Abstract. Osteosarcoma is the most common primary bone tumor and predominantly affects children and adolescents. The prognosis for patients with osteosarcoma is poor. Therefore, the development of novel treatments for osteosarcoma is required. Photodynamic therapy (PDT) is a disease site-specific treatment that utilizes a photosensitizing agent along with light to kill cancer cells. This agent only works following activation by certain wavelengths of light. After the agent is absorbed by the cancer cells, light is then applied to the area to be treated. The light causes the drug to react with oxygen, which produces radical and reactive oxygen species that kill the cells. However, the immune reaction that occurs following PDT remains unknown. The present study demonstrated that the necrosis of osteosarcoma cells inhibited the function of dendritic cells. However, treatment of osteosarcoma cells with PDT restored the function of dendritic cells by upregulating heat shock protein 70. Taken together, the results of the present study provided insight into the subsequent molecular reaction following PDT treatment of osteosarcoma at the molecular level.

Introduction

Osteosarcoma is the most common primary bone tumor and predominantly affects children and adolescents (1-3). The current treatment of osteosarcoma remains difficult, and osteosarcoma causes numerous mortalities due to its complex pathogenesis and resistance to conventional treatments (4).

Photodynamic therapy (PDT) is a disease site-specific treatment. It involves the local systemic administration of a photosensitizer followed by irradiation of the targeted lesion site with non-thermal visible light of an appropriate wavelength. In the presence of molecular oxygen, the light irradiation of the photosensitizer and energy transfer may lead to a number of photochemical reactions and generation of various cytotoxic species, thereby inducing apoptosis and necrosis of the targeted lesion (5). Osteosarcoma has been studied by our group for a number of years and have investigated the possibility of applying PDT to the treatment of osteosarcoma (6,7).

Previously, another study indicated that PDT causes acute inflammation, expression of heat-shock proteins, invasion and the infiltration of the tumor by leukocytes, and that it may increase the presentation of tumor-derived antigens to T cells by regulating dendritic cells (DCs) (8).

DCs are a heterogeneous population of antigen-presenting cells. Their main function is to process antigens and present them to T cells to promote immunity toward foreign antigens and tolerance toward self-antigens (9-15). It has been demonstrated that the inflammation milieu induced by PDT promoted the antigen-presenting function of DCs (16).

It has been previously reported that PDT treatment results in the induction of apoptotic and necrotic cell death, and that immature DCs co-cultured with PDT-treated tumor cells results in effective homing to regional and peripheral lymph nodes and stimulation of the cytotoxic activity of T and natural killer cells (17). However, the exact mechanism by which the DCs are initiated remains unclear. In the study, we firstly hypothesized that the necrosis of osteosarcoma cells inhibited the function of DCs and treatment with PDT restored the function of DCs. The present study may provide insight into the mechanism underlying the function of DCs following the treatment of osteosarcoma via PDT.

Materials and methods

Mice. A total of 20 C57BL/6 mice (6-8 weeks old, 21±2.1 g, male) were obtained from the Animal Center of the Second Military Medical University (Shanghai, China). A total of 10 DO11.10 OVA323-339-specific mice (6-8 weeks old, 21±2.1 g, male) with C57BL/6 background were obtained from the Jackson Laboratory (C57BL/6 x DO11.10). A total of 10 F1 mice (21±2.1 g, male) were prepared by crossing C57BL/6 mice with DO11.10 mice. All mice were maintained under specific pathogen-free conditions and used at 6-8 weeks of age.
age. Mice were provided with ad libitum access to food and water. Room conditions were controlled for humidity (40-70%) and temperature (22±3˚C) with a 12/12 h light/dark cycle. The use of the mice was approved by the Ethics Committee of Tongji University (Shanghai, China).

**Photodynamic therapy.** The photodynamic treatments were performed at the Department of Orthopedics of Shanghai Tenth People’s Hospital (Shanghai, China). The entire process was performed as previously described (18).

**Cell culture and treatment.** The murine osteosarcoma LM8 cell line was obtained from the Type Culture Collection of Chinese Academy of Sciences (Manassas, VA, USA) and was cultured in Dulbecco’s modified Eagle’s medium (catalog no. SH30022.01; HyClone, GE Healthcare Life Sciences, Logan, UT, USA), containing 10% fetal bovine serum (cat. no. 10100139; FBS; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), 1% penicillin and 1% streptomycin. Cells were placed at 37˚C in a humidified 5% CO₂ incubator for 24 h.

