Role of p21 in Prostaglandin A2-mediated Cellular Arrest and Death

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Abstract

Prostaglandin A2 (PGA2) treatment induces growth arrest of most cells, and we have recently shown that, for breast carcinoma MCF-7 cells, this is correlated with an induction of the cyclin-dependent kinase inhibitor p21 and reduced cyclin-dependent kinase 2 activity. In this study, we demonstrate that, in RKO cells, PGA2 treatment fails to induce growth arrest, but rather results in significant cell death. These effects are correlated with lack of p21 induction and enhanced cyclin-dependent kinase 2 activity. Reduction of endogenous p21 expression in MCF-7 cells through expression of antisense p21 resulted in a response pattern approaching that seen in RKO cells, characterized by diminished growth arrest and increased death. These findings support a role for p21 in PGA2-mediated growth arrest, which we propose serves to prevent cell death caused by inappropriate cell cycle progression.

Introduction

Cellular proliferation is a highly regulated process controlled by both positive and negative regulatory molecules that include cdks and cdk-inhibitory proteins. The best characterized cdk-inhibitory protein, p21Waf1/Cip1/Sdi1, is believed to exert its growth-inhibitory effect largely during G1, through binding to cdk4 and cdk2 (1). PGA2 transcription is highly elevated after DNA damage in cells with wild-type tumor suppressor p53 activity (2), and the p21 protein has been found to participate in the G1 checkpoint that follows DNA damage (3). p21 is also up-regulated via p53-independent mechanisms and we have recently shown that, for breast carcinoma MCF-7 cells, this is correlated with an induction of the cydlin-dependent kinase inhibitor p21 and reduced cyclin-dependent kinase 2 activity. In this study, we sought to further explore the relationship between p21 and PGA2 treatment of breast carcinoma MCF-7 cells results in the p53-independent induction of p21 expression concomitant with G1 growth arrest (19). However, we noticed that for some cells, PGA2 was extremely toxic, and in these cells p21 was not induced. Therefore, in this study, we sought to further explore the relationship between p21 expression and the cellular response (growth arrest versus cell death) to PGA2 treatment. Through the use of antisense expression vectors to lower endogenous p21 protein levels, we provide evidence that p21 participates directly in mediating PGA2-imposed cellular growth arrest. Our data suggest that enhanced p21 expression protects against cell death, perhaps by preventing the activation of cdks and subsequent progression of cells from G1 to S.

Materials and Methods

Cell Culture, Treatments, and Transfection. The human breast carcinoma MCF-7 and non-small cell lung carcinoma H-1299 cell lines were cultured in RPMI 1640 ( Gibco-BRL, Bethesda, MD). Human cervical carcinoma HeLa cells primary rat smooth muscle cells, mouse NIH 3T3 fibroblasts, and embryonal fibroblasts derived from a p53 knockout mouse (20) were maintained in DMEM ( Gibco-BRL). Human colorectal carcinoma RKO cells (21) were grown in MEM. All cells received 10% fetal bovine serum and 50 μg/ml gentamicin. 3T3 cells also received 10% calf serum. For starvation/synchronization experiments, cells at 50% confluence were placed in serum-free medium for 3 days, then released from starvation arrest by the addition of fetal bovine serum to a final concentration of 10%. PGA2 (Sigma Chemical Co., St. Louis, MO), prepared in ethanol, was added to cells at a final concentration of 36 μM unless otherwise indicated. Cell viability was assessed by trypan blue dye exclusion. The pCEP-WAF1(AS) (2) and pCEP4 plasmids were transfected by standard CaPO4 precipitation methods. Stable transfectants were selected in 250 μg/ml hygromycin B (Sigma), maintained in 150 μg/ml hygromycin B, and used within 4 weeks of selection.

