Exogenous HMGB1 Promotes the Proliferation and Metastasis of Pancreatic Cancer Cells

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Abstract

Background: Exogenous HMGB1 play a vital role in tumor recurrence which reside in the tumor microenvironment. However, the mechanism of action is still unknown. We studied the proliferation and metastasis effect of exogenous HMGB1 on human SW1990 and Panc-1 cells after radiotherapy and explored the possible molecular mechanism.

Materials and Methods: Residual Panc-1 cells and SW1990 cells were isolated after radiotherapy. The supernatant after radiotherapy was collected. The relative expression of HMGB1 was evaluated by Enzyme Linked Immunosorbent Assay (ELISA). The images of normal pancreatic cancer cells and residual pancreatic cancer cells were collected by Electron microscope (EMS). The proliferation of pancreatic cancer cells which were treated with difference groups was measured by Carboxy fluorescein succinimidylester (CFSE). The migration rates were measured by wound healing assays. The expression of related proteins were detected by Western Blot. In vivo, the subcutaneous pancreatic tumor models of nude mice were created and therapeutic capabilities were tested.

Results: HMGB1 was found in the supernatant of pancreatic cancer cells after radiotherapy. The results of CFSE showed that exogenous HMGB1 could promote the proliferation of pancreatic cancer cells. Meanwhile, HMGB1 also could promote the metastasis of PC cells. By western blot, HMGB1 could upregulation of p-GSK 3 β , N-CA, Bcl-2, and Ki67 and down-regulation of E-CA. In vivo, EP (HMGB1 inhibitor) could inhibit the growth of tumors and HMGB1 could promote the proliferation of tumors post-radiotherapy.

Conclusion: Radiotherapy could induce HMGB1 released into extracellular. Exogenous HMGB1 could promote the proliferation and metastasis of Panc-1 cells and SW1990 cells by upregulating p-GSK 3 β expression which might depend on Wnt pathway.

Introduction

Pancreatic cancer (PC) is a highly fatal malignancy, which is the seventh leading cause of cancer death. Meanwhile the incidence rates of pancreatic cancer is so high in males and females[1]. Due to the concealed onset, rapid development, and poor prognosis, PC is a serious threat to human health. Research shows that the 5-year survival rate of PC was less than 5%[2]. Sad to say, although we have made progress in the stabilization and management of other solid organ tumors, the diagnostic rate of PC has been increasing in the last 10 years. Pancreatic cancer is deemed to possess ability to resist conventional therapeutic agents. And no early detectable biomarkers of PC were observed[3]. Less than 20% of patients with pancreatic carcinoma can received surgery, however other patients are treated with chemotherapy and/or radiotherapy[4]. The patients still have a poor prognosis, even though the tumor has resected, and the incidence of cancer recurrence rate ranging from 20–60%. Study shows that about 30% of PC patients die of locally obstructive disease with few or no distant metastases. Although there are various treatment options such as surgical resection, chemotherapy, and radiotherapy, the outcome of PC

patients is still extremely poor[5]. These findings have highlighted the importance of finding a new therapy and mastering the pathogenesis in the treatment of pancreatic adenocarcinoma.

A group of non-histone nuclear proteins was discovered in 1973, and named High-Mobility Group (HMG) proteins[6]. The HMG proteins include three super-families which are respectively HMGB, HMGN, and HMGA [7]. High-mobility group box 1 (HMGB1) is the most well-studied and abundant HMG protein. And HMGB1 senses and coordinates the cellular stress response, which plays an important role not only inside of the cell as autophagy sustainer, a DNA chaperone and protector from apoptotic cell death, but also outside the cell as the damage associated molecular pattern molecule (DAMP)[6]. Human HMGB1 has 215 amino acid residues which forms two DNA binding domains (HMG A box, HMG B box) and a C-terminal acidic tail[8]. All of these peculiarities make HMGB1 as a critical molecular target in diversified human diseases including immune disorders, ischemia, infectious diseases, metabolic disorders, neurodegenerative diseases, and cancer[9]. There are 106 molecules of HMGB1 each cell approximately [10]. HMGB1 is released into the extracellular fluid massively by dead or dying cells[11]. Extracellular HMGB1 acts as a DAMP to inform the innate immune system [12]. These extracellular HMGB1 which has been activated are not only mediated by receptors, but also by its structure and Redox state [13]. Except for its nuclear and extracellular roles, cytoplasmic HMGB1 binds many proteins which involved in cancer progression, autophagy, and likely the unconventional secretory pathway[14].

