Retroviral Vector Design for Gene Therapy of Cancer: Specific Inhibition and Tagging of BCR-ABL^{p190} Cells

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ABSTRACT

Background: The main difficulty in providing effective treatment of patients with cancer is distinguishing between tumor and normal cells. The chimeric molecules created by cancer-associated chromosomal abnormalities (such as the BCR-ABL fusion protein) represent ideal therapeutic targets since they are unique to the disease state. A major challenge, however, is how to deliver the specific anti-tumor agent into every tumor cell.
Material and Methods: In this report we describe the use of a novel strategy to introduce specific anti-tumor reagents into every tumor cell. It uses retroviral vectors encoding both antisense transcripts specific for the BCR-ABL^{p190} fusion junction (the specific anti-tumor drug) and a truncated human CD5 cDNA, which allows selection of the infected cells. In order to coexpress the antisense molecule with the truncated human CD5 gene, the picornavirus internal ribosome-entry site was incorporated in the constructs.
Results: When the antisense transcripts in the CD5-retroviral vector were introduced into Ba/F3+p190 cells rendered interleukin 3 (IL-3) independent by expression of the BCR-ABL sequences, the cells died upon IL-3 withdrawal, as measured by the absence of CD5-positive cells. Control Ba/F3+p210 cells infected with the same virus did not die in the absence of IL-3.
Conclusions: These data suggest a novel strategy for cancer treatment which incorporates the use of a retrovirus coexpressing both a selectable surface marker and a tumor-specific agent.

INTRODUCTION

A key problem in the effective treatment of patients with cancer (both leukemia and solid tumors) is how to distinguish between tumor and normal cells. This largely explains why current cancer treatments are often ineffective. There have been remarkable advances in our understanding of the molecular biology of cancer, providing new mechanisms for selective tumor destruction (1–3). The molecular characterization of tumor-specific chromosomal abnormalities has revealed that fusion proteins are involved in the majority of cancers (4–6). These fusion proteins are encoded by chimaeric genes generated by the chromosomal rearrangement. These chimeric molecules represent ideal therapeutic targets since they are unique to the disease state (they only exist in the tumor cells, not in the normal cells of the patient) (1–3).

In the hematopoietic system, a well-characterized chromosomal rearrangement brings together the BCR and ABL genes in Philadelphia chromosome positive (Phl$^+$) chronic myelogenous leukemia (CML) and acute lymphoblastic leukemia (Phl$^+$-ALL) cells (7,8–16). Depending on the precise location of the translocation breakpoint within the BCR gene, BCR-ABL fusion proteins of either 210 kD (p210) or 190 kD (p190) in size are produced, possessing deregulated ABL tyrosine kinase activity. The p210 and p190 BCR-ABL oncogenes contain identical ABL-derived sequences, but differ in the number of BCR-encoded amino acid residues (8,10,11, 13,17–20). We have previously shown that the BCR-ABL oncogenes act by inhibiting apoptosis via a Bcl-2 pathway as a part of their oncogenic phenotype (21). Inhibition of p190 expression in

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the Ba/F3+p190 mouse hematopoetic cell line reverted this phenotype and cells died by apoptosis (3,21). This suggested that inhibition of p190 expression could similarly inhibit the leukemic phenotype of human Phl^+-ALL cells leading to a new therapeutic approach in this disease.

A major challenge is to deliver an anti-tumor drug into every tumor cell. If this is not achieved, any malignant cell that remains unaffected will emerge as a resistant clone. Several experiments have demonstrated the feasibility of using antisense oligonucleotides (1,2) to produce sequence-specific inhibition of gene expression. However, these oligonucleotides are unable to enter the majority of cell types with high efficiency, so that some type of delivery system is essential (22). In the present study we have employed a novel strategy to introduce specific antitumor drugs into tumor cells. It uses retroviral vectors encoding both antisense sequences specific for the BCR-ABL^{p190} fusion junction and a truncated human CD5 protein encoding only the extracellular and transmembrane domains and therefore defective in signal transduction. This marker allows the selection of all infected cells.

