Pituitary Tumor Transforming Gene Causes Aneuploidy and p53-dependent and p53-independent Apoptosis*

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The pituitary tumor transforming gene, PTTG, is abundantly expressed in several neoplasms. We recently showed that PTTG overexpression is associated with apoptosis and therefore have now studied the role of p53 in this process. In MCF-7 breast cancer cells that express wild type p53, PTTG overexpression caused apoptosis. p53 was translocated to the nuclei in cells expressing PTTG. Overexpression of p53, along with PTTG, augmented apoptosis, whereas expression of the human papillomavirus E6 protein inhibited PTTG-induced apoptosis. In MG-63 osteosarcoma cells that are deficient in p53, PTTG caused cell cycle arrest and subsequent apoptosis that was inhibited by caspase inhibitors. A proteasome inhibitor augmented PTTG expression in stable PTTG transfectants, suggesting that down-regulated PTTG expression is required for cell survival. Finally, MG-63 cells expressing PTTG showed signs of aneuploidy including the presence of micronuclei and multiple nuclei. These results indicate that PTTG overexpression causes p53-dependent and p53-independent apoptosis. In the absence of p53, PTTG causes aneuploidy. These results may provide a mechanism for PTTG-induced tumorigenesis whereby PTTG mediates aneuploidy and subsequent cell transformation.

Pituitary Tumor Transforming Gene (PTTG) is highly expressed in pituitary tumors and other neoplasms (1–3). In vitro, PTTG transforms 3T3 fibroblasts (1, 4), but the full mechanism of PTTG action has not been clarified. PTTG induces basic fibroblast growth factor secretion (1, 5) and trans-activates DNA transcription (6, 7). PTTG is a mammalian gene that maintains binding of sister chromatids during mitosis (8). We recently studied the cellular characteristics of PTTG (9) and showed that PTTG mRNA and protein expressions are cell cycle-dependent and peak at the G2/M phase. PTTG is localized to both the nucleus and cytoplasm and is degraded at the initiation of anaphase. PTTG overexpression causes apoptosis and inhibits mitosis (9).

Tumor suppressor p53 subjects cells with severe DNA damage or other stress to apoptosis by transactivating apoptosis-inducing genes. Expression of oncogenes such as myc (10–12) activates p53 and renders cells apoptotic. The same oncogenes, however, also activate p53-independent apoptosis (13, 14). In this report, we studied p53 involvement in PTTG-induced cell death in cells expressing or lacking wild type p53. Our results show that PTTG overexpression causes p53-dependent and p53-independent apoptosis. Aneuploidy arises as a result of PTTG overexpression in p53-negative cells. These results suggest that aneuploidy may be a mechanism for PTTG-induced tumorigenesis.

EXPERIMENTAL PROCEDURES

Cell Culture, Plasmids, and Transfection—MCF-7 and MG-63 cells were grown in Dulbecco’s modified Eagle’s medium with 10% fetal calf serum in a 37 °C, humidified incubator with 5% CO2. Cells were synchronized with double thymidine block (9). Cell cycle was analyzed with a fluorescence-activated cell sorter. Plasmids encoding wild type and an enhanced green fluorescent protein (EGFP)-tagged human PTTG and the parental plasmid pEGFP-N3 were described before (9). Plasmids encoding p53 and R175H p53 were gifts from Dr. C. W. Miller (Cedars-Sinai Medical Center, Los Angeles, CA). Plasmid encoding human papillomavirus (HPV) E6 protein was described before (15). Cells were transfected with FuGene (Hoffmann-La Roche). Stable MG-63 cell lines expressing EGFP or PTTG-EGFP were established by incubating cells with 1 mg/ml G418 after transfection. Caspase inhibitors I and III were from Calbiochem (La Jolla, CA). Apoptosis Assay—Cells transfected with EGFP or PTTG-EGFP were fixed with 4% paraformaldehyde and permeabilized with 0.6% Tween 20 and stained with Hoechst 33258 (1:10,000; Molecular Probes, Eugene, OR). Slides were observed with a 40× objective, and green cells with apoptotic nuclear characteristics such as nuclear condensation and fragmentation were scored as apoptotic. Apoptosis was confirmed by terminal dUTP nick-end labeling staining. 200–300 green cells were examined on each slide. Nuclear morphology of green cells was also examined for signs of aneuploidy such as micronuclei and macronuclei.

Immunofluorescent staining and fluorescence microscopy were performed as described (9). An antibody to p53 (1:1,000; Calbiochem) or to mdm2 (16) was used and detected with rhodamine-labeled goat anti-mouse IgG (1:500; Molecular Probes). Cells were finally stained with Hoechst 33258 (Molecular Probes). Several hundred cells were observed in each of the 2 to 3 staining experiments, and representative cells are depicted. Western blotting of PTTG-EGFP was performed as described (9).

Metaphase Karyotyping—Growing cells were treated with 100 ng/ml colcemid (Calbiochem) for 90 min, and G/M cells were isolated by brief trypsinization and gentle tapping. These cells were incubated with 75 mM KCl for 30 min and fixed with methanol-acetic acid (3:1) and dropped onto glass slides. Metaphase chromosomes were observed.

