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The aberrant expression of Smad6 and TGF-β in obesity linked cardiac disease

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Abstract. – OBJECTIVE: Obesity is a major health problem in modern society because their progression is always associated with many health issues. The major among them is developing the cardiovascular disease because as the obesity prolonged it results with cardiac remodelling and finally results in the dysfunction of the cardiac system. Many genes are associated with developing the obesity-linked cardiac dysfunction and it should be evaluated at different pathological stages of obesity.

MATERIALS AND METHODS: In the present studies, we analyzed the expression pattern of Smad6 and TGF-β using obesity-induced mice model which are *ob/ob–/–* deficient. The pathology of disease progression in initial and aggressive stage of cardiac dysfunction are studied together with Smad6 and TGF-β expression.

RESULTS: The mice develop initial stages of cardiac dysfunction on 3rd month and advanced stage of cardiac dysfunction on the 6th month. The results with histology show as the dysfunction progress it shows cellular lesions associated with enlarged cells. Immunochemistry with Smad6 represents that its expression positively regulate and repair the initial lesion but it has no role in the aggressive form of cardiac dysfunction and at that stage their expression downregulated. The results with TGF-β show initial upregulation in repairing the damage but in latter stage its expression many fold increases and it takes part in the inflammatory response.

CONCLUSIONS: Overall our results show aberrant expression of Smad6 and TGF-β at different stages of obesity linked cardiac dysfunction.

Key Words
Smad6, TGF-β, Obesity, ob/ob–/–.

Introduction

The occurrence of cardiac disease is a major health problem in the western world[1]. The reasons for developing the heart disease are due to smoking, intake of unhealthy diet; lack of physical exercise, diabetes, development of plaque in the artery which overall results with a heart attack. The studies using humans confirm that visceral obesity rather than peripheral obesity are the major cause behind the cardiac disease[2,3]. The adult heart is a well-differentiated organ and in that there is no active cell turnover[4]. Usually, the fat deposited in the cardiomyocytes are subjected to oxidative stress that results with the aging of heart and building up of cardiovascular complication[5,6].

The obesity persons are scientifically identified based on their height to weight ratio known as Body Mass Index (BMI)[7]. The people with higher BMI are at a risk of mortality associated with chronic heart failure[8], chronic obstructive pulmonary disease[9] and chronic renal failure[10]. Especially, the obesity associated with abdomen are showing a high risk for cardiac disease[11]. On the course of time, obesity result with many cardiovascular changes that scales from hyperdynamic circulation[12], structural remodelling of cardiac tissue[13] and finally to heart failure[14].

The health impact of obesity causes many molecular level changes that pay the way for developing the heart disease. Experiments in animal model reveal that many genes associated with cardiac are specifically activated as earlier of overfeeding[15]. But still, the complex pathways associated with obesity progression and cardiac dysfunction are needed to be understood in distinct stages. The function of Smad6 is maintaining the homeostasis condition of the adult cardiovascular system and the mutated form of Smad6 results with abnormal developmental as well as physiological functions of heart[16]. Similarly, transforming growth factor β (TGF-β) are take part in many physiological functions of heart from development to maintaining the internal homeostasis of heart tissue[17]. In the present study, we studied the expression of Smad6 and TGF-β in the context of obesity-associated heart disease.

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Materials and Methods

Mouse Model of Developed Cardiac Disease

The mice *ob/ob* are unable to express the leptin gene and was purchased from Jackson Laboratory. The mice lacking leptin gene are able to deposit the excess fat to develop obesity with diabetes, impaired healing ability and hypometabolic effects[18]. To study the cardiac inability in diverse pathological stages of obesity one group of *ob/ob*−/− deficient mice was allowed to grow up to three months and sacrificed to study the earlier effects associated with Smad6 and TGF-β. Another group of mice was maintained for five months and later was sacrificed to study the changes in advanced stages. All the experimental animals are maintained with care as per the guidelines and permission was granted by the Animal Ethical Committee of the host organization.

