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Ablation of either p21 or Bax prevents p53-dependent apoptosis induced by green tea polyphenol epigallocatechin-3-gallate

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SPECIFIC AIMS

Our previous study demonstrated that treatment with epigallocatechin-3-gallate (EGCG), a polyphenolic compound of green tea resulted in activation of p53 and induction of apoptosis in prostate cancer LnCaP cells. However, there has been no direct evidence to delineate the role of p53- and p53-dependent pathways in EGCG-mediated apoptosis. To understand the mechanism of negative growth regulation of prostate cancer cells by EGCG, we undertook a genetic approach and generated an isogenic pair of prostate carcinoma cells PC3 ($p53^{-/-}$) and PC3-p53 by stably introducing a cDNA encoding wild-type p53.

PRINCIPAL FINDINGS

1. Stabilization and activation of p53 by EGCG

To examine whether EGCG treatment modulated the levels of p53 in PC3-p53 cells, cell lysates of PC3 and PC3-p53 cells treated with 40 or 80 μM EGCG for 24 h or 48h were analyzed by Western blot. Treatment of PC3-p53 cells with EGCG resulted in an increase in the level of total p53 and phosphorylated p53 at serine 15. EGCG also increased the transcriptional activity in a dose-dependent manner in PC3-p53 but not in control (PC3) cells. Increase in the transcriptional activity and protein levels of p53 lead to a concomitant increase in the protein expression of p21 and Bax, two known transcriptional targets of p53. These observations demonstrate that EGCG treatment led to induction, modification, and activation of p53 as observed earlier in androgen-sensitive LnCaP cells.

2. p53-Dependent growth inhibition of PC3-p53 cells via cell cycle arrest and apoptosis

To investigate the role of p53 in negative regulation of growth upon EGCG treatment, we treated prostate carcinoma PC3 cells ($p53^{-/-}$) and their stable p53 transfectant PC3-p53 ($p53^{+/+}$) cells with 20–80 μM of EGCG for 24 or 48 h. In contrast to parental PC3 cells that upon treatment with EGCG continue to traverse through cell cycle, PC3-p53 cells expressing wild-type p53 showed loss of cell viability, a sustained G1 arrest and an increase in apoptosis as a function of dose and time of EGCG treatment. These observations suggest that restoration of wild-type p53 renders PC3 cells more sensitive to cell cycle arrest and subsequently killing by EGCG.

3. EGCG alters the ratio of Bax/Bcl-2 by up-regulating Bax and triggers caspase signaling in p53-dependent manner

Lysates from cells treated with 40 or 80 μM EGCG for 24 h or 48 h were subjected to Western blot analysis to determine the levels of Bax and Bcl-2. EGCG treatment resulted in a substantial decrease in the levels of Bcl-2, with a concomitant increase in Bax levels in PC3-p53 cells, but not in PC3 cells. Densitometry of the blots indicated an increase in the ratio of Bax/Bcl-2 within 24 h of treatment in PC3-p53 cells. This result corroborates with apoptosis seen in PC3-p53 cells within 24h of EGCG treatment. Since alteration in Bax/Bcl-2 is known to initiate caspase signaling, we sought to examine the activation of initiator caspase 9 and effector caspase 3. PC3 and PC3-p53 cells were treated with desired concentration of EGCG for 24 or 48 h and the cell lysates were subjected to Western blot analysis using antibodies against caspase 9 and 3. We observed that EGCG treatment caused activation of caspase 9 and 3 with concomitant cleavage of PARP in p53-expressing PC3-p53 cells but not in parental PC3 cells lacking p53.

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4. Ablation of Bax (by siRNA) can rescue p53-dependent apoptosis in EGCG-treated cells

To investigate whether up-regulation of Bax upon treatment with EGCG was important in inducing apoptosis in PC3-p53 cells, we sought to ablate Bax levels using siRNA. PC3-p53 cells were transfected with Bax siRNA or control siRNA followed by treatment with 40 μM EGCG for 24 and 48 h. Transfection with Bax siRNA, but not control siRNA, specifically reduced EGCG-mediated increase in Bax level in PC3-p53 cells. In addition, the EGCG-mediated increase in p53 level remained unaffected in these cells. Transfection with Bax siRNA prevented EGCG-induced apoptosis in PC3-p53 cells as evident from phase contrast microscopy and TUNEL. Transfection with Bax siRNA did not affect the profile of DNA content (cell cycle) in response to EGCG treatment (**Fig. 1**).

