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Complete List of Authors:	Tavakoli Dargani, Zahra ; University of Central Florida Burnett School of Biomedical Sciences, Division of Metabolic and Cardiovascular Sc Singla, Reetu; University of Central Florida Burnett School of Biomedical Sciences Johnson, Taylor; University of Central Florida Burnett School of Biomedical Sciences, Division of Metabolic and Cardiovascular Sciences Kukreja, Rakesh; Howard Hughes Medical Institute - Virginia Commonwealth University School of Medicine Singla, Dinender Kumar; University of Central Florida,
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Exosomes Derived from Embryonic Stem Cells inhibit Doxorubicin and Inflammation induced Pyroptosis in Muscle cells

Zahra Tavakoli Dargani* Reetu Singla*, Taylor Johnson, Rakesh Kukreja[#] and Dinender K. Singla

*Both authors contributed equally

[#]Virginia Commonwealth University, Richmond, Virginia,

Division of Metabolic and Cardiovascular Sciences, Burnett School of Biomedical Sciences, College of Medicine, University of Central Florida, Orlando, FL, 32816, USA,

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Address for Correspondence:

Dinender K. Singla, Ph.D.
Division of Metabolic and Cardiovascular Sciences
Burnett School of Biomedical Sciences
College of Medicine
University of Central Florida
4110 Libra Dr
Orlando, FL, 32816, USA

e-mail: dsingla@ucf.edu

Phone: 407-823-0953

Fax: 407-823-0956

Abstract

Doxorubicin (Dox) is an effective anticancer drug. Unfortunately, it causes cardio and muscle toxicity due to increased oxidative stress and inflammation; however, it remains unknown whether Dox induces “pyroptosis”- an inflammation mediated cell death. We investigated whether Dox induces pyroptosis in mouse soleus muscle (Sol 8) cells *in vitro* and to show the protective effect of embryonic stem cells-exosomes (ES-exos) on pyroptosis. Doxorubicin and inflammation induced *in vitro* model was generated. Pyroptosis was confirmed using immunohistochemistry and western blotting of putative markers caspase-1, IL1 β and pro-inflammatory cytokine IL-18. The results show significant increase in the expression of caspase-1, IL-1 β and IL-18 following treatment with Dox which was inhibited by ES-exos but not Mef-exosomes. Moreover, GW4869 compound inhibited functional activity of ES-exos suggesting these vesicles are key player in the inhibition of pyroptosis. These results suggest that Dox induces inflammatory pyroptosis in Sol8 cells which is attenuated by ES-exos *in vitro*.

Key words; Doxorubicin, Muscle , Pyroptosis, Inflammation

Introduction

Doxorubicin (Dox) is an antineoplastic therapeutic agent widely used for the treatment of a variety of cancers (Minotti et al., 2004; Singal and Iliskovic, 1998). However, the clinical use of this drug is limited due to a severe, dose-dependent, acute cardiac and muscle toxicity that may progress to irreversible chronic cardiomyopathy, congestive heart failure, muscle fatigue and death. DOX induced muscle toxicity (DIMIT) leads to adverse muscular remodeling; a complex, dynamic, and time-dependent process characterized by: a) the death of muscle cells; b) muscle atrophy; and c) fatigue, which is caused by various hormones, inflammation, oxidative stress, and is itself linked to apoptotic cell death. Doxorubicin induces inflammatory and metabolic alterations in skeletal muscle (Minotti et al., 2004), however, this remain unknown if Dox induces inflammation induced pyroptosis in soleus muscle. Therefore, we designed this study to develop cell culture model of Dox and inflammation to understand whether inflammation induces new form of cell death called pyroptosis.

