Bleomycin Suppresses the Proliferation and the Mobility of Human Gastric Cancer Cells Through the Smad Signaling Pathway

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Key Words
Bleomycin • Gastric cancer • Proliferation • Apoptosis • Migration

Abstract

Background/Aims: Extensive studies have demonstrated that Bleomycin (BLM) is a glycopeptide antibiotic that has been used as an anticancer chemotherapeutic reagent. It can induce both single- and double-strand DNA damage, inhibit synthesis of DNA, suppress proliferation, and induce apoptosis in cancer cells. Smad signaling transducers are considered as important molecules in tumor development and progression, and may closely be related to the biological behaviors of some malignant carcinomas, including gastric cancer. Methods: The effects of different concentrations of BLM on the proliferation, cell cycle, apoptosis, migration, and invasion on gastric cancer cell lines MKN45 and AGS were assayed by using CCK-8 assay, Annexin V/PI double staining, PI staining, and transwell assay. Western blot and Immunohistochemistry were applied to analyze the potential mechanism(s). Results: BLM treatment resulted in a low proliferation, high apoptosis, low migration and invasion in MKN45 and AGS cells. Furthermore, the possible mechanisms underlying that Smad3 activity could be changed after binding with BLM, and subsequently the Smad signaling pathway had a cascade response. Conclusion: These results highlight BLM as an exciting theme for gastric cancer treatment, which may represent an effective clinical therapeutic reagent for gastric cancer patients.

Introduction

Gastric cancer is associated with significant incidence and mortality worldwide, even though advanced diagnostic and operative techniques are widely applied in clinical practice.
[1-3]. Tumorigenesis and progression of gastric carcinoma is a multistage and multifactorial process. Identification of additional drugs for cancer biomarkers and novel therapeutic targets will improve the cancer diagnosis, treatment and prevention.

Bleomycin (BLM) is anti-tumor antibiotic glycopeptide produced by actinobacterium *Streptomyces verticillus* [4]. Cytotoxic activity of BLM is through oxidation in deoxyribose of thymidylate and other nucleotides, which produced single-strand and double-strand breaks in DNA, chromosomal aberration, gaps, fragments and translocation [4]. It has been reported that BLM was associated with other cytotoxic reagents for the treatment of testis cancer and Hodgkin disease, and the particularity of these two diseases is the high cure rate obtained by chemotherapy [5, 6]. BLM also shows anti-tumor activity in other malignancies, including head and neck, cervix, and vulva cancers [7-12]. However, the effects of BLM on proliferation, cell cycle, apoptosis, migration and invasion, and the potential mechanism of BLM in gastric cancer cells have not been reported.

Previous studies have reported that transforming growth factor β (TGF-β) initiates its diverse cellular responses by binding to and activating specific cell surface receptors which have intrinsic serine/threonine kinase activity [13]. These activated TGF-β receptors stimulate the phosphorylation of Smad2 (ser465/467) and Smad3 (ser423/425), which in turn form complexes with Smad4 that accumulate in the nucleus and regulate the transcription of target genes [14, 15]. The aberrant expression of Smad4 or disruption of Smad4 activity is a potential mechanism for the loss of tumor suppressor role of the Smad signaling pathway [16].

In the present study, we found BLM may have binding sites with Smad3 by the prediction of SYBYL-X 1.3 software. Therefore, we hypothesized that BLM may suppress the biological phenotype of gastric cancer cells through activating the Smad signaling pathway. And then we explored the expression of Smad2, phospho-Smad2 (ser465/467), Smad3, phospho-Smad3 (ser423/425), and Smad4 in gastric cancer cells to evaluate whether they were correlated with BLM-induced tumor suppression. These results may have valuable implications in the development of BLM as a new therapeutic reagent for gastric cancer patients.

**Materials and Methods**

**Cell culture and BLM treatment**

Gastric carcinoma cell lines AGS and MKN45 were purchased from Japanese Physical and Chemical Institute (Tokyo, Japan) and Cell bank of Chinese Academy of Sciences (Shanghai, China), respectively. They were maintained in Ham F12 or RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin, and 100 µg/ml streptomycin, in a humidified atmosphere of 5% CO$_2$ at 37°C. Cells were seeded on the plates over night and then exposed to increasing doses of BLM (0-20 µg/ml) (Selleck Chemicals, Boston, USA). After BLM treatment 24h, cell proliferation, cell cycle, apoptosis, migration and invasion assays were measured.

