Hypofractionated Radiation Induced the Immunogenic Death of Bladder Cancer Cells Leading to the Immune Sensitization of Dendritic Cells

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Abstract

Purpose

Radiotherapy is a commonly used method in the treatment of bladder cancer (BC). Radiation induced immunogenic death (ID) and antitumor immune response are related to the prognosis of radiotherapy. As the most powerful antigen-presenting cell in the body, the role of dendritic cells (DCs) is not very clear.

Methods

Apoptosis level, cell cycle analysis and expression levels of high mobility group protein 1 (HMGB1), calreticulin (CRT) and heat shock protein 70 (HSP70) were performed for bladder cancer cells after hypofractionated radiotherapy. The effects of the conditioned media on DCs for antitumor immune response activation were studied as well.

Results

The significantly increased apoptosis level, G2/M phase cell cycle arrest and significantly increased HMGB1, CRT and HSP70 expressions, and increased secretion of CCL5 and CCL21 in the supernatant of bladder cancer cells after hypofractionated radiotherapy. The expression of CD80, CD86, CCR5 and CCR7 on DCs was upregulated in the conditioned media of bladder cancer cells after hypofractionated radiotherapy.

Conclusion

Hypofractionated radiation blocked the cell cycle of BC cells in the G₂/M phase and induced ID occurrence, resulting in DCs immune sensitization, which is of great clinical significance in understanding the radiotherapy of BC and the immunoregulation function of DCs.

Introduction

BC is one of the most common tumors of the urinary system (Antoni et al. 2017). Radical cystectomy (RC) is the recommended treatment for BC in the world (Lenis et al. 2020, Taskovska, Kreft and Smrkolj 2020). However, the loss of bladder after RC can reduce the quality of life, negatively affects the intestinal and reproductive functions, and even increase the risk of postoperative death of the patients (Liu et al. 2018). Bladder preservation therapy for treating BC has gain significant awareness for improving patient's quality of life and reducing occurrence of complications in recent years (Hafeez et al. 2020, Byun et al. 2019).

Radiotherapy is one of the most important protocols in bladder preservation therapy. Radiotherapy not only directly kills tumor cells by damaging double-stranded DNA, blocking the cell cycle, and releasing reactive oxygen species, but also can induce the immune response against cancer through antigen-presenting cells (APCs), e.g. DCs, which capture cancer antigens in the local microenvironment and present them to naïve T cells in lymph node leading to cancer antigen-specific T cell proliferation (Rompré-Brodeur et al. 2020, Siva et al. 2015).

ID is a regulated cell death induced by stressful pressure. It could enhance the antigen presentation capacity of DCs by releasing damage-associated molecular patterns (DAMPs), which activates the adaptive immune response of CD8⁺T cells. DAMPs contained molecules including HMGB1, CRT, HSP70 and adenosine triphosphate (ATP) etc. HMGB1 can induce the maturation of DCs and promote the processing and presentation of tumor antigens by MHC-II by binding to TLR9 and TLR4 (Yang et al. 2007). CRT can act as a phagocytosis signal of DCs and send out a potential immunogenicity signal to recruit DCs and enhance their antigen presentation ability. HSP70 is rapidly recognized by APCs through CD91 and activates NF-κB, which promotes the release of proinflammatory cytokines by APCs and induces inflammatory responses (Zhou and Binder 2014).

Recently, several studies show that compared with conventional radiotherapy, hypofractionated radiation can induce cancer cell apoptosis and release more cancer antigens (Rodríguez-Barbeito et al. 2019) (Salimu et al. 2015). Moreover, it can also damage tumor blood vessels, making it easier for immune cells in the blood circulation to enter the tumor tissue and activate the immune response against cancer cells (Choudhury et al. 2021, Murthy et al. 2019, Hafeez et al. 2016, Huddart et al. 2013). Clinically, radiotherapy is the main treatment for BC patients with the goal of postoperative clearance of residual lesions and bladder preservation, but the roles of hypofractionated radiation-induced immune response and DCs in BC are still elusive. Therefore, in this study, human BC cells BT-B were radiated by different doses of X-rays, whose effect on their cell cycle and ID were investigated.

Materials And Methods

Cell culture

Human BC cells BT-B: BT-B cells were cultured in RPMI-1640 medium (Gibco) supplemented with 10% fetal bovine serum (BI) and 100 IU/mL penicillin/streptomycin (Gibco) and maintained in a humidified 5% CO₂ air incubator at 37 °C.

