An Antisense Oligodeoxynucleotide to p21<sup>Waf1/Cip1</sup> Causes Apoptosis in Human Breast Cancer Cells<sup>1</sup>

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Abstract
Whereas 40% of human breast carcinomas harbor mutations in the tumor suppressor protein p53, the use of tests demonstrating the presence of p53 mutations as a prognostic marker in breast cancer has not altered clinical management. Therefore, the search for new markers, especially among cell cycle-regulatory molecules, is a high priority, both in terms of prognostication and for identification of novel targets. p21 regulates the outcome of the p53 response to DNA damage, as might occur after administration of a chemotherapeutic agent, and we have shown that attenuation of p21 using an antisense oligodeoxynucleotide (ODN) inhibits cell proliferation in vitro and decreases growth of Met-1 mammary carcinomas in mice. In the current study, we extend this work to human cells and tissue. Three of eight human breast tumors that we obtained from a tissue bank showed markedly increased p21 levels, variably staining in the nucleus and cytosol. All corresponding normal tissues were p21 negative. In the three p21-positive tumors, the phosphatidylinositol 3'-kinase-relevant signaling proteins p85 and PTEN were also increased. To investigate whether p21 is a feasible target for attenuation in human breast cancer, we investigated two human carcinoma cell lines. When transfected with antisense p21 ODN, both MCF7 and T47D breast cancer cells exhibit dose-dependent attenuation of p21 levels, associated with apoptosis in the absence of an additional apoptotic stimulus. Because p21 regulates the cellular repair response to damaged DNA, our work suggests that attenuation of p21 using our antisense p21 ODN may be effective in modulating the progression of breast cancer in either the presence or absence of combination chemotherapy and sets the stage for future clinical trials.

Introduction
It is now widely accepted that cancer is a disease characterized by defects in cell cycle regulation. Whereas 40% of human breast carcinomas harbor mutations in the tumor suppressor protein p53, the use of tests demonstrating the presence of p53 mutations as a prognostic marker in breast cancer has not altered clinical management (1, 2). Therefore, the search for new markers is a high priority in breast cancer research, both in terms of prognostication and for identification of novel targets for therapy. Gene targets for antisense therapy of breast cancer have been suggested (reviewed in Ref. 3); in this study, we examine a novel candidate for such therapeutic intervention.

As a member of the cip/kip family of cyclin kinase inhibitors, p21 was initially described as an inhibitor of cell cycle transit. Recently, however, p21 has been shown to function as both a cyclin/cdk assembly factor (being required for G<sub>i</sub>→S transit) and as a survival protein downstream of PI3K. Through work in our laboratory and other laboratories, p21 has emerged as a potential stratifier of breast cancers in various population groups. However, whereas up-regulation of p21 has been linked to chemoresistance in ErbB-2-overexpressing cells (4), attempts to correlate increased levels of p21, as measured by immunohistochemical methods, with prognosis in breast cancer have shown inconsistent results (5–7).

We have demonstrated previously (8) that an antisense ODN to p21 attenuates growth in rat VSM cells, and others have shown (9) that a different antisense p21 ODN enhances Taxol-induced apoptosis in glioblastoma cells, consistent with the evolving concept of p21 as a “survival” protein lying downstream of PI3K (10, 11). In various cancers cell lines, cells lacking p21 show increased sensitivity to apoptosis induced by a variety of methods (12, 13). Consistent with a possible pathogenic role of p21 in growth induction, overexpression of p21 has been found to be an early event in development of cancer (14); we have shown a similar early increase in p21 occurring after mitogen stimulation in VSM cells (15).

