Basic Research Paper

Effects of a JAK inhibitor, AG490, on proliferation and apoptosis of human nasopharyngeal carcinoma cell line CNE-2Z

Min-Hua Wu,1 Xiao-Yi Chen2,* and Kang-Rong Cai3

1Department of Histology and Embryology; 2Department of Pathology; 3Analysis Center, Guangdong Medical College; Zhanjiang, Guangdong P.R. China

Key words: nasopharyngeal neoplasm, CNE-2Z cells, signal transduction and activator of transcription 3, apoptosis, survivin, Mcl-1, AG490

Background and Objective: Abnormal activation of Janus kinases/signal transducer and activator of transcription 3 (JAK-STAT3) signaling pathway is closely related to malignant transformation of cells. This study was to investigate the effects of a JAK inhibitor, AG490, on the proliferation, apoptosis, and expressions of apoptosis-related survivin and Mcl-1 genes, thus to explore the role of JAK-STAT3 signaling pathway in the regulation of cell apoptosis in nasopharyngeal carcinoma (NPC) cell line CNE-2Z. Methods: CNE-2Z cells were treated with different doses of AG490. The cell proliferation was measured using MTT array. Cell apoptosis was assessed by flow cytometry (FCM) and Hoechst33342 fluorescence staining. The mRNA levels of STAT3, survivin and Mcl-1 were detected by reverse transcription-polymerase chain reaction (RT-PCR). The protein contents of STAT3, phosphorlated STAT3 (p-STAT3), survivin and Mcl-1 were measured by western blot. Results: The inhibition rates of CNE-2Z cell proliferation by 100 μmol/L AG490 were 37.95% and 52.99% at 24 and 48 h after treatment. After incubation with 50 μmol/L and 100 μmol/L AG490 for 24 h, the apoptotic rates of CNE-2Z cells were increased from 1.37% to 9.30% and 9.00%, respectively (p < 0.01); typical changes in apoptotic morphology were observed under fluorescence microscopy; moreover, activation of STAT3 was inhibited, mRNA and protein levels of Mcl-1 and survivin were down-regulated. Conclusion: Blocking of JAK-STAT3 signaling pathway in CNE-2Z cells could remarkably inhibit cell proliferation and inactivate STAT3, as well as promote cell apoptosis by down-regulating Mcl-1 and survivin.

Abnormal activation of signal transducer and activator of transcription 3 (STAT3) signaling pathway is closely related to abnormal proliferation and malignant transformation of cells.1 In many cells, abnormal activation of STAT3 is due to the abnormal activation of Janus kinase (JAK kinases).2 Over-expression3 and constitutive activation4 of STAT3 are found in nasopharyngeal carcinoma (NPC) cells. This study investigated the effects of a JAK kinase inhibitor, AG490, on proliferation and apoptosis of CNE-2Z cells and the underlying mechanisms.

Materials and Methods

Materials. Poorly-differentiated human NPC cell line CNE-2Z was provided by Tumor Institute of Guangdong Medical College. AG490 was purchased from Alexis Inc. (USA). RNA extraction reagent (TRizol) was purchased from Invitrogen Inc. (USA). One-step RT-PCR kit was the product of Qiagen Inc. (Germany). PCR primers were synthesized by Shanghai ShengGong Biotechnology Services Co., Ltd. Mouse anti-human Mcl-1 monoclonal antibody was bought from NeoMarkers Inc. (USA), and rabbit anti-human STAT3 polyclonal antibody was purchased from Signalway Inc. (USA). Mouse anti-human survivin monoclonal antibody and mouse anti-human β-actin monoclonal antibody were products of Santa Cruz Inc. (USA). Horseradish peroxidase (HRP)-labeled goat anti-rabbit IgG and HRP-labeled goat anti-mouse IgG were purchased from Beijing ZhongShan Goldenbridge Biotechnology Co., Ltd. Cell protein extraction kit and the Bradford protein quantification kit were purchased from Beijing Apply Gene Technology Co., Ltd.

Methods. Cell culture. CNE-2Z cells were routinely recovered, plated in the culture flask with RPMI-1640 containing 10% neonate calf serum, 100 U/mL penicillin and 100μg/mL streptomycin at 37°C and an atmosphere of 5% CO2. Cells were digested and passaged upon 80% confluence.

