Abstract.
Nuclear factor-κB (NF-κB) is constitutively activated in most malignant gliomas and is involved in cancer progression and drug resistance to chemotherapy. Sulfasalazine (SAS) is a classic inhibitor of NF-κB. Apoptosis and autophagy were induced by SAS accompanied by inhibition of NF-κB signaling in U251 cells. Inhibition of autophagy by 3-MA suppressed the effects of SAS on NF-κB signaling and apoptosis in U251 cells. Multifunctional scaffold protein p62 is well known as an autophagy marker protein and provides crosstalk for important signaling pathways, including NF-κB signaling. SAS-induced decrease in the p62 protein levels may be the result of degradation through autophagy. SAS induced the inhibition of NF-κB signaling and apoptosis at least partly via a p62-dependent effect in U251 cells. Collectively, our data shed light on the link between p62 and the NF-κB signaling pathway, particularly in glioma cells. The results may facilitate the design of more effective targeted therapies for the treatment of tumors in which NF-κB signaling is altered.

Introduction

Nuclear factor-κB (NF-κB) is an important tumor-related nuclear transcription factor and is overexpressed and continuously activated in malignant glioma cells, thereby playing a role in promoting the survival of tumor cells (1,2). Sulfasalazine (SAS) has been widely used as an anti-inflammatory drug in the clinic. Because of its significant antitumor effect on human glioma, colon cancer, breast cancer, lymphoma and other malignant cells (3-7), it has been used in a phase 1/2 prospective, randomized study for the treatment of progressive malignant glioma (8). At present, most studies suggest that the antitumor mechanism of SAS is related to its effective inhibition of NF-κB signaling (9-11).

Autophagy is an evolutionarily conserved cellular metabolic process. It can promote cell survival and may also promote cell death via different mechanisms. Autophagy plays different roles depending on the drug, cell type or time of drug action and its mechanism is not fully understood (12-15). Therefore, study of the dual role of autophagy may provide new clues for tumor treatment.

Research has shown that NF-κB regulates autophagy by upregulating the mRNA and protein levels of the autophagy promoter Beclin 1; it can also inhibit autophagy by activating autophagy inhibitor mTOR (16). Other research has shown that autophagy can also inactivate NF-κB activity by degrading its upstream IκB kinases (IKKs) (17). Recent research found that SAS induced autophagy at the same time as causing the growth inhibition of human small cell lung cancer cell lines NCI-H69 and NCI-H82 (18). Yet, whether SAS induces autophagy in other types of tumor cells and the related mechanism of autophagy in the antitumor effect of SAS still require further investigation.

Our previous research demonstrated that the multifunctional protein p62/sequestosome 1 (encoded by SQSTM1) is involved in the autophagic degradation process of ubiquitinated proteins as an adaptor protein and therefore it plays an important role in the mechanism by which autophagy promotes human ovarian cancer cells to survive (19). p62 is a multiple domain protein, which can combine with ubiquitin through the UBA domain and can bind the autophagy regulatory protein LC3 via the LIR domain, resulting in these proteins performing autophagic degradation. Several studies have shown that autophagy can suppress the development and progression of tumors by downregulating the levels of p62 protein (20,21). In addition, p62 can also act with an apoptosis-
regulating molecular switch RIP1 by zinc-finger domains to form a signaling complex, which functions as a scaffold protein in the activation of IKKs, leading to degradation of the inhibitory molecule IκB and resulting in the activation of NF-κB signaling (22-25). A study demonstrated that knockdown of p62 with an antisense construct severely impaired the activation of NF-κB in response to TNFα (26). Duran et al also proved that p62 is necessary for the survival of human lung adenocarcinoma cells (27); therefore, high expression of p62 can be used as a marker of NF-κB signaling activation (28).

SAS can inhibit glioma growth, but whether it does this by influencing autophagy to inhibit the activity of NF-κB is not clear. Furthermore, it is important to elucidate whether the multifunctional protein p62 participates in the regulation of NF-κB signaling by SAS. Therefore, we inhibited autophagy by 3-MA and inhibited the mRNA and protein expression of p62 by RNA interference (RNAi) technology, to investigate whether both autophagy and p62 participate in the anti-glioma mechanism of SAS, in order to provide insight into a potential new target for the antitumor therapy of SAS.