MATERIALS AND METHODS

Plasmid Construction

The MFGA190CD5 and the MFGS190CD5 retroviral vectors are based on a simplified MFG retroviral vector, which does not contain a gene for a dominant selectable marker (23). The sequences subcloned into the MFG vector (23) were as follows: 61 nucleotides spanning the BCR-ABL junction either in antisense (MFGA190CD5 vector) or sense orientations (MFGS190CD5 vector); the picornaviral internal ribosome-entry site (IRES) sequence (24); and a 1218-bp sequence from the human CD5 cDNA encoding the complete extracytoplasmic region. CD5 DNA fragments were generated by reverse transcriptase polymerase chain reaction (PCR) using primers that included initiation and termination codons. The authenticity of the constructs generated was confirmed by DNA sequencing and revealed neither PCR errors nor cloning artifacts. The proviral structure of these vectors is shown in Fig. 1A below.

Cell Lines

The ecotropic retrovirus packaging cell line ψ-cre was used to generate helper-free recombinant retrovirus. The cell line was maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf serum. Viral packaging cell lines were maintained in the same medium supplemented with 1 mg/ml of the neomycin analog G418 (GIBCO/BRL). The interleukin 3 (IL-3)–independent mouse hematopoietic cell lines Ba/F3+p190 and Ba/F3+p210 expressing, respectively, BCR-ABL^{p190} and BCR-ABL^{p210}, were maintained in DMEM with 10% fetal calf serum (FCS). When required, 5% of WEHI-3B conditioned medium was included as a source of IL-3. All cells were cultured at 37°C in a humidified atmosphere of 5% CO_2 in air.

Retrovirus Production

ψ-cre cells were first transfected with 20 μg of either MFGA190CD5 or MFGS190CD5 retroviral vectors together with 1 μg of the pMC1neo vector by the calcium phosphate precipitation method. Following G418 selection (1 mg/ml), clones were selected based on the level of CD5 expression. BCR-ABL–expressing Ba/F3 cells were infected by 2 days of coculture with the MFGA190CD5 or the MFGS190CD5 viral producer cell lines in DMEM supplemented with 10% calf serum, Polybrene (8 μg/ml), and IL-3. After coculture, infected cells (as determined by CD5 expression) were tested for IL-3 dependence.

Labeling of Cells

Protein production was analyzed by immunofluorescence labeling as follows. Cells were fixed in 4% (w/v) paraformaldehyde for 15 min, washed in phosphate-buffered saline (PBS), and permeabilized in methanol for 2 min. After blocking in 5% FCS in PBS for 30 min, the FITC-directed conjugated anti-human CD5 monoclonal antibody (Pharmingen, San Diego, CA, U.S.A.) was added and incubated for 30 min. Fluorescent cells were visualized by epifluorescent microscopy. Images were recorded on the confocal scanning microscope. To sort CD5-positive cells, MFGS190CD5-infected cells were incubated on ice for 40 min with the FITC-directed conjugated anti-human CD5 monoclonal antibody (Pharmingen), washed twice with PBS with 1% FCS and resuspended in PBS with 1% FCS. Propidium iodide (Sigma Chemical Co., St. Louis, MO, U.S.A.) was included in the last wash to distinguish dead cells before analysis by flow cytometry using a FACScan cell analyzer (Beckton Dickinson, Mountain View, CA, U.S.A.). BCR-