Preparation of DCs: Human peripheral blood mononuclear cells (PBMCs) were isolated from the peripheral blood of healthy donors using the density gradient centrifugation method. After cells adhered to the wall of the culture flask, the non-adherent cells were removed. CD14⁺ monocytes were obtained from the collected adherent cells by immunomagnetic bead negative method. CD14⁺ monocytes were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (Gibco), 150 ng/ mL recombinant human granulocyte-macrophage colony-stimulating factor (rhGM-CSF, Peprotech) and 100

ng/ mL recombinant human interleukin-4 (rhIL-4, Peprotech) and maintained in a humidified 5% CO₂ air incubator at 37 °C for 5 days to induce imDCs.

Radiation treatment

The BT-B cells in the logarithmic growth phase were irradiated with 160 kV, 24 mA X-ray at the dose of 0 Gy, 2 Gy, 4 Gy, 10 Gy, 18 Gy (RS-2000, Rad Source Technologies, Inc).

BT -B cells and dendritic cells co-culturing

Irradiated 5×10^6 BT -B cells were cultured for 48 h, and the supernatant of the culture media was collected. The conditioned media was prepared by removing the cell debris at 2000 rpm for 5 min and filtered by 0.22 μ m membrane (Millipore). Then, 2×10^6 imDCs were cultured in the conditioned media containing RPMI-1640 with 20% fetal bovine serum and 1 mL supernatants of irradiated BT -B cells. The control group was 2×10^6 imDCs suspension and 1 mL Phosphate Buffer solution (PBS). The cells were maintained in a humidified 5% CO₂ air incubator at 37 °C for 48 h.

Cell cycle and apoptosis assay

After irradiation, BT-B cells were cultured routinely for 48 h. Cells were collected, fixed overnight with 70% ethanol, centrifuged at 1000 rpm for 5 min, the supernatant was discarded, washed with PBS three times, PI dye was added and incubated at room temperature for 15 min, and the cell cycle was detected by flow cytometry. The radiation treated cells were collected and fixed with 70% ethanol for 2 h, centrifuged at 1000 rpm for 5 min, the supernatant was discarded, washed with PBS three times, and Annexin V-FITC dye was added in the dark incubation for 30 min. PI dye was added before application, and the apoptosis of the cells was detected by flow cytometry.

Reverse transcription-polymerase chain reaction

After irradiation, BT -B cells were cultured routinely for 48 h to collect BT -B cells. The imDCs were co-cultured with BT-B cells culture supernatant for 48 h, then the dendritic cells were collected. Total RNA of the cells was extracted by TRIzol Reagent kit (Invitrogen, USA), and the total RNA of three replicates was quantified and evaluated for quality control by ultramicro spectrophotometer. In the 20 μ L PCR system, 1 μ g total RNA was reversed to cDNA by Fastking gDNA Dispelling RT Supermix (Tiangen, Beijing). Real-time PCR was performed using SYBR® Premix Ex Taq™ (TaKaRa, Japan) kit. The mRNA expression level of the detected gene was determined by the $\Delta\Delta C_t$ method. GAPDH was used as the internal reference gene, and the gene expression was calculated by the $2^{-\Delta\Delta C_t}$ method. The relative number of cells in the control group was set as 1, and the following specific primers were used, including forward (F) and reverse (R) primers (5' to 3'):

The primer	Primer sequences
GAPDH	F: GACCTGACCTGCCGTCTA
	R: AGGAGTGGGTGTCGCTGT
BAK1	F: GGACGACATCAACCGACGCTATG
	R: AACAGGCTGGTGGCAATCTTGG
CRT	F: AGATAAAGGTTTGCAGACAAGC
	R: CATGTCTGTCTGGTCCAACTA
Casp-1	F: TTGAAGGACAAACCGAAGGTG
	R: GTGGAAGAGCAGAAAGCGATAA
Casp-3	F: GGAACAAATGGACCTGTTGAC
	R: CTCAATGCCACAGTCCAGTTC
HMGB-1	F: AAATGAAAACCTATATCCCTCCC
	R: GGGCGATACTCAGAGCAGAAG
HSP70A1A	F: GACTCCCGTTGTCCCAAG
	R: CGGTTCCCTGCTCTCTGT
CD80	F: GTGGTCACAATGTTTCTGTTGA
	R: GTTCTTGTA CTGGGCCATATA
CD86	F: TGCTCATCTATACACGGTTACC
	R: TGCATAACACCATCATACTCGA
HLA-DR	F: CCAGAGACTACAGAGAATGTGG
	R: TTGATGATGAAGATGGTCCCAA
CCR5	F: GCAGCTCTCATTTTCCATACAG
	R: GACACCGAAGCAGAGTTTTTAG
CCR7	F: AGACCATGACCGATACCTACC
	R: GCAAAAGTGGACACCGAAGA
CD11c	F: AGCAGCCACGAACAATTCAC
	R: GAGACCTCCACATCCATCCA