Several mechanisms have been covered with regard to HMGB1 translocation from the cell nucleus to the cytoplasm and then subsequent release[15]. The Wnt signaling is the major pathway during embryonic development and continues throughout the life of an organism [16]. GSK-3 is a crucial kinase for this process [17]. The multifunctional serine/threonine kinase glycogen synthase kinase 3 β (GSK-3 β) is acted in multiple signaling pathways and the processes of pathophysiology, such as differentiation, EMT, proliferation, metabolism and inflammation. [18]. GSK-3 β participates in all kinds of diseases, including Alzheimer's disease, diabetes and cancer[19]. Some studies demonstrated that GSK-3 β could affect EMT by regulating the Wnt pathway [20]. However, the mechanism of GSK-3 β action in EMT has not been fully clear.

In this study, we conducted some studies by radiotherapy, ELISA, EMS, CFSE, wound healing assays, Western Blot and models of nude mice in order to know the proliferation and metastasis effect of exogenous HMGB1 on human residual pancreatic cancer SW1990 and Panc-1 cells after radiotherapy and explored the possible molecular mechanism. We hope to provide new ideas for the treatment of pancreatic carcinoma.

Materials And Methods

Materials

HMGB1 was purchased from Sigma(USA). HMGB1 was dissolved in double distilled water(DDW) to make a stock solution(3.7mg/ml) and stored at -80°C. Fetal bovine serum (FBS), DMEM/F12 were obtained

from Gibco (USA). HMGB1 ELISA Kit was purchased from R&D systems (USA). Ethyl pyruvate(EP) which is HMGB1 inhibitor was purchased by Sigma(USA).CFSE was purchased from BD(USA).Tris, glycine, TEMED, SDS, acrylamide were obtained from Amresco (USA).

Cell Culture

Pancreatic cancer cell lines (Panc-1 and SW1990) was purchased from Chinese Academy of Sciences Shanghai Branch Cell Bank (Shanghai, China). Panc-1 and SW1990 cells were cultured in DMEM/F12 medium supplemented with 10% FBS,5% penicillin, and 5% streptomycin at 37°C in a moist atmosphere with 5% CO₂. And cell passage dealt with when confluency of cells reached 70-80% by 0.25% trypsin.

Residual cells isolation

We collected residual Panc-1 cells after 1*4Gy radiation dose 24h later and residual SW1990 cells after 1*10Gy radiation dose 24h later. These still living Panc-1 and SW1990 cells after radiotherapy were defined as residual pancreatic cancer cells. These residual pancreatic cancer cells were cultured in DMEM/F12 medium which is supplemented with 10% FBS and cells grown as monolayers in a humidified atmosphere at 37°C,5% CO₂.Then we used these surviving cells to do some experiments at once.

Extraction of supernatant postradiotherapy

Firstly, Panc-1 and SW1990 cells were cultured about 2.5×10^5 per well. Secondly, Panc-1 and SW1990 cells cultivated 24h after radiotherapy which including 0Gy, 4Gy, 8Gy, 10Gy, and 12Gy. Thirdly, we collected the supernatant of Panc-1 and SW1990 cells using sterile EP tube.

Enzyme linked immunosorbent assay

The content of HMGB1 in the supernatant after radiotherapy was determined by using HMGB1 ELISA kit. All steps are performed in a sterile environment. Prepare all reagent and sample dilution in advance according to the instructions. Blank hole, standard hole and sample hole were set up respectively. The samples were supernatant of Panc-1 and SW1990 cells after radiotherapy which including 0Gy, 4Gy, 8Gy, 10Gy, and 12Gy. Add 100μl standard or sample per hole. Experiments were performed in triplicate.

CFSE staining

After Panc-1 and SW1990 cells stick to the wall, the experiment was divided into three groups (control group, HMGB1 group and HMGB1+EP group). After collect and wash by PBS, cells were incubated for

10min at 37°C in 5mM CFSE with fetal calf serum(FCS) free RPMI. Labelling was stopped with FBS and cells were washed 3 times prior to use. For the quantification of cell proliferation, cells were analysed by flow cytometry. Experiments were performed in triplicate.