MTT assay. CNE-2Z cells in the logarithmic phase of growth were digested and prepared as single cell suspension. Cell density was adjusted to 2.5 x 104/mL and plated into 96-well plates at 200 μL/well (about 5,000 cells). Culture medium was completely removed and replaced by fresh medium containing AG490 at final concentrations of 0, 25, 50, 75 or 100 μmol/L, respectively after cell attachment. Six replicate wells were performed per sample, and cells were cultured for 24 to 48 h. After culture medium was discarded, 20 μL 5 mg/mL MTT was added into each well and cells were continued to culture for 4 h. Then medium was carefully removed and 200 μL DMSO was added. After shaking, the absorbance value (A value) for each well was measured at 570 nm wavelength by an
Effects of a JAK inhibitor, AG490, on proliferation and apoptosis of human nasopharyngeal carcinoma cell line CNE-2Z.

Flow cytometry (FCM) When CNE-2Z cells were in the logarithmic phase of growth, medium was replaced by fresh medium containing different concentrations of AG490. Cells were cultured for 12, 24 and 48 h, digested, centrifuged at 180 x g for 8 min, washed by cold PBS twice, fixed by pre-cooled 70% ethanol and preserved at 4 °C overnight. Before FCM, ethanol was removed by centrifugation. Cells were washed by cold PBS twice and re-suspended in 0.2 mL cold PBS. Cells were stained by 0.6 mL PI in dark for 30 min at 4°C. FCM was then applied to detect cell apoptosis after cells were filtered by a nylon mesh strainer. Experiments were repeated three times.

Western blot Cells were processed as described for the preparation for RT-PCT. Total protein was extracted according to the protocol provided with the commercial kit. The Bradford assay was applied to determine the protein concentration. An equal amount of proteins was separated by 10% SDS polyacrylamide gel electrophoresis and transferred to a membrane. The membrane was blocked in 50 g/L non-fat milk at room temperature for 2 h and then incubated with the primary antibodies (Mcl-1 and survivin, 1:300; β-actin, STAT3 and p-STAT3, 1:1000) at 4 °C overnight. Then the secondary antibodies were added (1:5,000), and incubated at room temperature for 2 h. The film was developed according to the HPR luminescent method and β-actin was included as the internal control. The relative content of the target protein was determined by the ratio of the OD value of the target protein band to that of the internal control band. Experiments were repeated three times.

Discussion JAK-STAT3 signaling pathway is an important intracellular signal transduction pathway in the processes of cell growth, differentiation, apoptosis and function execution in many cells. Activation of JAK-STAT3 can induce expressions of many genes, such as apoptosis suppressor genes Bc1-xL and Mcl-1, cell cycle control genes c-myc, STAT3, survivin, p-STAT3, and others. In this study, we investigated the effects of AG490 on the proliferation and apoptosis of human nasopharyngeal carcinoma cell line CNE-2Z. The results showed that AG490 significantly inhibited cell proliferation at different concentrations. The inhibitory rate of 100 µmol/L AG490 on CNE-2Z cells at 24 and 48 h after treatment were 37.95% and 52.99%, respectively (Fig. 1). Moreover, AG490 significantly induced apoptosis in CNE-2Z cells. The apoptosis rate of cells treated with different concentrations of AG490 was significantly higher than that of the control group (p < 0.01). The apoptosis rate of cells treated with AG490 at 24 and 48 h were 37.95% and 52.99%, respectively (Fig. 2).

Western blot analysis showed that AG490 significantly inhibited the expression of STAT3, p-STAT3, survivin and Mcl-1 proteins. The mRNA expression levels of STAT3, survivin and Mcl-1 were also significantly reduced after treatment with AG490 (p < 0.01). These results suggested that AG490 could effectively inhibit the proliferation and induce apoptosis of CNE-2Z cells through the JAK-STAT3 signaling pathway.
Effects of a JAK inhibitor, AG490, on proliferation and apoptosis of human nasopharyngeal carcinoma cell line CNE-2Z

Table 1  Influence of AG490 on apoptosis of CNE-2Z cells detected by flow cytometry

<table>
<thead>
<tr>
<th>Group</th>
<th>Apoptotic rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>12 h</td>
</tr>
<tr>
<td>Control</td>
<td>0.83±0.40</td>
</tr>
<tr>
<td>25 μmol/L AG490</td>
<td>3.23±1.03a</td>
</tr>
<tr>
<td>50 μmol/L AG490</td>
<td>2.87±0.51a</td>
</tr>
<tr>
<td>100 μmol/L AG490</td>
<td>2.00±0.95</td>
</tr>
<tr>
<td>F value</td>
<td>6.019</td>
</tr>
<tr>
<td>P value</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

All values are presented as mean ± SD of three independent experiments. aP < 0.05 vs. control group; bP < 0.01 vs. control group.

Figure 1. Proliferation inhibition effects of AG490 on CNE-2Z cells detected by MTT assay. aP < 0.05 vs. control group. All values are presented as mean ± SD of three independent experiments.