5. Ablation of p21 (by siRNA) can rescue p53-dependent G1 arrest and apoptosis in EGCG-treated cells

To examine the mechanism underlying EGCG-induced G1 arrest and to assess the role of p53 in this process we depleted p21 transcripts by siRNA. PC3-p53 cells transfected with p21 siRNA or control siRNA were treated with EGCG for 24 or 48 h. Transfection of PC3-p53 cells with p21 siRNA, but not control siRNA, ablated EGCG-induced increase in p21 level. Cells transfected with p21 siRNA traversed through the cell cycle and failed to accumulate in G1 in response to EGCG treatment. In contrast, cells transfected with control siRNA and upon treatment with EGCG accumulated in G1. These data support our hypothesis that EGCG-induced G1 arrest was mediated by increased levels of p21. Microscopic examination and TUNEL assay indicated that ablation of p21 expression inhibited apoptosis in PC3-p53 cells while control cells expressing $p21^{WAF1}$ failed to resist apoptosis induced by EGCG. These results suggest that p21 may participate in EGCG-induced apoptosis. Our results indicate that both Bax and p21 are essential for EGCG-mediated apoptosis in prostate carcinoma cells (**Fig. 2**).

6. Inhibition of p53, p21, or bax by siRNA abrogated EGC-mediated apoptosis in LNCaP cells

To verify the role of p21 and bax in p53-dependent apoptosis, we employed another prostate cancer cell line, LNCaP that expresses wild-type p53. Expression of p53, p21, or bax was ablated by siRNA designed against each of these proteins, respectively. We found that cells infected with scrambled siRNA undergo apoptosis when treated with 80 μM EGCG for 48 h as measured by TUNEL assay. However, LNCaP cells infected with siRNA against p53, p21, or bax were resistant to EGCG-mediated apoptosis up to 48 h of treatment.

CONCLUSIONS AND SIGNIFICANCE

The current study was undertaken to establish the role of p53 and elucidate the mechanism thereof in EGCG-mediated cell cycle arrest and apoptosis. To achieve this goal, we employed human prostate carcinoma PC3 cells and generated stable transfectant of PC3 cells expressing wild-type p53 (PC3-p53). Treatment of PC3-p53 cells with EGCG resulted in G1 arrest followed by apoptosis. This observation was revalidated in another prostate cancer, LNCaP cells harboring wild-type p53. Inactivation of p53 by siRNA rendered these cells resistant to EGCG-mediated apoptosis. This agrees with the premise that since p53 is one of the major regulators of apoptosis, expression of this tumor suppressor

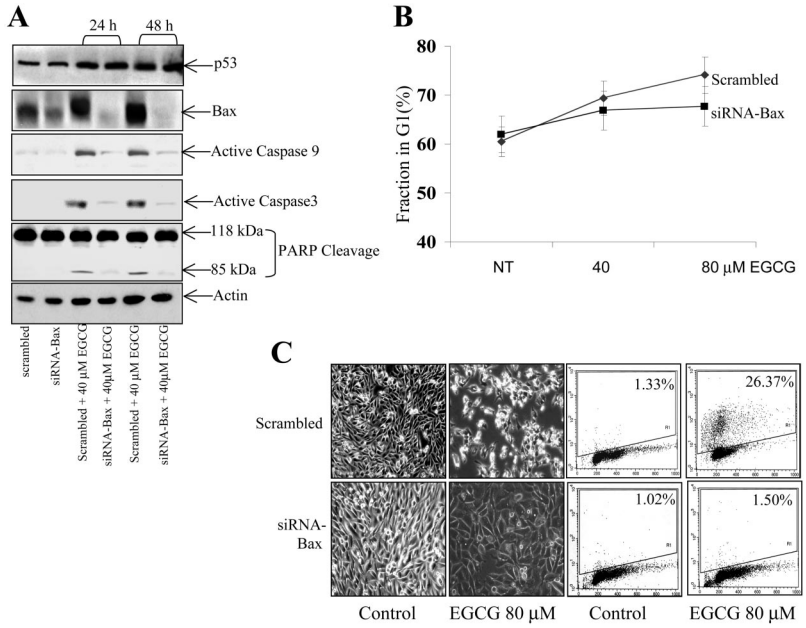

Figure 1. Ablation bax can rescue PC3-p53 cells from EGCG-mediated apoptosis. PC3-p53 cells transfected with 100 nM of Bax or scrambled siRNA and treated with 40 μM of EGCG for 24 or 48 h. *A)* Cell lysates were analyzed by immunoblotting using antibodies against bax, p53, caspase 9 and 3, PARP, and actin. *B)* EGCG-treated (40 or 80 μM for 48 h) PC3-p53 cells were fixed and stained with PI to measure DNA content by flow cytometry. % of cells in G1 was plotted vs. EGCG dose. *C)* PC3-p53 cells were treated with EGCG 80 μM for 48 h. After treatment, cells were visualized under phase contrast microscopy and images were captured using Optimas 6 software program. Apoptotic cells (TUNEL-positive) were determined by flow cytometry. The data shown here are representative of 3 independent experiments with similar results.

