Suppression of Acute Lymphoblastic Leukemia by the Human Wild-Type p53 Gene

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Abstract

Independent mutations in both alleles of the p53 tumor suppressor gene are a frequent finding in human T-cell acute lymphoblastic leukemia (T-ALL) cell lines and in the cells of some T-ALL patients in relapse. One major goal of studying the status of p53 (and other tumor suppressor genes) in human cancer is to facilitate the suppression of the tumorigenic phenotype through the restoration of the expression of the wild-type allele. While the efficient insertion of a suppressor into all cells of solid metastatic human tumors may at present be impossible, insertion into leukemia cells may be feasible due to the accessibility of the leukemia cells in the body. To examine the feasibility of suppressing the tumorigenicity of human T-leukemia cells, the human T-ALL cell line Be-13, which lacks endogenous p53 protein, was infected with a recombinant retrovirus encoding the wild-type allele of human p53 (hwtp53). Expression of p53 reduced the growth rate of infected Be-13 cells in vivo, suppressed colony formation in methylcellulose cultures, and abrogated their tumorigenic phenotype in nude mice in vivo. These results suggest that suppression of the leukemic phenotype of relapse T-ALL-derived Be-13 cells is feasible. Acute leukemia cell suppression via high infection with retroviruses encoding wtP53 may be feasible and beneficial in T-ALL cases as part of a bone marrow transplantation regimen in an effort to reduce the frequency of posttransplantation relapse.

Introduction

The gene encoding the Mr 53,000 cellular phosphoprotein p53 has the characteristics of a tumor-suppressor gene whose loss-of-function mutations are oncogenic (1). Wild-type forms of the tumor suppressor genes may function to suppress or inhibit cell transformation and tumorigenesis by one of several pathways. The p53 gene is frequently affected by mutations, rearrangements, or deletions which contribute to the genesis or progression of a wide variety of human cancers. Inactivation of the p53 gene has been demonstrated in human carcinomas of the colorectum (2), lung (3), liver (4), and bladder (5), in chronic myelogenous and myelocytic leukemias (6), in osteogenic sarcomas (7), and in T-cell acute lymphoblastic leukemias (8). In colorectal tumors, one allele of the p53 gene is frequently inactivated by mutation, while the other allele is lost through allelic deletion of chromosome 17p (9). In relapse T-ALL cells, both alleles frequently have been found to be independently mutated rather than being deleted (8). This suggests that an initial mutation in p53 endows the cell with a growth advantage through the induction of unscheduled proliferation, thereby creating an enlarged compartment of cells at risk. Inactivation of the second allele, by either mutation or deletion, is required to abrogate the suppressive activity of p53. Although, apparently, a single mutated p53 allele does not induce any abnormal phenotype in diploid cells (10, 11), some specific mutant forms of p53 represent gain-of-function mutations which may endow diploid cells with a growth advantage (12).

The role of p53 in the induction and progression of human cancer is suggested by the status of p53 in individuals with the Li-Fraumeni syndrome, in whom inherited (germline) heterozygous mutations in p53 are associated with a striking predisposition to a number of cancers (10, 11). Tumors in these individuals have lost the wild-type allele of p53, an observation reminiscent of the loss of heterozygosity of the retinoblastoma susceptibility gene in hereditary retinoblastoma and other cancers.

Recently, it has been shown that introduction of the wild-type p53 gene into tumor cell lines derived from colon carcinomas (13), glioblastomas (14), and osteosarcomas (15) resulted in the negative regulation of cell proliferation and/or the suppression of the tumorigenic phenotype. Hence, the wild-type p53 gene can suppress the tumorigenic phenotype of some cell lines derived from human solid tumors. It is difficult at this time to envision the efficient transfer of stably expressed and suitably regulated tumor suppressor gene constructs into solid or metastatic human tumors due to the inaccessibility of such tumors. In the case of human leukemias, however, the introduction of wild-type tumor suppressor gene constructs may be possible due to the relative accessibility of the leukemic cells in the body. As a first approach to the therapeutic suppression of the unregulated growth of human T-leukemia cells, one could visualize the introduction of tumor suppressor gene constructs in conjunction with an autologous bone marrow transplantation regime, in an effort to reduce the frequency of posttransplantation relapse.

