Activation of the JAK-STAT3 pathway is associated with the growth of colorectal carcinoma cells

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Abstract. Excessive activation of inflammatory signaling pathways facilitates colorectal carcinoma (CRC) malignancy. Continuous activation of the Janus kinase (JAK)/signal transducer and activator of transcription 3 (STAT3) pathway plays a central role in the development and progression of CRC. With the intent to explore whether attenuation of the JAK-STAT3 signaling axis inhibits cancer cell proliferation or induces apoptosis, a sophisticated oncolvtic adenoviral vector, AdCN305, carrying the SOCS3 gene was used to treat CRC cells. Our data revealed that i) in CRC cells, STAT3 was continuously activated by phosphorylation, and SOCS3 was at a relative low expression level; and ii) AdCN305-cppSOCS3 inhibited the continuous activation of the JAK/STAT3 pathway, suppressed CRC cell growth and induced apoptosis, in vitro and in vivo. We proved that SOCS3, a negative regulator of the JAK-STAT3 pathway, efficiently inhibited the activation of the pathway and decreased levels of downstream factors which regulate cell proliferation and the cell cycle.

Introduction

Epidemiological studies have revealed that chronic inflammation predisposes individuals to colorectal carcinoma (1) and increases the risk of carcinogenesis of colorectal tissue (2). Inflammatory cytokines (for example, IL-6 and INF- α) influence the behavior of colorectal cells in the inflammatory microenvironment through the JAK-STAT pathway (3). Signal transducer and activator of transcription (STAT) proteins play a key role in determining whether immune responses in the tumor microenvironment promote or inhibit tumorigenesis.

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Continuous activation of STAT3 signaling increases tumor cell proliferation, survival and invasion while the antitumor immunosystem is suppressed by the inflammatory microenviroment. The persistent activation of STAT3 also mediates tumor-promoting inflammation (4). Consequently, STAT3 has an important role in tumorigenesis and may be a promising target for cancer therapy.

Suppressor of cytokine signaling 3 (SOCS3) is one of the negative regulators of cytokine signaling that functions via the JAK/STAT pathway (5). SOCS3 binds the cytokine receptor with high affinity with its Src homology 2 protein domains and attenuates the activity of JAKs via its kinase inhibitory region in order to abolish STAT3 phosphorylation (6). In addition, it has been revealed that SOCS3 is frequently silenced in several types of tumor cells (7-9).

Our previous studies demonstrated that oncolytic adenoviral vectors carrying apoptosis-inducing genes induce strong antitumor activity (10-12). As we deleted the CR2 region of the vector genome which promotes the native E1A by hTERT, the oncolytic adenoviral vector was selectively amplified in most tumor cells. Meanwhile, endogenous major late promoter (MLP) in the adenoviral genome was found to promote cpp-SCOS3 expression, and the promoter contributed to the tumor-specific viral replication of adenoviral vectors and more effectively attenuated tumor cell malignancy (10).

In the present study, we treated colorectal carcinoma (CRC) cells with an oncolytic adenovirus that expressed the *SOCS3* gene and found that CRC cell growth was suppressed by overexpression of SOCS3. Finally, the data suggested that the recombinant adenovector has high efficacy in inhibiting CRC cell proliferation and promoting apoptosis *in vitro* and *in vivo*.

Materials and methods

Cell culture. The HEK293 cell line, the control cell line L02, and CRC cell lines HCT-116, HT-29 and SW620 were purchased from the Shanghai Cell Collection (Shanghai, China) and were cultured in RPMI-1640 or DMEM containing 10% fetal bovine serum (FBS; Gibco-Life Technologies, Grand Island, NY, USA) at 37°C in 5% CO₂. All cells were infected by the adenoviral vectors which expressed the genes of interest for further examination.

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Recombinant adenoviruses. The vectors AdCN305cppSOCS3, Ad-WT and AdCN305-HcRed were constructed as previously reported (10-12). Briefly, human SOCS3 CDS and fibroblast growth factor 4 cpp sequences were obtained from the cell line by PCR and inserted into pBS/IRES, and then the pBS/IRES-cppSOCS3 plasmid was constructed. Subsequently, the IRES-cppSOCS3 fragment was cloned into the pCZ305 plasmid, and the recombinant plasmid pCN305-cppSOCS3 was generated by the recombination of pCZ305-IRES-cppSOCS3 and pCN103 plasmid in *E coli*. CN305-HcRed was obtained followed the above protocol. The recombinant adenoviruses were amplified in HEK293 cells and purified by cesium chloride gradient ultracentrifugation. The recombinant adenoviruses were titrated by a plaque assay in HEK293 cells.

