Gene Therapy with Dominant-negative Stat3 Suppresses Growth of the Murine Melanoma B16 Tumor in Vivo

Guilian Niu, Richard Heller, Robyn Catlett-Falcone, Domenico Coppola, Mark Jaroszinski, William Dalton, Richard Jove, and Hua Yu

Immunology Program [G. N., H. Y.], Clinical Investigations Program [R. H., D. C., W. D.], and Molecular Oncology Program [R. C.-F., R. J.], H. Lee Moffitt Cancer Center and Research Institute, and Departments of Microbiology and Immunology [G. N., R. H., H. Y.], Surgery [R. H., M. J.], Pathology [R. C.-F., D. C.], Medicine [W. D.], and Biochemistry and Molecular Biology [R. J.], University of South Florida College of Medicine, Tampa, Florida 33612

Abstract

Whereas signal transducers and activators of transcription were originally discovered as mediators of normal cytokine signaling, constitutive activation of certain signal transducer and activator of transcription proteins, including Stat3, has been found in increasing numbers of human cancers. Recently, a causal role for Stat3 activation in oncogenesis has been demonstrated, suggesting that Stat3 represents a novel target for cancer therapy. We report here that in vitro expression of a Stat3 variant with dominant-negative properties, Stat3Δβ, induced cell death in murine B16 melanoma cells that harbored activated Stat3. By contrast, expression of Stat3Δβ had no effect on normal fibroblasts or the Stat3-negative murine tumor MethA, suggesting that only tumor cells with activated Stat3 have become dependent on this pathway for survival. Significantly, gene therapy by electroinjection of the Stat3Δβ expression vector into preexisting B16 tumors caused inhibition of tumor growth as well as tumor regression. This Stat3Δβ-induced antitumor effect is associated with apoptosis of the B16 tumor cells in vivo. These findings demonstrate for the first time that interfering with Stat3 signaling induces potent antitumor activity in vivo and thus identify Stat3 as a potential molecular target for therapy of human cancers harboring activated Stat3.

Introduction

STATs are latent cytoplasmic transcription factors that function as intracellular effectors of cytokine and growth factor signaling pathways. STAT proteins were originally defined in the context of normal cell signaling, where STATs have been implicated in the control of cell proliferation, differentiation, and apoptosis (1, 2). Whereas much progress has been made in understanding the normal roles of STATs in cytokine and growth factor signaling, aberrations in these signaling pathways associated with oncogenesis raise the possibility that unregulated activation of STAT signaling contributes to human cancers. Numerous studies have demonstrated that transformation of cells by oncoproteins, including the Src tyrosine kinase, results in constitutive activation of STATs (3, 4). In cells transformed by diverse oncogenes or tumor viruses, the most frequently activated STAT family members are Stat3, Stat5, and, to a lesser extent, Stat1 (5–10). Importantly, Stat3 signaling has been shown to be required for oncogenic transformation by Src, because the expression of dominant-negative forms of Stat3 interferes with Stat3-mediated gene regulation and blocks cell transformation (11, 12).

Mounting evidence directly implicates aberrant activation of Stat3 signaling in malignant progression of human cancers. Constitutive activation of Stat3 has been demonstrated in human breast carcinoma, multiple myeloma, lymphomas, leukemias, and head and neck carcinoma (8, 13–17). In the human myeloma cell line U266, interleukin-6-mediated constitutive activation of Stat3 signaling induces elevated expression of the antiapoptotic regulator Bcl-xL (17). Blocking Stat3 signaling in these myeloma cells down-regulates Bcl-xL expression and results in a dramatic induction of programmed cell death in vitro (17). These findings provide evidence that aberrant Stat3 signaling contributes to malignant progression of multiple myeloma by preventing apoptosis and suggest that Stat3 is a potential target for cancer therapy. However, it has not been determined whether blocking Stat3 signaling is sufficient to inhibit tumor growth in vivo. In this study, we used a gene therapy approach to inhibit activated Stat3 in vivo and assessed its effect on murine melanoma B16 tumor growth. Our results demonstrate that inhibition of activated Stat3 signaling by gene therapy with a dominant-negative Stat3 variant suppresses B16 tumor growth and induces apoptosis of B16 tumor cells in vivo.

