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Role of MLL3 in regulating cardiac stem cells following cardiac cachexia

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Abstract. – OBJECTIVE: Cardiac cachexia is a form of serious illness that results with terminal stage of heart failure. It is associated with unreasonable weight loss and muscle loss with poor prognosis. Cardiac stem cells play a major role in repairing, damaged cardiac tissue and they are regulated by different mechanisms. In the present study, we investigated the role of MLL3 in regulating cardiac stem cells following cardiac cachexia.

MATERIALS AND METHODS: To effectively study the cardiac cachexia, we established a Dahl rat model that produces symptoms similar to cachexia. Using histology, we analyzed the acute and chronic stage of cardiac cachexia. Immunohistochemistry and Western blotting were used to analyze the expression of MLL3 and Oct-4.

RESULTS: The rat develops an acute form of cachexia after 2 months of fed with high-salt (8% NaCl) diet, which is characterized by inflammation and tissue damage that are observed through the histological sectioning. The chronic cardiac cachexia developed after 5 months of high-salt diet and histologically it shows tissue loss. At the molecular level the stem cell marker, Oct-4, shows elevated expression at acute stage, but down regulated latter in the chronic stage of cardiac cachexia. Also, MLL3 shows a similar pattern of upregulated expression in acute stage of cachexia, but significantly down regulated in the chronic stage of cachexia that implies their role in regulating the cardiac stem cell and their proliferation.

CONCLUSIONS: Our results show that the cardiac stem cells in association with MLL3 support in maintaining homeostatic after initial pathological stages of cachexia but not in the chronic stage of cachexia.

Key Words:
Cardiac cachexia, Oct-4, MLL3, Dahl salt-sensitive rat.

Introduction

Cachexia is defined as muscle wasting that occurs due to chronic illness, and results with development of weight loss and chronic cardiac failure[1]. The clinical symptoms include loss of muscle strength, anorexia, fatigue, raise in inflammatory markers, anemia or reduction in serum albumin level[2]. Some recent investigations[3,4] claim that the loss of weight by >5%, regardless of any specific reason may be due to the development of cardiac cachexia. Due to the lack of specific therapies and poor aspects of disease management, cardiac cachexia emerged as a public health problem[5]. Cardiac cachexia is associated with multi-factors and link with complex systems like immune, neuromuscular, hormonal and metabolic imbalance[2]. Therefore, the treatment and prevention aspect of cardiac cachexia are complexes and not dependent on single agent. The drugs to manage cardiac cachexia are scarce; to date, only 19 drugs are recommended as therapeutic agents[6] and most of them work only as anti-inflammatory and orexigenic. To develop effective biomarkers, the cardiac cachexia is monitored in early stages as a patient shows limitation in performing daily activities, which itself represent tissue damage and initial sign of muscle wasting[7,8]. Usually, after muscle injury, muscle tissues are subjected to repair by activating many cellular and molecular pathways, which primarily involve the participation of stem cells[9]. It was reported that the normal physiological activity of stem cell was inhibited by enhanced expression of C/EBPβ, following cancer cachexia[10]. The proliferation and differentiation ability of the cardiac stem cells is vital to restore the damage that occurs in cardiac following cachexia. The better understanding of the pathophysiological conditions associated with cardiac stem cells and investigation of different proteins that regulate cardiac stem cells are more important to establish a novel strategy to handle cardiac cachexia. Mixed-lineage leukemia (MLL3) act as a histone methyl transferase that activates chromatin structures and play a role as coactivator for many transcription factors and thereby it regulates many gene expressions11-13.

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In this investigation we planned to find out the role of MLL3 in regulating cardiac stem cells following cardiac cachexia.

Materials and Methods

Dahl Salt-Sensitive Rats

To perform experiments, Dahl salt-sensitive rats (Male, age two months) were purchased from Charles River Laboratory (Wilmington, MA, USA). All animal experiments were approved by the animal Ethics Committee of Hubei University of Medicine, China (No. SMA/2015/01) and the given study followed the guidelines of Association for the Assessment and Accreditation of Laboratory Animal Care International (AAALAC) for experimentation and animal use. The rats were divided into three groups and each group was planned to have 6 rats. The first group of rat's acted as control and was fed with only 1% Nacl along with the diet. The second group of rats was fed with high-salt (8% NaCl) for a period of 2 months to develop an acute form of cardiac cachexia. The third group of rats was fed with high-salt (8% NaCl) for a period of 5 months to develop a chronic form of cardiac cachexia. All rats were maintained in the controlled conditions in the lab (temperature: 24-26ºC; humidity: 45-60%) and provided with tap water ad libitum. All the rats were scarified after completion of 4 months and their cardiac samples were dissected for further analysis.