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<sup>4</sup> The abbreviations used are: PI3K, phosphatidylinositol 3'-kinase; cdk, cyclin-dependent kinase; WCL, whole cell lysate; CKI, cyclin-dependent kinase inhibitor; NLS, nuclear localization signal; ODN, oligodeoxynucleotide; MAPK, mitogen-activated protein kinase; PARP, poly(ADP-ribose) polymerase; RT-PCR, reverse transcription-PCR; UCD, University of California, Davis; Ac-DEVD-CHO, N-acetyl-L-aspartyl-L-glutamyl-L-valinyl-L-aspartyl.
We have demonstrated previously (16) that daily s.c. injection of p21 antisense ODN attenuates breast cancer growth in mice. In the current study, as an extension of our work on antisense p21 ODNs in cell cycle control, we examined two human breast cancer cell lines as well as biopsy tissue from human breast cancers. We now show that p21 levels, as well as PI3K-relevant signaling proteins, are markedly increased in a subset of human breast cancers with poor prognostic features. In addition, T47D (ductal carcinoma) and MCF7 (adenocarcinoma) human breast cancer cell lines, which display relatively high levels of p21, show marked, dose-dependent attenuation of this protein and induction of apoptosis after transfection with antisense p21 ODN. These studies further demonstrate the feasibility of using antisense p21 ODNs as therapy of human breast cancer and suggest further avenues of research.

Materials and Methods

Materials. Mouse monoclonal anti-recombinant full-length p21\textsuperscript{Waf1/Cip1} antibody and HeLa cell nuclear extract were obtained from Upstate Biotechnology Inc. (Lake Placid, NY). Mouse monoclonal antihuman PTEN and antihuman PI3K (P85) antibodies, rabbit polyclonal anti-MAPK (extracellular signal-regulated kinase 1) antibody, and A-431 WCL, Caki-1 WCL, and Jurkat WCL were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse antihuman PARP purified antibody was obtained from BD Biosciences (San Diego, CA). For the immunohistochemistry, mouse monoclonal anti-full-length p21 antibody was obtained from Calbiochem (San Diego, CA). Goat antimouse and goat antirabbit horseradish peroxidase-conjugated IgG were obtained from Bio-Rad (Richmond, CA). Lipofectin was obtained from Invitrogen Life Technologies, Inc. (Carlsbad, CA). ECL Western Blotting Detection Reagents were obtained from Amersham Biosciences (Buckinghamshire, United Kingdom). CasPcE Assay System was obtained from Promega (Madison, WI). The avidin-biotin complex kit and 3,3′-diaminobenzidine tetrahydrochloride were obtained from Vector Laboratories (Burlingame, CA). All other reagents, including mouse anti-α-actin monoclonal antibody, were from Sigma (St. Louis, MO).

Cell Culture and DNA Synthesis. T47D (ductal carcinoma) and MCF7 (adenocarcinoma) human breast cancer cell lines were obtained from the American Type Culture Collection. All cell lines were maintained according to vendors’ recommendations. The cells were grown in serum- or platelet-derived growth factor-BB as indicated. 

Antisense Transfections. Human p21\textsuperscript{Waf1/Cip1} antisense and control ODNs were synthesized by Oligos Etc. (Wilsonville, OR). The human p21 antisense ODN sequence was 5′-ATC-CCC-AGG-CGG-CTT-TGA-CAT-3′. The randomly scrambled sequence of control ODN was 5′-TGG-ATC-CGA-CAT-GTC-AGA-3′, and the second control ODN (used for the experiment depicted in Fig. 8) was the sense human p21 sequence 5′-TAC-AGT-CTT-GGC-CGA-CCC-CTA-3′. For the transfection procedure, cells were grown to 70% confluence and washed with sterile PBS, and the ODNs (200 or 400 nm) were mixed with 6.6 μl Lipofectin/ml Opti-MEM media and added to the cells for 5 h at 37°C. Serum-free media (without ODNs) were added overnight, the media were changed in the morning, and the cells were stimulated with serum or platelet-derived growth factor-BB as indicated.

RT-PCR. RT-PCR was performed as described previously (18), using primers of sequence 5′-ACC-TCA-CCT-GCT-CTG-CTG-C-3′ (sense) and 5′-GAC-TGC-AGG-CTT-CCT-GTG-G-3′ (antisense) corresponding to the NLS region of human p21. The antisense primer is the 3′-flanking region of the coding sequence, with a PCR product size of approximately 230 bp because that is a convenient size for separation by agarose gel electrophoresis. Because the NLS amplification product contains the border between the second and third exons, the cDNA was used as a template rather than genomic DNA, as is standard practice (19). The PCR products from all tumors and controls were sequenced at the UCD Peptide Structure Laboratory.

