JBUON 2020; 25(2): 1076-1083
ISSN: 1107-0625, online ISSN: 2241-6293 • www.jbuon.com
Email: editorial_office@jbuon.com

ORIGINAL ARTICLE

Bleomycin inhibits proliferation and promotes apoptosis of brain glioma cells *via* TGF-β/Smad signaling pathway

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Summary

Purpose: *To investigate the influence of bleomycin (BLM) on the proliferation and apoptosis of brain glioma cells through transforming growth factor-β (TGF-β)/Smads signaling pathway.*

Methods: *The U87 brain glioma cells were cultured in vitro and reacted with different concentrations of BLM (5 and 10 mU/mL), and the cell growth status of each group was observed under a microscope. The cell proliferation activity was detected using Cell Counting Kit-8 (CCK-8) assay, the percentage of 5-Ethynyl-2'-deoxyuridine (EdU)-positive cells in each group was determined via EdU staining, and the apoptosis of U87 cells was tested by means of terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining. In addition, reverse transcription-polymerase chain reaction (RT-PCR) was performed to measure the messenger ribonucleic acid (mRNA) levels of genes related to proliferation, apoptosis and the TGF-β/Smads signaling pathway. Finally, western blotting assay was performed to analyze the expression of the TGF-β/Smads signaling pathway.*

Results: *In the 5 mU/mL BLM group, the glioma cells were in a poor growth status, with a low density, while the 10 mU/mL BLM group exhibited the poorest growth status and the lowest density, and the morphological structure trended toward normal. It was discovered via CCK-8 assay and EdU staining that the number of cells and proliferation activity were decreased markedly in the 10 mU/mL BLM group. According to TUNEL staining, 10 mU/mL BLM group had remarkably increased apoptotic cells, while negative control (NC) group had fewer apoptotic cells. The gene assay results revealed that the gene expressions of Bcl-2 and TGF-β1 declined notably in the 10 mU/mL BLM group but rose in the NC group, and the gene expression trends of Caspase-3 and Smad4 were the opposite. The protein assay results manifested that the expressions of TGF-β1 was obviously reduced, while that of Smad4 was evidently raised in the 10 mU/mL BLM group.*

Conclusion: *BLM at an appropriate concentration can inhibit the proliferation and promote apoptosis of brain glioma cells by repressing the TGF-β/Smads signaling pathway, thus ameliorating and treating brain glioma and other related diseases.*

Key words: *bleomycin, TGF-β/Smads signaling pathway, brain glioma cells, proliferation, apoptosis*

Introduction

Glioma, as the most common malignant tumor of the central nervous system, can be classified into low grade glioma (LGG) (grade I) and high grade glioma (HGG) (grade II) [1,2]. The relative survival rate of glioma is lower than 30%, and the 5-year survival rate remains at 5%, which have not been improved since the 1980s [3]. HGG is the most common primary brain tumor in adults at present [4,5]. However, the mean survival time of LGG patients is relatively longer. Operations are still the preferred therapeutic methods for glioma, but the glioma cannot be completely excised due to the tendency of glioma cells to expand to normal brain tissues, so 90% of the patients with

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Received: 07/09/2019; Accepted: 11/10/2019

marginal excision develop recurrent glioma [6,7]. Despite the comprehensive treatment, chemotherapy and radiation, the prognosis and treatment are still disappointing, and the therapeutic effects are usually far from satisfactory [8,9]. It has been discovered in recent years that bleomycin (BLM) is capable of ameliorating and treating brain glioma. As a type of glycopeptide antibiotic mixture with cytotoxicity isolated from Streptomyces vertillus, BLM is widely applied in antitumor therapies for testicular cancer, malignant lymphoma, head and neck squamous cell carcinoma, cervical cancer and skin cancer [10-12]. It is generally used in combination with other anticancer drugs because it will not cause apparent hepatotoxicity, nephrotoxicity and myelotoxicity [13]. Currently, it is critical to elaborate the roles of BLM in the development of brain glioma and further explore the underlying mechanism of action, which may be conducive to understanding the pathogenesis of the disease, providing theoretical supports for subsequent studies on the treatment of brain glioma-related diseases with BLM.