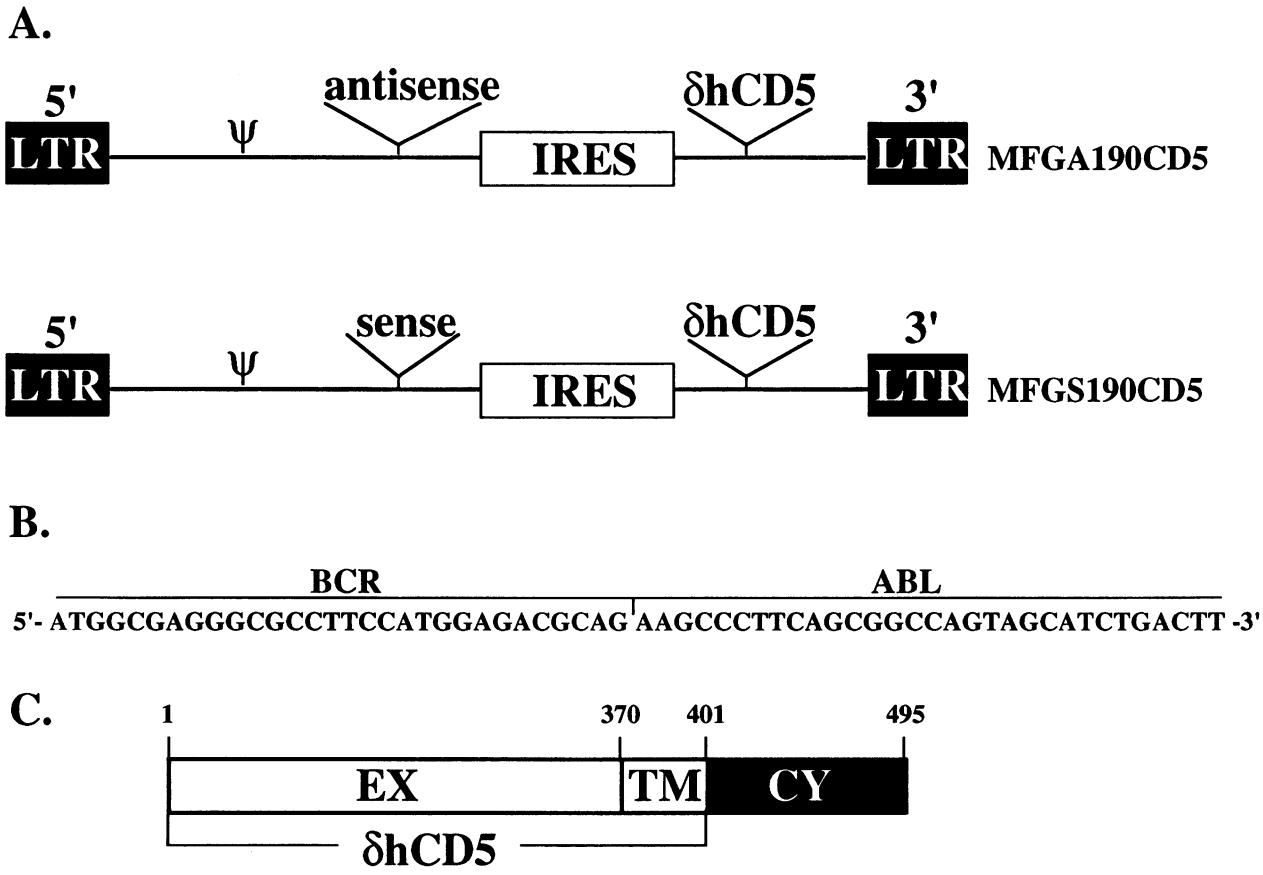

FIG. 1. CD5-recombinant retroviruses encoding BCR-ABL^{p190} antisense (MFGA190CD5) and sense transcripts (MFGS190CD5)

(A) Proviral structure of the vectors. The MFG retroviral vector (23) incorporates 61 nucleotides spanning the BCR-ABL fusion junction (A) and a 1218-bp sequence of the human CD5 cDNA encoding the complete extracytoplasmic region. The vector does not contain a selectable marker. (B) Nucleotide sequence of the BCR-ABL fusion junction carried by the retroviral vectors in either antisense or sense orientations. (C) Diagrammatic representation of the human CD5 protein. The portion included in the retroviral vectors is indicated (δhCD5). EX, extracytoplasmic domain; TM, transmembrane domain; CY, cytoplasmic domain.

ABL-expressing Ba/F3 cells cultured alone were used as a negative control. Cells were sorted on a FACStar$^+$ (Beckton Dickinson) equipped with a 5-W argon and a 30-mW helium neon laser. Cells were collected in sterile Eppendorf vials in medium with 50% FCS.

