Article

STAT3-Decoy ODN Inhibits Cytokine Autocrine of Murine Tumor Cells

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Tumor cells usually secrete soluble factors to improve their proliferation *via* autocrine network or to escape from immune surveillance by inhibiting antitumor immunity, among these factors IL-10 and IL-6 play more important roles. Since both cytokines' signal transductions are mediated through the STAT3 pathway, STAT3 becomes an attractive target for tumor therapy. In present study, STAT3 of murine tumor cell lines B16 and MCA-38 was constitutively activated. After treatment with STAT3-decoy ODN, the proliferation of these tumor cells was inhibited and the transcription of IL-10 or IL-6 in tumor cells was down-regulated. These results suggested that STAT3 is a good target candidate, and STAT3-decoy ODN may possibly be used as a strategy for breaking both tumor autocrine network and tumor immunotolerance. *Cellular & Molecular Immunology*. 2007;4(4):309-313.

Key Words: oligodeoxynucleotide, decoy, STAT3, cytokine

Introduction

Cytokines are important regulatory factors for immune response and play important roles in antitumor defense (1). Cytokines were mainly secreted by lymphocytes such as T cells, macrophages, NK cells and DCs. But many studies demonstrated that tumor cells secreted soluble factors to form a microenvironment which facilitated tumor cell escape from immune surveillance by suppression immune function during tumor development (2, 3). Our previous work (4) showed that tumor cells from solid or hematological malignant cell lines were type II (IL-4, IL-10 and IL-13 higher) predominance and were resistant to NK cytolysis. It was also clear that some key transcription factors control the expressions of several cytokine genes and lead to forming a cytokine profile. Among these transcription factors, STAT3 is a potential negative regulator of inflammatory responses and is commonly constitutively activated in diverse cancers including both hematopoietic and epithelial origin (5-9). Constitutively STAT3 activation enhances tumor cell proliferation and prevents apoptosis. Many tumor-associated factors are activators of STAT3, including IL-10 and IL-6, which secreted endogenously by tumor cells to form autocrine/paracrine loops (10). The association of STAT3 with various cancer types strongly suggests that cytokines play important roles in the development and growth of tumors.

In this study, we used STAT3-decoy ODN, which have high affinity for phosphorylated STAT3, to block STAT3 activation to see if transcription of IL-10 or IL-6 is influenced. The results demonstrated that proliferation of B16 and MCA-38 cells was decreased while IL-10 and IL-6 transcription was down-regulated. These results suggest the importance of STAT3 in production of IL-10 and IL-6 in tumor cell progression.

Materials and Methods

Cell culture

Murine melanoma cell line B16 and murine colon adenocarcinoma cell line MCA-38 conserved in our lab were cultured in RPMI 1640 medium (GIBCO) with 10% fetal bovine serum (FBS) inactivated at 56°C for 30 min. Cells were incubated in the condition of 37°C, 5% CO_2.

STAT3-decoy ODN and scramble control ODN

Phosphorothioated sense and antisense strands for STAT3-decoy ODN and scramble ODN were synthesized by Expedite™ Nucletic Acid Synthesis System (TaKaRa

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Abbreviations: STAT, signal transducer and activator of transcription; ODN, oligodeoxynucleotide; ds, double-stranded; IL, interleukin.

Table 1. RT-PCR primers used for cytokines

Primers	Sequences	Size
β-actin	5'-CTCCTTAATGTCACGCACGATTT-3' 5'-GTGGGGCGCCCCAGGCACCA-3'	539 bp
TNF-α	5'-ACTGGCAGAAGAGGCACTC-3' 5'-CTGGCACCACTAGTTGGTTG-3'	359 bp
IL-6	5'-AGAGACTTCCATCCAGTTGCC-3' 5'-TCTGAAGGACTCTGGCTTTGTC-3'	412 bp
IL-10	5'-AGCTGGACAACATACTGCTAACC-3' 5'-TCATTCATGGCCTTGTAGACAC3-3'	301 bp
IFN-γ	5'-AACGCTACACACTGCATCT-3' 5'-GAGCTCATTGAATGCTTGG-3'	399 bp