Cell viability assay. Cells were seeded on 96-well plates at a density of 1×10^4 /well in $100 \,\mu$ l complete medium. Twenty-four hours later, they were infected with a wild-type adenovirus (Ad-wt), adenovirus CN305 expressing HcRed fluorescence protein (AdCN305-HcRed) and AdCN305-cpp-SOCS3 at a multiplicity of infection (MOI) of 10, respectively. Twenty microliters of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma Chemical Co., St. Louis, MO, USA) solution (5 mg/ml) was added to each well at 24, 48, 72 and 96 h after infection. Plates were incubated at 37°C for 4 h, and then 150 μ l of DMSO was added to each well and shaken for 10 min. Finally, the absorbance was read at 595 nm with a DNA-Expert (Tecan).

Total RNA isolation and qRT-PCR. HCT-116, HT-29 and SW620 cell lines were grown to 80% confluence in 6-well plates and then infected by the three vectors (Ad-WT, AdCN305-HcRed and AdCN305-cppSOCS3). The culture medium was then replaced by RPMI-1640 medium for 48 h. RNA samples were then prepared, followed by complimentary DNA (cDNA) synthesis and qRT-PCR analysis which was performed on an ABI-7400 instrument. The following primers were employed: SOCS3-F (5'-gcacaagcacaaaaatccagc-3') and SOCS3-R (5'-agaa gccaatctgcccctg-3') for the SOCS3 gene; survivin-F (5'-ggcatgggt gccccgacgttg-3') and survivin-R (5'-cagaggcctcaatcccatggca-3'); for the survivin gene; Bcl-2-F (5'-tgtggccttctttgagttcg-3') and Bcl-2-R (5'-cacttgtggctcagatagg-3') for the Bcl-2 gene; c-Myc-F (5'-tggtcttcccctaccctctcaac-3') and c-Myc-R (5'-gatccagactct gaccttttgcc-3') for the c-Myc gene; cyclin D1-F (5'-ctgtgctgc gaagtggaaaccat-3') and cyclin D1-R (5'-ttcatggccaggcgggaagacc tc-3') for the cyclin D1 gene and GAPDH-F (5'-gacatcaagaaggtggt gaagc-3') and GAPDH-R (5'-gtccaccacctgttgctgtag-3') for the GAPDH gene. Transcript abundance was first normalized to the level of GAPDH mRNA and then compared to each other.

Protein preparation and western blot analyses. Total cellular proteins were prepared from the cells under different culture conditions using a previously described method (9). For western blot analyses, the sample proteins (50 μ g/well) were separated by electrophoresis in 10% sodium dodecylsulfatepolyacrylamide gel electrophoresis (SDS-PAGE), and the separated protein bands were transferred to a polyvinylidene difluoride membrane (Amersham, Buckinghamshire, UK). After the membrane was blocked with 5% skimmed milk in TBS-T (10 mM Tris-HCl, pH 8.0, 150 mM NaCl and 0.5% Tween-20) it was maintained at 4°C overnight, and then rinsed three times (10 min each time) with TBST, followed by a 3-h incubation at room temperature with the primary antibodies at appropriate concentrations. Incubation with HRP-conjugated anti-mouse or rabbit IgG was carried out for 1 h (Zymed Laboratories Inc., San Francisco, CA, USA). The bound antibody was detected using the enhanced chemiluminescence system (Roche GmbH, Mannheim, Germany). After removing the labeling signal by incubation with stripping buffer (62.5 mM Tris-HCl, pH 6.7, 100 mM 2-mercaptoethanol, 2% SDS) at 55°C for 30 min, the membrane was re-detected with other antibodies one by one following the same experimental procedure until all of the parameters were examined.

Apoptotic cell staining. The cells seeded in chamber slides were infected with the recombinant adenoviruses or phosphate-buffered saline (PBS). After a 48-h infection, the cells were incubated with Hoechst 33258 (Molecular Probes, Eugene, OR, USA) for 10 min, washed with PBS twice and observed under a fluorescence microscope and images were captured.