RNA Analysis

Total cytoplasmic RNA (10 μg) was glyoxylated and fractioned in 1.4% agarose gels in 10 mM Na_2HPO_4 buffer (pH 7.0). After electrophoresis, the gel was blotted onto Hybond-N (Amersham, Arlington Heights, IL, U.S.A.), UV–cross-linked, and hybridized to ^{32}P-labeled probes.

RESULTS

Plasmid Construction

To explore the possible use of coexpressing the antitumor agent (BCR-ABL^{p190} antisense molecules, in this case) and a selectable cell surface marker (CD5), the retroviral vectors depicted in Fig. 1 were constructed. These two vectors contained 61 nucleotides long sequences spanning the BCR-ABL^{p190} cDNA fusion junction either in antisense (MFGA190CD5) or sense (MFGS190CD5) orientations. They also carried a 1218-bp–long DNA sequence from a portion of the human CD5 cDNA encoding the complete extracytoplasmic region. The anti–BCR-ABL^{p190} sequences and the truncated human CD5 cDNA would be tran-

TABLE 1. Viability of CD5$^+$-cells after IL-3 withdrawal

Infected Cells	+IL-3	−IL-3
Ba/F3+p190 + MFGA190CD5	6.8%	0%
Ba/F3+p190 + MFGS190CD5	7.2%	7.2%
Ba/F3+p210 + MFGA190CD5	5.9%	5.9%

scribed from promoter/enhancer sequences in the retroviral long terminal repeat (LTR). In order to coexpress the anti–BCR-ABL^{p190} sequences with the extracytoplasmic portion from the human CD5 cDNA, we incorporated in the vectors the picornaviral internal ribosome-entry site (IRES). The IRES is a 500-nucleotides–long sequence which acts as ribosomal binding site and permits effective internal translation initiation in mammalian cells (24). MFGA190CD5 and MFGS190CD5 viral producing cells were generated using the ecotropic ψ-cri packaging cell line. Clones were selected based on high CD5 expression and had a titer of 0.5 (copy number) as determined by titering in NIH-3T3 cells.

Specific Inhibition of Ba/F3+p190 Cells Infected with MFGA190CD5 Retrovirus Following IL-3 Withdrawal

Mouse Ba/F3 cells expressing BCR-ABL^{p190} (designated Ba/F3+p190) were infected with CD5-retroviruses carrying either sense or antisense BCR-ABL^{p190} junction sequences. The BCR-ABL^{p190} oncogene renders the IL-3–dependent murine Ba/F3 cell line (25) growth factor independent (21,26). In turn, down-regulation of BCR-ABL oncogene expression restores IL-3 dependence (3,21).

Approximately, 7% of Ba/F3+p190 cells expressed high levels of surface CD5 antigen after 2 days of cocultivation with either MFGA190CD5 or MFGS190CD5 viral producers in the presence of IL-3 (Table 1 and Fig. 2A, top panels). CD5 expression provided a marker for cells infected with the retrovirus. Following IL-3 removal, the Ba/F3+p190 cells infected with retrovirus carrying antisense BCR-ABL^{p190} sequences died, as determined by lack of CD5-positive cells (Table 1 and Fig. 2A, bottom left panel). In contrast, Ba/F3+p190 cells infected with CD5-retrovirus carrying BCR-ABL^{p190} sequences in the sense orientation remained viable in the absence of exogenous IL-3 (Table 1 and Fig. 2A, bottom right panel). Therefore, the antisense RNA inhibited the function of the BCR-ABL^{p190} RNA and protein.

The specificity of the retrovirally transduced antisense transcript against BCR-ABL^{p190} junction sequence was tested by infecting Ba/F3 cells expressing the BCR-ABL^{p210} fusion protein with CD5-retrovirus encoding antisense BCR-ABL^{p190} junction transcript (Table 1 and Fig. 2B). The BCR-ABL^{p190} and BCR-ABL^{p210} oncogenes share 50% homology since the ABL sequences involved in the gene fusion are the same. However, the infected BCR-ABL^{p210}–expressing Ba/F3 cells did not die following IL-3 withdrawal, indicating that the BCR-ABL^{p190} antisense sequences are highly specific for BCR-ABL^{p190} and not for BCR-ABL^{p210}.

