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Exploring the Relationship Between HMGB1, CXCL12, CXCR4, and CXCR7 in the Context of Adriamycin-Induced Cardiotoxicity

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ABSTRACT

Objective: High-mobility group box-1 (HMGB1), known as an abundant and highly conserved nuclear protein, plays a pivotal role in initiating inflammation, tissue healing, and the immune response following various forms of cell damage. The chemokine C-X-C motif chemokine ligand 12 (CXCL12) forms a signaling axis known as CXCL12/ CXCR4/CXCR7, along with the receptors CXCR4 and CXCR7. Our study aimed to explore the connection between HMGB1 and the involved chemokine axis, CXCR4, CXCL12, and CXCR7, in the context of adriamycin-induced cardiotoxicity.

Materials and Methods: We performed RNA interference to suppress HMGB1 expression in H9c2 cardiac myoblast cells. Adriamycin, an anti-tumor antibiotic known for causing cardiotoxicity, was used in conjunction with HMGB1 suppression. We investigated the combined and individual effects of these factors. Gene expression analysis was conducted through qRT-PCR 36 and 48 h post-treatment.

Results: Adriamycin treatment increased the expression of CXCL12, CXCR4, and CXCR7. Notably, our study observed significant changes in gene expression when HMGB1 was downregulated and adriamycin was administered. These findings suggest potential molecular mechanisms associated with adriamycin-induced cardiotoxicity, emphasizing the significance of the CXCR4/CXCL12 axis and the impact of HMGB1 modulation.

Conclusion: Our study provides insights into the molecular interplay between HMGB1 and the CXCL12/CXCR4/CXCR7 ligand-receptor axis in the context of adriamycin-induced cardiotoxicity. The results shed light on further research to enhance therapeutic approaches or advance new strategies to address this cardiotoxicity.

Keywords: Adriamycin, cardiotoxicity, CXCL12, CXCR4, CXCR7, gene expression, HMGB1

INTRODUCTION

Chemokines, a family of cytokines, play a pivotal role in regulating various cellular functions. They are primarily known for their essential roles in immune cell recruitment, which is crucial for the body's response to infections and tissue damage. By directing immune cells to specific sites within the body where their actions are most needed, chemokines influence various pathological conditions such as inflammation, atherosclerosis, hematopoiesis, and cancer (1).

The chemokine superfamily is composed of numerous chemokines and chemokine receptors. To date, more

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than 50 chemokines and their receptors have been identified. Chemokines and their receptors are named using the term "chemokine" followed by an abbreviation for the ligand (L), and for chemokine receptors, the abbreviation (R) is used (2). The C-X-C motif chemokine ligand 12 (CXCL12) interacts with two seven-transmembrane domain G protein-coupled receptors: C-X-C chemokine receptor type 4 (CXCR4) and C-X-C chemokine receptor type 7 (CXCR7) (3). This interaction forms the CXCL12/CXCR4/CXCR7 axis, transmitting signals through its receptors; CXCR4 and CXCR7. High-mobility group box-1 (HMGB1), a highly conserved and abundant nuclear protein present in all eukaryotic cells, plays a key role in initiating inflammation, adaptive and innate immunity, as well as tissue healing following damage (4).

Unlike programmed apoptotic cell death, dead or dying cells release damage-associated molecular patterns (DAMPs). HMGB1 is the best-characterized DAMP, serving as a danger signal or alarm (5). Following cell death, HMGB1, a protein located inside the cell, is released (6). During stressful situations, HMGB1, a sensitive protein, is relocated from the nucleus to the secretory lysosomes in the cytoplasm or directly to the extracellular space. The released extracellular HMGB1 switches among different binding partners and receptors, triggering adaptive immunity and inflammation (5). Damaged tissue attracts immune cells, which then become active, and HMGB1 contributes to repair.

Post-transcriptional modifications in HMGB1 result in its functioning as different ligands for various receptors. For example, while acetylation doesn't appear to alter HMGB1's binding activity, it does induce changes in its redox state. Inside the cytoplasm and nucleus, HMGB1 has a strong reducing (redox) potential, but it becomes oxidized when it's outside the cell.