Tumor xenografts in nude mice. All animals used in these experiments were maintained at institutional facilities and received humane care according to the criteria outlined in the Guide for the Care and Use of Laboratory Animals. Female BALB/c nude mice (4-5 weeks of age) were obtained from the Animal Research Committee of the Institute of Biochemistry and Cell Biology (Shanghai, China). Mice were inoculated subcutaneously with SW620 cells ($2x10^6$ for each mouse). When the tumor volume reached 100-150 mm³, the inoculated mice were randomly divided into five groups. An intratumoral injection of each of the adenoviruses ($5x10^8$ PFU/dose) with 50 μ l of PBS was performed once every other day for a total of 4 times. After therapy, the tumor size was measured with a vernier caliper every 7 days. The tumor volume (mm³) was calculated as follows: (length * width²)/2.

Immunocytochemical staining. Sections from the frozen tumor samples (on day 6 after treatment) were stained with antibodies. Immunohistochemical staining was performed on the coverslips obtained from each of the experimental groups. The antibodies against STAT3, phosphorylated-STAT3 (p-STAT3), Bcl-2 were purchased from Cell Signaling Technology (Beverly, MA, USA) and were used according to the manufacturer's instructions. Briefly, the coverslips were washed with phosphate-buffered solution (PBS, pH 7.4), incubated for 10 min in 3% H_2O_2 and then diluted with the appropriate primary antibody at 37°C for 60 min in a humidity chamber, followed by the biotinylated peroxidase-conjugated streptavidin system (BioGenex Laboratories, San Ramon, CA, USA) and at last photographed (DP70 digital camera; Olympus, Tokyo, Japan) under a microscope (BX51; Olympus).

Statistical analysis. The statistical significance of the data was calculated with an analysis of variance and a one-sided Student's t-test with Microsoft Excel. Data were considered to indicate a statistically significant result at P<0.05.



Figure 1. Expression of SOCS3 and STAT3 in CRC cell lines, HCT-116, HT-29 and SW620, and the normal cell line, L02. The results of (A) western blot analysis and (B) real-time PCR of SOCS3 expression in the CRC cell lines compared to that in L02 cells.



Figure 2. Induction of cytotoxicity by the oncolytic adenoviral vectors *in vitro*. The CRC cell lines (HCT-116, HT-29 and SW620) and the normal cell line (L02) were infected with Ad-wt, AdCN305-HCRed and AdCN305-cppSOCS3, respectively. Cell viability was determined by MTT assay at different times after infection. The cell viability was calculated as a percentage with respect to cells without viral infection. The data are presented as the means \pm standard deviation of three independent experiments.

Results

Expression of SOCS3 in the CRC cell lines. To elucidate the the effect of SOCS3 expression on CRC cells, we analyzed expression of endogenous SOCS3 in different CRC cell lines and in a normal cell line (L02) by western blot analysis (Fig. 1A) and qRT-PCR (Fig. 1B). The expression of the SOCS3 transcripts was markedly reduced in the CRC cell lines HT-29, HCT-116 and SW620. In contrast, a high level of SOCS3 expression was noted in the L02 normal cell line (Fig. 1A).

Expression and the phosphorylation status of STAT3 in the CRC cell lines. SOCS3 is a negative regulatory factor of the JAK/STAT3 pathway in inflammation signaling. We examined the level of STAT3 expression and the phosphorylation status of STAT3. We found that the level of STAT3 expression was similar in all of the tested cell lines (CRC cell lines and the normal cell line). However, phosphorylation of STAT3 was much higher in the tumor cells than that in the normal cells (Fig. 1A). These data suggest that loss of SOCS3 expression correlates with persistent STAT3 phosphorylation and promotes the growth of tumor cells consistent with previous observations (13,14).

Cytotoxicity induced by the oncolytic adenoviral vectors in CRC cells. To analyze the antitumor efficacy of recombinant adenoviruses, three CRC cell lines (HCT-116, HT-29 and SW620) and a normal cell line (L02) were infected with AdCN305-cppSOCS3, AdCN305-HcRed or Ad-wt at an MOI of 10 with PBS as a negative control. Cytotoxicity was determined by MTT assay and Hoechst 33258 staining. As shown in Figs. 2 and 3, control vector AdCN305-HcRed induced a cytotoxic effect in the tumor cells similar to that induced by Ad-wt. However, AdCN305-cppSOCS3 markedly reduced the viability in all three CRC cell lines. Furthermore, AdCN305-cppSOCS3 induced even higher cytotoxicity in the tumor cells when compared with that induced by Ad-wt and AdCN305-HcRed. In addition, AdCN305-cppSOCS3 and AdCN305-HcRed did not induce obvious cytotoxicity to the normal cells due to the selective replication ability of the adenoviral vector (Figs. 2 and 3).