Materials and Methods

Cell Lines and Culture Medium. Mouse B16 melanoma cell line, MethA sarcoma cell line, and mammary carcinoma cell lines 4T1 and TSA (kindly provided by Dr. Ning-Sun Yang, University of Wisconsin Medical Center, Madison, WI) were grown in RPMI 1640 (Life Technologies, Inc., Grand Island, NY) supplemented with 10% fetal bovine serum, 2 mm l-glutamine, l sodium pyruvate, 1% MEM nonessential amino acids, and 100 IU/ml penicillin/streptomycin. NIH 3T3 (American Type Culture Collection) cells were grown in DMEM (Life Technologies, Inc.) supplemented with 5% calf serum.

Nuclear Extracts and EMSA. Nuclear extract preparation and EMSAs were performed essentially as described previously (3, 8, 17).

Plasmids. Stat3Δβ cDNA was kindly provided by E. Caldenhoven and R. de Groot (University Hospital, Utrecht, the Netherlands; Ref. 18). The backbone plasmid, pRRES-EGFP, was obtained from Clontech (Palo Alto, CA). The construction and characterization of pRRES-Stat3Δβ has been described previously (17). The pcDNA3 plasmid was obtained from Stratagene (La Jolla, CA). Stat3Δβ cDNA was also inserted into the pAdCMV vector (Quantum Biotechnologies, Montreal, Quebec, Canada). The ability of pAdCMV-Stat3Δβ to express Stat3Δβ protein was verified by Western blot analysis after transfection into NIH 3T3 cells.

Transfections and Flow Cytometric Analysis. Transfections in vitro were performed by the LipfectAMINE-mediated method (Life Technologies, Inc.). To determine transfection efficiency, relative fluorescence intensity was measured by fluorescence-activated cell sorting of both pRRES-EGFP/pSV.neo- and pRRES-Stat3Δβ.neo-transfected cells. For stable transfectants, one plate of transfected cells from each group was used to determine the transfection efficiency, and the remaining plates were allowed to grow in medium supplemented with 500 μg/ml G418. Two weeks later, the G418-resistant colonies were harvested and analyzed by flow cytometry.
were fixed in 4% paraformaldehyde, and the number of colonies was counted. GFP-positive colonies were counted (for B16 cells) or estimated (for NIH 3T3 cells) under fluorescence microscopy.

**Mice and Tumors.** Six-week-old female C57BL mice were purchased from the National Cancer Institute (Frederick, MD) and maintained in the institutional animal facilities approved by the American Association for Accreditation of Laboratory Animal Care. Mice were shaved in the left flank area and injected s.c. with $2 \times 10^6$ B16 cells in 100 µl of PBS. After 7–10 days, B16 tumors with a diameter of 3–6 mm were established. Animals were stratified so that the mean tumor sizes in all treatment groups were nearly identical. Tumor volume was calculated according to the formula $V = \frac{0.52}{2} \times a^2 \times b$ (a, smallest superficial diameter; b, largest superficial diameter).

**DNA Electroinjection in Vivo.** The gene delivery procedure was performed after the mice were anesthetized in an induction chamber infused with a mixture of 3% isoflurane and 97% oxygen. Procedures were then carried out using a supply of 2% isoflurane in oxygen to a standard rodent mask. One hundred µg of plasmid DNA in 100 µl saline were injected directly into the tumor using a 25-gauge, ½-inch-long needle. Electric pulses were delivered through custom-designed electrodes that were placed around the tumor using a PA 4000 DC generator (Cyto Pulse Sciences, Inc., Columbia, MD). Electroinjection of the tumor cells was accomplished by applying a total of fourteen 100-msec electric pulses at a nominal field strength of 1500 V/cm at 1-s intervals.

**Histochemistry and Immunohistochemistry.** Electroinjection with pIRE-S-EGFP or pIRE-Stat3β was carried out in 4–5-mm B16 tumors. Three days after in vivo transfection, mice were euthanized, and the tumors were excised and frozen immediately in liquid N₂. Serial sections of tumors were also fixed in formalin, stained with H&E, and processed for routine histological examination. The anti-GFP monoclonal antibody (Clontech) was applied to 3-µm sections from frozen sections of tumors using the avidin-biotin-peroxidase complex method (Vectastain Elite ABC kit; Vector Laboratories, Burlingame, CA). All slides were lightly counterstained with Mayer’s hematoxylin for 30 s before dehydration and mounting. Nonimmune protein (mouse IgG) negative controls were used for each section. For β-gal staining, tumor tissues were excised and fixed in 0.5% glutaraldehyde 3 days after electinjection of either the β-gal or pcDNA3 plasmids. Cryostat sections were mounted on poly-t-lysine-coated slides and fixed briefly in 0.5% glutaraldehyde. The X-gal reaction was carried out according to the supplier’s instructions (Boehringer Mannheim, Indianapolis, IN).