Stem cells have been shown to treat many diseases, including various muscle disorders (Meregalli et al., 2014). However, treatment with embryonic stem (ES) cells can potentially form teratoma, which limits the use of these cells for tissue repair. Accordingly, alternative approaches to ES cells use have been considered safer. Recent studies suggest that exosomes, which are extracellular vesicles released from stem cells, could be considered for therapeutic utility (Khan et al., 2015; Ono et al., 2014). Exosomes are 30-100nm size vesicles which consist of various proteins, lipids, growth factors and miRNAs (Khan et al.,

2015; Ono et al., 2014). Moreover, it has been suggested that cell type specific exosomes isolated from various types of stem cells including ES cells, CD34 stem cells, and mesenchymal stem cells have different properties such as anti-apoptotic, pro-angiogenic, and anti-fibrotic activities in the cardiac and skeletal muscle (Khan et al., 2015; Sahoo et al., 2011a; Sahoo and Losordo, 2014a; Zhao et al., 2017). However, it remains unknown whether exosomes derived from ES cells can inhibit Dox-induced inflammation and pyroptosis in muscle cells. To provide a proof of this concept, we examined the cytoprotective effect of exosomes, derived from ES cells, on Dox-induced inflammation and pyroptosis in slow-twitch soleus muscle Sol 8 cells. In addition, we used conditioned medium (CM) containing inflammatory components from THP-1 monocytes and used DOX treatment to induce pyroptosis in Sol 8 cells. These experimental conditions simulate Dox induced inflammatory conditions which are typical of DMT (Huang et al., 2017). Our results provide evidence that exosomes derived from ES cells inhibit pyroptosis in Sol 8 cells and these exosomes contain miRNAs which are protective in nature.

Materials and Methods

Cell lines, Exosomes and Conditioned Medium Preparation

Sol-8 cells (a mouse muscle cell line) were purchased from ATCC, USA and cultured in DM (Differentiation Medium) containing Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 20% fetal bovine serum (FBS), glutamine, sodium pyruvate and penicillin-streptomycin (P/S). To induce exogenous inflammation, we prepared conditioned medium (CM) from THP-1 monocytes

obtained from ATCC, USA. THP-1 cells were grown in suspension medium for 48 hours, centrifuged, supernatant filtered (using 0.22 µl filter, Millipore) and saved as THP-1 conditioned medium (THP-1 CM).

ES cells (CGR8, a mouse embryonic cell line) and mouse embryonic fibroblasts (MEF) were cultured as reported previously (Singla and McDonald, 2007; Lee et al., 2007). For isolation of exosomes, cell culture medium was replaced with serum free knockout DMEM supplemented with P/S and glutamine. ES and MEF exosomes were prepared using ExoQuick TC exosome isolation kit (SBI, USA) as per manufacturer's instructions and appropriate modifications were used as necessary. We used GW4869 compound which served as an exosome inhibitor.

Preparation of Pyroptotic Cell Culture Model

Regularly maintained Sol 8 cells were plated in the 8 well plates and/or in the 60mm plates. Cells were divided into 5 groups: Control, Dox+THP-1CM, Dox+THP-1CM+ES-exos, Dox+THP-1CM+Mef-exos and Dox+THP-1CM+ES-exos+Gw4869 compound. Sol-8 cells were treated with Dox (5µM) and THP-1 conditioned medium (33% vol/vol) for 4 hours followed by replacement with cell culture medium. Cells were treated with ES-Exos (10 µg), Mef-Exos (10 µg; cell line as a control) or ES-Exos+GW 4869 (10µM; to inhibit the function of ES-exos).

Detection of Pyroptosis

Pyroptosis was assessed using immunohistochemistry and Western blot analysis after 24 hours. Sol-8 cells were fixed with 4% PFA (20 minutes) and 0.3% Triton X-100 (15 minutes). The cells were blocked with 10% normal goat serum (Vector

lab) and incubated with primary antibodies for Caspase-1 (Abcam, 1:100), IL-1 β (Abcam, 1:100), and IL-18 (Abcam, 1:100) for overnight at 4°C. Primary incubation was followed by secondary incubation with Alexa 568 goat anti-rabbit antibodies for one hour at room temperature. Finally, DAPI was used to stain nuclei and mount coverslips on the slides. Pictures were captured through Olympus and Confocal microscopy and quantification performed using image J software.