GM-CSF (catalog no. 11904018, Thermo Fisher Scientific, Inc). The T-cell proliferation analysis.

**RNA sequencing (RNA-SEQ) and reverse transcription-quantitative polymerase chain reaction (RT-qPCR).** Total RNA were extracted from LM8 cells by TRizol® Reagent (catalog no. 15596018, Thermo Fisher Scientific, Inc.). RNA-SEQ was performed by Shanghai Shengong Engineering Technology Service, Ltd. (Shanghai, China). The expression levels of heat shock protein 70 (HSP70), activating transcription factor 3 (ATF3), B-cell lymphoma 2 (Bcl-2), tumor protein (P)53, P21, P16 and P27 levels were analyzed by RT-qPCR. RNA were reversely-transcribed by SuperScript™ First-Strand Synthesis System for RT-PCR (catalog no. 11904018, Thermo Fisher Scientific, Inc). The thermocycling conditions were 42˚C for 50 min, 70˚C for 15 min. PCR primers were constructed by Shanghai Shengong Engineering Technology Service, Ltd. qPCR reactions were performed in 96-well optical reaction plates using the cDNA equivalent of 20 ng total RNA for each sample in a total volume of 20 µl containing 1X SYBR® Green PCR master mix (24) (cat. no. A25741; Thermo Fisher Scientific) and forward and reverse primers were listed as following: HSP70, forward, 5’-GATGCCCAGCGTACAGTCC-3’, reverse, 5’-ACAGACATTGGTCACTGCTTTT-3’; ATF3, forward, 5’TGCAAAGAAGAATCGGACAG-3’, and reverse, 5’-CCTGCCCTGGATTTGAACGAT-3’; p53,
forward, 5'-CCCCTCCTGGCCCCCTGTCCATCCTTC-3', and reverse, 5'-GCCAGGCCTCAACACTCCGTCTCAT-3'; p21, forward, 5'-GAGCGGGCATGCTTGGGAGGAG-3', and reverse, 5'-GAGGCACTGTGGAGCTTCCGG, and reverse, 5'-CATGTTACTGCTCCTTGGTG-3'; p27, forward, 5'-GGTTAGCGGAGCAATGC-3', and reverse, 5'-TCCACAGAACCNGCATTTG-3'; GAPDH, forward, 5'-CGAGCCACTTGGTCAGCTCA-3', and reverse, 5'-AGGGGTCTACATGGCAACTG-3'. The thermocycling conditions were as follows: 95°C for 3 min and 40 cycles of amplification comprising 95°C for 12 sec, appropriate annealing temperature (60°C) for 30 sec, and 72°C for 30 sec (25-27).

**Statistical analysis.** A two-tailed unpaired Student's t-test was used to analyze the difference between two groups. Analysis of variance, followed by the least significant difference test, was used to analyze the difference among three groups. SPSS for Windows, v.16.0 (SPSS Inc., Chicago, IL, USA), was used to perform all statistical analyses. Values are expressed as the mean ± standard deviation of three independent tests. *P<0.05 was considered to indicate a statistically significant difference.

**Results**

The remnants of LM8 cells inhibit the function of DCs. To begin with, the effects of the lysis remnants of osteosarcoma
cells on the function of DCs were determined. The bone marrow-derived DCs were co-cultured with the remnants of osteosarcoma LM8 cells. DCs co-cultured with medium were used as control. After a 48-h co-culture, the DCs were isolated for phenotype and cytokine analysis, and their ability to stimulate the proliferation of T-cells was also investigated. It was revealed that the remnants of LM8 cells significantly inhibited the expression of major histocompatibility complex 2 (MHC-2), CD40, CD86, CD80 and C-C chemokine receptor type 7 (Fig. 1A). The treatment of LM8 cells decreased IL-12 levels and increased IL-10 levels (Fig. 1B). The mixed lymphocyte reaction analysis revealed that the treatment of LM8 cells decreased the ability of DCs to stimulate T-cell proliferation (Fig. 1C).