Inhibition of STAT3 phosphorylation and its downstream targets by overexpression of SOCS3. As previously stated, recombinant oncolytic adenoviruses harboring the gene cppSOCS3 suppress the growth of tumor cells *in vitro*, while



Figure 3. Induction of apoptosis by oncolytic adenoviral vectors (original magnification, x200). HT-29 cells were infected with AdCN305-cppSOCS3, Ad-wt or control vector AdCN305-HcRed at a MOI of 10 or were not infected. The cells were stained with Hoechst 33258 at 48 h after infection: (A) AdCN305-cppSOCS3, (B) AdCN305-HcRed, (C) Ad-wt and (D) PBS. The arrows indicate apoptotic cells; the original magnification was x200.



Figure 4. Expression of cell proliferation-associated (c-Myc, cyclin D1 and surviving) and cell cycle-associated factors (Bcl-2) in CRC cell lines (SW620 HT-29, HCT-116 and) at 72 h after infection by AdCN305-cppSOCS3.

the mechanisms of STAT3 activity which are negatively regulated by SOCS3 are not clear. In our experiment, in the three CRC cell lines transfected with AdCN305-cppSOCS3 or Ad-wt, several downstream factors of the JAK-STAT3 pathway were detection by qRT-PCR. Our results showed that AdCN305-cppSOCS3 inhibited STAT3 phosphorylation and induced apoptosis. The factors, survivin, cyclin D1, Bcl-2 and c-Myc are involved in promoting cancer cell proliferation and apoptosis, and all of these factors are regulated by the JAK-STAT3 pathway directly or indirectly. As shown in Fig. 4, all of the above factors exhibited a decreased expression level as revealed using qRT-PCR, and the results indicated that expression of SOCS3, the upregulated negative feedback factor of the JAK-STAT3 pathway, efficiently downregulated cancer proliferation-associated factors in CRC cells. These results demonstrated that the SOCS3 protein was a potent negative regulator of CRC cell growth and induced CRC cell death by reducing the expression of cancer proliferation-associated genes.

Antitumor activity of the oncolytic adenoviral vectors in an established tumor animal model. To evaluate the antitumor activity of the recombinant adenoviruses *in vivo*, a transplanted CRC mouse model was established based on implantation of human CRC cells (SW620) in nude mice. When the tumors reached 100-150 mm³, the animals were treated with an intra-



Figure 5. Antitumor activity by oncolytic adenoviral vectors in established tumors in an animal model. Tumors were established in nude mice by implantation of human SW620 tumor cells. When the tumors reached 100-150 mm³, the animals were treated with an intratumor injection of AdCN305-cpp-SOCS3, AdCN305-HcRed, Ad-wt or PBS as a control. The tumor size was measured, and the tumor volume was calculated as follows: length * width²/2. The data are expressed as the means of the tumor volume ± the standard deviation.

tumor injection of AdCN305-cppSOCS3, AdCN305-HcRed, Ad-wt or PBS.

In the control animals that received PBS, the tumors grew progressively during the course of the experiment. In contrast, the animals treated with control vector AdCN305-HcRed or Ad-wt exhibited a significant suppression of tumor development (P<0.01). Treatment with AdCN305-cppSOCS3 resulted in significant inhibition of tumor growth in comparison with animals treated with AdCN305-HcRed, Ad-wt or PBS (P<0.01) (Fig. 5).

In vivo STAT3 phosphorylation is further analyzed in transplanted tumor tissues by immunohistochemistry. As shown in Fig. 6, treatment with AdCN305-cppSOCS3 resulted in marked inhibition of STAT3 phosphorylation. In addition, histopathological analysis of tumor tissue sections revealed that treatment with AdCN305-cpp-SOCS3 resulted in a higher extent of necrosis than that induced by AdCN305-HcRed, Ad-wt and the PBS control.

Discussion

STAT3, a member of the STAT family, has been proven to act as a tumorigenesis driver, an oncogene. Researchers have previously assessed the efficicacy of STAT3 as a therapy for malignancies. For example, they performed repression of upstream-related receptors of the pathway (15), dimerization of STAT3 (16), transcription activation by nuclear entry of protein STAT3 and binding to target *cis* elements of the associated genes (17). Various factors which usually inhibit cancer cell apoptosis and promote progression of the cell cycle such as c-Myc, survivin and cyclin D1 (7,10,14) are commonly over-expressed as a results of STAT3 overactivity. Due to important



Figure 6. Immunohistochemical staining for phospho-Stat3 and histopathological examination of tumor tissues after treatment with oncolytic adenoviral vectors (original magnification, x200). Tumor-bearing animals were treated with AdCN305-SOCS3, AdCN305-cpp-SOCS3, Ad-wt or PBS as a control. Six days after treatment, the animals were sacrificed, and the tumors and livers were collected.

role that this pathway plays in cancer cell progression, more and more researchers have focused on JAK/STAT3 signaling. SOCS3, a member of the SOCS family that has a negative effect on the JAK/STAT pathway, indirectly suppresses the activity of JAKs and plays an important role in the immune response and embryonic development (18-21).