Histological Procedure

The cardiac samples obtained from control, acute and chronic form of cardiac cachexia were subjected to histological sectioning. Initially, the samples were fixed in 10% formalin solution for 48 h to retain the cellular structures. Following fixation, the tissues were subjected to dehydration with increasing concentration of ethyl alcohol (60%, 70%, 80%, 90% and 100%) and in each concentration the tissues were incubated for 1 h at 42ºC. Then, the tissues were subjected to clearing, using xylene, which helps to impregnate the embedding medium. After embedding with wax, the tissues were subjected to thin sectioning in microtome (6 µm) and finally processed to stain with haematoxylin and eosin (HE).

Immunohistology

For immunohistology, paraffin embedded cardiac tissue were subjected to thin sectioning using microtome (6 µm). The endogenous peroxidase activity was inactivated by incubating the sections in 10% H2O2 solutions for half an hour. The antigens were unmasked using trypsinization (0.1% trypsin in 1X PBS) at RT for 10 min. In order to restrict non-specific binding, the sections were blocked with 4% bovine serum albumin (BSA) solution for 2 h at RT. Immunohistological detection of MLL3, Oct-4 and actin were performed by incubating them with corresponding primary antibodies at 4°C for overnight. After throughout washing of non-specific binding primary antibody, the sections were incubated with horse radish peroxidase (HRP) conjugated secondary antibody. Following incubation with diaminobenzidine (DAB) kit, the chromogenic signals were obtained, which were counter stained with haematoxylin for documentation.

Western Blotting Analysis

The proteins were extracted from the cardiac samples by crushing them in a pre-cooled mortal and pastel with 2X protein sample buffer. The homogenized tissue samples were transferred to Eppendorf tube and subjected to short spin to remove the debris. The supernatant was collected in fresh tube and boiled in water bath for 10 min. The concentration of protein in samples were estimated using the Lowry method. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (10%) was performed by loading equal concentration of protein samples (60 µg) to isolate the proteins in the cardiac tissue. As the bromophenol blue reached the anode end, the plates were disassembled and the separated protein in the gel were transferred to nitrocellulose membrane. Then, the membrane was blocked using 5% BSA in Tris-buffered saline-tween (TBST) solution. The membrane was incubated with primary antibody (anti-MLL3, anti-c-kit or anti-actin) at 4ºC for overnight. The membranes were washed and incubated with HRP conjugated secondary antibody. The membrane was developed using DAB kit and the intensity of band obtained was further documented.

Statistical Analysis

To obtain concordant results the experimental procedures were repeated for three times and the results were specified as mean ± SEM. The variations among data were calculated using Student's *t*-test. Data was considered as significant when the $p<0.05$.

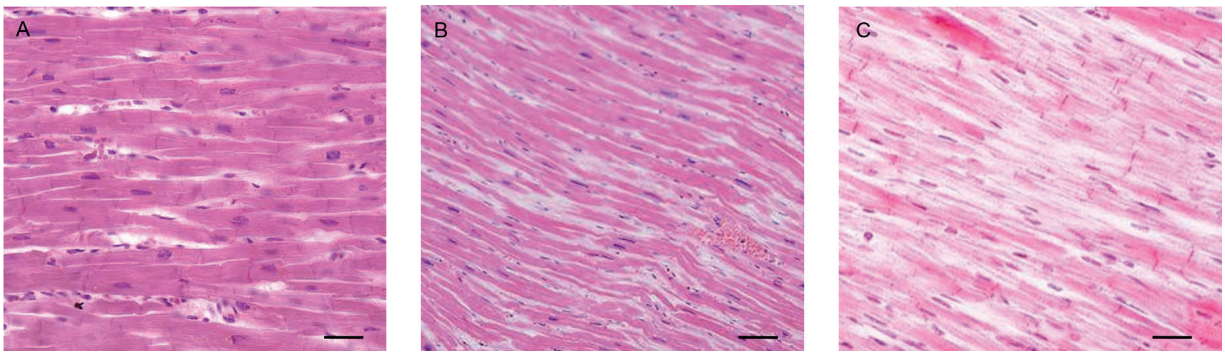

Figure 1. Rat developed with cardiac cachexia. ***A,*** Histological sectioning of normal cardiac muscle that are arranged in a compact structure. ***B,*** Cardiac tissue developed with acute cardiac cachexia shows initial muscle loss and inflammation. ***C,*** Cardiac tissue developed with chronic cardiac cachexia shows extensive damage with more muscle loss. Scale Bar represents 30 μm size.

Results

Developing Cardiac Cachexia Rat Model

In order to raise cardiac cachexia rat model, the Dahl salt-sensitive rats were fed with a high salt diet (8% Nacl). The rat that intake high salt diet for 2 months developed an acute form of cardiac cachexia and the rat subjected to high salt diet for a period of 5 months' time developed a chronic form of cardiac cachexia. The rat that developed these forms was confirmed through histological sectioning of their cardiac tissue (Figure 1A-C). The control cardiac tissue shows densely packed compact structures (Figure 1A) whereas in the acute form of cardiac tissue we observed initial form of cardiac muscle loss along with the inflammatory response (Figure 1B). In the chronic form, we observed extensive damage of the heart tissue with more cardiac tissue loss (Figure 1C).