Studies have manifested that transforming growth factor-β1 (TGF-β1)/Smad is involved in multiple physiological metabolism processes, and TGF-β1 is recognized as one of the most important factors regulating the metabolism of articular cartilage cells, which exerts regulatory effects on the matrix synthesis and metabolism as well as proliferation of those cells [14]. The activation of TGF-β1 can further activate its downstream protein Smad, and TGF-β1 plays a crucial role in the pathological process of articular cartilage destruction in the case of osteoarthritis [15]. There is evidence that the TGF-β1/Smads signaling pathway also participates in myocardial remodeling, in which TGF-β1 acts as a pivotal player [16]. Studies over the past few years have revealed that the TGF-β family, including subclasses TGF-β1 and TGF-β2, is associated with glioma all the time. Both TGF-β1 and TGF-β2 are expressed in mammalian tissues [17]. TGF-β2, the most powerful factor in the TGF-β family, is mainly implicated in the initiation and maintenance of glioma. Once activated, TGF-β binds to TGF-β receptor II and stimulates phosphorylation of downstream Smad2 and Smad3, and the phosphorylated Smad2, Smad3 and Smad4 form transcription complexes that aggregate in the nucleus, thereby controlling the transcription [18,19] and participating in the occurrence and development of glioma. It was conjectured in this research that BLM can affect the biological function of TGF-β1 by regulating the release of Smad4 in brain glioma patients, but its potential roles in the proliferation and apoptosis of glioma cells have not been clarified yet, and the action of BLM in glioma destruction through the Smad4-controlled TGF-β1/Smads signaling pathway and its mechanism have not been reported in studies, which need in-depth investigations.

Although BLM can influence the occurrence of glioma *via* the TGF-β1/Smad, the specific molecular mechanism of action during the treatment has not been elucidated completely. Therefore, it was proposed in this research that BLM is able to affect glioma *via* the TGF-β1/Smads signaling pathway. The cell culture combined with BLM, enzyme-linked immunosorbent assay (ELISA) and other methods were adopted to detect the content of inflammatory factors and observe the cell proliferation and apoptosis. Besides, gene and protein assays were conducted to measure the changes in pathway molecules in the cells.

This research aimed to reveal the therapeutic effects of BLM on brain glioma and investigate whether such effects regulate the proliferation and apoptosis of glioma cells through the TGF-β1/Smads signaling pathway, thereby providing experimental bases for developing new medicines and treatment methods in subsequent studies.

Methods

Cell culture and grouping

The glioma U87 cells, purchased from American type culture collection (ATCC, Manassas, VA, USA), were rapidly taken out of a liquid nitrogen tank and immediately thawed in 60°C sterile water prepared in advance, followed by centrifugation and elimination of the supernatant. After several repetitions, the cells were resuspended in Dulbecco's modified Eagle's medium (DMEM) and then seeded into a 6-well plate at a calculated density, followed by culture in a constant-temperature incubator, and the medium was replaced every other day. The second generation cells in good growth status were harvested and divided into negative control (NC) group, 5 mU/mL BLM group and 10 mU/mL BLM group, the morphological changes in each group of cells were observed, and the cell samples were collected at 24 h after stimulation.

Detection of cytokines in each group

Three groups of cells in good growth status after stimulation were selected from the incubator, and the DMEM containing 10% fetal bovine serum (FBS) was discarded. Then, the cells and supernatant in each group were collected using a cell scraper, followed by cell lysis with strong radio immunoprecipitation assay (RIPA) lysis buffer, centrifugation, separation and collection of the supernatant. Next, the ELISA (Novus, Littleton, CO, USA) kit was applied to determine the levels of tumor necrosis factor-α (TNF-α) and interferon-γ (INF-γ) in the cells in accordance with the practical situations and instructions. Finally, the absorbance in each group was detected using a microplate reader.

Cell Counting Kit-8 (CCK-8) proliferation assay

The cells in logarithmic growth phase in each group were inoculated into a 96-well plate and cultured in the constant-temperature incubator with 5% CO_2 at 37°C for 0, 24, 48 and 72 h. Then, the medium was discarded, and 110 μL of color developer was added into each well. After incubation in the constant-temperature incubator at 37°C for 1 h, the absorbance at 450 nm in each group was measured by virtue of an ultraviolet spectrophotometer, which was made into line charts to reflect the cell proliferation activity.