Immunohistochemistry and Antibody

The dissected cardiac samples obtained from control, earlier and advanced stages of cardiac disease are subjected to formalin fixation and embedded in paraffin for performing Immunohistochemistry[19]. Initially, the tissues are microdissected and placed in a clean glass slide along with floating water. The slides are heated in slide warming table to dewax the tissue and their endogenous peroxidase activity was blocked using 10% H_2O_2. After blocking with 4% bovine serum albumin (BSA) solution the tissue sections are incubated with primary antibody (from Abcam, Cambridge, UK), anti-Smad6 antibody, Abcam (ab80049) or anti-TGF-β antibody, Abcam (ab66043) for 8 hours at 4ºC. After washing with 1X phosphate buffered saline (PBS) for three times, the tissues are overlaid with secondary antibody for 1 hours in room temperature. The tissue sections are washed again and stained using diaminobenzidine (DAB) solution. The tissue develops signals and counterstains with haematoxylin and eosin.

Western Blot

The cell lysate was resolved in 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel as previously described[20]. The separated proteins samples are transferred to polyvinylidene difluoride membrane and blocked with 4% BSA solution. After blocking non-specific binding, the membrane is hybridized with primary antibody of anti-Smad6 antibody, Abcam (ab13727) or anti-TGF-β antibody, Abcam (ab66043) for overnight at 4ºC. To prevent non-specific signals, membranes are washed with 1X Tris buffered saline-tween (TBS-T) buffer and further with suitable secondary antibody for 2 hours at room temperature. Following incubation, the membranes are washed with 1X TBS-T buffer and developed.

Results

Mice Model for Obesity with Cardiac Disease

The mice model with adverse obesity shows structural as well as functional changes of the cardiac system[18]. The mice with cardiac dysfunction were established using *ob/ob* deficient, and it mimics pathological symptoms similar to human. The *ob/ob* deficient mice have the ability to form initial cardiac disease on 3rd month and its prolonged exposure up to 6th-month results with an aggressive form of cardiac abnormalities which are confirmed through histological sectioning as shown in the Figure 1A-C. The control cardiac tissue shows a regular uniform pattern of tissue arrangement with well-organized cells (Figure 1A). But the mice lacks *ob/ob*−/− shows an initial form of cardiac abnormalities on 3rd month with specific changes in the histological section with morphological abnormalities along with lesion development (Figure 1B). And when the *ob/ob*−/− mice maintained for another 3 months, the cardiac disease develops to more aggressive and it shows extensive lesions with enlarged cells (Figure 1C).

Aberrant Expression of Smad6 and its Relation with Obesity-linked Cardiac Disease

Smad6 plays a key role in the development and maintaining the homeostasis of heart tissue[16]. The association between Smad6 expression and their correlation with cardiac abnormalities in the context of obesity are more important to investigate. Immunohistochemical was used to examine the expression pattern of Smad6 in control, initial and aggressive cardiac disease associated with obesity. The normal cardiac tissue shows average expression of Smad6 (Figure 2A). The transition of normal tissue to initial cardiac disease on the 3rd week interestingly represent increased expression of Smad6 (Figure 2B). But their expression pattern shows downregulation on the 6th month in cardiac tissue with mice developed with aggressive cardiac disease (Figure 2C).