Expression of c-kit in Different Pathological Stages of Cardiac Cachexia

Oct-4 is an embryogenic stem cell marker that can be expressed even in adult heart tissue14 and it can be used as a cardiac stem cell markers15,16. In the present study, using immunohistology, the expression of Oct-4 was analyzed in control, acute and in chronic stage of cardiac cachexia (Figure 2A-C). The control cardiac tissue showed isolated pockets of Oct-4 expression and their expression was very limited (Figure 2A). While chasing the cardiac stem in the acute form of cardiac cachexia, we observed outstanding triggered expression of Oct-4 signals (Figure 2B), but their stability of expression lowered and reached a very meager amount as the cardiac cachexia proceed to chronic stage (Figure 2C).

Role of MLL3 in Cardiac Cachexia

Mixed Lineage Leukemia 3 (MLL3) is a type of histone methyl transferase, which regulates a large number of genes17. Usually methylation results with inactivation of genes and, thereby, it plays a major role in tumor suppression18,19. The link between MLL3 expression and cardiac stem cells are not well characterized and need to be investigated in detail at different pathological stages of cardiac disease. The immunohistological analysis of MLL3 shows prominent expression in the control cardiac tissue (Figure 2D) and we observed large changes in the expression pattern of MLL3 in the acute form of cardiac cachexia with significant over expression (Figure 2E). Nevertheless, in the critical stage of chronic form the expression of MLL3 shows significant suppression (Figure 2F).

Assessing the Level of Oct-4 and MLL3 Using Western Blotting

The expression pattern of Oct-4 and MLL3 was further assessed using Western blotting and the combined studies helped to evaluate the nominal pattern of expression. The protein lysate isolated from control, acute and chronic stage of cardiac tissue of cardiac cachexia are subjected to Western blotting as described in material and method section. From the results (Figure 3) we observed that the activity of Oct-4 and MLL3 correlates with the overexpression pattern in acute cardiac cachexia as obtained in immunohistological results. Similarly, we found that the expression of Oct-4 and MLL3 shows down regulated expression in the chronic stage.