Western blot was performed as reported previously (Abdelli and Singla, 2015). In brief, cells were lysed using RIPA lysis buffer, supernatant was collected, and protein concentration was estimated using Bio-Rad protein assay. 50 μ g of protein samples were loaded and run on SDS-PAGE (10 or 15 %) for 60-80 minutes. Proteins were transferred to PVDF membranes; membranes were blocked with 5% skim milk and incubated overnight at 4°C with the primary antibodies for Caspase-1 (Abcam, 1:1000), IL-1 β (Abcam, 1:1000) and β -actin (as a loading control, Cell Signaling, 1:1000). Following primary incubation, membranes were washed with 1XTBS-T and incubated with secondary antibody, goat anti-rabbit IgG (Cell Signaling, 1:1000) for 1 hour at room temperature. Finally, membranes were exposed to chemiluminescence ECL substrates and developed using X-Ray film. Densitometry analysis was performed using image J software.

Statistical Analysis

Data were expressed as the mean $\pm$ SEM and considered statistically significant at p-value <0.05 when examined through one-way analysis of variance (ANOVA) and Tukey test.

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Results and Discussion

Cell death is a natural phenomenon that is required for the survival and normal development of the organ in the body. Apoptosis and necrosis are two major forms of cell death which widely occurs in health as well as progression of disease in various organs. Apoptosis is programmed cell death which is regulated by caspase 3 and 9 (Kumar et al., 2001; Kumar et al., 2002). Necrosis is a form of cell injury that induces inflammation in the tissues (Kumar et al., 2001; Kumar et al., 2002). Damage-associated molecular pattern molecules (DAMPs) are released by apoptotic and necrotic dead cells which can lead to activation of inflammasome (TLR4, NLRP3), that activates caspase-1 and downstream release of cytokines including IL-1 β and IL-18 (Bergsbaken et al., 2009; Fantuzzi and Dinarello, 1999; Takahashi, 2014). Pyroptosis is inflammasome induced cell death which is regulated by caspase-1 following a range of microbial infections or a sterile inflammatory immune response (Takahashi, 2014). Non-microbial sterile inflammation stimulus enhances pyroptosis in myocardial infarction (Takahashi, 2014). In the present study, we generated a Dox and inflammation induced injury model in Sol 8 cells which is typical of *in vivo* Dox induced skeletal muscle toxicity (Huang et al., 2017; Supriya et al., 2016). We examined the presence of pyroptosis using specific markers such as caspase-1 and IL-1 β through immunohistochemistry and western blot analysis. Our data shows Dox and CM treatment stained positively for caspase-1 (Figure 1, panel A) and IL-1 β (Figure 1 panel C), indicating the presence of pyroptosis in these cells. Moreover, quantitative analysis shows that

the number of Sol 8 cells with caspase-1 and IL-1 β were significantly increased compared with the control group (Figure 1 B and D). Furthermore, our data shows significant increase of IL-18 (Figure 1, E and F), a cytokine which may be involved in inflammation induced pyroptosis (Fantuzzi and Dinarello, 1999). To strengthen the immunohistochemistry results, we also performed western blot analysis on IL-1 β and caspase-1 (Figure 2). Our data shows significant increase in the expression of IL-1 β and caspase-1 proteins following treatment with Dox and CM treatment as compared with control group. Densitometry analysis further corroborated these results. To our knowledge, this is the first report showing Dox and inflammation induced pyroptosis in a cell culture model which simulates in vivo Dox induced inflammation and pyroptosis in skeletal muscle. These data on the confirmation of pyroptosis is in agreement with the published studies as reported by others (Bergsbaken et al., 2009; Takahashi, 2014; Zheng et al., 2011).