Northern and Western Blot Analysis. Total RNA was isolated using STAT-60 (Tel-Test “B”, Friendswood, TX) following the manufacturer’s specifications and examined for p21 expression by Northern analysis. For the detection of p21 mRNA in the human cell lines, cDNA excised from pCEP-WAF1 (2) was used as a probe. An oligomer complementary to the mouse p21 mRNA (5 ’-ACGGTATCTGATCGTCTTCGAACC-3 ’) was used for the detection of p21 mRNA in rodent cells. The p21 oligomer and an oligomer that recognizes the 18S rRNA (5’-AAGCCATTATCGATCGTCTTCGAACC-3’; Integrated DNA Technologies, Coralville, IA) were 3’ end labeled. p21 hybridization signals were quantitated using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA) and normalized to 18 S signals obtained on the same blot to control for variations in loading and transfer among samples. p21 protein expression was examined by Western blot analysis using the enhanced chemiluminescence system (Amersham, Arlington Heights, IL) after incubation with monoclonal mouse antip21 human p21 (Oncogene Science).

Flow Cytometric Cell Cycle Analysis. Cell cycle distribution was analyzed using flow cytometry as described (22). Briefly, 2-5 × 106 cells were trypsinized, washed once with PBS, and fixed in 70% ethanol. Fixed cells were washed with PBS, incubated with 1 μg/ml RNase A for 30 min at 37°C, stained with propidium iodide (Boehringer Mannheim), and analyzed on a FACScan flow cytometer. The percentage of cells in the various cell cycle stages was determined using the MULTICYCLE software program (Phoenix Flow Systems, San Diego, CA).

Immunoprecipitation and Kinase Assays. For immunoprecipitation of endogenous cdks, cells were harvested and processed as described (3) using a rabbit polyclonal antihuman cdk2 antiseraum (PharmMingen, San Diego, CA). The cdk2 activity in the immunocomplex was assayed using histone H1 as a substrate, as described (3). The reaction products were subjected to electrophoresis in 12% SDS polyacrylamide gels, after which the gels were dried. The incorporation of 32P was visualized by autoradiography and quantitated with a PhosphorImager (Molecular Dynamics).

Results

Effect of PGA2 Treatment on Growth and p21 Expression in Various Cell Lines. Table 1 summarizes the results of a survey examining the growth-inhibitory effect of PGA2 and p21 induction in a number of cell lines. Exposure of most cell types to PGA2 resulted in both enhanced expression of p21 and inhibition of growth; in keeping with our recent studies in MCF-7 cells (19) this effect was not...
influenced by the p53 status of the cell. In two cell lines (RKO and mouse 3T3 fibroblasts), however, PGA2 failed to induce p21. In these cells, PGA2 treatment was found to be highly toxic, resulting in less than 10% cell survival by 48 h of treatment. Given the established role of p21 in regulating growth, we hypothesized that levels of p21 expression might be critical in determining the cellular outcome (arrest or death) that follows PGA2 treatment. Because MCF-7 and RKO cells exhibit these two opposing responses to PGA2, we carried out additional studies with these two cell types to test this hypothesis.

Changes in Cell Number and p21 Expression in MCF-7 and RKO Cells after PGA2 Treatment. Treatment of MCF-7 cells with 36 µM PGA2 resulted in complete growth inhibition with little evidence of cytotoxicity (Fig. 1A; > 90% viability with 48 h of exposure to PGA2). In contrast, PGA2-treated RKO cultures showed an initial increase in cell number (comparable to that which occurred in the untreated population), followed by a sharp decline in cell number at subsequent time points, reflecting extensive cell death (<10% of cells remained by 48 h posttreatment). FACS analysis revealed that ~90% of PGA2-treated MCF-7 cells were in G1, compared to 5% in the untreated population (Fig. 1B). In contrast, PGA2 treatment did not alter the cell cycle distribution of RKO cells, consistent with a lack of growth arrest.

Northern analysis was utilized to study the expression of p21 in MCF-7 and RKO cultures after a 12-h exposure to PGA2 (Fig. 1C). Whereas p21 mRNA was induced in MCF-7 cells in a dose-dependent manner (maximum induction occurred at 36–50 µM PGA2), it remained uninduced in RKO cells within the range of doses utilized.

