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A Novel Candidate Tumor Suppressor, INGI, Is Involved in the Regulation of Apoptosis

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Abstract

We have recently cloned a novel growth inhibitor and candidate tumor suppressor called p33INGI (I. Garkavtsev et al., Nature Genet., 14: 415–420, 1996). Because some tumor suppressors participate in the regulation of apoptosis, we hypothesized that the INGI gene may also play a role in this process. Our results show that p33INGI levels increase upon the induction of apoptosis in P19 teratocarcinoma cells by serum deprivation. Elevated expression of INGI in P19 and rodent fibroblast cells containing a tetracycline-controlled human c-myc gene enhanced the extent of serum starvation-induced apoptosis. This suggests that the pathway by which INGI modulates cell death is synergistic with Myc-dependent apoptosis. Conversely, constitutive expression of an antisense construct of INGI conferred protection against apoptosis in these cells. These data support the idea that loss of proper INGI function may facilitate tumorigenesis, in part, by reducing the cell’s sensitivity to apoptosis.

Introduction

Programmed cell death, or apoptosis, is characterized by distinct morphological changes such as chromatin condensation, nuclear fragmentation, cytoplasmic blebbing, and DNA fragmentation (1). Many diverse substances and growth conditions have been identified that induce apoptosis, including irradiation, antigen recognition, cytotoxic drugs, glucocorticoids, and withdrawal of serum growth and survival factors (1–3). A common feature of many of these response pathways is the requirement for p53 tumor suppressor function (4). Loss of p53 function results in inappropriate cell cycle progression, uncontrolled growth, and tumorigenesis, and p53 is the most frequently mutated gene in human tumors (5, 6). Reintroduction of p53 in deficient cells can induce either growth arrest (7–9) or apoptosis (10), depending upon cellular context. Some apoptotic events do not require p53, however, including glucocorticoid-mediated apoptosis of thymocytes (4, 11) and the developmentally programmed death of some embryonic cells (12, 13).

We have recently isolated INGI, a novel candidate tumor suppressor gene, using a method that combined subtractive hybridization of cDNAs from normal and cancerous cells with an in vivo selection assay (14). Because overexpression of the INGI gene efficiently arrests cells in the G2/M phase of the cell cycle, as does p53, we speculated that p33INGI might be similarly involved in modulating apoptosis.

We therefore tested the potential of INGI to modulate serum starvation-induced apoptosis in P19 teratocarcinoma cells and in rodent fibroblast cell lines that conditionally overexpress a native human c-myc protein. We demonstrate that overexpression of the p33INGI protein confers sensitivity to apoptosis in these cells, whereas decreasing INGI expression using an antisense construct protects them from apoptosis. We therefore suggest that functional loss of INGI may contribute to tumorigenesis by diminishing a cell’s capacity for apoptosis.

Materials and Methods

Cell Lines and Culture. P19 mouse teratocarcinoma cells were grown in α-MEM with 10% FCS (Life Technologies, Inc., Burlington, ON, Canada). For serum starvation experiments, the cells were washed twice in Ca2+/Mg2+-free PBS [137 mM NaCl, 2.7 mM KCl, 10.1 mM Na2HPO4, and 1.8 mM KH2PO4 (pH 7.4)], and serum-free α-MEM (Life Technologies, Inc.) was added. Rodent fibroblasts, either NIH 3T3- or rat 1-derived cells containing a tetracycline-controlled human c-myc gene (tet-myc cells) were maintained in high-glucose DMEM (Life Technologies, Inc.) supplemented with 10% FCS and 2 µg/ml tetracycline (Sigma, St. Louis, MO) to repress premature human c-myc gene repression. The cells were treated as described previously to induce c-myc expression and apoptosis. Briefly, this involves washing out the tetracycline inhibitor, elevating c-myc expression by up to 100-fold, and then transferring cells to medium without serum in which apoptosis rapidly ensues.