**Cell proliferation assay**

Cell proliferation was assessed by the cell counting kit-8 (CCK-8) assay according to the manufacturer’s protocol (Dojindo Laboratories, Gaithersburg, MD, USA). In brief, 1.0 × 10$^4$ cells/well were seeded on 96-well plates and allowed to adhere at 37°C, 5% CO$_2$. After 24 h of BLM (0-20 µg/ml) treatment, 10 µl of CCK-8 solution was added into each well of the plate and the plates were incubated for 2 h in the incubator, then measured at 450 nm using the Tecan Infinite 2000 Microplate Reader (San Diego, CA, USA).

**Apoptosis assay by flow cytometry**

After 24 h of 10 µg/ml BLM treatment, cells were washed twice with cold PBS, resuspended in 500 µl 1 × Binding Buffer at a concentration of 1 × 10$^5$ cells/ml, and incubated with 5 µl FITC- Annexin V and 5 µl PI. Samples were gently vortexed and incubated for 15 min at 25°C in the dark. Flow cytometry was performed within 1 hour by a FACScan flow cytometer (BD Biosciences, Baltimore, MD, USA).
Cell cycle analysis

1 × 10^6 cells treated with 10 µg/ml BLM for 24 h were collected, washed with PBS twice and fixed in cold 10 ml ethanol for more than 2 h at 4°C. And then, cells were washed with PBS twice and incubated with RNase at 37°C for 1 h. The tube with cells was added by PI to 50 µg/ml and incubated at 4°C in the dark for 30 min. Finally, FACS was employed to examine the PI signal.

Cell migration and invasion assays

For migration assay, after 10 µg/ml BLM added for 24 h, 2.5 × 10^5 cells were resuspended in serum-free Ham F12 or RPMI 1640, and seeded in the control-membrane insert on the top portion of the chamber (BD Bioscience). The lower compartment of the chamber contained 10% FBS as a chemo-attractant. After incubated for 20 h, cells on the membrane were scrubbed, washed with PBS and fixed in 100% methanol and stained with Giemsa dye. For invasive assay, the procedures were the same as above excluding the matrigel-coated insert (BD Bioscience). Images were visualized with a laser confocal microscope (Olympus, Tokyo, Japan).

Model of BLM bound to Smad3

The interaction of BLM and Smad 3 protein was analyzed by the modeling software SYBYL-X 1.3 (Tripos International, St. Louis, MO). The structure of Smad3 (PDB code: 1MK2) with crystallographic resolutions of less than 3.0 Å, were retrieved from the Protein Data Bank (http://www.rcsb.org). The molecular structure of BLM (CID: 5360373) was downloaded from PubChem Compound (http://www.ncbi.nlm.nih.gov/pccompound).

Immunohistochemistry

Cells were seeded on glass coverslips before exposing to increasing doses of BLM (0-20 µg/ml) and then fixed with PBS containing 4% formaldehyde for 10 min, and permeabilized with 0.2% Triton X-100 in PBS for 10 min at room temperature. After washing with PBS, endogenous peroxidase activity was suppressed by 3% H_2O_2 and blocked with goat serum or 5% BSA. Diluted primary polyclonal rabbit antibodies against Smad2, phospho-Smad2 (dilution 1:200, Cell Signaling, USA) and Smad3, phospho-Smad3 (dilution 1:200, Abcam, UK), mouse monoclonal antibody against Smad4 (dilution 1:200, Santa Cruz, USA) were added and incubated at 4°C overnight. As secondary reagents, biotin-labeled anti-IgG and an avidin-biotin horseradish peroxidase complex were used (Zhongshan, China), followed by staining with chromogen diaminobenzidine (Zhongshan, China) until a brown color developed. Slides were counterstained with Mayer’s hematoxylin and differentiated in a solution containing 1% hydrochloric acid and 99% ethanol. Cell nuclei were stained blue using lithium carbonate. Sections were dehydrated, and a transparent cover slip was added to enable observation by microscopy. All of the immunohistochemical photographs were analyzed using Image Pro Plus (IPP, USA). The mean optical density (OD), as a quantitative measure of the staining intensity, was analyzed to determine the average protein expression.