Observation of cell proliferation in each group via 5-Ethynyl-2'-deoxyuridine (EdU) staining

The cells in each intervention group were stained in accordance with the instructions of the Click-iT EdU staining kit. After that, the cells were photographed using a fluorescence microscope, and 3 fields of vision were randomly selected on each slide. Finally, the EdU-positive cells were counted.

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) apoptosis assay

In Situ Cell Death Detection Kit (Roche, Basel, Germany) was used to measure cell apoptosis in the paraffin-embedded sections. The specific steps were as follows: The sections were fixed, rinsed and permeabilized with 0.1% Triton X-100. Apoptotic deoxyribonucleic acid (DNA) fragments were subjected to fluorescein isothiocyanate (FITC)-end labeling *via* the TUNEL assay kit (Beyotime Institute of Biotechnology, Beijing, China), the images of FITC-labeled TUNEL-positive cells were observed under the fluorescence microscope, and 10 fields of vision were selected to count the TUNEL-positive cells.

Detection of expressions of apoptosis and pathway-related genes via reverse transcription-polymerase chain reaction (RT-PCR)

The ribonucleic acid (RNA) was extracted from harvested cells and then synthesized into DNA using kits (TaKaRa, Tokyo, Japan) according to the detailed steps in the instructions. After that, the DNA was amplified into single-stranded complementary DNA (cDNA) as per the conventional reaction system, followed by storage at -20°C and PCR amplification. The samples were amplified using the primers for genes to be detected and internal reference gene, with 3 replicates for each reaction. The amplification system (20 μL) was prepared with 2 μL of cDNA, 10 μL of quantitative real-time polymerase chain reaction (qRT-PCR) Mix, 2 μL of primer and 6 μL of ddH_2O, and PCR amplification was performed later. The primer sequences of target genes and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), an internal reference, were designed according to those on GenBank (Table 1). The expression levels of target genes were determined *via* qRT-PCR.

Table 1. Primer sequences

Target gene	*Primer sequence (F-R, 5'-3')*
GAPDH	GACATGCCGCCTGGAGAAAC
	AGCCCAGGATGCCCTTTAGT
B-cell lymphoma-2 (Bcl-2)	GGTGCTCTTGAGATCTCTGG
	CCATCGATCTTCAGAAGTCTC
Caspase-3	CTACCGCACCCGGTTACTAT
	TTCCGGTTAACACGAGTGAG
TGF-β1	GGCTCACCTTCTGCCCGTCT
	GTCTCGGTATCCCACGAAAGAAACG
Smad4	GGCAGGCTGACTTGTG
	CGCGGATCAACCGAGACATATACT

Western blotting assay

The cells in suitable density were collected from the three groups to extract proteins and measure the protein concentration. The total proteins extracted from the cells were subjected to water bath and centrifugation. After that, the Western blotting assay was performed in the sequence of preparation of 10% separation gel and 5% spacer gel, loading for electrophoresis, membrane transfer through semi-dry process, sealing, adding with primary antibody overnight and incubation with secondary antibody. Then, the protein bands were scanned and quantified using an Odyssey membrane scanner, and the level of proteins to be detected was corrected *via* GAPDH. Finally, the expression levels of proteins were calculated by grayscale scanning.

Statistics

All the raw data obtained from experiments were assessed using SPSS 20.0 (SPSS, Chicago, IL, USA) analysis software, the validity of the raw data was retained, and the data were subjected to multiple comparisons. The experimental results obtained were presented as mean±standard deviation ($\bar{x}$±SD), and $p<0.05$ suggested that the difference was statistically significant. The histograms were plotted by means of GraphPad Prism 7.0 (La Jolla, CA, USA).

Results

Observation of cell morphology

At about 24 h after processing of the 3 groups of cells, NC group exhibited faster, colonial and dendritic growth of cells, some cells extended irregular pseudopodia, and the cell body was enlarged with the prolongation of time (Figure 1A). Both 5 mU/mL and 10 mU/mL BLM groups had relatively uniform cell morphology, small density and slowed proliferation (Figures 1B & 1C).

