Under the Microscope

Inhibition of Erythroleukemia Cell Growth by Triplex-forming RNAs

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ABSTRACT

Objective: We have previously reported that oligodeoxyribonucleotides, designed to bind in a triplex fashion to a specific p53 binding site homology, inhibit the proliferation of colon cancer cells *in vitro* and *in vivo*. The present study was designed to extend these observations and to determine whether ribonucleic acid (RNA) generated from a retroviral vector (RVV) and possessing a corresponding triplex forming site can, in a similar fashion, inhibit proliferation of p53-null K-562 leukemia cells. Viral vectors may offer advantages over oligonucleotides for tumor treatment. RVVs have the potential to be taken up more efficiently than oligonucleotides and to be expressed continuously and long-term, circumventing the need for repeated and frequent oligomer administration.

Experimental Design: The p53-null human erythroleukemia cell line, K-562, was stably transfected with a tetracycline-repressible p53 expression construct (p53/pUHD10-3). p53 protein in these cells is expressed in the absence of tetracycline but downregulated upon tetracycline treatment. Triplex-forming oligonucleotides [Hoog 1 (experimental) and Hoog 3 (control)] were cloned into RVVs in order to generate triplex-forming fusion mRNAs. Naive K-562 cells and p53/pUHD10-3-transfected K-562 cells (with and without tetracycline treatment) were infected with viruses that express the triplex-forming RNAs. Cell growth was measured by BrdU incorporation into DNA.

Results: RVVs encoding Hoog 1, in both orientations, inhibit the growth of naive K-562 cells and p53-transfected, tet-repressed K-562 cells. p53 expression in K-562 cells decreases growth to the same extent as Hoog 1 RVV treatment. However, Hoog 1-RVV does not further inhibit growth of p53-expressing K-562 cells. Treatment with an RVV encoding the control, Hoog 3, has no growth inhibitory effect.

Conclusion: Triple helix-forming RNAs directed to a p53 consensus sequence homology reduce leukemia cell proliferation, suggesting a novel method of treatment.

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INTRODUCTION

Abnormal p53 function is commonly encountered in human tumors which may consequently escape apoptosis or programmed cell death (1). Novel methods for restoring p53 function in p53-deficient tumor cells have recently been reported (2). A p53 DNA-binding consensus sequence present in the human non-transcribed ribosomal spacer region was identified some years ago (3). Given the potential participation of ribosomal gene transcription in cellular proliferation, we elected to assess the effect of functionally substituting for p53 binding to this site (4,5). We developed several oligonucleotides in an effort to achieve this goal, one of which was designed to produce triple helical structures by Hoogsteen hydrogen bond base pairing at the target sequence (5,6). Electrophoretic mobility shift assays confirmed binding of oligonucleotide Hoog 1 to the target sequence, and cell culture studies revealed that application of this oligonucleotide markedly reduced the proliferation of COLO 201 and HCT 116 colon carcinoma cells—cells which contain mutant p53 protein. The proliferation of normal human lung fibroblasts was reduced much less (5). Cell death did not occur as was determined by trypan blue exclusion and lactate dehydrogenase (LDH) media assays.

We then extended these observations to the suppression of tumor growth in a nude mouse/subcutaneous tumor model. HCT 116 cells were used because they readily form tumors in mice. Although these cells contain normal p53 protein, our earlier studies revealed they also contain mutant p53 and that Hoog 1 reduces growth of these cells *in vitro* (5,7). Palpable tumors were injected with 100 ul (1 ug/ul) of experimental or control oligomer or with 100 ul of vehicle on a daily basis for two weeks. Daily administration of triplex-forming oligonucleotide, Hoog 1, produced a statistically significant reduction in tumor growth when compared either to vehicle or inactive oligonucleotide (Hoog 3) (8).

Because of the difficulties associated with performing daily injections over an indefinite period in the treatment of human tumors, we questioned whether a continuous long-term delivery system might be employed to similarly reduce proliferation. Ilan and colleagues (9,10) have shown that an RNA effector sequence designed to form a triplex with a homopurine-homopyrimidine sequence within the insulin-like growth factor type I receptor (IGF-IR) structural gene efficiently suppresses IGF-IR transcription in rat C6 glioblastoma cells and reduces tumorigenicity in an animal model.

Using a similar approach, we made RVVs designed to encode Hoog1 and Hoog3 sequences in both orientations as fusion mRNAs with the selectable marker, neomycin phosphotransferase. RNAs are potentially better triplex formers than DNAs and are potentially expressed continuously for long durations from expression vehicles.

MATERIALS AND METHODS
Cell Line
K562 p53-null (11,12) cells were obtained from American Type Culture Collection (ATCC, CCL 243). K-562 cells were originally isolated from the pleural effusion of a CML patient in terminal blast crisis (13). Cell surface properties have led to the conclusion that K-562 is a human erythroleukemia cell line that spontaneously differentiates into erythrocytic, granulocytic, and monocytic cells (14,15).