Immunoblotting. Breast cancer tissues were cut into small pieces and rapidly homogenized in lysis buffer (1 ml/ mg) at 4°C. Cells from cell lines were grown to confluence in 60-mm culture dishes, and after incubation under appropriate conditions, the cells were washed with PBS and lysed in lysis buffer at 4°C. The tissue homogenates or cell lysates were centrifuged (13,000 × g, 4°C, 10 min), and the supernatants were Western blotted as described previously (20). Densitometry was analyzed using NIH Image.

Immunohistochemistry. Formalin-fixed, paraffin-embedded tissue blocks of the human tumor samples were sectioned at 4–5-μm thickness, mounted on charged glass slides, baked for 1 h at 60°C, deparaffinized, and rehydrated. Also for immunohistochemistry, cultured cells were grown and treated on chambered microscope slides (Bio-Tek Instruments, Winooski, VT). At the end point, cells were washed twice with ice-cold PBS and fixed with 4% neutral buffered formalin, dehydrated with 95% ethanol, and air dried at room temperature.

Slides were blocked and microwaved for antigen retrieval in 10 mm citrate (pH 6.0). Slides were incubated in full-length p21 (Calbiochem OP64) primary antibody solution in a humidified chamber overnight at room temperature, followed by incubation with secondary biotinylated antimouse antibody solution for 1 h. The Vectastain ABC Elite Kit (Vector Laboratories) detection was performed according to the manufacturer’s instructions. Slides were counterstained in Mayer’s hematoxylin, dehydrated, cleared, and coverslipped. Slides were photographed with a Zeiss Axioskop light microscope and AxioCam digital camera.

Evaluation of Nuclear Morphology. Cells were seeded in 6-well culture dishes and treated with ODNs as described. The cells were then immersed in methanol for at least 10 min. Cells were stained in 1% nonfat dry milk. After staining for 8–10 min, the cells were rinsed in water and dried completely. Nuclear morphology was visually evaluated by fluorescence microscopy.

Caspase-3 Activity. The CaspACE Assay System was used to measure the activity of caspase-3. After incubation under appropriate conditions, the cells were lysed in Caspace lysis buffer, and protein concentration was determined by dye reagent protein assay (Bio-Rad, Hercules, CA).
The lysates with equal protein amounts were incubated with DEVD-p-nitroaniline substrate for 4 h at 37°C. The reaction products were detected at 405 nm using a PowerWave X automated plate reader (Bio-Tek Instruments).

Results

p21 Is Increased in a Subset of Breast Cancers and Is Associated with Aggressive Tumor Characteristics. p21 has been demonstrated to be a negative regulator of p53-dependent and -independent apoptosis and to convey the survival signal of PI3K to downstream signaling pathways (9, 21). However, studies on the prognostic role of p21 in a variety of cancers have not shown consistent results (5–7). In light of our findings that an antisense ODN to p21 attenuates growth of tumors in a mouse model of breast cancer (16) and the reports of other investigators showing involvement of p21 in cancer progression (13, 22, 23) and cell survival (9), we investigated whether p21 is a feasible marker and target for future therapeutic intervention in human breast cancer.

Initial studies were directed at determining whether a subset of human breast cancers can be identified that are associated with activation of this antiapoptotic pathway. We reasoned that, regardless of whether p21 levels directly correlate with patient outcome and in light of the antiapoptotic effect of intact p21, it is possible that (a) attenuation of p21 (as by antisense p21 ODN therapy) might result in a propensity of p21-attenuated cells to be more sensitive to the killing effects of DNA-damaging chemotherapy agents, and (b) identification of tumors expressing high levels of p21 might serve as a stratifier to identify patients who will respond to antisense p21 ODN treatment. In addition, it is possible that increased levels or cytosolic localization (vide infra) of p21 might be additionally useful in prognostication.