Detection results of cytokines in each group

For the purpose of detecting the occurrence of glioma in the early stage, the levels of inflammatory factors TNF-α and INF-γ were determined. As shown in Table 2, the NC group had remarkably higher levels than the other two groups, while the 10 mU/mL BLM group had obviously declined levels (p<0.05), suggesting that 10 mU/mL BLM can inhibit the production of inflammatory factors, further repressing the occurrence of brain glioma.

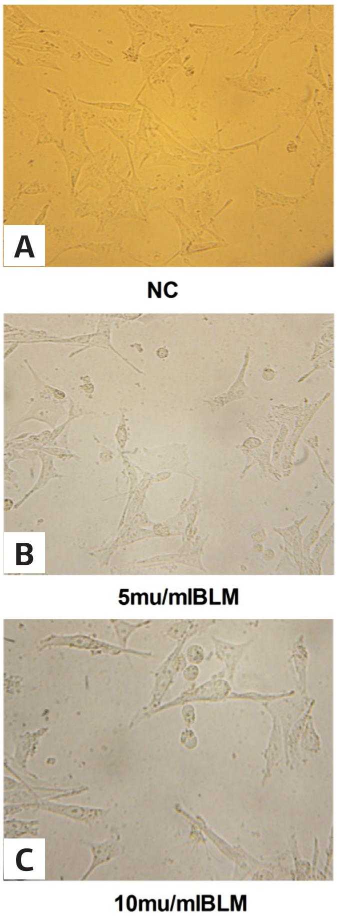

Figure 1. Observation of cell morphology. NC group exhibits faster, colonial and dendritic growth of cells, some cells extend irregular pseudopodia, and the cell body is enlarged with the prolongation of time (**A** ×40). Both 5 mU/mL and 10 mU/mL BLM groups have relatively uniform cell morphology, small density and slow proliferation (**B & C** ×40).

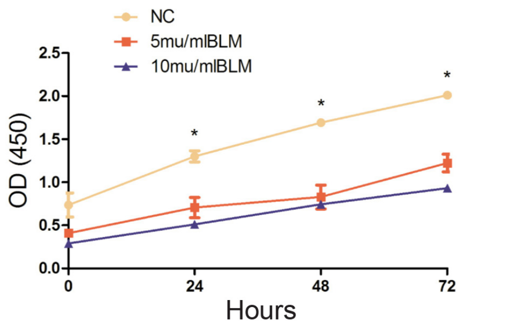

Figure 2. CCK-8 assay. The proliferative capacity of the glioma cells at 24, 48 and 72 h is enhanced markedly in NC group (p<0.05), *p<0.05.

Results of CCK-8 proliferation assay

CCK-8 proliferation assay was utilized to measure the absorbance at different time points among the three groups of cells. The results (Figure 2) manifested that the proliferative capacity of the glioma cells at 24, 48 and 72 h in the NC group was evidently stronger than that in the other two groups (p<0.05), and it was weakest in the 10 mU/mL BLM group (p<0.05).

Cell proliferation in each group observed via EdU staining

To further determine the impact of BLM on the proliferative capacity of glioma cells, EdU staining was utilized to assess the proliferative capacity of cells in the NC group and the 10 mU/mL BLM group. It was indicated that the number of EdU-positive cells in the 10 mU/mL BLM group was notably smaller than that in the NC group (p<0.05), and there was less cell proliferation (Figure 3).

Results of TUNEL apoptosis assay

The apoptosis level in each group of cells was detected *via* TUNEL staining in this research. According to the results (Figure 4), there were few apparent TUNEL-positive cells in the NC group, which could hardly be observed. The number of TUNEL-positive cells was increased prominently in both 5 mU/mL and 10 mU/mL BLM groups compared with that in the NC group, and the 10 mU/mL

Table 2. Detection results of cytokines

Group	*TNF-a (fmol/mL)*	*INF-γ (μg/L)*
NC	88.05 ± 2.35	30.21 ± 0.85
5 mU/mL BLM	60.52 ± 3.54[a]	15.32 ± 1.20[a]
10 mU/mL BLM	40.34 ± 2.14[b]	5.02 ± 1.34[b]