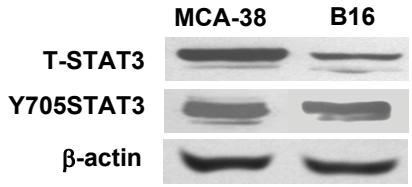

Figure 1. STAT3 was constitutively activated in both B16 and MCA-38 cells. Cell lysates and nuclear extracts were prepared from MCA-38 and B16 cells and were analyzed for STAT3 tyrosine phosphorylation and total STAT3. These extracts were subjected to Western-blot analysis using antibody of total STAT3 (upper panel), Y705 phosphorylated STAT3 (middle panel) and β-actin (lower panel), respectively.

Biotechnology, Dalian). The decoy ODN sequence target phosphorylated STAT3 (11) was 5'-CAT TTC CCG TAA ATC-3', 3'-GTA AAG GGC ATT TAG-5' and the scramble ODN sequence was 5'-CAT CTT GCC AAT ATC-3', 3'-GTA GAA CGG TTA TAG-5'. Both of the sense and antisense strands were annealed and purified by HPLC.

Transient transfections of ODN

Transfections were performed utilizing lipofectamine 2000 (Invitrogen Life Technologies, Carlsbad, CA) according to the manufacturer's instructions. B16 or MCA-38 cells (7×10^3/well) were seeded in 48-well plates in 400 μl medium. After 24 h, cells were transfected with lipofectamine 2000/vehicle (TE), lipofectamine 2000/decoy ODN or lipofectamine 2000/scramble ODN at the concentration of 25 nM to identify the effects of STAT3-decoy ODN on the proliferation of murine tumor cells or transfected with STAT3-decoy ODN at different doses of 12.5, 25 and 50 nM, respectively, to investigate the dose-effect manner.

Western blots

Cells were lysed in 20 mM Tris-HCl buffer (pH 7.4), which contained 150 mM NaCl, 1 mM Na_3VO_4, 1 mM EDTA, 1 mM PMSF, 50 mM NaF and 1% NP-40. Total protein was quantified by the Bradford method (7). The samples (30 μg/lane) were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membrane. Blots were probed with anti-STAT3, anti-phospho-specific STAT3 (Tyr705) or anti-β-actin antibody (Cell Signaling Technology, New England BioLabs Inc.). The proteins were detected by the enhanced chemiluminescence (ECL) system (Pierce, Rochford, IL), and then were exposed to X-ray film (Kodak).

Cell viability assay

B16 and MCA-38 cells were plated and transfected as described above. The cell viability of cells was determined by counting with a hemocytometer by trypan blue exclusion at the time of 24, 48 and 72 h after transfection.

Semi-quantitative RT-PCR assay

TRIzol reagent (Invitrogen, Carlsbad, CA) was used to extract total cellular RNA according to the instructions of the manufacturer. Concentration and quality of the extracted RNA were determined by measuring light absorbance at 260 nm (A_{260}) and the ratio of (A_{260}/A_{280}). Reverse transcription (RT) was performed with 2 μg total RNA according to the manufacturer's protocol of the M-MLV reverse transcriptase (Promega). PCR was carried out using 4× dNTP mixture, $MgCl_2$, Taq DNA polymerase with 10× reaction buffer and upstream primer. Primers for cytokines and the products' sizes were listed in Table 1. Semi-quantitative RT-PCR was performed using β-actin as an internal control. Results were obtained by electrophoresis and the relative light intensities of bands were analyzed by AlphaEaseFC software.

Statistical analysis

Data are expressed as mean ± SD of at least three independent experiments. The results were analyzed using the Student's *t* test. A p value < 0.05 was considered significant.