Retroviral Infection. Retroviral infection of target cells was used to introduce INGI expression constructs because we have previously demonstrated that INGI protein seems to block entry into S-phase of the cell cycle (14), thereby precluding the use of standard drug-resistance selection methods. Retroviral infection also has a higher efficiency than standard calcium phosphate transfection procedures. The retroviral vector, pLNCX (15), containing sense, antisense (nucleotides 942–1,124 of the INGI cDNA; Ref. 14), or vector alone was transduced into a highly efficient BOSC23 ecotropic virus-packaging cell line. The BOSC23 supernatant was then used to infect ecotropic producer cell lines. The target P19 and murine NIH 3T3 tet-myc cells were plated at 104 cells/10-cm plate and infected with undiluted viral supernatant because these cells were prone to clumping.

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Viability and Apoptosis Assays. Cell viability was assessed using a trypan blue dye exclusion assay. Cell suspensions were mixed 4:1 with 0.5% trypsin-blue saline solution (Life Technologies, Inc.). The cells were incubated at room temperature for 5 min and counted with a hemacytometer.

DNA laddering was assessed using the method of Smith et al. (16). Tet-myc cells were plated at equal densities as described above. At 72 h after exposure to 0.1% FCS, DNA was isolated from the floating cells on a per-plate basis. Equal volumes of lysate were run on a 2% agarose gel and stained with ethidium bromide.

Indirect Immunofluorescence. Tet-myc cells containing the retroviral constructs were seeded at a density of approximately 105 cells/cm2 on glass coverslips and grown at 37°C for 48 h before fixation and processing as described (17).

Microinjection Assays. Rat tet-myc cells were seeded on coverslips as described above and injected with about 25 µg/ml GST-p33INGI protein or

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4 The abbreviations used are: GST, glutathione S-transferase; CMV, cytomegalovirus.
controls (56% viable), suggesting that ING1 confers cellular susceptibility to death induced by serum starvation. These numbers represent a minimal estimate of the effects of ING1 and bcl-2 due to the modest transfection efficiency of P19 cells as outlined in “Materials and Methods.”

Because the infection efficiency was low for P19 cells, we turned to another model system of apoptosis developed in our laboratory,³ that of rodent fibroblasts (rat 1 and NIH 3T3) containing a tetracycline-controlled human c-myc gene (tet-myc cells). These tet-myc cells are maintained in 2 μg/ml tetracycline to repress the expression of a stably integrated human c-myc gene under the control of a minimal promoter and tetracycline operator sequences. Removal of tetracycline from the medium results in the rapid accumulation of human c-Myc protein (Fig. 2A) and subsequent apoptosis. The advantage of such a system is that control and experimental cells possess an identical genetic background, and the potential for cellular adaptation to constitutive c-myc overexpression is minimized. Because these cells form a monolayer, transfection efficiencies of greater than 90% are obtainable. The same ING1 retroviral constructs as described previously (antisense, sense, and vector alone) were used on the NIH 3T3 tet-myc cells, and similar p33ING1 expression levels were observed (Fig. 2, B–D) with localization of the protein to the nucleus (14, 16).

When myc was induced in the NIH 3T3 tet-myc cells by tetracycline withdrawal, and the cells were then serum-starved for 72 h, they showed a viability of 65% compared with controls in which myc was not induced (Fig. 3A). Apoptosis is minimized in cells coexpressing the antisense ING1 construct, with only 5% of cells dying upon serum withdrawal. Cells expressing ING1 protein alone show 70% viability after serum starvation, and apoptosis is magnified when c-myc is also expressed (40% viable). These cells exhibited several hallmarks of apoptosis including shrinkage, loss of substrate adhesion, and chromatin condensation. In addition, internucleosomal DNA fragmentation is greatly enhanced in tet-myc cells expressing p33ING1 compared to vector only and antisense ING1 or bcl-2-expressing tet-myc cells (Fig. 3B).