After obtaining protocol approval from the UCD Institutional Review Board, we obtained from the UCD Human Biological Specimen Repository breast cancers and corresponding control tissues from eight patients. Matched control tissues consisted of samples taken from each cancer biopsy that were chosen grossly and confirmed microscopically to be uninvolved by the tumor. Samples were numbered, and patient identifiers were stripped. Data collected for each sample were limited to the clinical pathological assessments of stage, grade, patient age, and routine marker studies. A portion of the cancer tissue from each biopsy sample as well as normal tissue from the same breast was homogenized, normalized for protein content, and subjected to immunoblotting for p21 using ß-actin as a loading control. The immunoblot showed several nonspecific protein bands; p21 was identified using a positive control supplied by the antibody vendor (HeLa cell nuclear lysate), and levels of p21 normalized to ß-actin were determined by densitometry. Increased p21 levels were seen in three of the tumor tissues (sample 526T, 652T, and 759T) and none of the normal tissues (labeled N, Fig. 1, A and B). Note that 652T did not have corresponding normal tissue; instead, two separate tumor samples were obtained. Pathology reports for tumors 526T and 652T gave a diagnosis of infiltrating ductal carcinoma, with a high combined histological grade of 3 (modified Bloom-Richardson, highest grade of 3). Both of these tumors had poor prognostic factors, including large tumor size, multiple lymph node involvement, and high grade. Tumor 759T, with the weakest p21 staining of the three positives by im-
munoblot, was also an infiltrating ductal carcinoma but had features of the mucinous carcinoma special type and had an intermediate combined histological grade of 2. No nodes were reported on sample 759. Grades of all of the tumor samples are shown in Fig. 1C.

We and others have shown that intracellular localization of p21 plays a role in dictating its effect on cell cycle progression and apoptosis (6, 9, 10, 21), thus we next asked whether such localization correlated with the findings in our samples. p21 immunostaining confirmed high levels of p21 in the three p21-positive tumors; however, the localization of p21 in these samples was variably predominantly cytosolic or nuclear (Fig. 1C). Whereas tissue was available for Western blotting on tumor 1420, no tumor appeared on the section analyzed. These results indicate that the use of p21 as a prognostic marker may be feasible, although it was not in the scope of the current study to correlate expression levels or localization with prognosis or therapeutic response. These correlations are being examined in larger cohorts by several groups, including ours.

A possible explanation for the increased aggressiveness of the tumors showing high levels of p21 is mutation of the NLS region encompassing the AKT binding consensus sequence, such that AKT binding does not occur, AKT is constitutively active, or p21 fails to localize to the nucleus, any of which might provide a tumor survival advantage (21, 24). There exist several reports of such mutations, one of which was reported in a single breast carcinoma (25). To examine this possibility in the three tumors that we identified which possessed high levels of p21, we performed RT-PCR of the tumor and control tissue of these three samples using primers encompassing the NLS region. In all of the three tumors expressing high levels of p21, there was a precise correlation with the GenBank human p21 sequence (data not shown), eliminating the possibility that the mechanism of tumor aggressiveness in these three patients was due to a mutation in the exon region corresponding to the NLS.

**PI3K-related Signaling Proteins Are Increased in Human Breast Cancers Expressing High Levels of p21.** There exist several mitogenic signaling pathways whose activation is important to the process of oncogenic transformation. The PI3K pathway is replete with oncogenically important components (26), being activated by the oncogenes HER2 and epidermal growth factor receptor. PI3K thus functions as a “survival” protein, having antiapoptotic properties when activated. In addition, PTEN, which attenuates PI3K activity by dephosphorylation of the lipid second messenger phosphatidylinositol-3,4,5-P3, a product of PI3K activity, functions to attenuate the proliferative effects of PI3K, such that PTEN-inactivating mutations are also oncogenic (27).

We examined all eight of the breast tumors and corresponding control tissues for levels of these signaling proteins by immunoblotting. The same quantity of lysate was loaded as described in Fig. 1A. All three of the tumors that overexpressed p21 (but none of the control samples) overexpressed the p85 catalytic subunit of PI3K (Fig. 2A). Surprisingly, all three tumors with high p21 expression (but none of the other tumor or control samples) also expressed high levels of PTEN (Fig. 2B); however, the magnitude of the increases in p85 and PTEN did not correlate in all cases with the levels of p21. PTEN induction may be occurring in a counter-regulatory or homeostatic fashion, as an attempt by the cell to subvert the antiapoptotic effect of p21 through PI3K.