Results

STAT3 was constitutively activated in both B16 and MCA-38 cells

Like human tumor cells, STAT3 in many murine tumors exhibited constitutively activation. As shown in Figure 1, total protein extracted from murine melanoma cell line B16 and murine colon adenocarcinoma cell line MCA-38 were analyzed by Western blot. Both cell lines showed high levels of phosphorylated STAT3 (Tyr705). This result demonstrated that JAK/STAT3 signaling pathway was activated in both cells.

Blockage of STAT3 activation induced inhibition of murine tumor cell proliferation

MCA-38 cells and B16 cells were transfected with STAT3-decoy ODN, scramble ODN or TE, followed by cell counting assay at different time points. Compared with scramble group, the proliferation of MCA-38 cells and B16 cells treated with STAT3-decoy ODN (25 nM) was significantly inhibited with

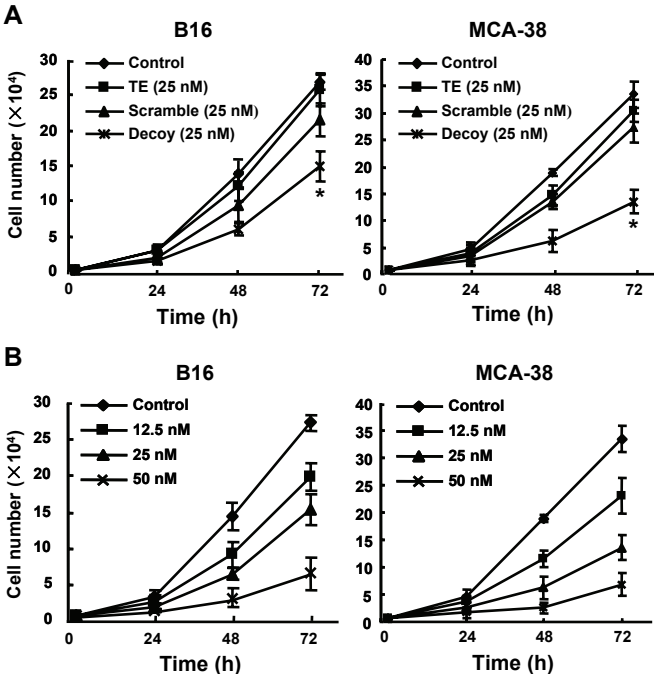

Figure 2. Blockage of STAT3 induced inhibition of murine tumor cell proliferation. (A) Cell numbers of MCA-38 and B16 cells treated with STAT3-decoy ODN were counted by trypan blue exclusion. Compared with scramble ODN or TE treatment, cells treated with STAT3-decoy ODN at the concentration of 25 nM exhibited significant growth inhibition. (B) When treated with different doses (12.5-50 nM) of STAT3-decoy ODN, both cell lines manifested a dose-dependent inhibition of proliferation. Each value represents mean ± SD of results from three independent experiments. *, $p < 0.05$ *vs* control/scramble.

