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Inhibition of the JAK-STAT3 pathway by andrographolide enhances chemosensitivity of cancer cells to doxorubicin

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ABSTRACT

Andrographolide (Andro), a diterpenoid lactone isolated from a traditional herbal medicine *Andrographis paniculata*, is known to possess potent anti-inflammatory and anticancer properties. In this study, we sought to examine the effect of Andro on signal transducer and activator of transcription 3 (STAT3) pathway and evaluate whether suppression of STAT3 activity by Andro could sensitize cancer cells to a chemotherapeutic drug doxorubicin. First, we demonstrated that Andro is able to significantly suppress both constitutively activated and IL-6-induced STAT3 phosphorylation and subsequent nuclear translocation in cancer cells. Such inhibition is found to be achieved through suppression of Janus-activated kinase (JAK)1/2 and interaction between STAT3 and gp130. For understanding the biological significance of the inhibitory effect of Andro on STAT3, we next investigated the effect of Andro on doxorubicin-induced apoptosis in human cancer cells. In our study the constitutive activation level of STAT3 was found to be correlated to the resistance of cancer cells to doxorubicin-induced apoptosis. Both the short-term MTT assay and the long-term colony formation assay showed that Andro dramatically promoted doxorubicin-induced cell death in cancer cells, indicating that Andro enhances the sensitivity of cancer cells to doxorubicin mainly via STAT3 suppression. These observations thus reveal a novel anticancer function of Andro and suggest a potential therapeutic strategy of using Andro in combination with chemotherapeutic agents for treatment of cancer.

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1. Introduction

Signal transducers and activators of transcription (STAT) proteins are a family of seven proteins (STATs 1, 2, 3, 4, 5a, 5b, and 6) that mediate signal transduction from extracellular signals to transcription of target genes. Among the STATs, STAT3 is the most intimately linked to tumorigenesis [1,2]. Numerous studies have shown a constitutive activation of the STAT3 pathway in a variety of human cancers, including breast carcinoma, colon cancer, cervical cancer, prostate cancer, melanoma, multiple myeloma, and leukemia [2,3].

STAT3 and its upstream Janus-activated kinase (JAK) signaling were originally identified as the signaling pathway for interferon (IFN). It mediates the immune responses of various cytokines as well as many growth factors and hormones, and thus participates in inflammation, cell growth and metastasis [4,5]. STAT3 can be activated by a number of cytokines, including interleukin-6 (IL-6), IL-11, ciliary neurotrophic factor, oncostatin M, and leukemia inhibitory factor, which all signal through a shared gp130 signal transducer receptor subunit [2]. For IL-6-induced STAT3 activation, upon IL-6 binding to IL-6R, gp130 is recruited to IL-6R, leading to binding and trans-phosphorylation of JAK. Activated JAK then phosphorylates the tyrosine residues of gp130. The phosphotyrosine side chains of gp130 serve as docking sites for latent transcription factors of the STAT3, resulting in homodimerization or heterodimerization and subsequently nuclear localization and DNA binding of STAT3 [4]. Fully activated STAT3 regulates transcription of specific target genes, including anti-apoptotic proteins (Bcl-xL, Mcl-1) [6,7] and proliferation regulatory proteins (cyclinD1, Myc and survivin) [8–10].

Abbreviations: Andro, andrographolide; DAPI, 4,6-diamidino-2-phenylindole; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; DN, dominant negative; FBS, fetal bovine serum; IFN, interferon; IL, interleukin; JAK, Janus kinase; MTT, 3(4,5-dimethylthiazol-2-yl)2,5-diphenyl-tetrazolium bromide N-acetylcysteine; NF-κB, nuclear transcription factor-kappaB; PARP, poly(ADP-ribose) polymerase; PBS, phosphate buffered saline; SDS, sodium dodecyl sulfate; STAT, signal transducers and activators of transcription; TRAIL, TNF-related apoptosis-inducing ligand; XIAP, X-linked inhibitor of apoptosis.

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STAT3 signaling pathway has recently been shown to confer resistance to chemotherapy-induced apoptosis in human tumors, due to its involvement in the proliferation, angiogenesis, immune evasion and anti-apoptosis of cancer cells and its aberrant activation in various tumor cells [4,11,12]. Thus, abrogation of STAT3 activation is believed to render tumor cells more susceptible to cancer therapeutic agents.

Andro is a diterpenoid lactone isolated from *Andrographis paniculata*, an anti-inflammation herbal medicine, which has been used for the treatment of various ailments including respiratory infection, bacterial dysentery and fever [13,14]. As the major active component contained in *Andrographis paniculata*, Andro has been shown to be responsible for the anti-inflammatory activity, mainly via its inhibitory effect on nuclear transcription factor-kappaB (NF-κB) [15]. In our previous studies, we demonstrated that Andro possesses potent anticancer property through promoting apoptosis in cancer cells. Andro alone or combined with TRAIL could induce both mitochondria-mediated and death receptor-mediated apoptotic cell death [16,17], supporting the development of Andro as an apoptosis inducer or chemo-sensitizer in combined therapy. However, whether Andro could inactivate other survival signaling pathways to achieve its anti-tumor effect remains unclear. In the present study, we sought to examine the effect of Andro on the JAK-STAT3 pathway and evaluate whether suppression of STAT3 activity by Andro could sensitize cancer cells to a common chemotherapeutic drug doxorubicin.