**PDT treatment reduces the inhibitory function of the LM8 remnants.** To investigate the role of PDT in the inflammation, we first treated LM8 cells with PDT and then collected the remnants of LM8 treated cells (PDT-LM8) for co-cultured with the bone marrow-derived DCs. We found that PDT-LM8 upregulated MHC-2 expression (Fig. 2A). We then analyzed the co-expression molecules and found that PDT-LM8 treated by PDT up-regulated the CD80, CD86, Ia, CD11c, CD40, and CCR7 (Fig. 2B). The mixed lymphocyte reaction analysis revealed that the PDT treatment may increase the ability of DCs in stimulating T cell proliferation (Fig. 2C).

**HSP70 is upregulated by PDT.** To identify the key molecular elements altered in osteosarcoma cells treated with PDT, the differential gene expression between LM8 cells treated with PDT and the control was determined by RNA-SEQ analysis. The data indicated that HSP70 was upregulated in the PDT-treated LM8 cells (Fig. 3A). As HSP70 serves an important role in the activation of DCs (28-30), the expression of HSP70 and related genes, including ATF3, BCL2, P53, P21, P16 and P27, was subsequently assessed by RT-qPCR. It was revealed that HSP70 was upregulated following PDT treatment (Fig. 3B). Next, the HSP70 levels were inhibited in
Figure 3. PDT treatment upregulated the HSP70 expression in LM8 cells and promoted upregulation of HSP70-activated DCs. (A) The LM8 cells with and without PDT pre-treatment were collected for RNA sequencing analysis. (B) The expression of HSP70, ATF3, Bcl-2, P53, P21, P16 and P27 was analyzed by reverse transcription-quantitative PCR. (C) LM8 cells were transfected with HSP70 small interfering RNA, and the DCs were then co-cultured with LM8 for 48 h, prior to being labeled with an antibody against CD86 for phenotypic analysis by flow cytometry. The numbers in the histograms indicate the geometric mean fluorescence intensity.

Data represent one of at least three experiments with similar results. *P<0.05 HSP70-si-PDT-LM8 vs PDT-LM8. PDT, photodynamic therapy; HSP70, heat shock protein 70; DCs, dendritic cells; ATF3, activating transcription factor 3; Bcl-2, B-cell lymphoma 2; PCR, polymerase chain reaction; CD, cluster of differentiation.
Discussion

PDT is a novel approach for the treatment of osteosarcoma. However, the underlying mechanisms affecting subsequent immune reaction remains unclear. The present study provided insights into these molecular mechanisms and revealed that the remnants of osteosarcoma cells inhibited the functions of DCs and that treatment with PDT reduced this inhibitory function. Notably, the upregulation of HSP70 was involved in the underlying mechanism regarding this phenomenon.

It has been established that the function of DCs in tumors is inhibited. In a previous study, authors revealed that the tumor microenvironment was able to induce DCs to differentiate into regulatory DCs with a CD11c(low) CD11b(high) Ia(low) phenotype and a high expression of IL-10, nitric oxide, vascular endothelial growth factor and arginase I. These tumor-educated regulatory DCs suppress T cell response (31). Notably, PDT treatment changed the gene expression of osteosarcoma cells and induced upregulation of HSP70. HSP70 has been demonstrated to activate the function of DCs (28-30). As it has been established that tumor-educated DCs develop into DCs with immune suppressive functions. The present study revealed that PDT treatment promoted the normal function of DCs.

However, for CD11c(low) CD11b(high) Ia(low) regulatory DCs, tumor-derived transforming growth factor β (TGF-β) and prostaglandin E2 (PGE2) are responsible for the generation of regulatory DCs. The present study revealed that upregulation of HSP70 reversed the inhibitory function of the tumor on DCs. Therefore, we concluded that there were two possible reasons for this: i) PDT treatment may prevent the process of tumor-educated DCs via HSP70, or ii) PDT treatment may re-educate the tumor-educated DCs in a HSP70-dependent manner.

In the process of inducing DCs with immune suppressive functions, the soluble factors TGF-β and PGE2 served central roles. Following PDT treatment, HSP70, an insoluble factor, was able to reverse the function of DCs. Therefore, the results of the present study indicated that soluble factors and other molecules expressed by tumors may affect the function of DCs.