Figure 2. Fluorescence staining of CNE-2Z cells before and after the treatment with AG490 (Hoechst33342 × 400). Compared with normal cells in control group (A), there are many apoptotic cells after the treatment of 50 μmol/L AG490 for 24 h (B) and 48 h (C), with the appearance of condensed nuclei, compacting chromatin at the margin of the nuclei and nuclear fragmentation.

cyclin D1 and p21, as well as the transcription of angiogenesis-related gene vascular endothelial growth factor (VEGF). Under normal physiological conditions, activation of STAT3 is prompt and temporary. Over-activation of STAT3 can induce abnormal up-regulation of the above mentioned key genes, thereby promote cell proliferation, malignant transformation, and impede apoptosis through various pathways, which would eventually lead to carcinogenesis.1

STAT3 is abnormally highly expressed in many tumor cells. There are evidences to show the involvement of STAT3 in tumorigenesis, indicating that STAT3 has the potential to become a new therapeutic target for cancer therapy. Since activation of STAT3 pathway in many cells is resulted from abnormal activation of its upstream tyrosine kinase JAK, JAK kinase inhibitors, such as AG490, can effectively block STAT3 signaling pathway. Studies reveal that after AG490 treatment, expression levels of p-STAT3 and survivin in breast cancer cells are significantly decreased.5 In addition, AG490 could block JAK/STAT3 signaling pathway in colorectal cancer cells, down-regulate Bcl-2 gene expression, and upregulate the gene expressions of p16, p21 and VEGF and so on,6 resulting in inhibition of cell proliferation and induction of apoptosis. Our findings show that after AG490 treatment for 24 and 48 h, AG490 significantly inhibited proliferation of CNE-2Z cells in a dose- and time-dependent manor. In addition, we also observed morphological changes typical of apoptosis in CNE-2Z cells after AG490 treatment. Moreover FCM detected a marked increase of apoptosis in cells treated by AG490. The apoptosis was more obvious at 24 h than after 48 h of treatment.

Survivin and Mcl-1 are both apoptotic suppressor genes. Survivin is a member of inhibitor of apoptosis proteins (IAPs) family. It suppresses apoptosis mainly through directly or indirectly interacting with cysteinyl aspartate-specific proteases (caspases), such as caspase-8, caspase-3 and caspase-7.7 Myeloid cell leukemia-1 (Mcl-1) is a member of bcl-2 gene family. Current researches on Mcl-1 focus on hematological malignancies. Mcl-1 is highly expressed in multiple myeloma and in a variety of leukemia and lymphoma cells,8 and it is an important survival factor for multiple myeloma cells. Zhang et al.9 claim that, Mcl-1 antisense oligonucleotides cause rapid down-regulation of Mcl-1 protein and induce apoptosis of myeloma cells. In addition, over-expression of Mcl-1 delays the apoptosis induced by actinomycin D, an antineoplastic antibiotic.

After AG490 treatment, we found that the protein contents of p-STAT3, Mcl-1 and survivin were decreased significantly, while that of STAT3 remained unchanged; similarly, mRNA expressions of Mcl-1 and survivin were reduced, while no obvious change in STAT3 mRNA level was detected. We propose that AG490 suppresses STAT3 activity by direct inhibition of STAT3 phosphorylation without affecting its gene expression. Meanwhile, our results also suggest that AG490 induces apoptosis in CNE-2Z cells partially through down-regulating the expression of Mcl-1 and survivin. Since the target genes of STAT3 also include many other apoptosis-related genes, such as bcl-2 and bcl-xL, whether these genes are involved in
Effects of a JAK inhibitor, AG490, on proliferation and apoptosis of human nasopharyngeal carcinoma cell line CNE-2Z

Figure 3. Apoptosis of CNE-2Z cells after the treatment with AG490 at different time points detected by flow cytometry. (A) Control group; (B) Cells treated with 50 μmol/L AG490 for 12 h; C: Cells treated with 50 μmol/L AG490 for 24 h; D: Cells treated with 50 μmol/L AG490 for 48 h.

Figure 4. Expressions of STAT3, Mcl-1 and survivin mRNA in CNE-2Z cells after AG490 treatment detected by RT-PCR. Lane M: DNA ladder; Lanes 1-4: CNE-2Z cells treated with 0, 25, 50, 100 μmol/L AG490 for 24 h, respectively.

Figure 5. Expressions of STAT3, Mcl-1 and survivin proteins in CNE-2Z cells after AG490 treatment detected by western blot. Lanes 1-4: CNE-2Z cells treated with 0, 25, 50, 100 μmol/L AG490 for 24 h, respectively.
AG490-induced apoptosis in NPC cells as well needs to be further investigated.

Acknowledgements
Grant: Natural Science Foundation of Guangdong Province (No. 031963)

References