Upon reduction, HMGB1 forms a hetero-complex with the chemokine CXCL12, facilitating its subsequent binding to CXCR4. This interaction influences the balance of various signaling pathways, including G proteins and calcium pathways, as well as a range of signaling molecules such as beta-arrestins, JAK, GRK, MAPK, and PI3K kinases (5, 7).

Adriamycin (ADR), an antibiotic with anti-tumor properties, is used in the treatment of various types of cancer, including solid tumors, leukemia, and lymphomas (8). Also known as doxorubicin, adriamycin is an effective drug that targets cancer cells to inhibit their growth and proliferation (9). However, this treatment can lead to cardiotoxicity, in addition to side effects such as nausea, vomiting, hair loss, and hematopoietic suppression (10). Symptoms can vary, ranging from arrhythmia to heart failure, during or after the treatment process (11). Luo et al. showed that HMGB1 contributes to ADR induced cardiotoxicity by upregulating autophagy (10).

In a previous study, we demonstrated that the inhibition of HMGB1 could prevent cardiac cell loss by suppressing the apoptotic pathway (12). The severe cardiotoxicity of ADR limits its clinical applications, and its mechanisms are not yet fully understood. Our study aimed to explore the relationship between HMGB1 and the receptors CXCR4 and CXCR7, in conjunction with the chemokine CXCL12, within the H9c2 cell line. The goal of this investigation was to shed light on the mechanisms underlying cardiotoxicity following adriamycin treatment.

MATERIALS AND METHODS

Cell Culture

The H9c2 cell line comprising cardiac myoblast cells was obtained from ATCC and cultured using DMEM as per ATCC recommendations. Cells were sub-cultured every 2–3 days upon reaching 70%–80% confluence.

Knockdown of HMGB1 by RNA Interference and Adriamycin Treatment

To test our hypothesis, we created four main experimental groups using exponentially growing H9c2 cardiac myoblast cells: (I) Control group treated with non-targeting small interfering RNA (siRNA); (II) siRNA group treated with 10 nM HMGB1 siRNA; (III) ADR group treated with 2 µM adriamycin, and (IV) siRNA + ADR group treated with both siRNA and adriamycin. Cells were transfected with HMGB1 siRNA (GE Healthcare Dharmacon, Lafayette, CO, United States) for knockdown (13), using non-targeting siRNA (Healthcare Dharmacon, Lafayette, CO, United States) as a negative control. Transfection was performed using a HiPerFect Transfection Reagent (Qiagen, Valencia, CA). We tested various ratios of siRNA and transfection reagent at different time points for transfection optimization. Based on these trials, we decided to use a 10 nM siRNA dose and 3 µL of transfection reagent (for a 24-well plate). Due to the ineffectiveness of achieving a significant knockdown within 24 h, we chose incubation times of 36 and 48 h for the experiments. siRNA and ADR applications were conducted simultaneously, and each group was replicated.

Expression Analysis

The real time–polymerase chain reaction (RT-PCR) technique was utilized to explore the effects of administered siRNA on gene expression and to determine the rate of gene knockdown in the cells. The PureLink RNA Mini Kit (Thermo Fisher Scientific, Waltham, Massachusetts) was used to isolate total RNA, adhering to the instructions provided by the manufacturer. The isolated RNA was then employed for cDNA synthesis using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Waltham, Massachusetts). The reverse transcription process was executed using random hexamers, following the manufacturer's instructions under suitable conditions. The synthesized cDNAs and primers, with sequences provided separately, were used to perform RT-PCR. The LightCycler 480 Instrument II device (Roche Diagnostic, Mannheim, Germany) and SYBR detection method were used for expression analysis.