RVVs
LN∆SX was created from the RVV, LNSX [(16), stands for LTR-neomycin phosphotransferase-SV40 promoter-cDNA of choice], by excising the *Bam* HI/*Avr* II fragment which contains the SV40 promoter. We ligated into the unique *Bcl* I site within LN∆SX (in the 5′-untranslated region of the neomycin phosphotransferase transcript), oligomer duplexes so that the encoded RNAs possess p53-binding site triplex potential. We then established producer cell lines and measured effectiveness of virus produced by these in slowing growth of wild-type p53-deficient K-562 cells or genetically engineered p53-possessing K-562 cells. Primers are as follows:

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Hoog1-forward:  5′-GATCATTTCTTTCTTTCTTTCTTTCTTTTCCT-3′
Hoog1-reverse:  5′-GATCAGGAAAAGAAAGAAAGAAAGAAAGAAAT-3′
Hoog3-forward:  5′-GATCACCCTTTTTTCCTTTTTTTTCTTTTTCTT-3′
Hoog3-reverse:  5′-GATCAAGAAAAAGAAAAAAAGGAAAAAAGGGT-3′
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Hoog1 forward and reverse primers were annealed, ligated into *Bcl*-I-digested LN∆SX. Forward and reverse orientations of the duplex are referred to as Hoog1-CT (pyrimidine-encoding) and Hoog1-AG (purine-encoding), respectively. Hoog3 forward and reverse primers were likewise annealed, ligated into *Bcl*-I-digested LN∆SX. Forward and reverse orientations of this duplex are referred to as Hoog3-CT and Hoog3-AG. Clones were identified, sequenced to verify fidelity, and grown large scale.

p53 clone
The human p53 cDNA was isolated as a *Bam*H I fragment from pHp53B (ATCC 57254) and ligated to *Bam*H I-digested pUHD10-3 (17), transcriptional regulation of which is controlled by the *E. coli* tet repressor.

Virus Production and Infection of Target Cells
Clones were transfected into the PA317 amphotropic retrovirus packaging cell line (16). Colonies were established by neomycin phosphotransferase resistance selection. Virus was collected from producer cells and delivered to K-562 target cells at a multiplicity of infection of 5. Target cells were then examined for growth effects using bromodeoxyuridine incorporation into nuclei as a measure of proliferation.

Statistics
Groups were compared using a one-way analysis of variance (ANOVA) with Tukey-Kramer or Bonferroni multiple comparisons post-hoc tests.

RESULTS
RVVs encoding both Hoog1-CT and Hoog1-AG was effective in reducing the growth rate of naive (untransfected, p53-null) K-562 cells, suggesting a functional compensation for p53 protein [as we found previously with triplex oligomers (8)]. Neither Hoog3-CT (control) nor vehicle had significant effects on growth of naive cells (Fig. 1).

Hoog1-CT and Hoog1-AG were similarly effective in repressing growth of p53/pUHD10-3-transfected cells which were tet-treated (p53-repressed). For each given treatment, the effects of these oligonucleotides on the naive K-562 and tet-repressed p53/pUHD10-3-transfected K-562 cells are statistically indistinguishable.

Release from tet-repression (tet) leads to p53 expression and a concurrent reduction in basal growth

Figure 1. Expression of both p53 and triplex-forming RNAs of Hoog1-CT and Hoog1-AG, effectively and to a similar extent, reduce the growth rate of K-562 cells. BrdU-labeled cells were measured at 36 h post-infection.
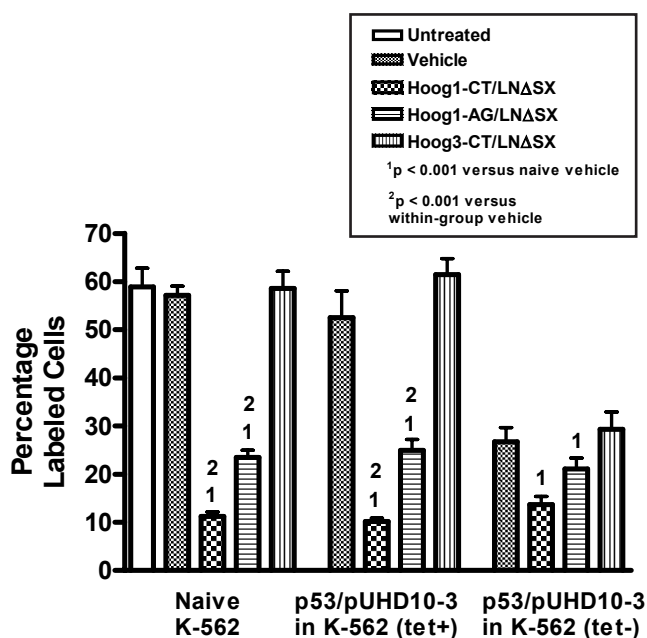
rate of p53/pUHD10-3-transfected cells (see vehicle and control oligomer, Hoog3-CT). Infection of these cells with Hoog1-CT and Hoog1-AG does not further reduce growth significantly, consistent with our contention that p53 and Hoog1 function through a common pathway.

CONCLUSIONS

In the present study we show that fusion mRNAs containing a triplex-forming effector sequence are as potent in suppressing growth as the corresponding triplex oligonucleotides *in vitro*. RVV delivery allows for the infection of mitotically active cells for long-duration expression. Vector-mediated delivery of triplex-forming ribonucleotide effector sequences could be superior to the use of exogenously added oligonucleotides, which may lose their biological activity over hours to days. It is also possible that triplexes containing RNA rather than DNA as the Hoogsteen-paired third strand are more stable, possibly through an additional hydrogen bond between the 2′-hydroxyl proton of DNA and a phosphate oxygen on the backbone of the purine RNA strand (9).

Our studies suggest this approach may be useful for treating wild-type p53-deficient malignancies of various cellular derivations. Additional vectors of, for instance, lentiviral, adenoviral, or adeno-associated viral origin may offer advantages for targeting specific tumor types.

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