The levels of INF-γ and TNF-α are increased remarkably in NC group, while they decline obviously in 10 mU/mL BLM group (p<0.05). [a]p<0.05 *vs.* NC, [b]p<0.05 vs. 5 mU/mL BLM group

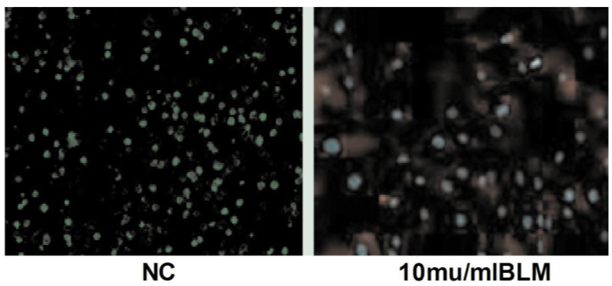

Figure 3. EdU staining results for each group of cells. The number of EdU-positive cells in the BLM group is notably smaller than that in the NC group, and there is statistically less cell proliferation (p<0.05).

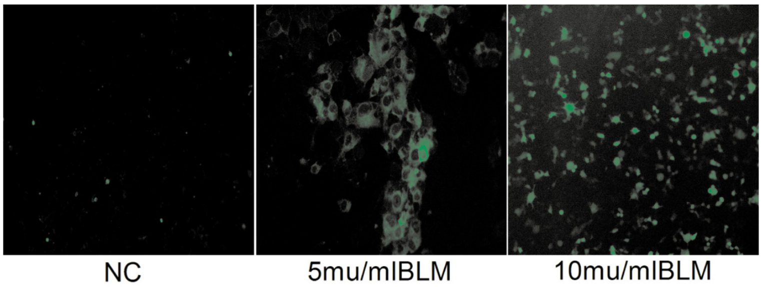

Figure 4. Apoptosis level in each group of cells detected via TUNEL staining. There are few apparent TUNEL-positive cells in the NC group, while the number of TUNEL-positive cells is increased prominently in both 5 mU/mL and 10 mU/mL BLM groups compared with that in the NC group (p<0.05).

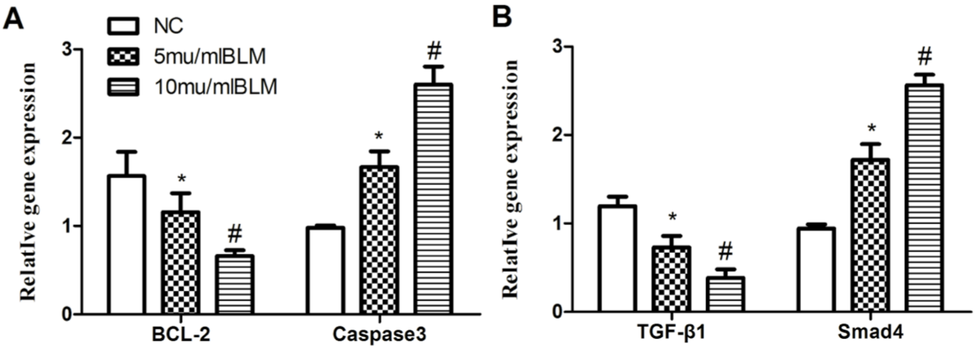

Figure 5. Gene detection results. The mRNA levels of Bcl-2 and TGF-β1 are lowered evidently in 5 mU/mL and 10 mU/mL BLM groups (p<0.05), while the mRNA expression levels of Caspase-3 and Smad4 are elevated remarkably (p<0.05). *p<0.05 *vs.* NC, #p<0.05 *vs.* 5 mU/mL BLM.