Effect of PGA2 Treatment on the Cell Cycle Distribution of MCF-7 and RKO Cultures Synchronized by Serum Starvation. We have recently found that a 12-h PGA2 treatment can block the serum-stimulated entry into S phase of MCF-7 cells synchronized in G1 by serum starvation (19). Because PGA2 treatment failed to arrest asynchronously growing RKO cells, we examined the effect of PGA2 on the cell cycle distribution of synchronous cultures after serum addition to obtain clearer evidence for whether PGA2 affected cell cycle progression of these cells. As shown in Fig. 2A, the progression of synchronous, serum-stimulated RKO cells from G1 into S was not prevented by pretreatment with PGA2. In fact, even before serum stimulation, a substantial percentage of the PGA2-treated cells were found in the S phase (compared with non-PGA2-treated, serum-starved cells) indicating that PGA2 treatment itself induced cell cycle progression in serum-starved RKO cells.

Progression of cells from G1 into S phase was also studied by monitoring the activity of cdk2 (which is active from mid G1 through the end of S) with an immunocomplex-kinase assay using histone H1 as a substrate (Fig. 2B). In previous studies, we had demonstrated a physical association between cdk2 and p21 proteins in lysates of PGA2-treated MCF-7 cells (19) which was correlated with loss of cdk2 activity. In keeping with these findings, PGA2 treatment of synchronous MCF-7 cells completely prevented cdk2 activation by serum stimulation, whereas in non-PGA2-treated MCF-7 cells, cdk2

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* Effect of PGA2 on different cell types. Cells were either left untreated or were treated with 36 µM PGA2. Cellular growth arrest was determined by cell counting methods using a hemocytometer, and cell viability was assessed by trypan blue exclusion. p21 expression was studied by Northern blot analysis, as described in "Materials and Methods." 

** Fold induction of p21 mRNA was determined by comparing p21 mRNA signals on Northern blots from control (untreated) and PGA2-treated cells (36 µM, 12 h). 

### Table 1: Growth arrest and induction of p21 mRNA in cell lines with different p53 status

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### Fig. 1. Effect of PGA2 on cell number and FACS distribution of MCF-7 and RKO cultures after a 12-h exposure to PGA2 (Fig. 1C). Whereas p21 mRNA was induced in MCF-7 cells in a dose-dependent manner (maximum induction occurred at 36–50 µM PGA2), it remained uninduced in RKO cells within the range of doses utilized.

Fig. 1. Effect of PGA2 on cell number and FACS distribution of MCF-7 and RKO cultures. A. MCF-7 and RKO cultures with functional p53 (neo) or transfected with an E6 vector to functionally inactivate p53 (E6) were treated with 36 µM PGA2 and the cells were counted every 12 h using a hemocytometer. B. FACS analysis of MCF-7 and RKO cultures that were either left untreated (control) or treated with 36 µM PGA2. Cells were treated for 48 h before FACS analysis, as described in "Materials and Methods." C. The expression of p21 in the expression of p21 in MCF-7 and RKO cells. Dose-response changes in p21 mRNA after PGA2 treatment of MCF-7 and RKO cells. Cells were treated with the indicated doses of PGA2 for 12 h, and the expression of p21 mRNA was analyzed on Northern blots, as described in "Materials and Methods." 18 S rRNA signal was used to normalize for differences in loading and transferring of the RNA samples.
untransfected cells (not shown). Whereas none of the hygromycin selection in hygromycin, was indistinguishable from that of parental, MCF-7 cells transfected with the empty pCEP4 vector, followed by mycin-selectable plasmid vector (pCEP4) that expresses high levels of growth arrest. To more directly assess the relative contribution of p21 p21 in the inactivation of cdk2 complexes during PGA2-mediated before the addition of serum, and remained high after serum stimulation in MCF-7 cells through their stable transfection with a hygro.