Fluorescence-Activated Cell Sorter Selection of CD5-Transduced Ba/F3+p190 Cells

Viable cells infected with the sense BCR-ABL^{p190} junction sequences expressed high levels of truncated human CD5, which is not expressed in the parental mouse cell line, allowing selection of infected cells by fluorescence-activated cell sorting (FACS) and subsequent propagation of viable CD5-positive cells (Fig. 3). A representative FACS profile from one experiment shows that approximately 7% of the Ba/F3+p190 cells recovered after infection were positive for the CD5 cell surface antigen (Fig. 3 B and C). In three independent experiments, 90–95% of the Ba/F3+p190 cells recovered in the CD5-positive fraction were CD5 positive (Fig. 3 D and E) compared with 7% in the unsorted population (Fig. 3C). Thus, retrovirally transduced Ba/F3+p190 cells could be successfully enriched based on their immediate expression of the CD5 gene in vivo. Moreover, as the fraction of CD5-positive cells did not decrease with extended propagation, the surface marker does not have deleterious effects on cell growth and viability.

In order to ensure that the level of BCR-ABL^{p190} RNA was indeed reduced in Ba/F3+p190 cells following infection with the antisense viral construct, CD5-positive cells were selected by FACS sorting in the presence of IL-3 and the level of BCR-ABL^{p190} mRNA was analyzed by Northern blot analysis. As illustrated in Fig. 4, BCR-ABL^{p190} RNA levels were significantly reduced in selected Ba/F3+p190 cells infected with the antisense vector. Residual BCR-