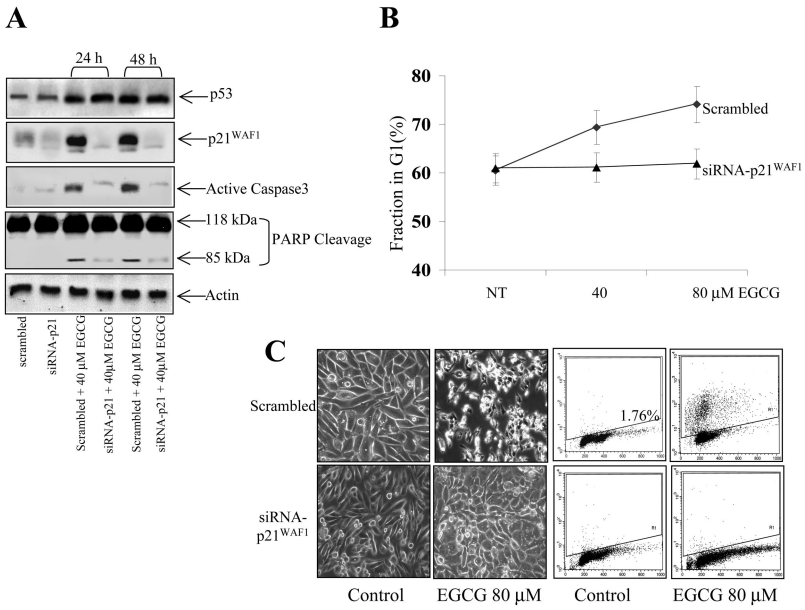

Figure 2. Ablation p21 can rescue PC3-p53 cells from EGCG-mediated growth arrest and apoptosis. PC3-p53 cells were transfected with either 100 nM of p21 siRNA or scrambled siRNA for 48 h, then treated with 40 μM EGCG for 24 and 48 h. *A)* Cell lysates were analyzed for protein levels of p21-p53, caspase 3, and PARP. *B)* Fixed cells were stained with PI to measure DNA content by flow cytometry. Percentage of cells in G1 as a function of EGCG dose (48 h treatment) is plotted. *C)* Cells were treated with EGCG 80 μM for 48 h. After treatment, cells were visualized under phase contrast microscopy and images were captured using Optimas 6 software program. Apoptotic cells (TUNEL-positive) were determined by flow cytometry.

sensitizes cells to apoptosis in response to stress. Treatment of PC3-p53 with EGCG led to an increase in the transcriptional activity and protein level of tumor suppressor p53 protein which was accompanied by an increase in the levels of its two transcriptional targets $p21^{WAF1}$ and Bax. Increase in p53 and its dependent targets exacerbated both G1 arrest and apoptosis in PC3-p53 cells in response to EGCG treatment, while PC3 cells lacking p53 continued to cycle and did not undergo apoptosis upon treatment with similar concentrations of EGCG, thus establishing the action of EGCG in a p53-dependent manner. While p21 is up-regulated during p53-mediated G1 arrest, this event is not a prerequisite for p53-induced apoptosis. To decipher the role of p21, we examined the effects of EGCG in PC3-p53 cells transfected with p21 siRNA. Ablation of p21 protein prevented G1 arrest and apoptosis in PC3-p53 cells. The p53-dependent increase in Bax expression altered the Bax/Bcl-2 ratio and paralleled the activation of caspase 9 and 3 and cleavage of PARP. Transfection of cells with Bax siRNA abolished these effects and inhibited apoptosis but did not affect the accumulation of the cells in G1. In summary, we propose that EGCG-mediated growth arrest and apoptosis occurs primarily via p53-dependent pathways. One intriguing finding of our study is that depletion of p21 or Bax in PC3-p53 cells prevented apoptosis in these cells. On the basis of this observation, we hypothesize that the function of p21 and Bax may be necessary to induce apoptosis via the p53-dependent pathway.

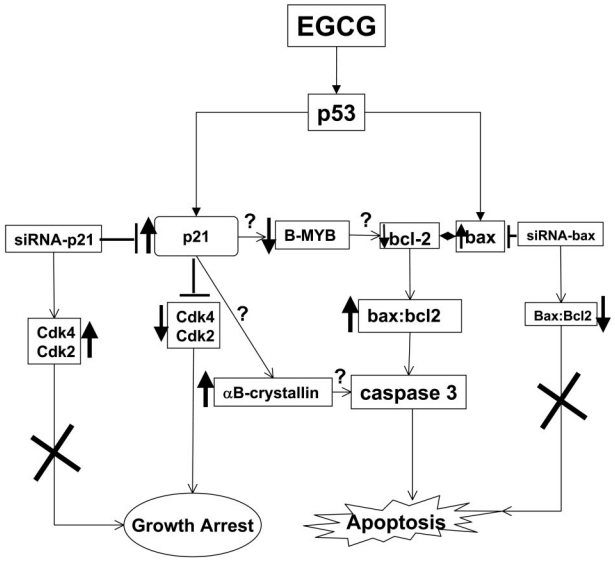

Figure 3. Possible mechanism of p53-dependent G1 arrest and apoptosis by EGCG: role of p21 and bax in induction growth arrest and apoptosis.