RESULTS

PTTG Causes Apoptosis in MCF-7 and MG-63 Cells—Human breast cancer MCF-7 cells express wild type p53 (17), and human osteosarcoma MG-63 cells are deficient in p53 (18). Both MCF-7 and MG-63 cells express PTTG mRNA (data not shown). EGFP had no significant effect on apoptosis in either MCF-7 or MG-63 cells (Fig. 1). Expression of PTTG-EGFP caused apoptosis in both MCF-7 and MG-63 cells. In MCF-7
both blocking cell cycle at G2/M phase. We used the total blockade on exit from mitosis.

Corresponding smaller G1 phase were observed, suggesting a decrease in cell number of green cells and the number of live green cells on day 5 after transfection with PTTG-EGFP as indicators of cell survival. Both caspase inhibitor I (Z-VAD-FMK) and III (Boc-d-FMK) promoted survival of PTTG-EGFP-expressing cells (Fig. 3c).

**PTTG Protein Is Continuously Degraded**—7 h after treatment with a proteasome inhibitor, LLnL, there was no significant change in EGFP fluorescence (Fig. 4a) or in EGFP protein level, revealed by Western blotting of MG-63 cells stably expressing EGFP (Fig. 4b). In cells stably expressing PTTG-EGFP, PTTG-EGFP fluorescence and PTTG-EGFP protein level were both enhanced after LLnL treatment. These results show that PTTG-EGFP was continuously degraded through the ubiquitin pathway and suggested that cells stably expressing PTTG escape apoptosis by down-regulating PTTG.

**PTTG Overexpression Causes Aneuploidy**—Because PTTG is a mammalian securin that helps keep sister chromatids together, its overexpression may cause abnormal chromosomal separation. In both the parental MG-63 cells and in cells expressing EGFP, signs of aneuploidy were uncommon. In MG-63 cells transiently or stably expressing PTTG-EGFP, the severity and frequency of aneuploidy signs such as micronuclei, macro-nuclei, or chromosomal bridges were both enhanced (Fig. 5). In
transfection efficiency was higher. In one representative experiment, we counted the rate of aneuploidy only in JEG-3 cells where PTTG-EGFP and HPV E6 protein were stably expressed. The lack of differing in chromosome numbers may be because most aneuploid cells did not divide or because of the low frequency of aneuploid cells.

PTTG-EGFP did not induce aneuploidy in MCF-7 and human choriocarcinoma JEG-3 cells, which also express wild type p53 (data not shown). Aneuploidy was observed in some MCF-7 and JEG-3 cells transfected with PTTG-EGFP and HPV E6 protein. Because the transfection efficiency in MCF-7 cells was low, we counted the rate of aneuploidy only in JEG-3 cells where transfection efficiency was higher. In one representative experiment, 24 of 650 cells (3.7%) expressing PTTG-EGFP and HPV E6 protein were aneuploid, whereas only 5 of 647 cells (0.8%) expressing EGFP and HPV E6 protein were aneuploid. These results suggested that p53 prevents aneuploidy induced by PTTG.

**DISCUSSION**

In this study, we have elucidated mechanisms for PTTG-induced apoptosis. Although all results shown were derived from the comparison of PTTG-EGFP and EGFP, PTTG-EGFP appears to faithfully represent PTTG, because they exhibit similar cellular characteristics (9) and caused similar cell death (data not shown). Several lines of evidence from our study indicate that p53 mediates PTTG-induced apoptosis in MCF-7 cells. PTTG up-regulated and translocated p53 to the nucleus, overexpression of p53 augmented PTTG-induced apoptosis, and the HPV E6 protein, a p53 inactivator, prevented PTTG-induced apoptosis. It is not clear, however, how p53 is activated by PTTG. myc and ras activate p53 by activating ARF, a tumor suppressor protein (12, 21) that in turn causes nuclear accumulation of mdm2, preventing p53 nuclear export and subsequent degradation (22, 23). We did not observe a simultaneous nuclear accumulation of p53 and mdm2, suggesting that the ARF mechanism may not apply to PTTG-induced apoptosis, yet PTTG may induce p53 nuclear accumulation by inhibiting mdm2 expression through other mechanisms (23). The dominant negative p53 mutant did not inhibit PTTG-induced apoptosis in MCF-7 cells, possibly indicating that the mutant p53 may not completely inhibit endogenous p53 activity as effectively as the HPV E6 protein. It is also likely that mechanisms other than p53 are also involved in PTTG-induced apoptosis.

PTTG caused apoptosis in p53-negative MG-63 cells, demonstrating that PTTG-induced apoptosis can be p53-independent. It appeared that PTTG causes cell cycle arrest prior to apoptosis. This was evident inasmuch as cells expressing PTTG did not divide after release from double thymidine block, and cycling PTTG-expressing cells were also partially blocked at G2/M. The PTTG effect on the cell cycle may therefore be both...
and doubling times, therefore suggesting an additional indirect effect of PTTG on apoptosis and aneuploidy. This model implies that PTTG-induced tumorigenesis is a slow process, and tumors do not form until apoptosis surveillance systems fail. On the other hand, if PTTG expression is down-regulated by the ubiquitin pathway as we show in this paper, the resultant slightly increased PTTG expression may still escape surveillance and cause aneuploidy. Thus PTTG potentially can contribute to tumorigenesis even when the apoptosis surveillance systems are intact. Because aneuploidy is assumed to be less severe when PTTG is down-regulated, tumorigenesis should also be slow.

In summary, we show that PTTG induced both apoptosis and aneuploidy, and the results suggest that apoptosis may play an important role in preventing tumorigenesis. We have now established mice deficient in PTTG and will more directly address the mechanism of PTTG-induced tumorigenesis in these animals.

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