Cell migration assay

We studied the migration of Panc-1 and SW1990 cells by wound scratch assay. Cells seeded in a 6-well plate until the confluence rate up to 60%. Through all night incubation, we made a scratch at the center of each well by a 200µl pipette tip. After the cells were incubated with PBS, 100ng/ml HMGB1 and 100ng/ml HMGB1+5µM EP-HMGB1 inhibitor for 24 hours, we use pipette scratch across the confluent cell layer. Cells were washed twice in order to remove detached cells and debris. Then, the size of wounds were observed and measured after 0h, 12h, and 24h by EMS. Each experiment was manipulated in triplicate.

Western Blot Analysis

Cultured cancer cells were washed three times in phosphate-buffered saline (PBS) and lysed with RIPA buffer. Proteins were isolated by 10% sodium dodecyl sulfate–polyacrylamide gel (SDS-PAGE). And then transferred to nitrocellulose membranes and incubated with primary antibodies all night at 4°C followed by secondary antibodies for 1h at room temperature. Blots were visualized using an ECL detection system (Amersham, Piscataway, NJ, USA). LANE-1D Analyzer (Bjsage, BJ, China) was used to quantify the intensity of protein bands. The relative intensity of β -actin, total GSK3 β and Histone 4 was calculated by normalization. Histone 4 was used as a loading control. Experiments were manipulated in triplicate.

Nude mice

Male nude mice (6 weeks old) were purchased from Yangzhou University in Jiangsu (China). All animal experimental protocols were approved conducted in accordance with the guidelines of the animal care committee of Yangzhou University. All male nude mice were fostered in animal experiment center of Jiangsu University where have the condition of constant temperature 25-27°C, constant humidity(45%-50%), fresh air, and without special pathogenic bacteria. We set up the pancreatic tumor model of nude mice by injecting SW1990 cells (2×10^6 per mouse) into the flanks of the mice subcutaneously. When the tumor reached about 5 mm in diameter, all subcutaneous tumor of nude mice were treated by radiotherapy (3*10Gy). After radiotherapy for 6 days, nine nude mice were divided into three groups and then received hypodermic injection of physiological saline, 100 ng/ml HMGB1, and 100 ng/ml HMGB1+ 5mmol/L EP around subcutaneous tumor. All tumor were measured with a ruler every 3 days. After 2 weeks treatments, all nude mice were sacrificed and then tumors were collected. Experiments were manipulated in triplicate.

Statistical analysis

All statistical analyses were performed using GraphPad Prism 5 (GraphPad Software Inc. San Diego, CA, USA). Data were presented as the mean $\pm$ standard deviation (SD). Between two groups, the statistical analysis was performed by using two-tailed unpaired t test. Among three or more group, the statistical analysis was performed by using one-way analysis of variance (ANOVA). And $p < 0.05$ suggested a statistically significant difference.

Results

HMGB1 was found in the supernatant of PC cells after radiotherapy

After treatment with different radiation dose (0,4,8,10 or 12 Gy), the content of HMGB1 in the supernatant of pancreatic cancer cell lines (SW1990, Panc-1) were assessed via ELISA. As shown in Fig. 1A, the content of HMGB1 in the supernatant of PC cells after radiotherapy was apparently higher than control group (0 Gy). And the optimal radiation dose of SW1990 cells was 10 Gy, meanwhile the optimal radiation dose of Panc-1 cells was 4 Gy (Fig. 1A; $P < 0.05$). When radiation dose was 10 Gy, the content of HMGB1 in the supernatant of SW1990 cells was (217.3 ± 35.0) ng/ml. When radiation dose was 4 Gy, the content of HMGB1 in the supernatant of Panc-1 cells was (229.9 ± 18.3) ng/ml.

Under normal culture conditions, the shape of SW1990 and Pan-1 cells were fusiform (Fig. 2A,C). However, after cultured in the supernatant of corresponding optimal radiation dose 24 h later, residual SW1990 and Pan-1 cells grew faster and larger than normal group (Fig. 2B,D).

Exogenous HMGB1 can promote the proliferation of PC cells

SW1990 and Pan-1 cells were collected and cultured in different medium (PBS, 100 ng/ml HMGB1 and 100 ng/ml HMGB1 + 5 μ M EP) respectively. Then corresponding cells were collected 96 h later and detected by using CFSE staining. The results showed that the proliferation rate of HMGB1 group was significantly higher than control group, while the proliferation rate of EP (HMGB1 inhibitor) group was down-regulated (Fig. 2A,B, $P < 0.05$). Thus, exogenous HMGB1 play a critical role in the proliferation of SW1990 and Pan-1 cells in radiation injury.