Materials and Methods

Preparation of hwtp53- and lac-encoding Virus and Infection of T-ALL Cells. Cultures of Be-13 cells were infected for 3 h, three times in succession, with >2 × 10⁴ helper-free virus encoding either hwtp53 or luciferase in the presence of 10 μg/ml polybrene. A mock (medium only) control was included as well. Both viral constructs contained the neosresistance gene as a selectable marker. Following the third infection the cultures were grown for 24 h in complete medium and then selected in complete medium containing 500 μg/ml of the antibiotic G418. All cells of the mock-infected cultures died in 7 days. Approximately 10% of the infected cells became G418 resistant following this infection procedure. Infected cultures were maintained in the presence of 400 μg/ml G418 and were expanded as separate infected cultures. Since we wished to avoid studying the characteristics of selected clones of p53-infected cells, the infected cultures were not cloned but were studied as separate mass cultures. To generate amphotropic helper-free virus, 20 μg of the vector DNA were transfected into the ecotropic packaging cell line psi2 by calcium phosphate coprecipitation. Culture medium...
was collected 36 h after transfection and used to infect the amphotropic packaging cell line PA317 (for references see Ref. 16). Single colonies of virus-producer cells were isolated by selection of infected PA317 cells in G418-containing medium and expanded into mass culture. To determine virus titers, aliquots of cell-free culture medium from each producer line were applied to rat 208F cells in the presence of 4 μg/ml polybrene. Selection was applied 24 h after infection, and neoresistant colonies were stained with Giemsa and counted after 2 weeks.

Immunoprecipitation. For immunoprecipitation, 5 × 10^6 cells were labeled for 3 h in 100 μCi/ml [35S]methionine/[35S]cysteine translabel (ICN). Cells were lysed in radioimmunoprecipitation assay buffer (150 mM NaCl, 10 mM NaPO4, 1% NP40, 0.1% sodium dodecyl sulfate, 1% aprotinin, 1% deoxycholic acid, 500 μM phenylmethylsulfonyl fluoride, 1 mM EDTA) for 15 min on ice. The lysate was centrifuged at 100,000 × g for 30 min, and the pellet was discarded. Equal amounts of radioactive material were reacted with the p53-specific antibody G59–12 (Pharmingen, San Diego, CA) for 4 h at 4°C. The immune complexes were collected on immobilized recombinant protein A (Repligen), washed three times with radioimmunoprecipitation assay buffer and once with phosphate-buffered saline, and boiled for 5 min in sample buffer. Samples were then analyzed on a 10% sodium dodecyl sulfate-polyacrylamide gel.

Colony Formation in Methylcellulose Cultures: Methylcellulose cloning. Agarose (1%) plates containing 5 ml agarose mercaptoethanol (SeaKem) in DMEM (10% fetal bovine serum) in 6-cm dishes were prepared. Methylcellulose was made by mixing 2.4 g of methylcellulose with 50 ml of H2O. After sterilization, the mixture was made up to DMEM strength with 2x DMEM. For cloning, 1.5 ml of DMEM + 10% fetal bovine serum containing 1000 cells were mixed with 1.5 ml of 2.4% methylcellulose and poured on top of agar plates. Colonies were counted under a binocular microscope after 2 weeks of growth in a 37°C CO2 incubator and were photographed under an inverted microscope at a magnification of ×100.