Western blot

After increasing doses of BLM (0, 10 and 20 µg/ml) treatment for 24 h, the whole cell extracts were prepared using the Total Protein Extraction Kit (KeyGEN, Nanjing, China) according to the manufacture’s instruction. Protein concentrations were measured using a BCA protein assay kit (Boster, China). Total proteins (50 µg) were separated by SDS-PAGE and blotted onto Hybond membrane (Amersham Biosciences, Westborough, MA, USA). After blockade of nonspecific protein binding with 5% milk, the membrane was incubated at 4°C overnight with primary antibodies diluted in Tris buffered saline with Tween20 (TBST). Rabbit polyclonal antibody recognizing Smad2, phospho-Smad2 (ser465/467), Smad3, phospho-Smad3 (ser423/425) were used at 1:1000, mouse monoclonal antibody directed against Smad4 was used at 1:500, and mouse monoclonal antibody against β-actin (Santa Cruz, USA) was used at 1:500. Following incubation with primary antibodies, the blots were washed three times in TBS/Tween20 before incubation for 1 h in goat anti-mouse or goat anti-rabbit horseradish peroxidase (DAKO, Glostrup, Denmark) conjugate antibody at 1:5000 dilution in TBS/Tween20 containing 5% skim milk. After extensive washing in TBS/Tween-20, the blots were washed with distilled water and processed for antigen detection of antigen using the ECL-chemiluminescent kit (ECL-plus, Thermo Scientific, USA). β-actin probing was used as loading control. The band intensity was determined by Image J software (version 10.2, Bethesda, MD). Experiments were repeated at least three times from three independent protein extracts.
Statistical Analysis

Statistical evaluation was performed using Fisher’s exact test to compare the different rates. SPSS 13.0 software was applied to analyze all data and \( P<0.05 \) was considered statistically significant.

Results

The effects of BLM on biological phenotypes of human gastric cancer cells

Firstly, the viability of MKN45 and AGS cells treated with different concentration BLM (0, 5, 10 and 20 µg/ml) for 24h was analyzed, and median lethal dose of BLM was around...
10 µg/ml for the two cell lines (Fig. 1a). In order to investigate the role of BLM in biological phenotypes of MKN45 and AGS cells, cell cycle, apoptosis, migration and invasion were assessed after treatment with 10 µg/ml BLM for 24h. A slower growth was observed in cells with BLM treatment than that in untreated ones (P<0.05, Fig. 1a). Cell cycle analysis indicated G2 arrest and there was a higher apoptosis evidenced by Annexin-V in AGS and MKN45 cells after adding 10 µg/ml BLM for 24h (P<0.05, Fig. 1b and 1c). Additionally, the treatment of BLM could obviously suppress migration and invasion of AGS and MKN45 cells (P<0.05, Fig. 1d).

**BLM acts as a tumor inhibitor through Smad signaling pathway in gastric cancer cells**

Then, we applied the modeling software SYBYL-X 1.3 and found that BLM could dock into Smad3 (Fig. 2). In order to further investigate the mechanism of suppression by BLM in the development and progression of gastric cancer, we detected the protein expression of phospho-Smad2 (ser465/467), Smad2, phospho-Smad3 (ser423/425), Smad3, and Smad4. By western blot analysis, we found that MKN45 cells showed an increasing trend...
of phosphorylated smad2 (ser465/467) and phosphorylated Smad3 (ser423/425) after BLM treatment, and while the total smad2/smad3 expression showed no obvious changes (P<0.05, Fig. 3). Furthermore, we found that smad4 protein levels increased significantly in BLM-treated cells compared with untreated ones (P<0.05, Fig. 3). Further analysis by immunohistochemistry of cell slides showed that the concentration of BLM increased from 10 to 20 μg/ml, BLM significantly increased the expression of phosphorylated smad2 (ser465/467), phosphorylated Smad3 (ser423/425) and Smad4 protein.