In the present study, we employed a selectively replicating adenoviral vector (AdCN305) expressing SOCS3 which exhibits strong activity against hepatocarcinoma cells (10). Replication of the AdCN305 vector system depends on overexpression of hTERT and dysfunction of the retinoblastoma tumor-suppressor gene in vector-infected cells. Simultaneously, the SOCS3 gene was fused with the cpp gene (cell-penetrating peptide), which has the capacity to deliver peptides, proteins, and oligonucleotides into intact cells (22,23). In addition, in the backbone of the adenovector, expression of the SOCS gene was controlled by an endogenous adenoviral major late promoter (MLP) which restricts SOCS3 expression within the tumor microenvironment due to tumor-specific viral replication (24). A previous study showed that this recombination manipulation of the oncolytic vector did not affect replication of the virus (10). As a result, we aimed to ascertain whether overexpression of SOCS3 affects CRC which is believed to be initiated partially by local inflammation.

Our data suggest that the recombinant vector effectively suppressed proliferation of CRC cells. In contrast to the control vectors, AdCN305-cppSOCS3 exhibited strong cytotoxicity in CRC cells, and this effect was even higher than that induced by Ad-wt. More importantly, the vectors did not induce irrelevant cytotoxicity in normal cells as did AdCN305-HcRed (red fluorescence protein).

The positive effect induced by AdCN305-cppSOCS3 may be partially due to the cppSOCS3 fusion protein which can enter the surrounding tumor cells, which are not infected by oncolytic adenoviral vectors directly, with the help of the cpp peptide. The application of cpp for intercellular traffic of proteins has been successfully explored *in vitro* and *in vivo* (10,23).

To decipher the mechanism of the inhibition of CRC growth by the vector, we analyzed the expression of several factors downstream of the STAT3 pathway with qRT-PCR. The results showed that AdCN305-cppSOCS3 resulted in high expression of SOCS3, and a normal level of SOCS3 effectively suppressed hyperphosphorylation of the STAT3 protein and blocked the proliferation of CRC cells. We also found that several oncogenes (c-Myc, cyclin D1 and survivin) and an anti-apoptosis gene (Bcl-2) were expressed at a high level in the CRC cells, while the oncolytic vector carrying the SOCS3 gene decreased the expression of these two types of genes and facilitated apoptosis of the tumor cells.

To further examine the therapeutic potential of the AdCN305-cppSOCS3 vectors on CRC *in vivo*, we treated the established tumors in an SCID mouse model with the vector. Consistent with the *in vitro* results, a dramatic inhibition of STAT3 phosphorylation was noted in the xenograft tumor cells that were infected with AdCN305-cppSOCS3. The infection of tumor cells with AdCN305-cppSOCS3 also resulted in a reduction in the expression of Bcl-2 and induced apoptosis of tumor cells. These data indicate that transfer of the SOCS3 gene into the tumors suppressed tumor growth and inhibited activation of STAT3.

In summary, we found that overexpression of SOCS3 inhibited JAK/STAT3 signaling and induced programmed apoptosis in CRC cells. At the same time, various downstream genes of STAT3 pathway, such as survivin, cyclin D1 and c-Myc were also downregulated. These results suggest that hyperphosphorylation of STAT3 and overactivity of STAT3 signaling may have an important role in the malignancy maintaining of colorectal cells.

In colorectal carcinoma cells, decreased SOCS3 expression definitely exists, accompanied by hypophosphorylation of STAT3. As a result, the associated cell signals of the inflammatory pathway promote and maintain CRC cell growth. Recombinant oncolytic adenovirus AdCN-305-cppSOCS3 was found to selectively replicate in CRC cells *in vitro* and is relative safe when used *in vivo*. Restoration of SOCS3 was obviously achieved by expression of the adenoviral vector in cells, and a simultaneous antitumor effect was achieved by inhibiting constitutive STAT3 phosphorylation *in vitro* and *in vivo*. The present study provides a method for developing a novel cancer gene therapeutic strategy.

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