Mouse Injections. Five-week-old female NCI nude mice (IM-DYNE, San Diego, CA) were treated with 300 cGy 60Co irradiation, and 3–7 days later live culture-grown Be-13-p53 cells and Be-13-lux cells (or, in some experiments, Be-13 cells) were injected s.c. Cells (5 × 10^5–5 × 10^6) in 0.2 ml buffer were tested for tumor formation. Be-13 or Be-13-lux cells (10^6) routinely grew to produce a palpable (>8 mm³) tumor in 10 days. In most experiments mice received injections of 3 × 10^6 and 3 × 10^5 live control (Be-13-lux) and suppressed (Be-13-p53) cells on the left and right flanks, respectively. On three occasions mice received injections of 5 × 10^5 Be-13-p53 cells only, to test for the limits of in vivo suppression by hwt53. Eight weeks after injection of 5 × 10^5 suppressed (Be-13-p53) cells no tumors had developed. The mouse in Fig. 5 had been injected with 3 × 10^6 and 3 × 10^5 Be-13-lux and Be-13-p53 cells, respectively. Nine days after injection palpable tumors were visible on the left flank, while 28 days postinjection 3 × 10^6 control cells had grown to a tumor of >20 mm in diameter.

Results and Discussion

Because of the potential clinical benefit of gene therapy with T-ALL, we have examined the suppression of the leukemic phenotype of human T-ALL (relapse) cells by the introduction of a human wild-type p53 gene and chose the well-characterized human T-ALL cell line Be-13 (17) as the model system. Be-13 cells synthesize no p53 protein. The recombinant retrovirus pLhp53RNL was constructed, in which the human wild-type p53 (hwt53) complementary DNA is expressed under the control of the Moloney long terminal repeat promoter, and the drug resistance gene neo is expressed under the promoter of the Rous sarcoma virus (Fig. 1). An identical retroviral construct in which the luciferase gene was substituted for the hwt53 gene was used as the control (Fig. 1).

Be-13 cells were infected with the helper-free amphotropic pLhp53RNL retrovirus encoding hwt53 and with pLLRNL control virus encoding the luciferase (lux) gene. Introduction of the p53 gene into T-ALL cells by infection rather than by transfection or electroporation has the advantage of efficient gene transfer and results in the introduction into each cell of only a single or a few copies of the transgene. Infected cultures were selected in the presence of 400 μg/ml G418. All four cultures of Be-13 cells infected with virus encoding hwt53 expressed p53 protein, as assayed by immunoprecipitation of metabolically labeled cells. Assay for expression of hwt53 at the single-cell level would be useful but is hampered by the short half-life of the wt53 protein. Cells infected with the virus encoding the luciferase gene and noninfected Be-13 cells did not express detectable levels of p53 protein (Fig. 2). Lux-virus-infected cells were assayed for the expression of the luciferase

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**Fig. 1.** Construction and generation of the retroviral vectors encoding the wild-type human p53 gene. To construct pLhp53RNL, the complementary DNA encoding the human wild-type p53 gene in plRbrNL was removed by BamHI digestion and replaced with the 2.1-kilobase BamHI fragment containing the human wild-type p53 complementary DNA derived from php53BAM. Construction of the pLLRNL retroviral construct has been described previously (16). LTR, long terminal repeat of Moloney leukemia virus; RSV, promoter of the Rous sarcoma virus, Neo, gene encoding neomycin phosphotransferase. Arrows, position of the promoters and the direction of transcription.

**Fig. 2.** Expression of p53 protein in infected and control T-ALL cells. Be-13 cells were infected with p53 virus (pLhp53RNL amphotropic virus; Lanes 1 and 2) or lux virus (pLLRNL amphotropic virus; Lane 3) and grown in DMEM + 10% fetal calf serum with 400 μg/ml of G418 for 4 weeks. Noninfected Be-13 cells (Lane 4) and the T-ALL cell line A3/Kawakami (Lane 5; Ref. 8) were used as additional negative and positive controls, respectively. The cells were labeled with [35S]methionine/[35S]cysteine, lysed, and immunoprecipitated. Lane 1, Be-13-p53 mass culture no. 1; Lane 2, Be-13-p53 mass culture no. 2; Lane 3, Be-13-lux cells; Lane 4, Be-13 parental cells; Lane 5, A3K cells; Lane 6, molecular weight markers in kilodaltons.
Table 1  Tumorigenic properties of Be-13 cells infected with a retrovirus expressing human wt p53 (pLhpS3RNL) and with the control virus expressing the luciferase gene (pLLuxRNL)