To confirm that ING1 enhances apoptosis during serum starvation, we microinjected p33ING1 or a CMV-ING1 expression construct into rat tet-myc fibroblasts and counted the number of remaining injected cells at various times after serum deprivation. Because the history of each injected cell could be followed, a more dramatic effect of ING1 expression compared to the previous experimental approaches was apparent (45% viable at 24 h; Fig. 3C), with a further decrease in surviving cells when c-myc and ING1 were coexpressed (9% viable at 24 h). These results support the data obtained with both the P19 and NIH 3T3 tet-myc cells and show that p33ING1 is involved in regulation of apoptosis in a manner that is synergistic with the action of myc.

It therefore seems that at least three independent cell lines, ING1 expression confers an increased susceptibility to death upon serum starvation. Conversely, decreasing endogenous ING1 levels afforded some protection against cell death. ING1 markedly influences the outcome of c-myc-induced apoptosis and may therefore participate in guiding the response pathway whereby c-myc overexpression activates either apoptosis or tumorigenesis. Thus, a loss of p33ING1 or its function seems to have similar consequences to those observed for p53 (2, 20). Because ING1 seems to be important in the control of the G1 to S-phase transition (14), it is possible that ING1 could modulate or be modulated by p53. Conversely, ING1 could act independently, perhaps providing an activity in which p53-independent mechanisms are at work. We are currently investigating these possibilities.

Several pieces of evidence suggest that ING1 loss is of important biological consequence. Northern blot and reverse transcription-PCR analyses reveal a marked decrease in ING1 RNA expression in a wide

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Fig. 2. Characterization of human c-myc and INGI expression in NIH 3T3 tet-myc cells. A. NIH 3T3 tet-myc cells were retrovirally infected with constructs containing vector only (V), antisense (aS), and sense (S) INGI constructs grown in the presence (+) or absence (−) of tetracycline. Removal of tetracycline from the medium results in a substantial accumulation of human c-myc protein as detected by a 1–9E10 mouse monoclonal antibody on Western blots of isolated nuclear proteins. Constitutive retroviral expression of INGI reveals that the p33INGJ protein is exclusively found in the nucleus through immunohistochemical analyses of tet-myc cells containing vector only (B), antisense (C), and sense (D) INGI constructs. Polyclonal anti-ING1 antibody (14, 21) was used. Note the conspicuous absence of nuclear staining in C, which was overexposed to reveal background cytoplasmic fluorescence.

Fig. 3. Expression of INGI enhances c-myc-induced apoptosis. A, cell viability was determined using a trypan blue dye exclusion assay on NIH 3T3 tet-myc cells containing antisense (aS) or sense (S) INGI or vector-only (V) constructs after 72 h in 0.1% FCS in the presence (−myc) or absence (+myc) of tetracycline. The data show the results of three independent experiments and were normalized to vector-only cells not expressing c-myc. B, tet-myc cells expressing sense INGI (S) show a greater amount of DNA laddering than antisense (aS), vector only (V), and bcl-2-overexpressing tet-myc cells. DNA was isolated from the floating cells on a per-plate basis as described in “Materials and Methods” and separated on a 2% agarose gel. A positive control (C) from rat tet-myc fibroblasts undergoing apoptosis was also included for comparison. C, microinjection of GST-p33INGJ protein or a CMV-ING1 construct affects cell viability. One hundred rat tet-myc cells were injected with control GST fusion protein or CMV construct alone or with GST-p33INGJ protein or CMV-ING1 constructs (+ING1) in the presence (−myc) or absence (+myc) of tetracycline. After a brief recovery period, the injected cells were serum-starved in the presence (−myc) or absence (+myc) of tetracycline. Surviving cells were identified by immunostaining of a coinjected nonspecific antibody and counted at the indicated time points.
variety of tumor cell lines and primary tumors, and in addition, several tumor cells show mutations in the ING1 gene (Ref. 14). We therefore suggest that alterations in the proper functioning of p33ING1 may contribute to tumorigenesis by rendering cells refractory to normal apoptotic pathways.

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References

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