Materials and methods

Cell lines. The human glioma U251 cell line was obtained from the Department of Pathophysiology, Jilin University Norman Bethune Medical College. The cells were cultured in Iscove's modified Dulbecco's medium (IMDM; Gibco-BRL, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Invitrogen Life Technologies, Carlsbad, CA, USA) and cultured at 37°C in 5% CO₂ with high humidity.

Cell viability assays. Cells were plated at a density of 0.7x10⁵ cells/well in 96-well plates. The next day, different concentrations of SAS were added to the wells and incubated for 4, 8 and 24 h. Each treatment was repeated in 5 wells. To each well, we added 20 µl MTT (Sigma-Aldrich, St. Louis, MO, USA) and incubated the plates for 4 h; 150 µl dimethyl-sulphoxide was then added to dissolve the formazan crystals. Absorbance was measured with a VMax microplate reader (Molecular Devices, Sunnyvale, CA, USA) at a wavelength of 570 nm.

Flow cytometry. After exposure to different experimental conditions, cells were trypsinized and incubated with propidium iodide (PI; 1 µg/ml) and Annexin V-FITC (1 µg/ml; Invitrogen) for 15 min at room temperature. Samples were then analyzed for apoptosis by a FACSscan flow cytometer (Becton-Dickinson, Franklin Lakes, NJ, USA) within 1 h.

Western blot analysis. Lysate proteins (30-50 µg) were separated by 12% w/v SDS-polyacrylamide gel electrophoresis and transferred onto PVDF membranes (Millipore, Bedford, MA, USA). Membranes were blocked with 5% nonfat dry milk in buffer [10 µM Tris-HCl (pH 7.6), 100 µM NaCl and 0.1% Tween-20] for 1 h at room temperature, incubated with the desired primary antibody overnight at 4°C and then incubated with the horseradish peroxidase-conjugated secondary antibody (Thermo Scientific, Waltham, MA, USA) at a 1:2,000 dilution for 1 h at room temperature. Immunoreactive bands were visualized using the DAB (Sigma-Aldrich) coloration method. Protein levels were quantified by densitometry using Quantity One software (Bio-Rad).

Indirect immunofluorescence staining and confocal laser microscopy. Cells were fixed with 4% paraformaldehyde, stained with Hoechst 33258 (2 µg/ml, Sigma-Aldrich) for 30 min and examined using confocal laser microscopy to observe apoptotic nuclei. For indirect immunofluorescence staining, cells were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100 and blocked with bovine serum albumin. They were then incubated with primary antibodies against p65 and p50 (1:50 dilution) overnight at 4°C, and then in FITC/Rhodamine Red-conjugated secondary antibodies (1:400 dilution) (all antibodies, Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 0.5 h and stained with Hoechst 33258 (2 µg/ml) for 2 min and examined by confocal fluorescence microscopy.

p62 knockdown by small interfering RNA (siRNA). siRNA sequences targeting human p62/SQSTM1 (GeneBank Accession NM_003900) and a non-target sequence were constructed by Genechem (Shanghai, China). The p62 siRNA (si-p62) sequence was GAC-ATC-TTC-CGAATC-TAC-A and that of non-target siRNA (scramble) was TTC-TCC-GAA-CGT-TG-TC-3'; GAPDH: forward, 5'-GGG-TGA-TGC-TGG-TGC-CTA-CAG-AT-3' and reverse, 5'-CGA-TGT-CAT-AGT-TCT-TGA-GTA-TGC-3'. PCR products were run on 1% agarose gel. Reverse transcription was performed to generate cDNA, which was then amplified by PCR. The sequences of the primers as follows: p62: forward, 5'-GAA-CTC-CAG-TCC-GAA-CTC-TGT-GTA-GTA-AGT-3' and reverse 5'-AAG-AAT-GGG-AGT-TGA-GTA-TGC-3'; GAPDH: forward, 5'-GGG-TGA-TGC-TGG-TGC-CTA-CAG-AT-3' and reverse, 5'-CGA-TGT-CAT-AGT-TCT-TGA-GTA-TGC-3'. PCR products were run on 1% agarose gel electrophoresis containing ethidium bromide, visualized by figure gel image processing system and analyzed by GIB ID gel image system software (Tanon, Shanghai, China). The ratio of p62 and GAPDH reflected the changes in p62 levels.