Western Blot

The BT-B cells were irradiated and cultured for 48 h. The cells were collected and the total protein of the cells was extracted with RIPA cell lysis solution (SolarBio, R0010) and PMSF (SolarBio, P0100). After the protein concentration was determined by the BCA method, 50 µg of total protein was extracted by 12% digestion SDS-PAGE separation and transferred to nitrocellulose membrane (Millipore, Boston, MA, USA). The nitrocellulose membrane was sealed with 5% skim milk powder for 1 h, washed three times with TBST, and specific antibodies against GAPDH (Proteintech, 10494-1-AP), HMGB1 (BBI, D260488), CRT (Abcam, AB92516), HSP70 (Abcam, AB181606) were added, incubated overnight at 4 °C, washed with TBST for three times, horseradish peroxidase-conjugated anti-rabbit IgG (ZSGB-BIO, ZB-2306) was added, incubated at room temperature for 1 h, washed with TBST for three times. Finally, the protein bands were detected with the ECL kit (Beyotime Biotechnology, P0018S). GAPDH was used as the internal reference protein.

Enzyme-linked immunosorbent assay (ELISA)

Human CCL5 and CCL21(4A Biotech, CHE0092, CHE0140) were used for ELISA detection. We added 100 µL of standard or samples to the corresponding well of specific antibody-coated ELISA plates and incubate for 90 min at 37 °C. Each well was aspirated and washed with washing buffer (300 µL), and repeated the process three times. Next, 100 µL of biotin-antibody diluent was added to the corresponding well and incubated for 60 min at 37 °C, followed by four washes with washing buffer. Enzyme binding diluent was added 100 µL to the corresponding well and incubated for 30 min at 37 °C, followed by four washes with washing buffer. Added chromogenic agent 100 µL/well with protection from light and incubated for 20 min at 37 °C. Finally, 100 µL of stop reagent was added to the corresponding well, the reagent was run on the microplate reader (Bio-Rad) and the measurement was immediately conducted at 450 nm.

Statistical analysis

Statistical analysis was performed using GraphPad Prism version 7.0 (GraphPad Software, Inc, USA) and image data were analyzed using Image J (NIH, USA). Statistic data were expressed as the mean ± standard deviation from at least three independent experiments, and comparisons between groups were made using Student's t-test. P values less than 0.05 were considered significant.

Result

Hypofractionated radiation promoted the apoptosis of bladder cancer cells

After normal culture for 48 h, the apoptosis of BT-B cells was found closely correlated to the radiation dose (Fig. 1). The highest apoptosis level of BT-B cells was observed at the dose of 10 Gy. Meanwhile, we analyzed some apoptosis marker proteins expression for verification (Fig. 2). It was found that the expressions of apoptosis-related proteins in BT-B cells were up-regulated after irradiation.

Hypofractionated radiation altered the cell cycle of bladder cancer

The cycle progression of BT-B cells showed a dose-dependent relationship with radiation (Fig. 3). With the rise of radiation dose, the ratio of BT-B cells in the G2/G1 phase increased, with significantly increased number of cells staying in the G2 phase.

Immunogenic death of bladder cancer cells induced by hypofractionated radiation

We verified the signature proteins of ID using by analyzing both mRNA levels and proteins levels. It was found that the expression of HMGB1 and CRT in BT-B cells rose to the highest expression after 4 Gy radiation (Fig. 4). The expression of HSP70 and HMGB1 in BT-B cells at the protein level were most expressed after 10 Gy radiation (Fig. 5). In general, radiation can induce the occurrence of the ID of BT-B cells.

Hypofractionated radiation increased the secretion of CCL5 and CCL21 in bladder cancer cells

After radiation, the concentration of CCL5 and CCL21 in the supernatant of BT-B cells increased gradually with hypofractionated radiation. In BT-B cells, the secretion of CCL5 and CCL21 both reached the highest level under 10 Gy radiation (Fig. 6).