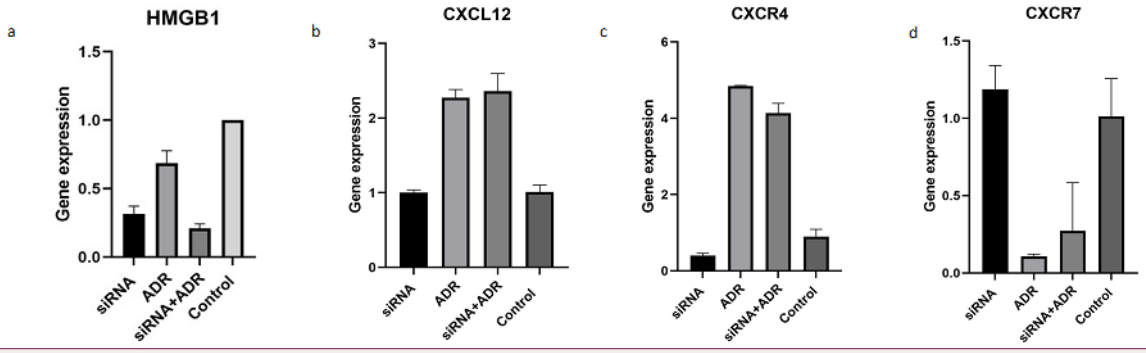

Figure 1. Expression of HMGB1 (a), CXCL12 (b), CXCR4 (c), and CXCR7 (d) in H9c2 cells after transfection of HMGB1 siRNA and/or ADR treatment. The expression levels of genes were detected 36 h after transfection by quantitative PCR.
siRNA: cells were transfected with HMGB1 siRNA; ADR: cells were treated with adriamycin; HMGB1; siRNA + ADR: cells were transfected with HMGB1 siRNA and treated with ADR; Control: untreated cells.

Each sample was replicated, and the mean value was computed. The $2^{-(\Delta\Delta ct)}$ method (14) was employed for gene expression analysis. The expression levels of the genes were normalized to those of the housekeeping gene, beta-actin. The primers were designed based on the *Rattus norvegicus* reference genome, Rnor_6.0.

B-actin F: 5′-TCTGAACCCTAAGGCCAACCGTG-3′

B-actin R: 5′-AACACAGCCTGGATGGCTACGT-3′

HMGB1 F: 5′-AAACATGGGCAAAGGAGATCC-3′

HMGB1 R: 5′-AGTTGACAGAAGCATCCGGGT-3′

CXCL12 F: 5′-CCAGAGCCAACGTCAAACAT-3′

CXCL12 R: 5′-GTTGTTGCTTTTCAGCCTTGC-3′

CXCR4 F: 5′-GTTGTTGCTTTTCAGCCTTGC-3′

CXCR4 R: 5′-AAGAGTGTCCACCCCGTTTC-3′

CXCR7 F: 5′-TTGCTGTCCCCTTCACCATC-3′

CXCR7 R: 5′-CAAAATGGTACGGCAGCCAA-3′

Statistical Analyses

All data analyses were conducted using the Statistical Package for Social Sciences (SPSS version 21.0, SPSS Inc., Chicago, IL, USA). To determine if there was a statistically significant difference in gene expression levels, we employed the Kolmogorov-Smirnov test. $p<0.05$ was considered statistically significant for determining significance.

RESULTS

Gene Expression Changes in 36 Hours

We investigated the role of ADR in chemokines through the HMBG1 gene. The knockdown level of HMGB1 was determined using RT-PCR. A decrease in HMGB1 expression by 69% was observed 36 h post-siRNA transfection in H9c2 cells (Figure 1a). The expression of the chemokine ligand CXCL12 was not significantly changed in HMGB1 knockdown cells. However, it increased by 2.3-fold in cells treated with ADR. In cells treated with both ADR and siRNA, the increase was slightly higher at 2.35-fold ($p<0.05$; Figure 1b).

The expression of the chemokine receptor CXCR4 was decreased by 0.61-fold, compared to the control in HMGB1 knockdown cells. Conversely, it was increased by 4.8-fold in cells treated with ADR. In cells treated with both ADR and siRNA, the Increase was slightly lower at 4.1-fold ($p<0.05$; Figure 1c).

In HMGB1 knockdown cells, there was no significant change in the expression of the CXCR7 receptor. However, in cells treated with ADR and both ADR and siRNA, similar decreases of 0.9-fold and 0.7-fold, respectively, were observed ($p<0.05$; Figure 1d).