Statistical analysis. Experiments were performed at least 3 times and data are presented as mean ± SD. Statistical analysis of the data was performed using one-way ANOVA; differences between treatment means were examined with
Dunnett’s tests. P<0.05 was considered to indicate a statistically significant difference.

Results

Apoptosis and autophagy are induced by sulfasalazine (SAS) in U251 cells. U251 cells were treated with different doses of SAS and stained with Hoechst 33258. Cell morphology was observed by laser scanning confocal microscopy (scale bar, 10 µm; arrows indicate apoptotic cells). From the resulting IC₅₀ values, we selected 1.5 µM SAS for the treatment of U251 cells at different time intervals.

We observed cell nuclei stained with Hoechst 33258 using confocal laser scanning microscopy. The results revealed that SAS induced apoptosis in the U251 cells (Fig. 1B). Research suggests that autophagy may be involved in the antitumor effect of SAS (18). Therefore, we analyzed the protein expression of the autophagy marker protein LC3 in response to 1.5 µM SAS by western blot analysis and showed that the protein expression ratio of LC3-II to LC3-I was significantly increased by SAS treatment for 8 h (Fig. 1C and D). Furthermore, indirect immunofluorescence showed that LC3 had translocated to the cytoplasm, forming punctate aggregates and the fluorescence intensity of LC3 was also enhanced (Fig. 1E), suggesting that SAS can induce autophagy in U251 cells.

Most studies suggest that SAS has an antitumor effect by inhibiting NF-κB signaling (9-11). Therefore, we investigated the expression of IκBα in response to 1.5 µM SAS by western blot analysis. The results showed that the ratio of p-IκBα to IκBα protein expression was significantly decreased following treatment with SAS for 8 h (Fig. 2A and B). In addition, the transcriptional activity of NF-κB was reduced by 1.5 µM SAS in U251 cells as detected by dual luciferase reporter assays (Fig. 2C). The intracellular localizations of the NF-κB subunits p65 and p50 were detected by indirect immunofluorescence, and the results showed that p65 and p50 were mainly distributed in the cytoplasm following SAS treatment for 8 h (Fig. 2D). This finding suggests that SAS can effectively inhibit NF-κB signaling.
Inhibition of autophagy by 3-MA suppresses the effects of SAS on NF-κB signaling and apoptosis in U251 cells. 3-MA as a classic autophagy inhibitor has been widely used in related research (29,30). Previous research used 3-MA to inhibit autophagy and prove that autophagy is involved in the growth inhibition of hepatoma cells induced by SAS (31). Therefore, we treated U251 cells with a combination of 5 μM 3-MA and 1.5 μM SAS for 8 h and detected the protein expression of LC3 by western blot analysis; SAS combined with 3-MA resulted in a reduction in the protein expression ratio of LC3-II to LC3-I compared with this ratio following SAS alone (Fig. 3A and B). This suggests that 3-MA effectively inhibits SAS-induced autophagy. The results of MTT assays showed that the viability of U251 cells was significantly increased by SAS combined with 3-MA (Fig. 3D and E). Therefore, inhibition of autophagy by 3-MA reduced the apoptosis induced by SAS in U251 cells.

These findings motivated us to ascertain whether inhibition of autophagy by 3-MA can also impact NF-κB signaling at the same time. Compared with the cells treated with SAS alone, the protein expression ratio of p-IκBα to IκBα was slightly increased in the cells treated with SAS combined with 3-MA (Fig. 3F and G). Moreover, dual luciferase assays demonstrated that the transcriptional activity of NF-κB was significantly enhanced (Fig. 3H), suggesting that inhibition of autophagy can weaken SAS-induced inhibition of NF-κB signaling.