**Antisense ODN to p21 Attenuated p21 Levels and Causes Apoptosis in Two Human Breast Cancer Cell Lines.** We have shown previously that attenuation of p21 in a mouse model of breast cancer results in attenuation of growth of implanted Met-1 cells (which are derived from a high metastatic potential tumor in transgenic mice expressing polyomavirus middle T oncogene; Refs. 16 and 28), likely due to induction of apoptosis in the tumor or blood vessels supplying it by this ODN. To begin to translate this work to human breast cancer, we first examined two human breast cancer cell lines, T47D (ductal carcinoma) and MCF-7 (adenocarcinoma), for basal levels of p21. Surprisingly, both of these cell lines, whether serum starved or grown in serum-containing medium, displayed constitutively elevated levels of p21 (Fig. 3; compare with MAPK levels in control lanes of Fig. 4, in which the same quantity of protein was loaded), a property that may explain their transformed phenotype.

Due to the high levels of p21 in these two human breast cancer cell lines, we reasoned that, in a manner similar to

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dependent attenuation of p21 (Fig. 4, after transfection, both cell lines showed marked, dose-

Figure 3

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p21 is expressed at high levels in MCF7 and T47D cells. T47D and MCF7 cells were grown to confluence in serum-containing media, serum starved for 24 h, and then serum stimulated for the times indicated. The cells were lysed, and equal protein quantities (20 μg) were subjected to immunoblotting with full-length p21 antibody.

their effect in VSM cells (8), antisense ODNs to p21 may result in increased apoptosis and/or cell cycle arrest. This phenomenon might explain the effects of the antisense p21 ODN that we observed in nude mice and suggest future therapeutic possibilities for the use of this ODN in human disease. T47D and MCF7 cells were lipofected with either antisense p21 ODN (200 or 400 nM), the control ODN at the same concentrations, or lipofectin alone (no DNA). Control cells (Fig. 4, cont) were not transfected. When examined 24 h after transfection, both cell lines showed marked, dose-dependent attenuation of p21 (Fig. 4, A and B). Whereas there was a moderate decrease in p21 in the MCF7 cells after transfection with the control ODN, dose-dependent attenuation of p21 in response to antisense p21 ODN was substantially greater; this effect parallels the apoptotic effect of two control ODNs (see Figs. 6 and 8) and is likely due to exquisite sensitivity of the MCF7 cells to ODN transfection. There was no effect of lipofectin alone when compared with no transfection (control). Specificity of antisense p21 ODN to p21 has been shown in prior studies (8, 15, 29) and is further supported by the lack of modulation of MAPK (or PARP; see Fig. 6) levels in the ODN-transfected cells as compared with controls. However, an inhibitory effect of the control ODN on p21 levels occurred in both cell lines (although it was more pronounced in the MCF-7 cells), likely due to a nonspecific toxic effect of ODN transfections in general in these cells.

To determine whether a decrease in total p21 levels by immunoblotting parallels changes in cellular levels of p21 in breast cancer cells, we performed immunohistochemistry of both T47D and MCF7 cells after transfection with the antisense p21 ODN. The MCF7 cells become sparse after transfection with the antisense p21 ODN (Fig. 4C, a and b), as expected given their increased propensity to apoptosis. Both the MCF7 and the T47D cells show decreased p21 staining in the antisense p21 transfected cells as compared with the random sequence ODN-transfected cells (Fig. 4C), consistent with the immunoblots of these cells (Fig. 4, A and B).

Because cancer is characterized by disordered cell cycling, it is reasonable to assume that p21, which carries out growth-arresting orders of p53 as well as the proapoptotic effects of some mitogens, may play a role in the origin or progression of this disease and its response to conventional treatment. Furthermore, if this assumption is correct, it follows logically that manipulation of this protein, as with antisense ODNs, may be of use in cancer therapy. In fact, decreasing p21 has been shown to trigger apoptosis in human cancer cells likely by disallowing faithful repair of damaged DNA (30, 31), and the use of antisense ODNs against other targets has already shown promise in treatment of breast cancer (3). The current dogma of cancer chemotherapy is that these drugs ultimately generate signals that activate or open apoptotic metabolic pathways (2, 32); thus, because p21 influences the outcome of the p53 response to cell damage from these proapoptotic agents (cell cycle arrest versus apoptosis; Ref. 33), we next assessed whether attenuation of p21 results in apoptosis in the two breast cancer cell lines.