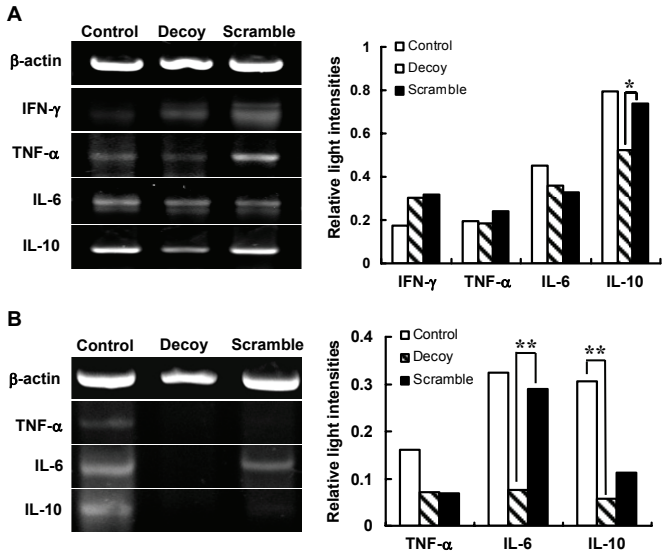

Figure 3. Blockage of STAT3 down-regulated the mRNA levels of IL-10 and IL-6 in MCA-38 and B16 cell lines. As described in Materials and Methods, MCA-38 (A) and B16 (B) cells were transfected with decoy ODN (decoy) or scramble ODN (scramble) at same dose. Twenty-four hours later, total RNA was prepared and mRNA for IL-10, IL-6, IFN-γ, TNF-α and β-actin were amplified by RT-PCR. RT-PCR products were analyzed by agarose gel electrophoresis and were semi-quantitated by relative light intensities with β-actin as an internal control. *, $p < 0.05$; **, $p < 0.01$ *vs* control/scramble.

about 60% and 47%, respectively (Figure 2A). By contrast, STAT3 scramble ODN showed no significant changes. The two cell lines were also transfected with STAT3-decoy ODN at different doses (12.5-50 nM) and cell number was determined at indicated time points. As shown in Figure 2B, the proliferation inhibitory effects of STAT3-decoy ODN were in a dose-dependent manner for both cell lines.

Blockage of STAT3 activation down-regulated the mRNA levels of IL-10 and IL-6 in MCA-38 and B16 cell lines

In mammals, the JAK/STAT pathway is the principal signaling mechanism for a wide array of cytokines and growth factors. Many researches reported that IL-6 and IL-10 signal transduction is mediated through the JAK/STAT pathway. In order to determine whether blockage of STAT3 could break IL-6/IL-10 signal transduction pathway, total RNA was prepared from MCA-38 and B16 cells 24 h post transfection. Then RT-PCR assay was performed and the transcription levels of IL-6, IL-10, IFN-γ and TNF-α were detected. The results showed that type II cytokine mRNAs of IL-6 and IL-10 were higher than that of type I cytokine IFN-γ and TNF-α, to some extent, in the control group of both cell lines (Figure 3). In MCA-38 cells, IL-10 mRNA level was significantly decreased by the treatment of STAT3-decoy ODN about 34.4% (Figure 3A), while IL-6 mRNA in B16 cells showed about 76% decrease (Figure 3B). Although IL-10 mRNA levels in decoy and scramble treated MCA-38 cells showed no significant differences, it was dramatically reduced when compared with control MCA-38 cells. Interestingly, IFN-γ mRNA levels in MCA-38 cells showed a little increase. It seems that the transfection of STAT3-decoy ODN had no significant effect on the expressions of TNF-α.

Discussion

Through binding to their specific receptors, cytokines trigger activation of intracellular signaling cascades and exert multiple biological functions. Based on their biological functions, cytokines were classified by type I (IL-2, IFN-γ) and type II (IL-4, 6, 10, 13). Type II cytokines (IL-4 or IL-13 and IL-10) are inhibitors of type I responses (12-14) and predominance in tumor microenvironment resulted in the inhibition of cell-mediated immune response which are the most suitable for the eradication of malignant cells.

Interleukin-10 is a governor of inflammation produced by various immunocompetent cells, and by tumor cell lines derived from many different cancer types, such as carcinoma of breast, pancreas, kidney, and colon, as well as neuroblastoma (15, 16), activated to regulate the pro-

inflammatory activity of macrophages and dendritic cells which express the highest levels of IL-10 receptor (IL-10R). IL-10 serves as a potent factor for limiting the differentiation and maturation of monocyte-derived DCs and the initiation type 1 response (17-20). IL-10 was also shown to be a useful marker for predicting both surgical removal of the tumor and tumor recurrence (21). IL-6 is a potent, pleiotropic, inflammatory cytokine which is produced by a variety of normal and malignant cells and has been reported to enhance tumor cell growth in myelomas (22) and can promote tumor cell motility *in vitro* (23).