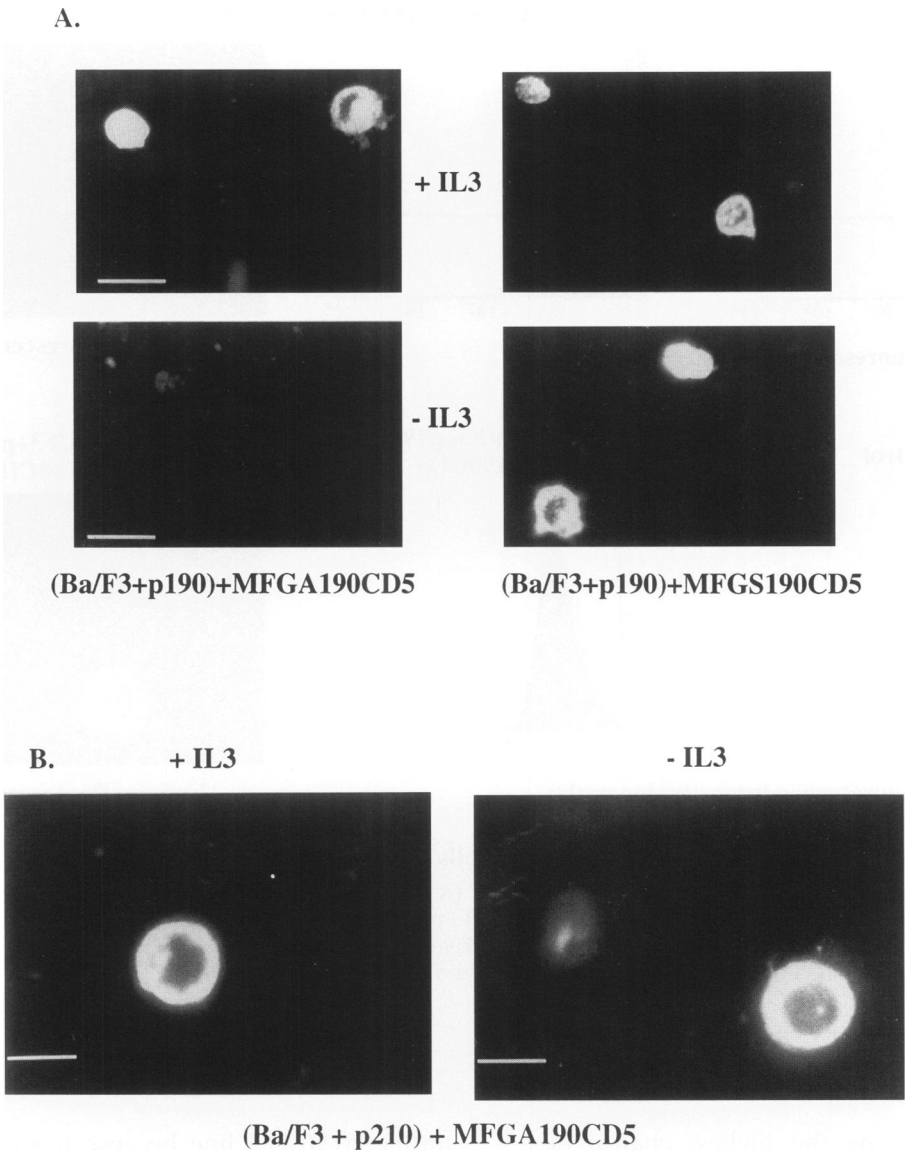

FIG. 2. (A) Specific growth inhibition of Ba/F3+p190 cells infected with the MFGA190CD5 retrovirus following IL-3 withdrawal, and (B) The viability of Ba/F3+p210 cells is not affected by MFGA190CD5 virus

(A) Ba/F3+p190 cells were infected with recombinant MFGA190CD5 (left panels) or MFGS190CD5 (right panels). The viability of infected cells was assessed by staining with anti-human CD5 antibody in the absence of IL-3 (bottom panels). (B) Ba/F3+p210 cells were infected with the virus encoding BCR-ABL^{p190} antisense transcripts and the truncated human CD5 surface marker. Infected cells (left panel) were assessed by their viability after IL-3 withdrawal (right panel).

ABL^{p190} mRNA corresponded to uninfected CD5-negative cells which were not efficiently sorted by FACS (data not shown). In contrast, BCR-ABL^{p190} mRNA production was not affected in CD5-positive selected cells infected with the sense vector.

The data suggest a possible strategy treating Philadelphia positive ALL or CML. The strategy makes use of retrovirus which enables dual expression of both a selectable marker (truncated CD5) and a tumor-specific agent (antisense oligonucleotides specific for tumor-associated fusion proteins). In this way, following ex vivo targeting, only cells expressing both a tumor-specific agent and a selectable cell surface marker would be used for bone marrow reconstitution in a