Cell proliferation was compared between control group, HMGB1 group and HMGB1 + EP group by flow cytometry. **B** data of proliferation rates are statistically analyzed between control group, HMGB1 group and HMGB1 + EP group. $*p < 0.05$, $**p < 0.01$ vs. control group

Exogenous HMGB1 can promote the migration of PC cells

The effect of exogenous HMGB1 on the migration of PC cells was evaluated via wound healing. As shown in Fig. 3A and C, with the culture time gone by, the migration distance of HMGB1 group (100 ng/ml HMGB1) in SW1990 and Panc-1 cells was bigger than control group (PBS) and HMGB1 + EP group (100 ng/ml HMGB1 + 5 μ M EP). While, the migrative ability of HMGB1 + EP group was significantly decreased, compared with that in the HMGB1 group (Fig. 3B,D; $p < 0.05$).

EMT of PC cells is affected by exogenous HMGB1

Epithelial-mesenchymal transition (EMT) is an early event in tumor migration [21]. When the EMT change occurs in cells, E-cadherin gene expression is downregulated, while N-cadherin gene expression is upregulated [22]. Ki67 is a marker commonly used to measure proliferation [23]. Glycogen synthase kinase (GSK-3 β) plays a vital role in Wnt signaling pathway [17]. As shown in Fig. 4A-B, after treatment with 100 ng/ml exogenous HMGB1, E-CA expression was downregulated and N-CA, Ki67, p-GSK 3 β were upregulated. While, HMGB1 + EP group (100 ng/ml HMGB1 + 5 μ M EP) showed the opposite results.

Exogenous HMGB1 promotes the proliferation of tumor postradiotherapy in vivo

2×10^6 SW1990 cells was injected into nude mice to detect whether exogenous HMGB1 affects the proliferation of residual tumor. In Fig. 5A, we used local radiotherapy for tumors of nude mice. As shown in Fig. 5B, exogenous HMGB1 treatment significantly promoted the proliferation of tumors postradiotherapy compared with the control. However, the proliferation of tumors was significantly inhibited in HMGB1 + EP group compared with the control group. A difference from each group was representatively shown in Fig. 5C. The analysis result of tumor volume in different groups shown that exogenous HMGB1 promotes residual tumor proliferation in vivo.

Discussion

As a highly malignant tumor, the prognosis of pancreatic cancer is poor very much due to its insidious onset, deep anatomic position, difficulty in early diagnosis, inoperability and poor response to radio-chemotherapy [24]. People often considered that radiotherapy was beneficial to eliminate pancreatic cancer. However, our research showed another phenomenon. With an estimated 232 000 new cases per year, pancreatic cancer is among the most common malignancies all around the world [25]. Sad to say, the cure rate for these “curable” PC patients is only about 20%[26]. So far the efficacy of radiotherapy alone is highly unsatisfactory [27]. Radiotherapy can really kill most of pancreatic cancer cells, but a few of PC cells can survive. This is associated with radio-resistance of residual PC cells. Meanwhile these apoptotic cells will release plenty of HMGB1 into culture supernatant after radiotherapy. These exogenous HMGB1 will play a vital role in growth and development of residual PC cells.

HMGB1 is not only a chromatin-associated nuclear protein, but also an extracellular damage-associated molecular-pattern molecule. HMGB1 is overexpressed in tumor cells and triggers inflammation, tumor

migration and cell migration [28]. Some studies have shown that HMGB1 play an important role by RAGE or the toll-like receptors TLR2 and TLR4, and the activation of these receptors can result in the activation of NF- κ B. Then, these activated signals will enhance the productions of angiogenic factors and proinflammatory cytokines in hematopoietic and endothelial cells [29]. HMGB1 has contribution in the regenerative and survival response [30]. Pancreatic cancer cells showed that the highest release of HMGB1 compared to different cancer cell lines (e.g., breast, lung and bladder) [31]. HMGB1 plays an important role in the progression and development of pancreatic cancer[32].

EMT is associated with tumor growth and especially dissemination, which has currently been found in malignant tumors such as liver cancer, pancreatic cancer, gastric cancer, colorectal cancer, and breast cancer [33, 34]. In order to colonize distant organs, cells experiencing EMT not only acquire the ability to migrate, but also undergo a lot of changes together with cell cycle exit and resistance to apoptosis [35]. In human pancreatic tumor samples, the expression of E-cadherin is decreased, with a corresponding increase in N-cadherin expression [36]. E-cadherin has been regarded as a tumor metastasis suppressor gene as well as a key gene in the EMT process [37]. It is found in this study that the expression of E-cadherin was downregulation with the upregulation of N-cadherin in HMGB1 group (($p < 0.05$).