Gene Expression Changes in 48 Hours

The expression of HMGB1 was downregulated by 72% 48 h after siRNA transfection in H9c2 cells (Figure 2a). The expression of the CXCL12 ligand was increased by 2.7-fold in cells treated with ADR. In cells treated with a combination of ADR and siRNA, the increase was slightly higher at 31-fold ($p<0.05$; Figure 2b). The expression of the CXCR4 receptor had a significant increase in ADR-treated cells, with a 53-fold increase. In cells treated with both ADR and siRNA, the increase was slightly lower at 31-fold ($p<0.05$; Figure 2c). The expression of the CXCR7 receptor was decreased by 0.91-fold in ADR-treated cells and by 0.93-fold in cells treated with both ADR and siRNA ($p<0.05$; Figure 2d).

DISCUSSION

Adriamycin, a chemotherapy drug used in the treatment of leukemia, neuroblastoma, breast cancer, lymphoma, Ewing's syndrome, and osteosarcoma, disrupts DNA repair by intercalating into DNA via topoisomerase II, generating free radicals that cause DNA and protein damage. However, its clinical use can induce cardiac toxicity by triggering an

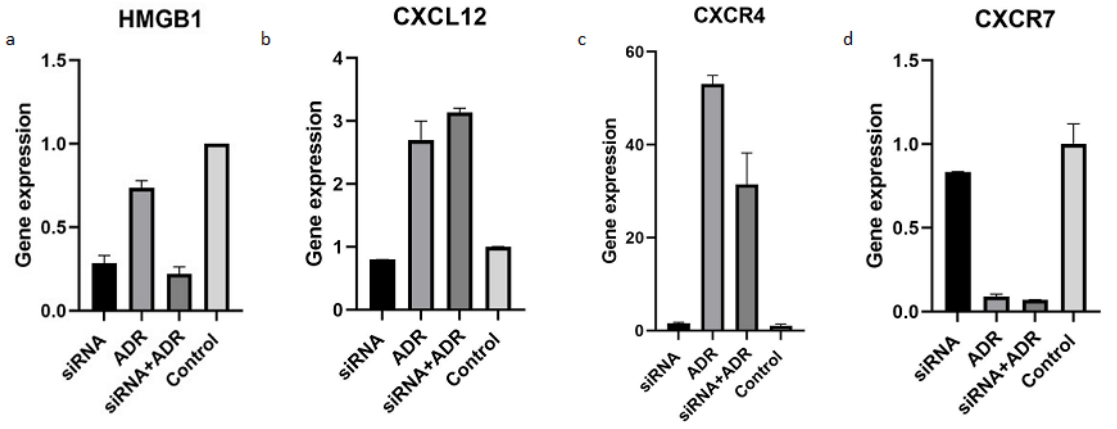

Figure 2. Expression of HMGB1 (a), CXCL12 (b), CXCR4 (c), and CXCR7 (d) in H9c2 cells after transfection of HMGB1 siRNA and/or adriamycin (ADR) treatment. The expression levels of genes were detected 48 h after transfection by quantitative PCR.
siRNA: cells were transfected with HMGB1 siRNA; ADR: cells were treated with adriamycin; HMGB1; siRNA + ADR: cells were transfected with HMGB1 siRNA and treated with ADR; Control: untreated cells.

inflammatory response through immune cells, thereby increasing the risk of cardiac failure (15).

When tissues are damaged, endogenous molecules known as DAMPs are released, with HMGB1 being one of these DAMPs associated with cellular damage (5). Innate immune cells recognize DAMPs through specific pattern recognition receptors (PRRs), including Toll-like receptors, nucleotide-binding oligomerization domain (NOD)-like receptors, and C-type lectin receptors. Activation of these receptors upon sensing DAMPs initiates inflammatory pathways, leading to the release of chemokines (15).

Inflammatory mediators and chemokines release signal for neutrophils to detach from circulation and migrate to the site of damage. Neutrophils, constituting the majority of granulocytes in peripheral blood (50%–70%), play a vital role in the innate immune system's defense mechanisms.The process of neutrophil formation, known as granulopoiesis, involves maturation stages regulated by the cytokine G-CSF, disrupting the CXCR4-CXCL12 interaction and assisting in retaining neutrophils in the bone marrow. G-CSF induces the release of circulating neutrophils by reducing CXCL12 expression in the bone marrow and decreasing CXCR4 on neutrophils (16). This mechanism disrupts the retention of neutrophils in the bone marrow, leading to their release into circulation.