Apoptosis is the end result of an elaborate cascade of molecular events that ultimately result in programmed cell death; thus, a variety of tests are generally used to determine whether apoptosis is occurring. We assessed apoptosis in three ways: (a) assessment of nuclear morphology; (b) PARP cleavage; and (c) caspase-3 cleavage. Serum-starved cells were transfected with antisense p21 ODN or the control ODN, fixed, stained with Hoechst 33258, and examined under UV light. Both MCF7 and T47D cells showed nuclear morphological changes consistent with apoptosis (Fig. 5) in response to antisense p21 ODN.

As a further measure of apoptosis, we examined cleavage of PARP. This protein is cleaved by caspases and results in appearance of M, 85,000 and M, 24,000 fragments, the latter of which binds irreversibly to broken ends of DNA, which assures irreversibility of apoptosis. Both breast cancer cell lines were transfected with antisense p21 and random sequence ODNs and immunoblotted with PARP antibody. There was marked PARP cleavage, as evidenced by appearance of the M, 85,000 cleavage product, in the cells transfected with antisense p21 ODN (Fig. 6). Similar to what was seen in Fig. 4, a proapoptotic effect of the control ODN on p21 levels was seen in both cell lines, likely due to a nonspecific toxic effect of ODN transfections in these cells. Indeed, it is possible that the proapoptotic effect of the control ODNs is a consequence of their inhibitory effect on p21 levels (Fig. 4), given what is known about the function of p21 as a survival protein.

As another confirmatory test for antisense p21 ODN-mediated apoptosis, we assessed caspase-3 activation. Members of the caspase family of proteases are essential components of an evolutionarily conserved cell death pathway in multicellular eukaryotes and play key roles in inflammation and apoptosis in mammalian cells. Caspase-3 has substrate specificity for the amino acid sequence DEVD (Asp-Glu-Val-Asp) and is inhibited by the tetrapeptide inhibitor Ac-DEVD-CHO; its catalytic activity was assessed colorimetrically using the labeled Ac-DEVD-pNA substrate. T47D cells, after transfection with the antisense p-nitroaniline p21 ODN, showed marked dose-dependent caspase-3 cleavage, with no change after control ODN transfection (Fig. 7). The MCF-7 cells lack caspase-3 and thus did not show significant caspase-3 cleavage products (data not shown).

To further demonstrate apoptosis in the breast cancer cells in response to antisense p21 ODN, we assessed [3H]thymidine incorporation in cells transfected with the
ODNs. Apoptotic cells will not incorporate thymidine into DNA during S phase, but \[^{3}H\]thymidine incorporation experiments will not distinguish between G1-S arrest and apoptosis. Serum-starved cells were transfected with 200 nM antisense p21 and control ODN, stimulated with serum-containing media, and examined for their ability to incorporate \[^{3}H\]thymidine into DNA. Due to the significant attenuation of \[^{3}H\]thymidine incorporation in control ODN-transfected cells, we used two different control ODNs: (a) the scrambled sequence ODN used in the preceding experiment; and (b) another control ODN that had the sense p21 sequence. In both cell lines, antisense p21 ODN transfection resulted in significant attenuation of DNA incorporation as compared with both control ODNs at 200 nM (Fig. 8). Both control ODNs (only one is shown in Fig. 8) showed similar attenuation, suggesting that these cancer cells are extremely sensitive to small ODN transfections or to p21 attenuation (compare with Figs. 4 and 6). This thymidine data show a marked resem-

Fig. 4. Breast cancer cells transfected with antisense p21 ODN show reduced levels of p21. MCF7 and T47D cells were grown to confluence, serum starved for 24 h, and lipofected with antisense p21 or control ODN or lipofectin only. cont refers to nontransfected cells. The cells were subsequently serum stimulated for another 24 h. A and B, the cells were lysed, and equal protein quantities (20 μg) were electrophoresed and immunoblotted with p21 antibody. The same lysate was immunoblotted with MAPK antibody as a loading control. Density of p21 and MAPK bands was determined and reported as a ratio. C, the cells were transfected as described in A, fixed, subjected to immunohistochemistry with p21 antibody as described in "Materials and Methods," and examined under visible light at ×400.