In PGA2-mediated growth inhibition, we sought to inhibit p21 expres in this report, we have addressed the role of p21 in mediating growth arrest that ensues in most cell types after treatment with PGA2, and have further explored the relationship between p21 expression and cytotoxicity in PGA2-treated cells. Therefore, a comparative analysis of MCF-7 and RKO cells indicated that, whereas MCF-7 cells respond to PGA2 treatment with a G1 arrest, high levels of p21 expression, and inhibition of cdk2 activity, RKO cells fail to show an increase in p21 expression, show enhanced cdk2 activity, and do not arrest but rather undergo cell death. The effect of PGA2 treatment on MCF-7 cells was significantly altered by reducing their level of p21 expression through stable transfection with p21 antisense expression vectors, such that their response to PGA2 more closely resembled that seen in RKO cells. That is, they were less growth inhibited, showed greater cdk2 activity, and exhibited reduced cell survival after PGA2 treatment than did parental cells. Taken together, these findings implicate p21 in regulating growth arrest in response to PGA2, and they suggest that either p21 itself or the associated growth arrest (resulting, at least in part, from p21 expression) protects cells from the cytotoxic effects of the prostaglandin. These findings are consistent with recent observations by Canman et al. (7), who observed that in mouse Baf-3 hematopoietic cells, growth factor withdrawal resulted in greater sensitivity to radiation-induced apoptosis, which correlated with reduced p21 expression. Whether PGA2-imposed cell death occurs through an apoptotic mechanism is not clear. Although the cells exhibit morphological characteristics of apoptotic cell death (i.e., chromatin condensation) and present a sub-G1 population, particularly after the addition of serum (Fig. 3C). These findings are consistent with the notion that cells expressing less p21 are able to escape the G1 arrest imposed by PGA2 treatment. Taken together, these results support our proposed model of PGA2 action whereby the induction of p21 contributes to arresting the cells in G1, and lack of sufficient p21 to elicit this effect would result in the observed enhanced cdk2 activity and the progression of these cells out of G1 into S.

Finally, we examined the cytotoxicity of PGA2 treatment of AS.3 and AS.4 cultures compared to that of parental MCF-7 cultures. As shown in Fig. 3D, 48 h of continuous exposure to PGA2 resulted in significant cytotoxicity in AS.3 and AS.4 clones (55–60% survival) compared to the parental cells (>90% survival). However, PGA2 was not as cytotoxic for those cells as it was for RKO cells, which contain even lower levels of p21. This finding strongly supports our model that p21 exerts a protective function by mediating cellular arrest, and failure to carry out this function results in enhanced toxicity of the prostaglandin.

Discussion

In this report, we have addressed the role of p21 in mediating growth arrest that ensues in most cell types after treatment with PGA2, and have further explored the relationship between p21 expression and cytotoxicity in PGA2-treated cells. Therefore, a comparative analysis of MCF-7 and RKO cells indicated that, whereas MCF-7 cells respond to PGA2 treatment with a G1 arrest, high levels of p21 expression, and inhibition of cdk2 activity, RKO cells fail to show an increase in p21 expression, show enhanced cdk2 activity, and do not arrest but rather undergo cell death. The effect of PGA2 treatment on MCF-7 cells was significantly altered by reducing their level of p21 expression through stable transfection with p21 antisense expression vectors, such that their response to PGA2 more closely resembled that seen in RKO cells. That is, they were less growth inhibited, showed greater cdk2 activity, and exhibited reduced cell survival after PGA2 treatment than did parental cells. Taken together, these findings implicate p21 in regulating growth arrest in response to PGA2, and they suggest that either p21 itself or the associated growth arrest (resulting, at least in part, from p21 expression) protects cells from the cytotoxic effects of the prostaglandin. These findings are consistent with recent observations by Canman et al. (7), who observed that in mouse Baf-3 hematopoietic cells, growth factor withdrawal resulted in greater sensitivity to radiation-induced apoptosis, which correlated with reduced p21 expression. Whether PGA2-imposed cell death occurs through an apoptotic mechanism is not clear. Although the cells exhibit morphological characteristics of apoptotic cell death (i.e., chromatin condensation) and present a sub-G1 compartment in FACS analysis, we have been unable to obtain clear evidence of internucleosomal DNA fragmentation in agarose gels. Although it remains to be determined why PGA2 is cytotoxic for RKO cells, the differential responsiveness to PGA2 with respect to