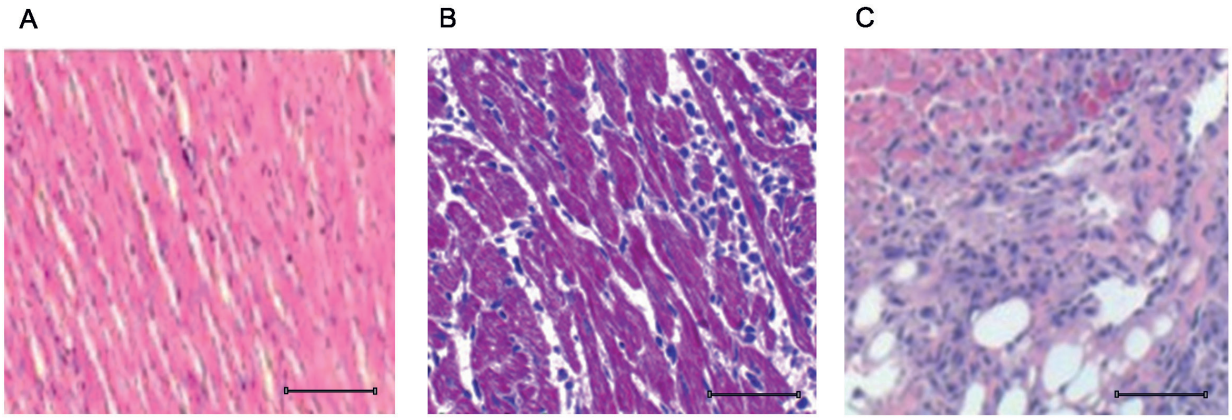

Figure 1. Mice model with obesity associated cardiac disease. **A**, The figure represents the histological image of control cardiac tissue with a regular pattern of cell arrangement. **B**, The variation observed in the initial form of cardiac disease tissue with lesions at many areas. C, aggressive cardiac disease showing several lesions together with enlarged cells. Scale Bar represents 100 μm size.

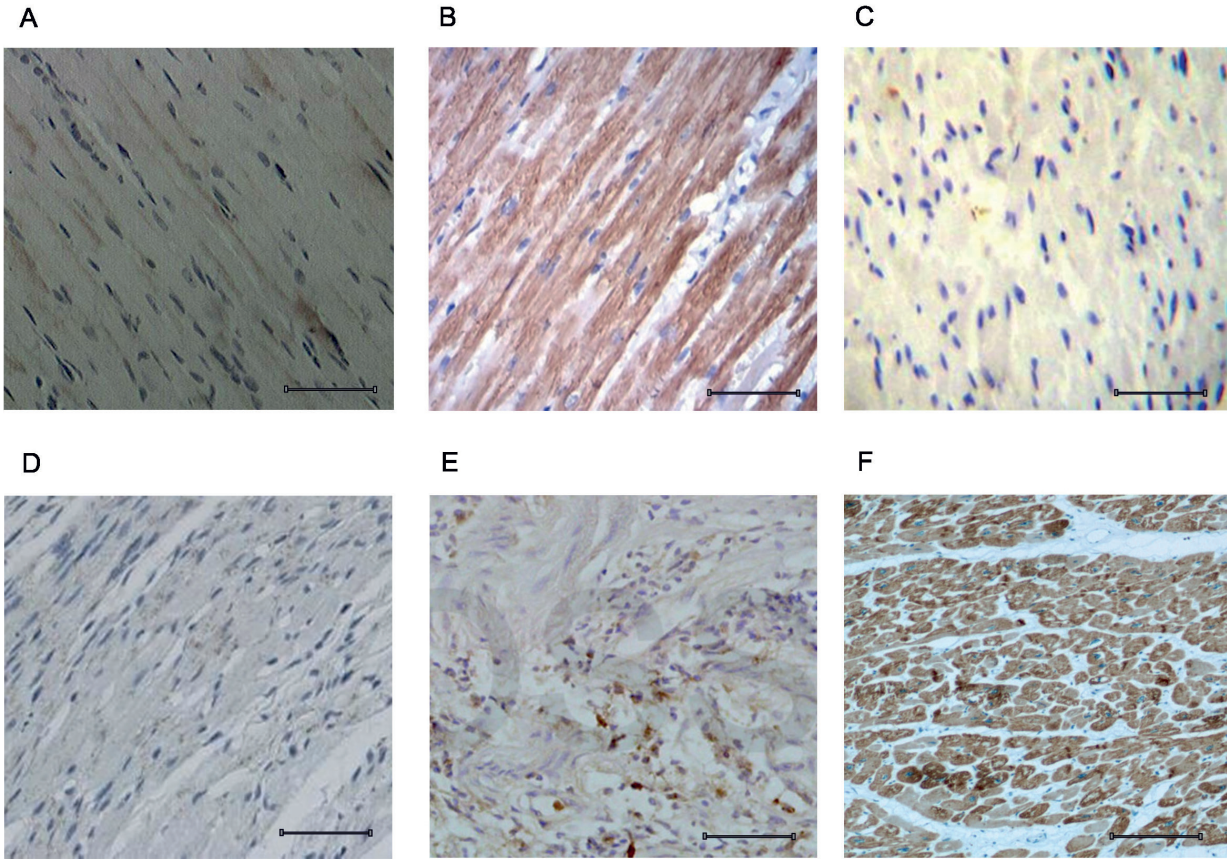

Figure 2. Aberrant expression of Smad6 and TGF-β. **A**, Smad6 that regulates normal functions of the heart shows slight expression in the control tissue. **B**, Immunohistological image showing increased expression of Smad6 in Initial cardiac disease. **C**, Aggressive cardiac disease showing a significant decrease in expression of Smad6. **D**, Control tissue with a mild expression of TGF-β. **E**, Initial stage of cardiac disease with a slight increasing expression of TGF-β. **F**, Aggressive stage of cardiac disease with overexpression of TGF-β. Scale Bar represents 100 μm size.