Fig. 5. Lipofection of antisense p21 results in apoptotic morphological changes in breast cancer cells. MCF7 and T47D cells were grown to confluence, serum starved for 24 h, and lipofected with antisense p21 or control ODN. The cells were subsequently serum stimulated for another 24 h, fixed, stained with Hoechst 33258, and examined under UV light at ×400.
to the PARP cleavage data (Fig. 6); whereas a decrease in DNA synthesis can be the result of growth arrest, it is more likely, based on the apoptotic data shown above, that it is due in this case to promotion of apoptosis. Thus, antisense p21 ODN transfection results in apoptotic changes in two human breast cancer cell lines, in the absence of a chemotherapeutic stimulus; our results suggest that this ODN may be of therapeutic benefit in human disease.

Discussion
Whereas the CKIs have long been assumed to play a growth-inhibitory role in cell cycle progression, recent data have demonstrated that these proteins act as both positive and negative regulators of G1-S events (34). One of the functions of p21 is to act as an assembly factor for cyclin/cdk pairs (8, 35), which likely explains the increase in p21 seen soon after growth factor stimulation (15, 36) as well as in proliferating breast (7) and pancreatic (14) cancer cells.

In addition to its role as a cell cycle regulator, p21 functions to prevent apoptosis, mediating the survival function of PI3K through phosphorylation by AKT (9, 21). It is likely that p21, under conditions where it is induced by p53, functions to keep DNA-damaged cells from becoming apoptotic while p53-mediated repair processes are under way; recent data has suggested that Myc may be important in regulating this decision (33). Forced cytosolic localization, also important in the function of this protein in cell cycle regulation (vide infra), has been shown to be apoptosis inhibitory in human cells (10).

The role of p21 in oncogenesis is not clear. Although initially described as a mediator of p53 tumor suppression, recent studies point to cytosolic localization of the cip/kip CKIs as being important in promoting cell cycle transit, and thus this property of p21 may be important in cancer progression. For example, cytosolic localization of p21 as the result of overexpression of HER2/neu may enhance cell proliferation (21), and cytosolic localization of the CKI p27 attenuates its growth-inhibitory properties (37). We have shown that forced cytosolic localization of p21 increases cell cycle transit in VSM cells. Interestingly, p21 has been shown to be increased early in the development of pancreatic cancers (14), suggesting a role of this protein, perhaps related in malignancy to its intracellular localization, in growth progres-
sion. Consistent with our data presented here, one author (38) has suggested and provides several lines of evidence that cytoplasmic p21 and p27 may even be oncoproteins.

Reports of the function of p21 in human breast cancer have been inconsistent at best. In one study, p21 expression was significantly associated with the presence of node metastases (7), but in another, p21 expression in primary node-positive breast carcinoma did not have prognostic significance (5). One group has found a significant association between p21 and ER expression using multivariate analysis (39), and other investigators found that p21 immunoreactivity is higher in male as compared with female breast cancers (40). In studies using malignant cells in culture, AKT-induced phosphorylation of p21 (a) enhances proliferation in a HER2/neu-overexpressing breast cancer cell line (21) and (b) promotes survival in a glioblastoma cell line (9). Also consistent with its promitogenic function, induction of p21 was found necessary for G1-phase progression in ErbB-2-overexpressing human breast cancer cells (41).

The current dogma of cancer chemotherapy is that these drugs ultimately generate signals that activate or open apoptotic metabolic pathways (2, 32). We have shown that an antisense ODN to p21 homologous to the one used in the current study attenuates growth of Met-1 tumors in nude mice (16); whether this is due to a direct effect on the tumors or an indirect one on tumor angiogenesis is not known and is under current investigation in our laboratory. In light of the now accepted function of p21 in inhibiting apoptosis, it is reasonable to investigate means of decreasing p21 levels as possible adjuvant therapy ultimately of use in human breast cancer patients. Given that antisense therapy is becoming a quite reasonable avenue of clinical investigation in human breast cancer (3), the data presented in the current study (that antisense ODN to p21 promotes apoptosis in two established breast cancer cell lines even in the absence of conventional chemotherapeutic agent exposure) enhance the feasibility of this prospect.