Inhibition of autophagy by 3-MA weakens p62 reduction by SAS in U251 cells. Multifunctional scaffold protein p62 is well known as an autophagy marker protein and provides crosstalk for important signaling pathways, including NF-κB signaling (24,32). We detected the expression of p62 in
response to 1.5 µM SAS by western blot analysis and found that p62 expression was decreased in a time-dependent manner (Fig. 4A and B). However, RT-PCR results showed that the mRNA levels of p62 remained basically unchanged (Fig. 4C and D). Therefore, we speculated that the SAS-induced decrease in p62 protein levels may not be the result of reduced p62 transcript levels, but may be caused by other processes, such as post-translational modifications. Compared with the cells treated with SAS alone, the protein expression of p62 was increased in the cells treated with SAS combined with 3-MA (Fig. 4E and F). These results indicate that inhibition of autophagy can result in increased p62 protein expression.

SAS induces NF-κB signaling inhibition and apoptosis via a p62-dependent effect in U251 cells. Since 3-MA can enhance the protein expression of p62 by inhibiting autophagy, the
changing trend was similar to the transcriptional activity of NF-κB. We applied RNAi technology to inhibit p62 expression (12). After the si-p62 recombinant plasmids were transfected into U251 cells for 24 h, we found that p62 protein expression was significantly decreased as detected by western blot analysis (Fig. 5A and B). RT-PCR analysis showed that the mRNA levels of p62 were also significantly decreased (Fig. 5C and D).

In order to identify whether autophagy is involved in the regulation of NF-κB signaling in a p62-dependent manner, we applied RNAi technology to inhibit p62 expression, 5 µM 3-MA to inhibit autophagy and then 1.5 µM SAS to treat U251 cells for 8 h and detected the protein expression of IκBα by western blot analysis.

Knockdown of p62 did not influence the expression ratio of p-IκBα and IκBα. Yet, p62 inhibition inverted the enhancement of the ratio of p-IκBα to IκBα by 3-MA in the SAS-treated U251 cells (Fig. 5E and F). In addition, p62 suppression further decreased the transcriptional activity of NF-κB in the U251 cells treated with SAS and 3-MA (Fig. 5G). Similarly, we detected the viability of U251 cells by MTT assays and found that knockdown of p62 reversed the enhancement of cell viability by 3-MA in the SAS-treated U251 cells (Fig. 5H). These results demonstrate that the effects of 3-MA on SAS-treated U251 cells are dependent on p62.

Discussion

NF-κB is one of the most important nuclear transcription factors related to inflammation and tumors. Activation of the NF-κB signaling pathway is involved in cancer progression and chemotherapy drug resistance (33). NF-κB is constitutively activated in most malignant gliomas (1,2,9,34,35). Zanotto-Filho et al used siRNA to knockdown NF-κB-p65 and proved that inhibition of NF-κB induces the apoptosis of glioma U138MG cells (34). Furthermore, Robe et al showed that SAS effectively inhibited NF-κB and induced apoptosis of glial stromal tumor U87 and LN18 and human glioma U251 cell lines (9). Thus, in-depth study concerning the regulatory mechanism of the NF-κB signaling pathway is of significance to further understand glioma biological characteristics and to develop new glioma therapeutic strategies. In the present study, we utilized SAS, a drug that can effectively inhibit the activity of NF-κB, and has recently been considered as a potential anti-tumor drug (36,37). The results showed that SAS effectively inhibited NF-κB signaling. SAS also induced apoptosis and autophagy in the U251 cells.

As an evolutionarily conserved cellular metabolic process, autophagy has conflicting roles in cell death according to the context in different research. Therefore, we were interested in the role of autophagy induced by SAS in U251 cells. Based on previous studies, we utilized classic autophagy inhibitor 3-MA in our research. We found that inhibition of autophagy by 3-MA suppressed the apoptosis induced by SAS in U251 cells. We also detected the activation of NF-κB signaling. The inhibition of the transcriptional activity of NF-κB by SAS was weakened by 3-MA. Thus, we aimed to ascertain how suppression of autophagy affects the NF-κB signaling pathway.