Furthermore, these data also indicated that that p53 was upregulated following treatment of LM8 cells with PDT (Fig. 2B). However, it was previously demonstrated that the p53 gene was most often mutated/deleted in human osteosarcoma (32,33), and it is possible that the OS cell lines used in those studies lacked p53 expression. Due to the fact that the results of the present study cannot confirm that p53 was upregulated by PDT treatment, this will be investigated in future studies.

In conclusion, the results of the present study revealed that the remnants of osteosarcoma treated with PDT induced the activation of DCs, and that the molecular mechanism involved upregulation of HSP70 expression induced by PDT. Therefore, the present study may provide novel insight into the treatment of osteosarcoma via PDT and its effects on the function of DCs.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

FZ and YZ collected patient data and performed cell experiments. GF performed the PCR molecular experiment and FACS. SH contributed to the study design and manuscript writing.

Ethics statement and consent to participate

The present study was approved by Ethics Committee of Tongji University (Shanghai, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Reference

1. Mirabello L, Troisi RJ and Savage SA: Osteosarcoma incidence and survival rates from 1973 to 2004: Data from the surveillance, epidemiology, and end results program. Cancer 115: 1531-1545, 2009.
2. Kansara M and Thomas DM: Molecular pathogenesis of osteosarcoma. DNA Cell Biol 26: 1-18, 2007.
3. Ottaviani G and Jaffe N: The epidemiology of osteosarcoma. Cancer Treat Res 152: 3-13, 2009.
4. Miao J, Wu S, Peng Z, Tania M and Zhang C: MicroRNAs in osteosarcoma: Diagnostic and therapeutic aspects. Tumor Biol 34: 2093-2098, 2013.
5. Huang Z: A review of progress in clinical photodynamic therapy. Technol Cancer Res Treat 4: 283-293, 2005.
6. Gong HY, Sun MX, Hu S, Tao YY, Gao B, Li GD, Cai ZD and Yao JZ: Benzochloroporphyrin derivative induced cytotoxicity and inhibition of tumor recurrence during photodynamic therapy for osteosarcoma. Asian Pac J Cancer Prev 14: 3351-3355, 2013.
7. Zeng H, Sun M, Zhou C, Yin F, Wang Z, Hua Y and Cai Z: Hematoporphyrin monomethyl ether-mediated photodynamic therapy selectively kills sarcomas by inducing apoptosis. PloS One 8: e77727, 2013.
8. Castano AP, Mroz P and Hamblin MR: Photodynamic therapy and anti-tumour immunity. Nat Rev Cancer 6: 535-545, 2006.

9. Merad M: Dendritic cells: Controllers of adaptive immunity. Nat Rev Immunol 11: 1-2, 2011.

10. Schuler G, Schuler-Thurner B and Steinman RM: The use of dendritic cells in cancer immunotherapy. Curr Opin Immunol 15: 138-147, 2003.

11. Merad M, Sathe P, Helft J, Miller J and Mortha A: The dendritic cell lineage: Ontogeny and function of dendritic cells and their subsets in the steady state and the inflamed setting. Annu Rev Immunol 31: 563-604, 2013.

12. Steinman RM: Decisions about dendritic cells: Past, present, and future. Annu Rev Immunol 30: 1-22, 2012.

13. Chen R, Deng X, Wu H, Peng P, Wen B, Li F and Li F: Combined immunotherapy with dendritic cells and cytokine-induced killer cells for malignant tumors: A systematic review and meta-analysis. Int Immunopharmacol 22: 451-464, 2014.

14. Guilliams M, Ginhoux F, Jakubzick C, Naik SH, Onai N, Schraml BU, Segura E, Tussiwand R and Yona S: Dendritic cells, monocytes and macrophages: A unified nomenclature based on ontogeny. Nat Rev Immunol 14: 571-578, 2014.

15. Anguille S, Smits EL, Lion E, van Tendeloo VF and Berneman ZN: Clinical use of dendritic cells for cancer therapy. Lancet Oncol 15: e257-e267, 2014.

16. Pizzo V, Tomankova K, Daskova A, Binder S, Bajgar R and Kolarova H: Photodynamic therapy for enhancing antitumour immunity. Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub 156: 93-102, 2012.