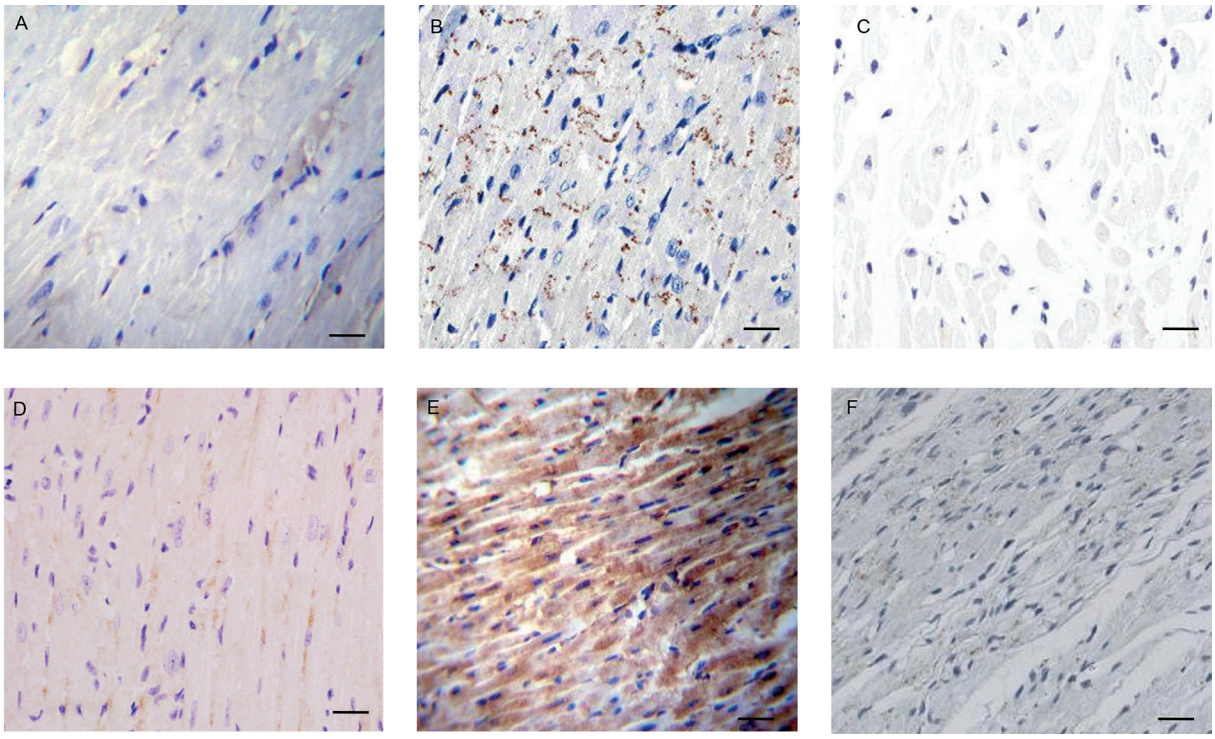

Figure 2. Expression of Oct-4 and MLL3 in different pathophysiological stages of cardiac cachexia. ***A,*** Expression of transcriptional factor, Oct-4 in isolated pockets of normal cardiac tissue of rat. ***B,*** Upregulated expression of Oct-4 in acute stage of cardiac cachexia in rat. ***C,*** Significant decreased expression of Oct-4 in chronic stage of cardiac cachexia. ***D,*** Control cardiac tissue of rat with prominent expression of MLL3. ***E,*** Acute stage of cardiac cachexia with higher magnitude expression of MLL3. ***F,*** Chronic stage of cardiac cachexia represent a lower expression of MLL3. Scale Bar represents 30 μm size.

Discussion

Cardiac cachexia results with cardiac tissue loss, which affects the normal physiological function of the heart. Here we successfully develop cardiac cachexia using Dahl salt-sensitive rats as previously described[20]. In our study, the rat responds well and develops typical features (Figure 1A-C). As the cardiac cachexia progress, we observed significant loss of cardiac tissue that may be due to increased catabolism, apoptosis and inflammatory response[21,22]. Our results with histology confirm development of peculiar features as that of cardiac cachexia, and this developed pathological feature helps to accurately assess the expression of different proteins. The endogenous presence of cardiac stem cells is the key that regulates repair mechanism following cardiac injury[23]. The response of cardiac stem cells in a critical stage of cardiac cachexia is an interesting aspect to investigate. Our result suggests that the re-population of resident cardiac stem cells occurs in the acute stage (Figure 2B) and higher proliferation of cardiac stem cells may help to restore the normal physiology of the heart by regenerating new cardio myocytes as that occurs after transplanting cardiac stem cells[24]. The capacity of cardiac stem cell proliferation restricted largely in the chronic stage (Figure 2C), which implies the limited ability of cardiac repair[25]. The metabolic dysfunctions associated with cachexia are regulated by many factors like hormonal, immune, hyper catabolism and disturbed muscle homeostasis[26-29]. MLL3 regulates many protein expressions by acting as a type of histone methyl transferase[17]. We observed that in the acute stage of cardiac cachexia the MLL3 expression was upregulated (Figure 2E); this may help to regulate Oct-4 expression to potentially repair the initial cardiac damage. Nevertheless, in the chronic stage their expression was down regulated (Figure 2F) when compared with the control cardiac tissue (Figure 2D) that helps to deregulate Oct-4 expression. The accuracy of the results and the tool of immunohistological data are cross-verified using Western blotting analysis (Figure 3).

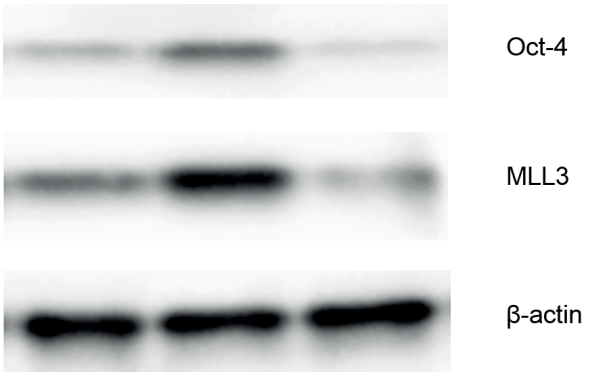

Figure 3. Western blot analysis of Oct-4 and MLL3 expression. (Row 1) Lane 1: expression of transcriptional factor, Oct-4 in control cardiac rat tissue. Lane 2: overexpression of Oct-4 in acute stage of cardiac cachexia. Lane 3: down-regulated expression of Oct-4 in chronic stage of cardiac cachexia. (Row 2) Lane 1: expression of protein, MLL3 in control cardiac tissue. Lane 2: increased expression of MLL3 in acute stage of cardiac cachexia. Lane 3: decreased expression of MLL3 in chronic stage of cardiac cachexia. β-actin was used as a loading control.

Conclusions

Overall, our results help to understand the response of the cardiac stem cells in different pathophysiological of cardiac cachexia. Also, we evaluated the role of MLL3 in association with cardiac stem cells in altered physiological stages of cardiac cachexia.

Conflict of interest

The authors declare no conflicts of interest.

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