Discussion

Bleomycin is a cytotoxic antibiotic and applied clinically for anticancer chemotherapy. This drug has specific functional components which can interact with DNA and induce DNA damage effects: the one is represented by two thiazole rings and the other is by the pyrimidine-imidazole moiety [17, 18]. The cytotoxic activity of BLM has often been attributed to its ability to mediate double-strand cleavage of DNA in targeting tumor cells [19]. The previous study reported BLM was used to treat different types of malignant neoplasms, such as germ cell tumor and lymphoma [18]. Nonetheless, the application of BLM in patients with gastric cancer has yet to be clarified.

Our data clearly indicated that treatment with BLM (10 μg/ml) caused a lower proliferation, migration and invasion, and induced apoptosis and G2 arrest of gastric carcinoma cells. Taken together the previous findings, BLM can delay the growth, and promote the apoptosis of AML cells by mitochondrial DNA (mtDNA) damage [8]. BLM induces both apoptosis and cellular senescence in alveolar epithelial cells, leading to suppression of tumor growth through modulating caveolin-1 [20]. In addition, it is reported that BLM can reduce tumor cell growth and viability in Her-2+ breast cancer cells through liposome delivery system [21]. BLM also can inhibit the progression and development of hepatocellular carcinoma through the comet of DNA damage, G2/M phase arrest and the activation of...
apoptotic caspase pathways, and increase of apoptosis-modulated protein expression [22]. Moreover, BLM can induce apoptosis in NT2 cell by activating a mitochondrial pathway of apoptosis and cause resistance to development of malignant testicular germ cell tumor [23]. However, the application and outcome of different doses of BLM treatment in these cancer cells are still poorly understood, it may need to be further explored in the prospective study.

Furthermore, our predicted result showed that BLM docks into Smad3, which is a member of the Smad signaling pathway. And it may induce phosphorylation of Smad2/3 and activate the Smad signaling pathway. In order to address this issue, we examined the expression of phospho-Smad2 (ser465/467), Smad2, phospho-Smad3 (ser423/425), Smad3, and Smad4 in gastric cancer cells. And the results suggested that BLM plays a tumor-suppressive role in MKN45 and AGS cells through the Smad signaling pathway. The previous study indicated that Smads are critical integrators of different tumor-suppressive and tumorigenesis molecule signaling transduction system [14]. It can also be activated or regulated by the members of TGF-β superfamily including TGF-βs, activins, inhibins and bone morphogenic proteins (BMP)/Growth differentiation factor (GDF) [24, 25]. And the activated Smad protein complex translocates into the nucleus and interacts with transcriptional co-activators or co-repressors to regulate promoter activity to positively or negatively control gene expression [26]. In our findings, BLM induces phosphorylation of Smad2/3 and activates the Smad signaling pathway to prevent the malignant progression of gastric cancer cells. It has been reported that Smad proteins, such as phosphorylated Smad3, are the intracellular signaling effectors for the mediation of intracellular signaling of TGF-β, and the expression of it provides significant prognostic power for gastric cancer patients [27]. Kim et al. [28] reported that the prognosis of patients with Smad4-positive gastric carcinomas was significantly better than that of patients with a negative tumor. Smad3 phosphorylated at the COOH-terminal (ser423/425) region inhibits human colorectal cancer cell proliferation [14]. These results confirmed that the activation of Smad proteins is critical for preventing tumorigenesis in gastric carcinoma cells.

In conclusion, BLM represses the aggressive biological phenotypes of MKN45 and AGS cells through regulating the Smad signaling pathway and plays a suppressive role in tumorigenesis and progression of gastric cancer. Our results provide new evidence for further exploring the action mechanism of BLM as a new suppressive reagent or a novel therapeutic drug for gastric carcinogenesis and subsequent invasion. However, it is difficult to avoid the side effects and drug resistance for BLM induced lung toxicity, rash, etc. And the dual role of Smad signaling in tumor-suppressive and tumor-promoting in cancer initiation or progression is complex and has not been elucidated. Thus, the precise tumor-suppressive mechanism between BLM and Smad signaling pathway, the biological function and the moderate dose applied in clinical therapy of BLM in gastric carcinomas need further investigations in vitro or in vivo.

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Disclosure Statement

None.
References