As stated elsewhere, exosomes have been reported to be cytoprotective in various disease conditions including cardiac and skeletal muscle (Ono et al., 2014; Sahoo et al., 2011b; Sahoo and Losordo, 2014b; Zhao et al., 2017). However, whether ES-Exos are protective against Dox and inflammation induced pyroptosis in vitro has never been examined. Our data suggest that treatment with ES-exos significantly reduced IL-1 β and capase-1 positive cells as in Sol 8 cells incubated with Dox and CM (Figure-1 A-D). Moreover, the number of IL-18 pro-inflammatory cytokine positive cells was also reduced following treatment with ES-exos (Figure 1, E and F). The immunohistochemistry results were

confirmed with western blot analysis showing significantly reduced protein expression levels of IL-1 β and caspase-1 following treatment with ES-Exos. Most importantly, we also used Mef-exos as control cell line to determine whether protective effects exerted by ES-exos are cell specific and unique in nature or exosomes prepared from other cell lines can also protect Dox and CM induced pyroptosis in Sol 8 cells. Our results confirmed that Mef-exos treatment is not protective in the Sol 8 induced pyroptosis model. This data shows that this may be due to different composition of growth factors and miRNAs as compared with ES-Exos containing protective components against pyroptosis induced cell death in this cell culture model. These results further support published studies showing ES-exos are cytoprotective (Ono et al., 2014; Sahoo et al., 2011b; Sahoo and Losordo, 2014b; Wang et al., 2015; Zhao et al., 2017)

GW4869 is a compound reported to form a complex with exos, which neutralizes their functional effects (Ibrahim et al., 2014). In the present study we examined the effect of GW4869 to show whether the therapeutic benefit of ES-exos is inhibited in terms of attenuation in IL-1 β and caspase 1 expression. Our data shows that pre-treatment with GW4869 blocked protective effects of ES-exos on inhibition of pyroptosis and decreased expression of IL-1 β (Figure 1 and 2), as well as caspase 1 (Figure 1 and 2) which is confirmed through both immunostaining and Western blot analysis. Interestingly, GW4869 treatment also blocked decreased levels of pro-inflammatory cytokine IL-18 as we previously observed with ES-exos.

In conclusion, our data provides compelling evidence that Dox and inflammatory CM induce pyroptosis in muscle Sol 8 cells. Moreover, our results show that Dox and inflammation induced pyroptosis is inhibited by ES-exos. These observations are unique because of their novel mechanistic insights on the potential role of pyroptosis in Dox induced acute and chronic muscle toxicity. However it remains unknown as to how pyroptosis plays a role in the progression of Dox induced muscle toxicity. Moreover, further studies are required to analyze which protective growth factors and miRNAs may have contributed directly or indirectly in the cytoprotective effect of ES-exos.

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Figure Legends

Figure 1. Effects of ES-Exo on inflammation induced pyroptosis in SM-Sol 8

cells following Dox toxicity. Representative images showing Dox-induced increase in positive cells for caspase-1 (left panel), IL-1 β (mid panel) and IL-18 cells (right panel) as compared to control. ES-Exo treatment reduced staining for caspase-1, IL-1 β and IL-18 while no effect of Mef-Exo are observed in the inhibition of caspase-1, IL-1 β and IL-18. Blocking ES-Exo production following treatment with GW compound inhibited protective effects by ES-Exo. Panel A, C and E show caspase-1, IL-1 β and IL-18 cells in red (a, d, g), DAPI in blue (b, e, h) and merged images (c, f, i). B, D and F show quantitative analysis and histograms of caspase-1, IL-1 β and IL-18 cells over DAPI. * $p < 0.05$ vs. control, # $p < 0.05$ vs. Dox, @ $p < 0.05$ vs. Dox+ ES-exos, \$ $p < 0.05$ vs Dox, NS=Non-significant. Scale bar = 100 μ m, n=4.

Figure 2. Effect of ES-Exo on inflammation induced pyroptosis in DIMIT

model. (A) Upper panel shows representative pictures of Western Blot (WB) for IL-1 β , caspase-1 and β -actin. (B and C) Bottom histograms show densitometric analysis of WB bands for IL-1 β and caspase-1. Note a significant enhancement of these markers following exposure to Dox and conditioned medium (CM) in cells treated with ES-exos. * $p < 0.05$ vs. control, # $p < 0.05$ vs. Dox, and \$ $p < 0.05$ vs Dox, NS=Non-significant.