In contrast to p53, mutations in p21 are rare in cancer, and p21-null animals are phenotypically normal (42), although they may be more susceptible to chemically induced skin carcinoma, and they have a higher incidence of pituitary tumors (43). These data further support the viability of p21 targeting in the therapy of human disease: whereas p53 attenuation in vivo would be forbidden due to its cancer-protective effects, p21 manipulation appears clinically more attractive.

Our findings that levels of the p85-regulatory subunit of PI3K are increased in those cancers that display high levels of p21 is not unexpected, given the location of this signaling protein upstream of p21 (9, 37) and its importance in oncogenic signaling (reviewed in Ref. 44). However, in a study of 33 breast cancer patients, whereas 79% of the samples showed increased levels of p85, there was no correlation with patient prognosis (45). Increased expression of PTEN in the p21-overexpressing tumors is puzzling because this protein is a negative regulator of PI3K (44). Although there is evidence that PTEN deficiency plays a role in mammary carcinogenesis (46), it is possible that levels of PTEN are increased in an attempt by the cell to counteract the oncogenic propensity of PI3K. A recent study showing that PTEN causes transcriptional activation of p21 (47) is consistent with our data and suggests a direct link between these two proteins.

The use of members of the cyclin/cdk system as targets for breast cancer has been entertained before, but the use of attenuators of p21 in this disease, prior to our studies, has not been investigated. The cyclins lies downstream of p21, and p21 functions as an assembly factor for cyclin D and cdk4 (8) as well as cyclin A and cdk2 (48), such that these molecules can be brought together in order for cdk4 to become catalytically active. Consistent with our data on p21, cyclin D1-deficient mice have been shown to be resistant to certain breast cancers (49), and high levels of cyclin E correlate with poor breast cancer patient outcome (50). Whether the cyclins in these cases represent a further distal target along the p21 pathway or an entirely distinct mitogenic signaling cascade that may be exploited for therapeutic benefit remains to be elucidated.

Emphasizing the potential pathogenetic role of p21 in breast cancer, there have been several reports of polymorphisms in p21. In one study, a single-nucleotide polymorphism in intron 2 of human p21 was associated with a slightly increased breast cancer risk (51). Another group has found an Arg39→Trp mutation in an invasive ductal breast carcinoma that resulted in an impaired ability of p21 to inhibit cdk4 (25). Others have shown the existence of polymorphisms in p21 in human esophageal (52) and oral (53) cancers that may play a role in tumorigenesis. Of interest in the latter studies, the mutation was in the COOH terminus (codon 149), which is important for both proliferating cell nuclear antigen binding and nuclear localization. In addition, phosphorylation of Thr145 and Ser146 by AKT has been shown to enhance p21 stability and promote cell survival (9). Consistent with reported findings of cytosolic localization of p21 by AKT activation in breast cancer cells (21), we have shown that forced cytosolic overexpression of ∆NLS-p21 results in vigorous growth of vascular cells. However, in our small sample size reported in this paper, we were unable to find mutations in the NLS region of the tumors expressing high levels of p21 by RT-PCR.

Whereas p21 attenuation in human breast cancer has not been clinically attempted, our data, as well as other published studies, strongly support the feasibility of this idea. There have been numerous reports of sensitization to chemotherapy in p21(−/−) cells (12, 13, 54) or antisense p21 ODN transiently transfected (23) or antisense p21 plasmid stably transfected (55) cell lines. Our demonstration of a proapoptotic effect in two breast cancer cell lines, in the absence of conventional chemotherapy, of antisense p21 using an ODN that we have previously shown to be systemically active in an animal model of breast cancer is the first such report. Potential advantages of the use of ONDs rather than stably transfected p21 plasmids are as follows: (a) there is a very high transfection efficiency at least in vitro using our 21-bp antisense p21 ODN; and (b) attenuation of p21 using antisense ONDs will be transient in time and could conceivably be targeted at a specified time in the cell cycle i.e., when p21 is increased early after a mitogenic stimulus or soon after a
DNA-damaging agent or procedure) when p21 serves its assembly factor role. Thus, our work has set the stage for a clinical trial of antisense p21 ODN in human breast cancer, an idea for which the time is now right.

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References


Molecular Cancer Therapeutics

An Antisense Oligodeoxynucleotide to \(^{\text{Waf1/Cip1}}\) Causes Apoptosis in Human Breast Cancer Cells

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