**TUNEL Assay.** B16 tumors that received either pIRE-S-EGFP or pIRE-Stat3β electroinjections were used for this assay. Three-µm sections from paraffin-embedded tissues were dewaxed and rehydrated according to standard protocols. After incubation with proteinase K (30 min at 21°C), the TUNEL reaction mixture (Boehringer Mannheim) was added to rinsed slides and incubated in a humidified chamber for 60 s at 37°C. This was followed by an incubation with Converter-AP (50 µl) and substrate solution (50 µl). The reaction was visualized by light microscopy.

**Results**

**Stat3 Is Constitutively Activated in Murine Tumor Cells.** Four murine tumor cell lines, including melanoma B16, mammary carcinoma TSA and 4T1, and sarcoma MethA, were evaluated for STAT DNA-binding activity by EMSA. An oligonucleotide probe corresponding to a high-affinity mutant of the sis-inducible element (hSIE), which binds activated Stat1 and Stat3 (3, 8, 17), was used to determine whether the nuclear extracts from these tumor cells contain constitutively activated Stat3 protein. With the exception of MethA, all of the other murine tumor cells contained elevated hSIE binding activity corresponding to Stat3 homodimers (Fig. 1, a and b). Because B16 is a poorly immunogenic s.c. tumor, it provides an excellent model for testing local gene therapy. We therefore focused our studies on this tumor model to evaluate whether Stat3 could be used as a molecular target for the therapy of cancers with constitutively activated Stat3.

**Overexpression of a Stat3 Dominant-negative Protein, Stat3β, Induces Cell Death in B16 Tumor Cells in Vitro.** Stat3β is a naturally occurring splice variant of Stat3 that lacks the COOH-terminal transcriptional activation domain and hence functions as a dominant-negative form of Stat3 in many cellular contexts (11, 18). Recent experiments demonstrated that Stat3β expression induces apoptosis in cultured human U266 myeloma cells, which have constitutively activated Stat3 (17). To determine whether Stat3 signaling is essential for B16 cell survival in vitro, we examined the effects of Stat3β overexpression on B16 cells. B16 cells were cotransfected with pSVneo and either a vector encoding both EGFP and human Stat3β (pIRE-Stat3β) or the empty vector encoding only EGFP (pIRE-EGFP). Because the pIRE-Stat3β construct contains an internal ribosomal entry site to allow translation of Stat3β and EGFP from a single bicistronic mRNA, detection of EGFP can be used as a marker for Stat3β expression in the same cell.

In all of the experiments shown in Table 1, transfection efficiencies with pIRE-EGFP or pIRE-Stat3β vectors were very similar, as determined by the percentage of cells that exhibit green fluorescence at 36 h after transfection (fluorescence-activated cell-sorting analysis). The remaining transfected plates were selected in medium supplemented with G418. Because the transfection efficiencies of the two constructs in each experiment were nearly the same, >95% of the B16 cells that received the Stat3β construct did not survive (only 6 colonies survived, as compared with 138 colonies in B16 cells transfected with the empty vector). In the six surviving colonies, the intensity of green fluorescence was also much dimmer than that seen in those transfected with the empty vector (data not shown). To determine whether expression of Stat3β could mediate the cell death of other murine tumor cells with activated Stat3, transfection was carried out in the TSA murine breast carcinoma cell line. Consistent with the B16 cells, a marked reduction in the number of viable cells was observed in Stat3β-transfected TSA tumor cells when compared with empty vector-transfected control cells (data not shown).