Fig. 2. Cell cycle profiles and cdk2 kinase activity of serum-starved MCF-7 and RKO cultures after serum addition in the presence or absence of PGA2. A, after a 72-h starvation period, MCF-7 and RKO cells received PGA2 for 12 h (PGA2) or were left untreated (control) before serum addition for the times indicated. Fixed cells were subjected to FACScan analysis to determine the distribution of cells throughout G1, S and G2-M. Columns, percentage of cells in S and G2-M after serum addition to the PGA2-treated (ﬁl) and control (□) cultures. B, after serum starvation, MCF-7 and RKO cells received PGA2 for 12 h (PGA2) or were left untreated (control) before serum addition for the indicated times. cdk2 was immunoprecipitated from 1 mg of cell lysates, and the kinase activity was assayed using histone H1 as a substrate. 32P-labeled histone H1 is shown.

was readily activated by the addition of serum as the cells proceeded through late G1 and into the subsequent stages of the cell cycle. In contrast, and consistent with the FACS analysis described above, cdk2 activity in RKO cells was elevated in PGA2-treated cultures even before the addition of serum, and remained high after serum stimulation.

Antisense p21-expressing MCF-7: FACS and cdk2 Activity. Taken together, the experiments described thus far support a role for p21 in the inactivation of cdk2 complexes during PGA2-mediated growth arrest. To more directly assess the relative contribution of p21 in PGA2-mediated growth inhibition, we sought to inhibit p21 expression in MCF-7 cells through their stable transfection with a hygromycin-selectable plasmid vector (pCPE4) that expresses high levels of the antisense p21 transcript (2). The response to PGA2 elicited by MCF-7 cells transfected with the empty pCPE4 vector, followed by selection in hygromycin, was indistinguishable from that of parental, untransfected cells (not shown). Whereas none of the hygromycin-resistant clones selected from cells transfected with the antisense p21-expression vector showed an absence of p21 expression, several clones displayed substantially lower levels of basal and inducible p21 protein. Two of these clones (AS.3 and AS.4), showing reduced p21 protein levels in response to PGA2 treatment (Fig. 3A), were chosen for further analysis.

In keeping with their lower p21 expression, a reduced cdk2 inhibition by PGA2 was observed after stimulation of AS.3 and AS.4 clones, relative to that seen in the parental cultures (Fig. 3B). Comparative FACS analysis examining the effect of PGA2 treatment on cell cycle distribution of synchronous, serum-stimulated AS.3, AS.4, and parental MCF-7 cells revealed that a substantial percentage of AS.3 and AS.4 cells were found in the S phase relative to the parental population, particularly after the addition of serum (Fig. 3C). These findings are consistent with the notion that cells expressing less p21 are able to escape the G1 arrest imposed by PGA2 treatment. Taken together, these results support our proposed model of PGA2 action whereby the induction of p21 contributes to arresting the cells in G1, and lack of sufficient p21 to elicit this effect would result in the observed enhanced cdk2 activity and the progression of these cells out of G1 into S.

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cell type could have practical significance with respect to the utility of this experimental chemotherapeutic agent. Further understanding of how PGA2 mediates growth arrest could lead to chemotherapeutic strategies using PGA2 in combination with agents that act at different points in the cell cycle to inhibit growth. However, the prostaglandin is likely to prove to be most beneficial for treatment of tumors that respond to PGA2 with cell death similar to RKO cells. In fact, the responsiveness of specific tumors to PGA2 (arrest of death) could perhaps be identified before treatment through examination of small biopsy specimens. Finally, given the findings presented here for PGA2, and the fact that many chemotherapeutic agents currently in use induce high levels of p21 expression (perhaps thereby allowing some degree of protection), strategies to preferentially block p21 activities in human fibroblasts during radiation-induced G1 arrest. Cell, 75: 817–825, 1993.


References


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