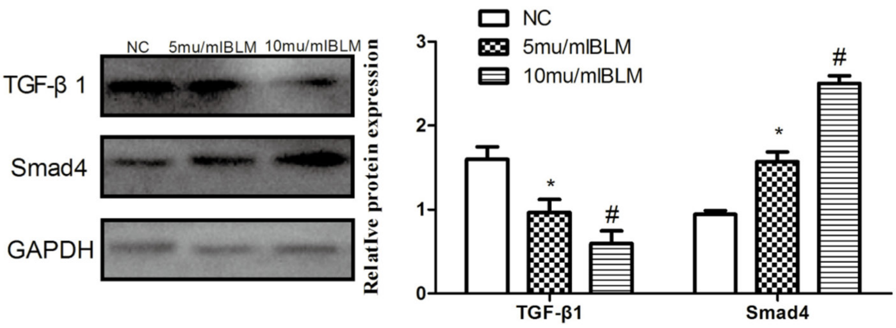

Figure 6. Protein expressions. The protein expression level of TGF-β1 is reduced obviously in 5 mU/mL and 10 mU/mL BLM groups (p*<0.05), while that of Smad4 is raised notably (p<0.05). *p<0.05 *vs.* NC, #p<0.05 *vs.* 5 mU/mL BLM.

BLM group had the greatest number of TUNEL-positive cells (p<0.05), illustrating that BLM is able to facilitate the apoptosis of glioma cells.

QRT-PCR assay results

The results of RT-PCR (Figure 5) manifested that the messenger RNA (mRNA) levels of Bcl-2 and TGF-β1 were lowered evidently in the 5 mU/mL and the 10 mU/mL BLM groups (p<0.05), while the mRNA expression levels of Caspase-3 and Smad4 were elevated remarkably (p<0.05). Opposite results of the expression levels of those genes were obtained in the NC group, implying that BLM represses cell proliferation and promotes cell apoptosis, further suppressing the progression of brain glioma.

Western blotting assay results

It was revealed in Western blotting results (Figure 6) that the protein expression level of TGF-β1 was reduced obviously in the 5 mU/mL and 10 mU/mL BLM groups (p<0.05), while that of Smad4 was raised notably (p<0.05). The proteins in the NC group showed the opposite expressions trends. These results suggest that BLM inhibits cell proliferation and promotes cell apoptosis by repressing the TGF-β/Smads signaling pathway, further suppressing the progression of brain glioma.

Discussion

Glioma is the most common primary intracranial malignancy, and high grade glioma (HGG) is the most malignant type, accounting for about 70% of malignant brain tumors in adults [20]. Although progress has been achieved in treatment, including chemotherapy together with radiotherapy after surgical resection, HGG is still an incurable and life-threatening disease, with an overall survival of only about 9-15 months after diagnosis [21]. In this research, the *in vitro* cell culture was employed to observe the influence of BLM on the proliferation and apoptosis of glioma cells. It was shown that at about 24 h after processing of the 3 groups of cells, both 5 mU/mL and 10 mU/mL BLM groups had relatively uniform cell morphology, small density and slow proliferation. NC group exhibited faster, colonial and dendritic growth of cells, some cells extended irregular pseudopodia, and the cell body was enlarged with the prolongation of time. Later, the absorbance at different time points among the three groups of cells was detected *via* CCK-8 proliferation assay. The results indicated that the proliferative capacity of the glioma cells at 24, 48 and 72 h in the NC group was evidently stronger than that in the other two groups, and it was weakest in the 10 mU/mL BLM group. In order to further determine the impact of BLM on the proliferative capacity of the cells, EdU staining was applied to assess the proliferative capacity of each group of cells. It was revealed that the number of EdU-positive cells in BLM groups was notably smaller than that in the NC group, displaying less cell proliferation, suggesting that BLM inhibits the growth of glioma cells. In addition, the levels of inflammatory factors TNF-α and INF-γ were determined, so as to detect the incidence of glioma in the early stage. It was found that the NC group had remarkably higher levels than the other two groups, while the 10 mU/mL BLM group had obviously declined levels, suggesting that 10 mU/mL BLM can inhibit the production of inflammatory factors, further repressing the occurrence of brain glioma. These findings imply that the increased TNF-α level can further stimulate the development of brain glioma, thus aggravating the inflammatory responses. However, the level declined after the treatment with BLM, indicating that the symptoms are improved after treatment with BLM, and that BLM has favorable therapeutic effects on brain glioma. TNF-α occupies an indispensable position in the occurrence and development of inflammation. IL-6 can stimulate the overproduction of INF-γ, another inflammatory mediator, increasing the damage of pulmonary diseases [22,23]. The results in this research were consistent with previous studies [24,25], illustrating that BLM can inhibit the excessive production of inflammatory cytokines and prevent the cells from irrecoverable injuries of such overproduction. As a metabolic pathway, apoptosis can clear up harmful substances in cells. Besides, it can respond relevantly to invasion to cell bodies, and apoptotic response is initiated rapidly in case of lethal threats, thereby timely eliminating garbage produced due to maintenance of life activities in cells. As a defender in the body, apoptosis can supply energy for the generation of subcellular structures and metabolism and maintain cell stability [24]. However, the mechanism of action of apoptosis in physiological metabolism in organisms has not been completely defined, but the elaborated mechanisms of action and pathways can serve as important guidelines for related clinical diseases such as tumor and rheumatic arthritis. In this research, the apoptosis level in each group of cells was detected *via* TUNEL staining, and it was shown that a few apparent TUNEL-positive cells were observed in the NC group, which could hardly be observed. Both 5 mU/mL and 10 mU/mL BLM groups had obviously more TUNEL-positive cells than NC group, illustrating that BLM is able to facilitate the apoptosis of glioma cells. According to the RT-PCR results, the mRNA expression level of Bcl-2 was lowered evidently, while that of Caspase-3 was elevated remarkably in the 5 mU/mL and 10 mU/mL BLM groups. Opposite results of the expression levels of those genes were observed in the NC group, implying that BLM represses cell proliferation and promotes cell apoptosis, further suppressing the progression of brain glioma.