2. Materials and methods

2.1. Cell culture and reagents

Andro was purchased from Sigma (Cat #365645) as a pure compound, as described in our earlier studies [16,17]. Human cancer cells, HCT116, MDA-MB-231, HepG2 and HeLa, were purchased from ATCC. Human papillary thyroid cancer cells (TPC-1) and human anaplastic thyroid cancer cells (ARO) have been described previously [18]. All cell lines were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. 4,6-diamidino-2-phenylindole (DAPI), ANTI-FLAG® antibody and other common chemicals were all purchased from Sigma–Aldrich (St. Louis, MO). A JAK specific inhibitor, Pyridone 6, anti-human phospho-(Tyr1007/1008)-JAK2 antibody, and recombinant human IL-6 were purchased from Merck (San Diego, CA). pSTAT3 luciferase vector was obtained from Panomics (Redwood City, CA). Anti-human phospho-(Tyr705)-STAT3, phospho-(Ser727)-STAT3, STAT3, Bcl-xL, caspase-3 and PARP antibodies were from Cell Signaling (Beverly, MA). Anti-human Mcl-1, cyclinD1, phospho-(Tyr1022)-JAK1, gp130 (C20) and tubulin were from Santa Cruz (Santa Cruz, CA).

2.2. Detection of cytotoxicity and apoptosis

The general cytotoxicity of doxorubicin and Andro on different human cancer cells was detected by the MTT test as described previously [19] and the results were presented as the relative cell viability compared with the control group. Cells undergoing apoptosis were evaluated by DAPI staining for morphological changes including chromatin condensation and nuclear shrinkage, as previously reported [20]. Briefly, at the end of designated experiments, the cells were fixed with 70% ethanol at room temperature for 10 min and stained with 0.3 μg/mL DAPI (in PBS) at room temperature for another 10 min and visualized under an inverted fluorescence microscope. The cells with condensed nucleus were visualized and counted under an inverted fluorescent microscope (Nikon ECLIPSE TE2000-S, Nikon Instruments, Tokyo, Japan).

2.3. Colony formation assay

Cancer cells (HCT116, MDA-MB-231, HeLa, and HepG2) were treated as designated for 12 h before reseeded in six-well plates (5000 cells/well), respectively. After 2 weeks, the survival clones were stained by 0.5% crystal violet for 1 h and photos were taken using digital camera.

2.4. Luciferase assay

The transient transfection of pSTAT3 luciferase vector was done in MDA-MB-231 cells using LipofectAMINE PLUS transfection reagent according to the manufacturer's protocols. *Renilla* luciferase vector was used as a transfection control. The luciferase activity was measured in the cellular extracts using a Dual-Luciferase Reporter Assay System (Promega) based on the protocol provided by the manufacturer. Briefly, following the treatments, the cell lysate was collected from each well after the addition of cell lysis reagent. After adding the luciferase assay substrate, the firefly luciferase activity was determined using a luminometer (Promega) and the *Renilla* luciferase activity was then measured by adding the Stop&Glo substrate.

2.5. Preparation of nuclear and cytosolic extracts

Nuclear extracts and cytosolic extracts were prepared by NE-PER® nuclear and cytoplasmic extraction reagents (Pierce) according to the manufacturer's protocol. Briefly, after washed with PBS, cell pellets were collected by centrifugation at 500 × *g* for 3 min, and removed the supernatant. Cell pellets were incubated with cytoplasmic extract reagent I for 10 min then added with ice-cold cytoplasmic extract reagent II. After 5 min centrifugation at maximum speed, supernatant was collected and considered as cytoplasmic extract. The insoluble fraction was resuspended with ice-cold nuclear extract reagent and incubated for a total of 40 min, and centrifuged at maximum speed for 10 min. The supernatant was collected immediately as nuclear extract. Protein concentration was determined using Bio-Rad Protein Assay reagent.

2.6. Transient transfection and RNA interference

HCT116 cells were transfected with Flag-STAT3 vectors (wild type STAT3 and dominant negative STAT3 vectors) as described previously [18], using LipofectAMINE PLUS (Invitrogen) following the manufacturer's instructions. Cells were treated as indicated for 24 h after transfection. For the RNA interference study, synthetic small interfering RNAs (scrambled siRNA and STAT3 siRNA) were from Santa Cruz (Santa Cruz, CA). The cellular delivery of siRNA was carried out by using LipofectAMINE PLUS and optimized with various doses and post-transfection time and evaluated by Western blot experiment in HeLa cells.

2.7. Immunoprecipitation and Western blot

Immunoprecipitation was performed briefly by following the instructions of the manufacturers of the respective antibodies. In brief, the cells were washed three times with cold PBS and lysated in 1 mL RIPA buffer (150 mM NaCl, 50 mM Tris–HCl [pH 7.2], 1% Triton X-100, 0.1% SDS, 0.25 mM EDTA [pH 8.0]) containing protease inhibitor cocktails (Roche). Cell lysates containing 3 mg of protein were incubated with appropriate antibody at 4 °C overnight or 50 μl ANTI-FLAG® M1 Monoclonal Antibody-Agarose Affinity Gel (Sigma). Immune complexes were captured by adding 50 μl of protein A/G agarose beads and rotated as 4 °C for 3 h (this step was omitted when ANTI-FLAG® M1 Monoclonal Antibody-Agarose Affinity Gel was used). After five times washing of protein

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