Infected cells were grown under drug selection for at least 6 weeks in the presence of 400 μg/ml G418. Live Be-13-lux cells (1 x 10⁶) were injected s.c. into the left flanks of nude mice, while the same number of live Be-13-p53 cells were injected into the right flanks of the same nude mice. Tumor formation was scored 28 days later. In assays of colony formation of Be-13-p53 and Be-13-lux cells in methylcellulose cultures, total colony numbers were determined after 2 weeks of growth.

<table>
<thead>
<tr>
<th>Cells</th>
<th>Tumorigenicity assay (no. of mice with tumor/ no. of mice injected)</th>
<th>Methylcellulose assay (no. of colonies/10⁴ cells seeded)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Be-13 parental</td>
<td>2/2</td>
<td>ND*</td>
</tr>
<tr>
<td>Be-13-lux</td>
<td>6/6</td>
<td>489 + 47.8</td>
</tr>
<tr>
<td>Be-13-p53 (culture no. 1)</td>
<td>0/7</td>
<td>60 + 8.8</td>
</tr>
<tr>
<td>Be-13-p53 (culture no. 2)</td>
<td>0/2</td>
<td>78 + 11.7</td>
</tr>
</tbody>
</table>

* ND, not determined.

Fig. 3. Growth rate of Be-13-p53 (○) and Be-13-Lux (●) cells. Be-13 cells infected with p53 or lux virus were selected in 400 μg/ml G418 and grown for 8 weeks. Cells (2 x 10⁶) of each type were seeded in 1 ml of DMEM + 10% fetal calf serum in individual wells of 24-well tissue culture cluster plates. Cells from three wells were counted each day for 5 days. Mean cell numbers per well were plotted. Bars, one SD determined for three wells. The reduced growth rate of Be-13-p53 cells was determined in four separate experiments.

Fig. 4. Colony formation of Be-13-p53 and Be-13-lux cells in methylcellulose. Cells infected with p53 or lux virus were grown in medium containing 400 μg/ml G418 for 8 weeks. One thousand cells were seeded in duplicate in 1.2% methylcellulose layered on 1% agarose medium and grown for 2 weeks. p53-infected Be-13 cells (B) produced 6-8 times fewer colonies than lux-infected control cells (A). Individual Be-13-p53 colonies contained 20-30 times fewer cells than control colonies.

Suppression of the tumorigenic phenotype of Be-13 cells by the expression of hwt p53 was tested by s.c. injection of Be-13-p53 cells and Be-13-lux control cells into immunosuppressed nude mice. Nude mice received an initial injection of 10⁷ Be-13-p53 cells in the right flank and a simultaneous injection of an equal number of Be-13-lux or Be-13 control cells in the left flank, 3-7 days after irradiation with 300 cGy. Tumors, first palpable 9 days after injection, were induced only by the Be-13 parental or Be-13-lux cells (Table 1). Fig. 5 shows the result of a similar experiment using 3 x 10⁶ and 3 x 10⁷ cells at 16 days postinjection. Tumors induced by the control cells were first palpable 9 days after injection and grew to diameters of 20 mm and 50 mm after 28 days. After the same length of time, growth-suppressed Be-13-p53 cells had not produced any palpable tumors. The growth-suppressed Be-13-p53 cells did not produce tumors even when 5 x 10⁷ cells were injected, as late as 56 days after injection (in mice not challenged with Be-13-lux control cells). Thus, expression of hwt p53 protein was capable of suppressing the tumorigenic phenotype of Be-13 T-ALL cells when
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Fig. 5. Nude mouse that received an injection on its left flank of lux-virus-infected (control) Be-13 leukemia cells and an injection on its right flank of wtp53-virus-infected Be-13 cells. Replicating cells (3 x 10^5) of each kind were injected s.c. just behind the neck, and 3 x 10^5 live cells of each kind were injected in the back just above the hind foot. In this photograph, taken 16 days postinjection, the leukemia cells, injected in the left flank of the mouse, produced tumors of 12 and 5 mm, while the wtp53-infected, growth-suppressed cells, injected in the right flank of the mouse, produced no tumors. After 28 days, the p53-reconstituted, growth-suppressed cells still had not produced any palpable tumors, whereas the p53-infected (control) Be-13 leukemia cells and an injection on its right flank of wtp53-virus-infected Be-13 cells. Replicating cells (3 x 10^5) of each kind were reconstituted, growth-suppressed cells still had not produced any palpable tumors, even with 3 x 10^5 injected cells, while 3 x 10^5 leukemic cells produced a tumor >20 mm in size.