17. Jalili A, Makowski M, Switaj T, Nowis D, Wilczynski GM, Wiczek E, Chorazy-Massalska A, Radzikowska A, Maslinski W, Bialy L, et al: Effective photoimmunotherapy of murine colon carcinoma induced by the combination of photodynamic therapy and dendritic cells. Clin Cancer Res 10: 4498-4508, 2004.

18. Szczko J, Nowak M, Skolacka N, Kulbacka J and Kotul ska M: The effects of the electro-photodynamic in vitro treatment on human lung adenocarcinoma cells. Bioelectrochemistry 79: 90-94, 2010.

19. Zhang M, Tang H, Guo Z, An H, Zhu X, Song W, Guo J, Huang X, Chen T, Wang J and Cao X: Splenic stroma drives mature dendritic cells to differentiate into regulatory dendritic cells. Nat Immunol 5: 1124-1133, 2004.

20. Tang H, Guo Z, Zhang M, Wang J, Chen G and Cao X: Endothelial stroma programs hematopoietic stem cells to differentiate into regulatory dendritic cells through IL-10. Blood 108: 1189-1197, 2006.

21. Xia S, Guo Z, Xu X, Yi H, Wang Q and Cao X: Hepatic microenvironment programs hematopoietic progenitor differentiation into regulatory dendritic cells, maintaining liver tolerance. Blood 112: 3175-3185, 2008.

22. Li Q, Guo Z, Xu X, Xia S and Cao X: Pulmonary stromal cells induce the generation of regulatory DC attenuating T-cell-mediated lung inflammation. Eur J Immunol 38: 2751-2761, 2008.

23. Lutz MB, Kukutsch N, Ogilvie AL, Rössner S, Koch F, Romani N and Schuler G: An advanced culture method for generating large quantities of highly pure dendritic cells from mouse bone marrow. J Immunol Methods 223: 77-92, 1999.

24. Morrison TB, Weis JJ and Wittwer CT: Quantification of low-copy transcripts by continuous SYBR Green I monitoring during amplification. Biotechniques 24: 954-958, 960, 962, 1998.

25. Chidlow G, Wood JP and Casson RJ: Expression of inducible heat shock proteins Hsp27 and Hsp70 in the visual pathway of rats subjected to various models of retinal ganglion cell injury. PLoS One 9: e114838, 2014.

26. Dorak MT: Real-time PCR. Taylor & Francis 2007.

27. Fraga D, Meulia T and Fenster S: Real-time PCR. Current protocols essential laboratory techniques: 10.13. 11-10.13. 40, 2008.

28. Floto RA, MacAry PA, Boname JM, Mien TS, Kampmann B, Hair JR, Huey OS, Houben EN, Pieters J, Day C, et al: Dendritic cell stimulation by mycobacterial Hsp70 is mediated through CCR5. Science 314: 454-458, 2006.

29. MacAry PA, Javid B, Floto RA, Smith KG, Oehlmann W, Singh M and Lehner PJ: HSP70 peptide binding mutants separate antigen delivery from dendritic cell stimulation. Immunity 20: 95-106, 2004.

30. Wu Y, Wan T, Zhou X, Wang B, Yang F, Li N, Chen G, Dai S, Liu S, Zhang M and Cao X: Hsp70-like protein 1 fusion protein enhances induction of carcinoembryonic antigen-specific CD8+ CTL response by dendritic cell vaccine. Cancer Res 65: 4947-4954, 2005.

31. Liu Q, Zhang C, Sun A, Zheng Y, Wang L and Cao X: Tumor-educated CD11bhighIalow regulatory dendritic cells suppress T cell response through arginase I. J Immunol 182: 6207-6216, 2009.

32. Kansara M, Teng MW, Smyth MJ and Thomas DM: Translational biology of osteosarcoma. Nat Rev Cancer 14: 722-735, 2014.

33. Chen X, Bahrami A, Pappo A, Easton J, Dalton J, Hedlund E, Ellison D, Shurtleff S, Wu G, Wei L, et al: Recurrent somatic structural variations contribute to tumorigenesis in pediatric osteosarcoma. Cell Rep 7: 104-112, 2014.