintillation counting, as described previously (16). C, relative percentage of cells in different phases of the cell cycle for each cell line was calculated from flow cytometric measurements of DNA content.
resulting in a near constitutive pattern of histone H1 phosphorylation of cells. Our data indicate that striking differences exist among the expression of four CKI mRNAs and proteins including both the p21(CIP1/WAF1) and Ink4 families in normal and tumor breast epithelial cells. Our data indicate that striking differences exist among the expression of four CKI mRNAs and proteins including both the p21(CIP1/WAF1) and Ink4 families in normal and tumor breast epithelial cells. We have found that the drug lovastatin is capable of inducing p21 and p27 protein in a cell-specific manner via a cell cycle-independent mechanism. Lovastatin, an inhibitor of HMG CoA reductase (the first enzyme in the isoprenyl lipid biosynthetic pathway), is a widely used drug for the treatment of hypercholesterolemia. Lovastatin prevents the first step of cholesterol synthesis, which is the conversion of HMG into mevalonic acid. The blockage of this pathway also prevents the isoprenylation of several proteins such as Ras, Rap, and G by farnesyl, a downstream product of the pathway. This inhibition of isoprenylation blocks the function of the aforementioned proteins (26, 27). Apart from its inhibitory action on HMG CoA reductase, lovastatin has also been used as an effective agent in cell synchronization for both tumor and normal cells (12, 13).

Recently, Hengst et al. (9, 10) reported an elevation of p27 in lovastatin-arrested HeLa cells. This increase was attributed to a cell cycle effect since a similar increase was observed in cells synchronized by density-mediated arrest and thymidine and nocodazole blocks (10). However, the increase in p27 levels, in density-mediated arrested fibroblasts, was much lower than that in lovastatin-treated HeLa cells. This was attributed to imperfect synchronization of the fibroblast cell cycle, indicating that the increases in p27 levels seen might be due to experimental design. Furthermore, HeLa cells are derived from a cervical carcinoma and carry the human adenopapilloma virus, whereas most cancer cell lines in general do not carry this virus.

We have shown here that although lovastatin is capable of inducing both p21 and p27 in human breast tumor cells, this induction is not due to cell cycle synchronization effects of lovastatin. The induction of the inhibitors was not observed using other methods of cell synchronization such as double-thymidine block in normal and tumor cells or growth factor deprivation in normal cells. Furthermore, we used both normal and tumor cells that were derived from human mammary epithelial cells which are representative of most types of cancer, since more than 90% of all human cancers are of epithelial origin. Although lovastatin is capable of inducing these inhibitors in human epithelial cells, the mechanism of induction is not through arrest of cells in a specific phase of the cell cycle, but through a lovastatin, drug-mediated effect. Whether the CKI induction following lovastatin treatment is due to inhibition of any one reaction of the cholesterol biosynthesis pathway remains to be elucidated.

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References

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