Bladder cancer cells affected the function of immature dendritic cells after radiation

After co-cultured with BT-B cells supernatant after different radiation doses, the expression of CD80, CD86 and CCR7 on imDCs were upregulated and the expression of HLA-DR was down-regulated, the expression level of CCR5 peaked in 18 Gy group, while CD80 and CD86 peaked in 10 Gy group and CCR7 peaked in 4 Gy group, respectively (Fig. 7).

Discussion

Radiotherapy provides an alternative treatment for BC for patients who refuse or not allow to take RC (Storozynsky and Hitt 2020). In this study, we observed the highest BT-B cells apoptosis level in 10 Gy irradiation group using flow cytometry (Fig. 1). Caspase-1 promotes apoptosis by activating IL-1 β and IL-18 (Sun and Scott 2016, Miao, Rajan and Aderem 2011). BAK1 promotes apoptosis by leading to the reduction of membrane potential and the release of cytochrome C (Uren et al. 2017, Guttà et al. 2020). The protein encoded by Caspase-3 cleaves cellular proteins, such as cytoskeletal protein and proteins of repairing DNA, causing apoptosis (Zhou et al. 2018, Liu et al. 2017). The expression levels of Caspase-1, Caspase-3 and BAK1 increased after hypofractionated radiotherapy correlated well with the results from apoptosis assay detected by flow cytometry (Fig. 2), thereby confirmed the apoptosis of bladder cancer

cells at the molecular and cellular levels. On the other hand, we found a dose-dependent relationship between BT-B cell cycle progression and radiotherapy dose (Fig. 3). After hypofractionated radiation, the number of BC cells remaining in the G2 phase increased significantly, indicating that the G2/M checkpoint blocked the cell cycle progression. Compared to G1 and S phases, cells in M and G2 phases have higher radiosensitivity (Kastan and Bartek 2004, Bhoora and Punchoo 2020, Williams and Stoeber 2012), indicating that hypofractionated radiation not only promoted the apoptosis of BC cells but also helped improve the sensitivity of BC cells to radiation.

Classic radiation oncology theory valued the radiation-induced DNA damage to malignant cells, while recent studies have shown that the immune response triggered in the irradiated tissue played the key roles in driving the efficacy of radiation therapy *in vivo* (Dar, Henson and Shiao 2018). Savage T et al. show that the expression of CRT and HMGB1 increase in breast cancer tumor tissues of mice after hypofractionated radiation, enhancing tumor immunogenicity (Savage, Pandey and Guha 2020, Golden et al. 2014). We found that the expressions of HSP70, HMGB1 and CRT in BT-B cells after hypofractionated radiation were all higher than those after conventional radiation (Fig. 4, Fig. 5). It showed that HSP70 can be rapidly recognized by APCs through cell surface receptors CD91 to promote the release of pro-inflammatory cytokines by APCs and trigger the Th17 response (Zhou and Binder 2014, Choudhury et al. 2021, Pawaria and Binder 2011). HMGB1 is a histone-chromatin binding protein released from radiation-damaged tumor cells, and its release amount is related to the degree of apoptosis (Venereau et al. 2012). Our results confirm the correctness of the previous research, that the HMGB1 expression and apoptosis of BC cells reached the highest level at 10 Gy irradiation dose. HMGB1 can also bind to TLR4 and TLR9 to up-regulate the expression of DCs surface molecules and promote the maturation of DCs (Kamo et al. 2013, Andersson and Tracey 2011, Yang et al. 2007). CRT exposure on the cell surface can act as DCs phagocytosis signal, promote DCs recruitment and enhance DCs antigen phagocytosis and presentation ability (Schcolnik-Cabrera et al. 2019, Venkateswaran et al. 2018). The increased expression of HSP70, HMGB1 and CRT in BC cells after hypofractionated radiation indicated the occurrence of ID.