To ensure that the lack of survival in cells that received Stat3β was not due to nonspecific toxicity, the same cotransfection conditions and G418 selection were performed with normal mouse...
NIH 3T3 fibroblasts. The number of G418-resistant clones were the same in both empty vector- and Stat3β-transfected cells (Table 1), and no differences in the number of GFP-positive clones or the intensity of green fluorescence were observed. To assess whether the sensitivity to Stat3β expression in B16 cells is due to transformation in general or requires activated Stat3 signaling, MethA tumor cells that do not harbor constitutively activated Stat3 were transfected with either pIRES-Stat3β or pIRES-EGFP. Whereas the number of live B16 cells decreased dramatically as a result of Stat3β transient transfection, the number of live MethA cells in both the Stat3β and vector control groups remained the same 48 h after transfection (data not shown).

Table 1: STAT3β kills B16 tumor cells but not normal NIH 3T3 fibroblasts

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Transfection efficiency (%)</th>
<th>No. of G418&lt;sup&gt;b&lt;/sup&gt; clones&lt;sup&gt;c&lt;/sup&gt;</th>
<th>No. of GFP-positive clones</th>
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<tr>
<td>B16 exp. 1&lt;sup&gt;d&lt;/sup&gt;</td>
<td>68.7</td>
<td>81.7</td>
<td>21</td>
</tr>
<tr>
<td>B16 exp. 2</td>
<td>61.4</td>
<td>58.7</td>
<td>31</td>
</tr>
<tr>
<td>B16 exp. 3</td>
<td>36.6</td>
<td>41.3</td>
<td>122</td>
</tr>
<tr>
<td>B16 exp. 4</td>
<td>36.8</td>
<td>47.5</td>
<td>56</td>
</tr>
<tr>
<td>Sum</td>
<td></td>
<td>220</td>
<td>138</td>
</tr>
<tr>
<td>NIH 3T3</td>
<td>30.1</td>
<td>27.1</td>
<td>129</td>
</tr>
</tbody>
</table>

<sup>a</sup> G418<sup>b</sup>, G418-resistant.
<sup>b</sup> exp., experiment.
<sup>c</sup> ND, not determined (no difference in GFP-positive clones was observed between the two groups).

Intratumoral Electroinjection of Stat3β Vector Leads to Suppression of Tumor Growth in Vivo. Electroinjection for gene delivery in vivo has been reported previously (19, 20). We first determined the efficacy of gene delivery into 4–5-mm (average) B16 tumors by examining the percentage of tumor cells positive for GFP or β-gal after electroinjection with the respective vectors. Approximately 15% of the tumor cells were scored as positive for β-gal expression (Fig. 2a), and similar results were obtained for GFP expression (data not shown).

To determine the effects of Stat3β expression on in vivo tumor growth, electroinjected 3–6-mm B16 tumors with either pRES-EGFP or pRES-Stat3β plasmids. Of the 15 mice that received empty vector by electroinjection (pRES-EGFP, 10 mice; pcDNA3, 5 mice), only 1 mouse temporarily regressed its tumor (Fig. 2c). In contrast, 11 of 20 tumors that received Stat3β expression vectors (either pRES-Stat3β or pAdCMV-Stat3β) regressed (compare Fig. 2, c and d). Five of these 11 tumors demonstrated continued response because no tumor regrowth was observed at the original tumor site at sacrifice, although new tumors emerged near the original tumors. In all of the experiments, the growth of B16 tumors was clearly inhibited by Stat3β gene therapy in a majority of the mice (Fig. 2b). However, injection of pIRES-Stat3β intratumorally without electroporation had no inhibitory effect on tumor growth (data not shown).

Stat3β-mediated Tumor Suppression Involves Apoptosis in Vivo. To determine the mechanism of tumor cell killing in vivo, B16 tumors (from experiment 3 in Fig. 2b) treated with either the empty vector or the Stat3β vectors were excised for H&E staining and TUNEL assays. All 5 of the control tumors and all 10 of the Stat3β-treated tumors were stained with H&E. Whereas none of the five control tumors showed more than 10% apoptotic cells, many of the Stat3β-treated tumors had undergone massive apoptosis (Fig. 3, a and b). Of the 10 Stat3β-treated tumors, 5 regressing tumors had more than 50% apoptotic cells (2 of them had greater than 90%). TUNEL/alkaline phosphatase assays for apoptosis confirmed that Stat3β treatment induced extensive apoptosis in B16 tumors (Fig. 3, c and d). In
addition to apoptosis, infiltrating inflammatory cells in the apoptotic tumors were observed in Stat3β-treated tumors (data not shown).