Multifunctional scaffold protein p62 is well known as an autophagy marker protein and provides crosstalk for important signaling pathways, including NF-κB signaling (24,32). Accumulating research has confirmed that p62 participates in the regulation of NF-κB signaling, yet the mechanism is not fully understood (27,38). Our previous study indicated that p62 is involved in the mechanism of cisplatin-resistance through
Autophagy in ovarian cancer cells (19). Thus, we focused on the role of p62 in the suppression of NF-κB signaling induced by SAS. We detected the protein expression of p62 in response to SAS and found that p62 expression was decreased in a time-dependent manner. However, RT-PCR results showed that the mRNA levels of p62 remained basically unchanged. Therefore, we speculated that SAS-induced decrease in p62 protein levels may not be the result of reduced transcript levels.

Figure 5. Sulfasalazine (SAS) induces NF-κB signaling inhibition and apoptosis via a p62-dependent effect in U251 cells. (A) Cells were transfected with p62 siRNA (si-p62) or non-target sequence siRNA (si-Scramble) recombinant plasmid for 24 h, and the protein expression of p62 was detected by western blot analysis. (B) Quantitation of p62 protein. *P<0.05, compared with the control group; n=3. (C) U251 cells were transfected with si-p62 or si-Scramble and the mRNA expression of p62 was detected by RT-PCR analysis. (D) Quantitation of p62 mRNA. *P<0.05, compared with the control group; n=3. (E) U251 cells were transfected with si-p62 for 24 h and treated with 1.5 µM SAS for 8 h with or without 5 µM 3-MA. The protein expression levels of p-IκBα and IκBα were detected by western blot analysis. (F) Quantitation of the ratio of p-IκBα to IκBα. *P<0.05, compared with each other; n=3. (G) U251 cells were transfected with si-p62 for 24 h and treated with 1.5 µM SAS for 8 h with or without 5 µM 3-MA. NF-κB transcriptional activity was analyzed by luciferase reporter assays. *P<0.05, compared with each other; n=3. (H) U251 cells were transfected with si-p62 for 24 h and treated with 1.5 µM SAS for 8 h with or without 5 µM 3-MA. Cell viability was analyzed by MTT assays. *P<0.05, compared with each other; n=3. (I) U251 cells were transfected with si-p62 for 24 h and treated with 1.5 µM SAS for 8 h with or without 5 µM 3-MA, then stained with Hoechst 33258. Cell morphology was observed by laser scanning confocal microscopy (scale bar, 10 µm; arrows indicate apoptotic cells).
but may be caused by other processes, such as degradation. Inhibition of autophagy by 3-MA reversed the decrease in p62 induced by SAS. Furthermore, the altered tendency of p62 is in keeping with the transcriptional activity of NF-κB.

To test our hypothesis, we utilized RNAi technology to inhibit p62 expression. The results showed that knockdown of p62 weakened the effects of 3-MA on NF-κB signaling inhibition induced by SAS in U251 cells. Consistent with this, after inhibition of p62, the protective effect of 3-MA on SAS-treated U251 cells disappeared; the viability of the cells was decreased and apoptotic cells were increased.

This indicates that SAS induces NF-κB signaling inhibition and apoptosis at least partly via a p62-dependent effect in U251 cells. The results based on the in vitro study indicate that p62 is an important regulatory factor of NF-κB signaling transduction process. Given that the NF-κB signaling pathway involves autophagy and apoptosis, this finding is consistent with our results. Yet, further studies are needed to confirm whether p62 provides signaling activation center through aggregation or promotes IκBα degradation through protein degradation pathways including autophagy.

This study demonstrated that apoptosis and autophagy were induced by SAS accompanied by inhibition of NF-κB signaling in U251 cells. Inhibition of autophagy by 3-MA can suppress the effects of SAS on NF-κB signaling and apoptosis in U251 cells. p62 is involved in the mechanism of NF-κB signaling inhibited by SAS in U251 cells.

In conclusion, the identification of the association of p62 and NF-κB signaling sheds light on the link between p62 and the NF-κB signaling pathway, specifically in tumors. Therefore, p62 can act to nucleate different signaling molecules to ensure the efficiency and selectivity of the signal transduction process. Given that the NF-κB signaling pathway is commonly deregulated in cancer, the recent identification of p62 as a critical step in this pathway may help in the design of better targeted therapies for the treatment of tumors in which NF-κB signaling is altered.

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References