Wnt signaling is the major pathway of embryogenesis and cancer development, while GSK-3 is a crucial kinase for this process [38, 39]. GSK-3 β play a role by the phosphorylation procedure and p-GSK 3 β regulate the downstream target genes [40]. It is reported that HMGB1 underwent EMT through GSK-3 β signaling pathway [41]. In this study, we found that p-GSK 3 β is upregulated in HMGB1 group and downregulated in HMGB1 + EP group.

By a series of experiments, we guess that radiation, which is a kind of external stimulus, induced the activation of HMGB1 in most pancreatic cancer cells. After hydroxylation of HMGB1 happened, activated HMGB1 would be released into the extracellular by the form of vesicles. Most radiation injured cells could release a lot of HMGB1 outside of the cell. There are many receptors on the cytomembrane of residual PC cells. And then GSK-3 β would be phosphorylated in these residual PC cells. Then a series of reactions would be happened (Fig. 6).

Conclusion

Our data suggest that exogenous HMGB1 can promotes the proliferation and metastasis of pancreatic cancer cells after radiotherapy. The HMGB1 in residual PC cells may lead to the activation of EMT by increasing p-GSK 3 β activity and activating Wnt signal path. We will aim at the identification of more HMGB1 target proteins in future experiment, which are necessary for elucidate the molecular mechanisms. Meanwhile, we will select more pancreatic cancer cell lines in the follow-up experiment. We hope to provide a new idea in the treatment of pancreatic cancer recurrence postradiotherapy.

Abbreviations

PC: Pancreatic cancer

HMGB1: High mobility group box-1 protein;

FBS: Fetal Bovine Serum

PBS: Phosphate Buffered Saline

ELISA: Enzyme-linked immunosorbent assay

EMT: Epithelial-Mesenchymal Transition

E-CA: E-cadherin

N-CA: N-cadherin.

Declarations

Availability of data and materials

Not applicable.

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Contributions

LZ, WQ, LS and SR performed the literature search and drafted the manuscript; Tao You prepared the figures; ZW and DW designed the review, revised the manuscript, and acquired funding.

All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

Figure 1

HMGB1 was found in the supernatant of PC cells after radiotherapy. A the content of HMGB1 in the supernatant were compared between control group and radiotherapy groups (0,4,8,10 or 12Gy) by using ELISA assay. B The images of normal pancreatic cancer and pancreatic cancer in radiation injury were collected by electron microscope. * $p < 0.05$, ** $p < 0.01$ vs. control group

Figure 2

Impact of exogenous HMGB1 on the proliferation of PC cells. A Cell proliferation was compared between control group, HMGB1 group and HMGB1+EP group by flow cytometry. B data of proliferation rates are statistically analyzed between control group, HMGB1 group and HMGB1+EP group. * $p < 0.05$, ** $p < 0.01$ vs. control group

Figure 3

Exogenous HMGB1 promote the migration of PC cells. A-B the migration of SW1990 cells was executed by using wound healing. The ability of migration was compared among control group, HMGB1 group, HMGB1+EP group for 0,24,48h. C-D the migration of Panc-1 cells was determined by using wound healing. The migrative ability was compared among control group, HMGB1 group, HMGB1+EP group for 0,24,48h. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. control group

Figure 4

Impact of exogenous HMGB1 on EMT and Wnt pathway of pancreatic cancer cells. A Western blots for E-CA, N-CA, p-GSK 3 β and Ki67 levels in control group, HMGB1 group or HMGB1+EP group. Actin, total GSK and Histone 4 were used as internal control. B Data are presented as the mean $\pm$ SD, n=3. *p<0.05, **p<0.01 vs. control group

Figure 5

exogenous HMGB1 promotes residual tumor proliferation in vivo. A Nude mice fixed and used local radiotherapy. B-C The tumor volumes of nude mice in different group (control group, HMGB1 group, HMGB1+EP group). **P<0.01, ***P<0.001 vs. control group

Radiation

Figure 6

HMGB1 promoted the proliferation and migration through upregulation of GSK-3 β in residual pancreatic cancer cells after radiotherapy