The TGF-β1/Smad4 signaling pathway exerts crucial effects in the regulation of normal and cancer cells, but its detailed effects in brain glioma and other aspects need to be explored [25]. The expression levels of Smad2 and Smad3 in tumor specimens and glioma cell lines were investigated in previous studies [28,29], but no consistent results were obtained. In previous investigations, the expressions of downstream components (such as Smad2 and Smad3) related to the expression level of TGF-β receptor in glioma cell lines were detected and verified, and the results indicated that the protein expression levels of Smad2 and Smad3 in glioma cell lines are lower than those in normal astrocytes [26]. Similarly, Kjellman et al analyzed the mRNA expression levels of Smad2 and Smad4 in 23 cases of glioma tissue specimens, and observed the mRNA expression levels of Smad2 and Smad4 decline, which are associated with the malignancy [27]. Some studies [29,30] have also demonstrated that the knockout of Smad2 and Smad3 can accelerate the cell proliferation, suggesting that Smad2 and Smad3 have inhibitory effects on the proliferation of glioma cell lines. The study of Zhang et al [28] manifested that the protein expression levels of Smad2 and Smad3 in glioma cell lines are lower than those in normal astrocytes. According to the RT-PCR results in this research, the mRNA expression level of TGF-β1 was decreased distinctly, while that of Smad4 was increased obviously in the 5 mU/mL and 10 mU/mL BLM groups. Moreover, opposite expression levels of those genes were observed in the NC group, implying that BLM represses cell proliferation and promotes cell apoptosis, further inhibiting the progression of brain glioma.

The results of Western blotting assay indicated that both 5 mU/mL and 10 mU/mL BLM groups exhibited notably reduced protein expression level of TGF-β1 and evidently raised protein expression level of Smad4, while the NC group showed the opposite expression trends. This research is in consistence with the aforementioned studies, illustrating that BLM inhibits the proliferation and promotes the apoptosis of glioma cells by repressing the TGF-β/Smads signaling pathway, thereby repressing the occurrence of brain glioma. In subsequent studies, changes in the expressions of various factors during treatment can be dynamically observed, and more genes and proteins in the signaling pathway can be detected, so as to verify such effects.

Conclusions

In conclusion, BLM may regulate the proliferation and apoptosis of glioma cells by repressing TGF-β1/Smad4, and the TGF-β1/Smad4 axis probably plays a role in the pathogenesis of brain glioma, which can be explored through more techniques in subsequent studies. The test results enrich and perfect the theoretical basis for the impact of BLM on the proliferation and apoptosis of glioma cells as well as the TGF-β1/Smads signaling pathway, and provide a theoretical basis for the research and development of new anticancer drugs.

Conflict of interests

The authors declare no conflict of interests.

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