Discussion

In this study, we used a syngeneic mouse tumor model system involving the poorly immunogenic murine B16 tumor to evaluate whether constitutively activated Stat3 is a valid molecular target for novel cancer gene therapy. Our results show that inhibition of activated Stat3 by its dominant-negative variant, Stat3b, leads to a significant inhibition of tumor growth mediated by tumor cell apoptosis in vivo.

The high incidence of Stat3 activation in human cancers from diverse origins implicates Stat3 signaling in neoplastic transformation (4, 8, 13–17). Although the mechanisms of Stat3 activation in most cancers are not known, our results indicate that Stat3 is also constitutively activated with high incidence in murine tumors, highlighting the importance of Stat3 signaling in oncogenesis. Compared with the human myeloma cell line U266, the levels of activated Stat3 in the B16 tumor cell line are relatively low (Fig. 1). Such low levels of Stat3 activation have also been observed frequently in human tumor cell lines and tissues, including myeloma and breast cancer (8, 17). The fact that expression of Stat3β killed nearly all of the B16 tumor cells in vitro suggests that low levels of constitutively activated Stat3 are sufficient to maintain tumor cell survival. These results also imply that human tumors with low levels of constitutively activated Stat3 are potential candidates for Stat3-targeted therapy. In contrast to B16 and TSA tumor cells, expression of Stat3β had no detectable effect on the survival of normal NIH 3T3 fibroblasts or the Stat3-negative MethA tumor cells, suggesting that cells lacking constitutively activated Stat3 are resistant to Stat3-targeted therapy. We speculate that in contrast to normal cells or Stat3-negative tumor cells, cells with constitutively activated Stat3 have become dependent on this pathway for survival.

Whereas the precise mechanism by which Stat3β mediates apoptosis in B16 cells is still under investigation, our recent studies in human myeloma cells demonstrated that Stat3β inhibits expression of the Bcl-xL protein (17). These experiments have provided evidence that Stat3β can be a proapoptosis regulator in cells that require Stat3 function for survival. The tumor suppressor protein p53 is another apoptosis regulator capable of mediating repression of Bcl-2 family genes (21). Ectopic expression of high levels of wild-type p53 has been shown to induce apoptosis of various tumor cell lines in vitro. Gene therapy (predominantly using adenoviral vectors) with wild-type p53 has been shown to inhibit tumor growth and, in some cases, induce complete tumor regression in vivo (22–24). However, most of these experiments were performed with human tumor cells in nude mice. In one syngeneic murine model of breast cancer, p53 gene therapy resulted in tumor growth delay, but not in tumor regression (25). The antitumor effect mediated by Stat3β that we report here is more pronounced compared with p53 gene therapy in the murine breast cancer model. Nonetheless, initial reports of p53 gene therapy in clinical trials have described antitumor responses in patients with advanced non-small cell lung cancer (26). Therefore, it is conceivable that Stat3 gene therapy in cancer patients could be successful, as seen for p53 gene therapy.

Although antitumor bystander effects have been observed in tumors treated with p53 gene therapy (24, 27), in vivo studies demonstrating the precise mechanism of the p53-mediated bystander effect are lacking. Nevertheless, a recent report demonstrated 29% growth inhibition of nontransduced cells after p53-transduced and nontransduced cells were cocultured in vitro (27). In the case of B16 tumors treated with the Stat3β gene via electroinjection, the number of apoptotic cells also exceeds the number of cells transfected, consistent with antitumor bystander effects. Experiments are under way to investigate the mechanism by which Stat3β gene therapy may induce antitumor bystander effects. It is also notable that tumor infiltration by acute and chronic inflammatory cells was observed after Stat3β expression. These inflammatory cells may participate in killing of residual tumor cells, suggesting that one strategy to improve the efficacy of anti-Stat3-based therapy is to combine it with immunotherapy. Whereas p53 treatment alone failed to induce impressive tumor regression in a murine breast cancer model, gene therapy with combined interleukin 2 and p53 treatment has been shown to achieve

5 Unpublished data.
long-term and significantly greater antitumor effects than either one alone (25).

Our results provide in vivo proof of principle that Stat3 is a valid molecular target for developing novel therapies against cancers harboring constitutively activated Stat3. In addition to gene therapy, the development of small molecule drugs that specifically inhibit Stat3 signaling would also be highly desirable.

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References

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