As members of the interleukin classification of signaling proteins, IL-6 and IL-10 signal transduction is mediated *via* the JAK/STAT pathway (24). IL-6 activates predominantly STAT3 (25), and STAT3 is essential for all known functions of IL-10 (26, 27). IL-6 induces IL-10 production through the STAT3 signaling pathway, that IL-6 secreted by macrophages was responsible for IL-10 production by colon adenomacarcinoma cells (28). STAT3 activation was involved in both cytokine signaling pathways and implicated in tumor growth and function, making an attractive target for intervention therapy in tumor cells. Targeting STAT3 may block the expression of many tumor-associated factors, neutralize the tumor-induced immunosuppressive microenvironment and thereby contribute to antitumor immunity. $STAT3^{-/-}$ promotes T cells infiltrating tumors and secreting IFN-γ, reduces Treg cells in tumor, and enhances NK cell cytolysis (29).

In the present work, the control roles of STAT3 were investigated in two murine tumor cell lines B16 and MCA-38. Both cell lines showed high levels of phosphorylated STAT3 (Tyr705), which demonstrated that JAK/STAT3 signaling pathway was consistently activated in both cells. Blocking STAT3 by decoy ODN could inhibit cell growth, and the inhibitory effects showed a dose-dependent manner. At the same time, IL-6/IL-10 transcription levels were also down-regulated. These results suggested that STAT3 may be an ideal target for tumor therapy, and STAT3-decoy ODN may possibly be used as a strategy for breaking both tumor autocrine network and tumor immunotolerance.