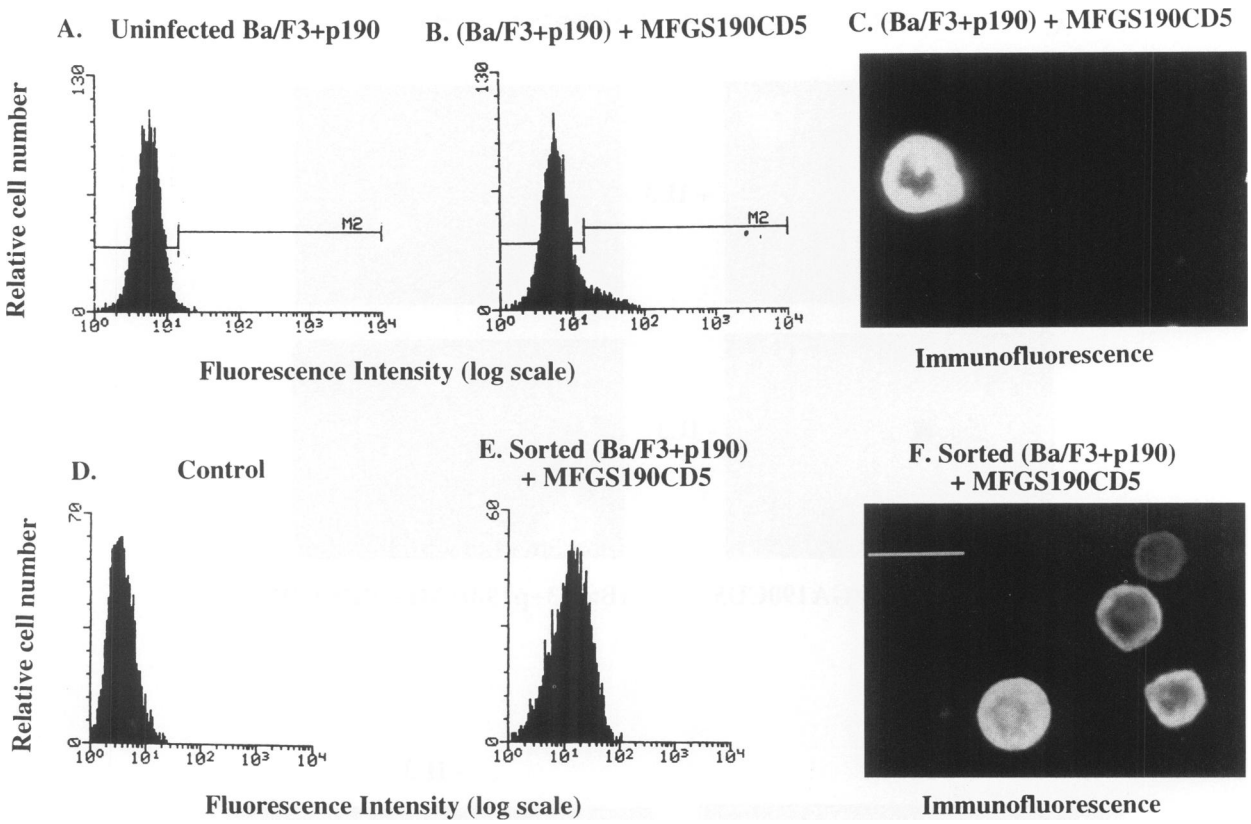

FIG. 3. Selection of CD5 virus-infected Ba/F3+p190 cells by FACS

The CD5 expression profiles of uninfected Ba/F3+p190 cells (A and D), Ba/F3+p190 cells cocultured with CD5 viral producer cells 48 hr postinfection (B), and sorted Ba/F3+p190 cells (E). (C and F) The immunofluorescence analysis of Ba/F3+p190 cells before and after sorting, respectively. Cells were stained with anti-human CD5 antibody and analyzed by flow cytometry (A, B, D, and E) and immunofluorescence (C and F).

patient, thus ensuring the highest chance of eradicating the tumor.

DISCUSSION

Use of Retroviral Vectors to Deliver Antisense Oligonucleotides in the BCR-ABL–Expressing Ba/F3 Model

Gene therapy promises to be an effective strategy for the treatment of cancer. Recombinant retroviruses provide an attractive vehicle for gene transfer because of their capacity for high and efficient infection, and nontoxic integration into the genome in a wide range of cell types. We report a novel strategy to target tumor-associated fusion proteins using retrovirally transduced antisense sequences targeted against the BCR-ABL fusion mRNA in Ba/F3+p190 cells. We chose the Ba/F3+p190 cell line because it was rendered IL-3 independent by the expression of p190-coding sequences. Following inhibition of p190 expression by antisense treatment, which causes cell death in a culture medium deprived of IL-3, cells can be rescued by addition of exogenous IL-3. This system is therefore well suited for evaluating the efficacy of the technique.