The occurrence of ID can induce DCs to mature and infiltrate into tumor tissue and activate the anti-tumor immune response (Zhou et al. 2019, Derer et al. 2015, Rapoport and Anderson 2019). Therefore, we analyzed the concentration of DCs chemokines CCL5 and CCL21 in the supernatant of BC cell culture after radiation, as well as the expression of DCs surface molecules after co-culture with the supernatant (Fig. 6, Fig. 7). Studies show that the concentration of CCL5 in the adult kidney is 97.51 pg/mL (Gawłowska-Marciniak and Niedzielski 2013), and that in the supernatant of human peripheral nerve cells is 359.2 pg/mL (Tianyi and Zhiyuan 2017). Our study found that hypofractionated radiation increased the secretion of CCL5. The highest concentration of CCL5 was secreted by BT-B cells at 10 Gy radiation, reaching 2430 pg/mL. When DCs receives antigenic stimulation, imDCs gradually transform into mDCs, which manifest as the upregulated expression of co-stimulatory molecule CD80, CD86 and chemokine receptor CCR7, and chemotactic migration to secondary lymphoid tissue along the concentration gradient of CCL19 and CCL21 (Marsland et al. 2005). In our study, the expression of CD80, CD86 and CCR7 on imDCs was significantly increased after co-culture with BT-B cells after hypofractionated radiation. This is consistent with the findings of Kulzer L et al. in colorectal cancer after 5 Gy radiation (Kulzer et al. 2014,

Rodríguez-Barbeito et al. 2019). At the same time, radiation increased the secretion of CCL21 in BC cells, which reached the highest at 10 Gy radiation. The above results suggested that the BC cells after hypofractionated radiation were more conducive to the maturation and of migration abilities DCs.

In conclusion, compared with conventional radiotherapy (2 Gy), hypofractionated radiation can induce stronger apoptosis, cell cycle arrest and immunogenic cell death of BT-B bladder cancer cells and promote maturation and migration abilities of DCs to serve better anti-tumor immunity effect. Such findings may contribute to the improvement of radiotherapy protocols for the most beneficial induction of anti-tumor immunity in bladder cancer treatments.

Declarations

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Conflicts of interest

The authors declare that there are no conflicts of interest.

Ethics approval

This is an in vitro study. No ethics approval was required.

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Figures

Figure 1

Effects of different radiation doses on the apoptosis of BT-B cells. The apoptosis of BT-B cells irradiated by 0, 2, 4, 10, 18 Gy was analyzed by flow cytometry. (NS>0.05; *P<0.05; **P<0.01)

Figure 2

Gene expression of BT-B cells apoptosis-related proteins at different radiation doses. The mRNA expression levels of Casp-1 (a), Casp-3 (b) and BAK1 in BT-B cells irradiated by 0, 2, 4, 10 and 18 Gy (c). (NS>0.05; *P<0.05; **P<0.01; ***P<0.001)

Figure 3

Effects of different radiation doses on the BT-B cell cycle. The cell cycle of BT-B cells irradiated by 0, 2, 4, 10, 18 Gy was analyzed by flow cytometry. (NS>0.05; *P<0.05; **P<0.01)

Figure 4

The gene expression of ID related proteins in BT-B cells at different radiation doses. The mRNA expression of HSP70A1A (a), HMGB1 (b) and CRT (c) in BT-B cells after irradiation. (NS>0.05; *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001)

Figure 5

Relative expression levels of ID related proteins in BT -B cells at different radiation doses. The protein expression of HSP70, HMGB-1 and CRT in BT-B cells after irradiation. (NS>0.05; *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001)

Figure 6

Concentration of BT-B cells chemokines at different radiation doses. The concentration of CCL5 (a) and CCL21 (b) in the conditioned medium; In the statistical graph. 0 Gy denoted the conditioned media prepared after 48 h culture with BT-B cells without radiation treatment. 2 Gy, 4 Gy, 10 Gy and 18 Gy respectively denoted the conditioned media prepared after 48 h culture with BT-B cells treated with 2 Gy, 4 Gy, 10 Gy and 18 Gy radiation. (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$)

Figure 7

Expression of molecular genes on the surface of dendritic cells after co-culture with BT-B at different radiation doses. The mRNA expression of HLA-DR(a) CD11c(b) CD80(c) CD86(d) CCR5(e) CCR7(f) in dendritic cells after co-culture for 48 h is shown above. In the statistical graph, B denoted the control group, the dendritic cells without co-culture with BT-B cells. 0 Gy denoted dendritic cells co-cultured with BT-B cells without radiation treatment for 48 h. 2 Gy, 4 Gy, 10 Gy and 18 Gy respectively denoted dendritic cells co-cultured with 2 Gy, 4 Gy, 10 Gy and 18 Gy radiated BT-B cells for 48 h. (NS>0.05; *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001)