Acknowledgements

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References

1. Sveinbjornsson B, Rushfeldt C, Olsen R, Smedsrød B, Seljelid R. Cytotoxic effect of cytokines on murine colon carcinoma cells involves TNF mediated apoptosis. Biochem Biophys Res Commun. 1997;233:270-275.
2. Mann EA, Spiro JD, Chen LL, Kreutzer DL. Cytokine expression by head and neck squamous cell carcinomas. Am J Surg. 1992;164:567-573.
3. Luczynski W, Stasiak-Barmuta A, Ilendo E, et al. Low expression of costimulatory molecules and mRNA for cytokines are important mechanisms of immunosuppression in acute lymphoblastic leukemia in children? Neoplasma. 2006;53:301-304.
4. Zhang J, Sun R, Liu J, Wang L, Tian Z. Reverse of NK cytolysis resistance of type II cytokine predominant-human tumor cells. Int Immunopharmacol. 2006;6:1176-1180.
5. Catlett-Falcone R, Landowski TH, Oshiro MM, et al. Constitutive activation of STAT3 signaling confers resistance to apoptosis in human U266 myeloma cells. Immunity. 1999;10: 105-115.
6. Gouilleux-Gruart V, Gouilleux F, Desaint C, et al. STAT-related transcription factors are constitutively activated in peripheral blood cells from acute leukemia patients. Blood. 1996;87:1692-1697.
7. Gao B, Shen X, Kunos G, et al. Constitutive activation of JAK-STAT3 signaling by BRCA1 in human prostate cancer cells. FEBS Lett. 2001;488:179-184.
8. Grandis JR, Drenning SD, Zeng Q, et al. Constitutive activation of STAT3 signaling abrogates apoptosis in squamous cell carcinogenesis *in vivo*. Proc Natl Acad Sci U S A. 2000;97: 4227-4232.
9. Hodge DR, Hurt EM, Farrar WL. The role of IL-6 and STAT3 in inflammation and cancer. Eur J Cancer. 2005;41:2502-2512.
10. Fang M, Dai H, Yu G, Gong F. Gene delivery of SOCS3 protects mice from lethal endotoxic shock. Cell Mol Immunol. 2005;2: 373-377.
11. Leong PL, Andrews GA, Johnson DE, et al. Targeted inhibition of STAT3 with a decoy oligonucleotide abrogates head and neck cancer cell growth. Proc Natl Acad Sci U S A. 2003;100: 4138- 4143.
12. Paul WE. Interleukin-4: a prototypic immunoregulatory lymphokine. Blood. 1991;77:1859-1870.
13. Zurawski G, de Vries JE. Interleukin 13 elicits a subset of the activities of its close relative interleukin 4. Stem Cells. 1994; 12:169-174.
14. Moore KW, de Waal Malefyt R, Coffman RL, O'Garra A. Interleukin-10 and the interleukin-10 receptor. Annu Rev Immunol. 2001;19:683-765.
15. Gastl GA, Abrams JS, Nanus DM, et al. Interleukin-10 production by human carcinoma cell lines and its relationship to interleukin-6 expression. Int J Cancer. 1993;55:96-101.
16. Kim J, Modlin RL, Moy RL, et al. IL-10 production in cutaneous basal and squamous cell carcinomas: a mechanism for evading the local T cell immune response. J Immunol. 1995; 155:2240-2247.
17. Corinti S, Albanesi C, la Sala A, Pastore S, Girolomoni G. Regulatory activity of autocrine IL-10 on dendritic cell functions. J Immunol. 2001;166:4312-4318.
18. Morel AS, Quaratino S, Douek DC, et al. Split activity of interleukin-10 on antigen capture and antigen presentation by human dendritic cells: definition of a maturative step. Eur J Immunol. 1997;27:26-34.
19. De Smedt T, Van Mechelen M, De Becker G, Londei M. Effect of interleukin-10 on dendritic cell maturation and function. Eur J Immunol. 1997;27:1229-1235.
20. Buelens C, Verhasselt V, De Groote D, Thielemans K, Goldman M, Willems F. Interleukin-10 prevents the generation of dendritic cells from human peripheral blood mononuclear cells cultured with interleukin-4 and granulocyte/macrophage-colony-stimulating factor. Eur J Immunol. 1997;27:756-762.
21. Galizia G, Orditura M, Romano C, et al. Prognostic significance of circulating IL-10 and IL-6 serum levels in colon cancer

patients undergoing surgery. Clin Immunol. 2002;102:169-178.
22. Bataille R, Barlogie B, Lu ZY, et al. Biologic effects of anti-interleukin-6 murine monoclonal antibody in advanced multiple myeloma. Blood. 1995;86:685-691.
23. Asschert JG, Vellenga E, Ruiters MH, de Vries EG. Regulation of spontaneous and TNF/IFN-induced IL-6 expression in two human ovariancarcinoma cell lines. Int J Cancer. 1999;82: 244-249.
24. Heim MH. The Jak-STAT pathway: cytokine signaling from the receptor to the nucleus. J Recept Signal Transduct Res. 1999;19: 75-120.
25. Ahmed ST, Ivashkiv LB. Inhibition of IL-6 and IL-10 signaling and Stat activation by inflammatory and stress pathways. J Immunol. 2000;165:5227-5237.
26. Murray PJ. Understanding and exploiting the endogenous interleukin-10/STAT3-mediated anti-inflammatory response. Curr Opin Pharmacol. 2006;6:379-386
27. Gao B. Cytokines, STATs and liver disease. Cell Mol Immunol. 2005;2:92-100.
28. Herbeuval JP, Lelievre E, Lambert C, Dy M, Genin C. Recruitment of STAT3 for production of IL-10 by colon carcinoma cells induced by macrophage-derived IL-6. J Immunol. 2004;172:4630-4636.
29. Kortylewski M, Kujawski M, Wang T, et al. Inhibiting Stat3 signaling in the hematopoietic system elicit multicomponent antitumor immunity. Nat Med. 2005;11:1314-1321.