Ba/F3+p190 cells were infected by a retroviral vector carrying selected antisense sequences designed against the BCR-ABL fusion junction. The antisense transcripts generated under the control of the viral promoter specifically killed Ba/F3+p190 cells in the absence of IL-3. This effect was specific since control Ba/F3+p210 cells infected with the same viral constructs did not die following IL-3 withdrawal. In addition, the level of BCR-ABL^{p190} mRNA was specifically reduced in selected Ba/F3+p190 cells infected

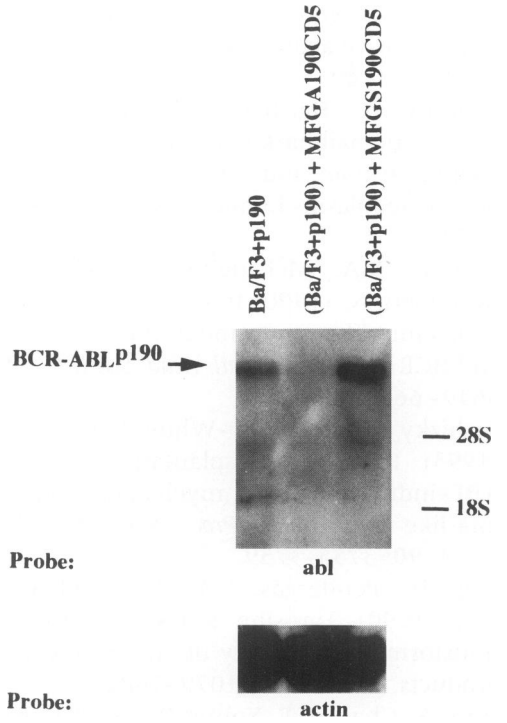

FIG. 4. Specific suppression of BCR-ABL^{p190} mRNA transcripts in selected Ba/F3+p190 cells infected with the antisense virus

Total RNA was isolated from Ba/F3+p190 cells (Lane 1), selected Ba/F3+p190 cells infected with the antisense virus (Lane 2), and selected Ba/F3+p190 cells infected with the sense virus (Lane 3). Cellular RNA was hybridized to a human ABL probe (top) and to a mouse β-actin cDNA (bottom). Autoradiography was for 9 hr at −70°C.

with the antisense virus. We conclude that this approach successfully achieves suppression of the oncogenic protein p190. The effect on fresh Ph[1]-ALL cells in bone marrow cultures must be assessed before this technique may be used to suppress leukemic hematopoiesis in vitro. While antisense oligonucleotides are unable to enter most cell types with high efficiency (22), this study indicates that retroviral vectors are a suitable delivery system for antisense oligonucleotides to produce sequence-specific gene inhibition.

Novel Strategy for Cancer Treatment: Dual Expression of Both the Tumor-Specific Agent and the Selectable Tagging Molecule

Although the efficiency for retrovirus-mediated gene transfer approaches 100%, this may not be realized either because of low viral titers or because of the failure to stimulate cells to divide, which is required for successful integration. Moreover, expression of the transferred gene(s) may not reach the desired levels or be sustained in cells. Such problems are especially evident in current efforts to apply the technique to the gene therapy of cancer; if the anti-tumor vector is not delivered to every tumor cell, any malignant cell that is not affected will emerge as a resistant clone. In this study we have coexpressed the specific anti-tumor agent (antisense molecules against the tumor-associated fusion proteins) with a cDNA encoding a truncated human cell surface antigen (CD5) which allows immediate postinfection selection of the hematopoietic cells transduced with the retrovirus. FACS analysis in combination with functional studies showed that under the conditions used, all Ba/F3+p190 cells selected expressed CD5 by 48 hr following infection. Moreover, the fraction of CD5-positive cells did not decrease with their extended propagation, indicating that the surface marker does not have deleterious effects on cell growth and viability. The use of the truncated CD5 cell surface antigen as a selectable marker of gene transfer offers significant advantages over other selectable markers which confer resistance to toxic compounds. They include rapid and quantitative detection by flow cytometry of expression of the transfected genes in the desired target cell population and the efficient and nontoxic selection of transduced target cells by FACS.

These results suggest a new approach for in vitro gene therapy of hematopoietic cancer: autografting patients with bone marrow cells infected in vitro with the retrovirus expressing sequences targeted specifically against the tumor-associated fusion junctions and a selectable marker, followed by nontoxic selection, ensures that all reinfused stem cells will contain integrated antisense sequences. This strategy would thus make it possible to restore cancer-negative hematopoesis in patients.

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