assayed for tumor formation under the skin of nude mice.

Expression of the wtp53 gene in a human leukemia cell line derived from a T-ALL patient in relapse leads to negative growth regulation in vitro and a powerful suppression of the tumorigenic phenotype in vivo without evidence of significant toxic effects in the reconstituted cells. The Be-13 cell line represents a fully progressed leukemic T-cell which does not produce any detectable p53 protein but which shows evidence of additional cytogenetic and other abnormalities (8, 17), abnormalities which can reasonably be expected to contribute to its tumorigenic phenotype. Nevertheless, the leukemic phenotype was effectively suppressible by the introduction and expression of the wtp53 gene. Our experiments suggest that restoration of the expression of tumor suppressor genes holds potential promise in the quest for a genetically based suppression of the tumorigenic phenotype in some forms of human leukemia.

The hypothesis underlying the suppression of tumor-forming ability by the introduction and expression in human leukemic cells of a wtp53 gene is that the p53 tumor suppressor gene plays a central role in growth control and differentiation and, by inference, in oncogenesis (18). The specific role of wtp53 in growth control and differentiation has not been delineated; hence its role in the suppression of cell proliferation in vitro, in colony formation in methylcellulose cultures, and in the suppression of tumorigenesis in vivo is not understood. It is interesting that expression of wtp53 slowed down, to about one-half, the rate of Be-13-p53 cell proliferation in culture and reduced its cloning efficiency in methylcellulose, whereas it completely abolished leukemia cell growth in nude mice, even when 3 x 10^5 cells were injected (Fig. 5). This difference between the observed in vitro and in vivo effects may reflect the ability of Be-13-p53 cells to differentiate in vivo, as was shown for hybrids between HeLa cells and keratinocytes (19). The observed differences may signify that the antiproliferative activity of wtp53 in vitro (e.g., inhibition of entry of Go/G1 cells into S phase) is only one aspect of the tumor-suppressive activity of wtp53 in vivo. Further experiments are needed to elucidate the mechanism(s) whereby wtp53 exerts differential effects on leukemic cells in vitro and in vivo. Such experiments are in progress in our laboratories.

A potential problem in the reversal of the leukemic phenotype by the introduction of the wtp53 gene is that reversion of the suppressed cells back to their fully leukemic state may occur by the mutation of the transfectected gene and may even lead to a more malignant cell (20). The risk of such an eventuality should be reduced, although it is not clear at this point how this could be accomplished.

These studies have not approached the question of the extent to which a wtp53 allele is dominant in T-ALL cells over different mutant p53 alleles. Since relapse T-ALL cells and their derived cell lines may possess a variety of p53 mutations (8), it is important to demonstrate that the wild-type gene is capable of suppressing the neoplastic phenotype of relapse leukemia cells expressing mutant p53 alleles. Preliminary studies in our laboratory suggest that fully progressed T-ALL cells possessing independently mutated p53 alleles (8) may also be suppressible by the introduction of the wtp53 gene, while immortalized normal diploid lymphoid cells are refractory to growth inhibition by wtp53.

References

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