Our study contributes to understanding the CXCR4-CXCL12 relationship and its relevance to neutrophil release (16), thereby impacting the inflammatory process and their recruitment to sites of tissue damage. This understanding holds significance for various pathological conditions characterized by inflammation and tissue damage, such as cancer, autoimmune diseases, and infections.

Our study aimed to down-regulate HMGB1 and examine its relationship with the chemokine CXCL12, as well as the CXCL12 receptors CXCR4 and CXCR7. We also sought to understand the effects of the chemotherapy ADR. We analyzed the expression of these genes in rat myoblast cells treated with ADR alone and in combination with HMGB1 siRNA. Interestingly, we observed no significant change in HMGB1 gene expression in cells treated solely with ADR. This finding aligns with Luo et al.'s demonstration that ADR treatment increased HMGB1 release and that HMGB1 silencing could potentially reverse ADR-induced cardiac toxicity. However, since our analysis was conducted at the RNA level, we did not observe an increase in HMGB1 expression (10).

In our examination of CXCL12 expression in cells treated with HMGB1 siRNA, we found that the down-regulation of HMGB1 at 36 and 48 h did not lead to a significant change. However, the application of ADR increased CXCL12 expression in both 36 and 48h samples. When we compared samples treated with both siRNA and ADR to those treated with only ADR, we observed that HMGB1 suppression did not significantly affect CXCL12 expression. This finding is consistent with a study by Beji and colleagues on human cardiac mesenchymal progenitor cells, which showed that ADR application increased the expression of this chemokine (17).

In our study, we observed an increase in the expression of the chemokine receptor CXCR4 in cells treated with ADR. However, in cells subjected to a combined treatment of siRNA and ADR, the expression of CXCR4 decreased compared to cells treated solely with ADR. This decrease in CXCR4 expression may be attributed to the reduction in heterocomplex formation that occurs when HMGB1 is downregulated. A study conducted by Schiraldi et al. demonstrated that the recruitment of inflammatory cells induced by HMGB1 depends on CXCL12. HMGB1 and CXCL12 form a heterocomplex that exerts its

effects through the CXCR4 receptor. This study revealed that the HMGB1-CXCL12 heterocomplex induces. distinct conformational re-arrangements of CXCR4 compared to CXCL12 alone (7). When examining the expression of the CXCR7 receptor, we observed a significant decrease in expression in samples treated solely with ADR and in samples treated with a combination of siRNA and ADR, compared to the control.

In our study, we found that the knockdown of HMGB1 did not cause any change in the expression of CXCR7, while it did affect the expression of CXCR4. CXCR7 plays a role in decreasing CXCL12 levels in mammalian cells and signals independently. Through various mechanisms, CXCR7 modulates the expression and activity of CXCR4 and CXCL12. Interestingly, CXCL12 initially has a higher affinity for CXCR7 than CXCR4. However, the binding and dissociation rates of CXCL12 for CXCR7 are slower than those for CXCR4, favoring kinetic binding to CXCR4 (18).

Our study demonstrated that the expression of CXCR4 changes depending on HMGB1 expression. These results suggest that in the context of ADR-induced cardiotoxicity, the expression of CXCL12 occurs through the CXCL12/CXCR4 axis. In conclusion, ADR treatment reveals new potential molecular mechanisms associated with cardiotoxicity. This finding opens the possibility for further research to enhance existing therapeutic approaches or develop new strategies for managing cardiotoxicity.

Ethics Committee Approval: Ethics committee approval is not required due to the use of commercial cell lines.

Peer-review: Externally peer-reviewed.

Author Contributions: Conception/Design of Study- Z.E., E.T., C.G, N.A; Consultant: Z.E.; Experiments: Z.E., S.P., M.S.; Drafting Manuscript- Z.E., N.A.; Critical Revision of Manuscript- N.A.; Final Approval and Accountability- Z.E., E.T., C.G, N.A., S.P., M.S.

Conflicts of Interests: The authors declare that they have no competing interests.

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