Expression of TGF-β in Different Pathological Stages of Obesity-linked Cardiac Disease

TGF-β is a cytokine with a wide range of functions that begins with development, cell growth, differentiation and repair of multiple tissues[21,22]. In the present study initially the expression pattern of TGF-β was analyzed, and it shows optimum expression similar to Smad6 (Figure 2D) but its expression seems increasing in initial cardiac disease tissue (Figure 2E). The results imply the role of TGF-β as a key regulator that has a control over the repair mechanism. But interestingly, their expression remarkably increased too many folds in the aggressive stage of cardiac disease (Figure 2F).

Western Blotting Analysis

The data that are obtained through Immunohistochemistry are further validated using western blotting analysis. The protein lysate is initially separated; then, using Western blotting with specific antibody the expression pattern of Smad6 and TGF-β are analyzed. The results through Western blotting helps to evaluate the expression pattern which represents dramatic increasing expression pattern of Smad6 in the initial stage of the cardiac disease and shows downregulated pattern of expression in the advanced stage of obesity-linked cardiac disease (Figure 3, Upper Lane). Similarly, as in immunohistochemistry, the TGF-β represent remarkable increasing expression as the cardiac disease progress (Figure 3, Middle Lane).

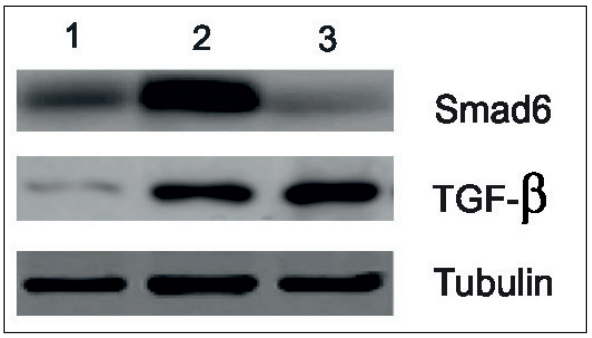

Figure 3. Western blot analysis. Lane 1: Smad6 expression in control cardiac tissue. Lane 2: Smad6 expression in the initial form of cardiac disease. Lane 3: Smad6 expression in advanced stages of the cardiac disease. Lane 4: TGF-β expression in normal cardiac tissue. Lane 5: increased expression of TGF-β in initial cardiac disease. Lane 6: TGF-β expression in aggressive cardiac disease. For control purpose tubulin was used.

Discussion

Recent studies with obesity strongly suggest their link with cardiovascular disease. Obesity that progress with hypertension increase stroke volume and it generate wall stress[23,24]. As the obesity progress or prolonged many changes happened in a cardiac system which includes dilation, hypertrophy, progressive dysfunction and finally ends up with cardiac failure[25,26]. The gene expression associated with developing the cardiac disease upon the progression of obesity is still less understood because it involves complex networks.

In the present study, we used *ob/ob−/−* deficient mice that show increase in obesity as the duration increase. The pathological changes as the cardiac disease progress are visualized using histological techniques. As the disease progress, we clearly able to point out the aggressive nature of lesion together with enlarged cells as shown in Figure 1(A-C). Many inflammatory and chemical mediators are involved in this damage and in developing hypertrophy[27].

At the next level, we investigate the expression pattern of Smad6 and TGF-β associated with pathological changes. Interestingly, we observed the over expression of Smad6 in the initial stage of cardiac dysfunction which suggest that the initial damage and associated repair are controlled by Smad6 (Figure 2B). But perhaps it expression becomes limited as it attains aggressive nature (Figure 2C). Recent studies with Smad6[28] support our finding that in the absence of Smad6, it results with the development of an aggressive form of Rheumatic heart disease when injected with group A streptococcal recombinant M5 protein.

TGF-β role in cardiac damage is a controversial one, and some studies[29] define its roles as an inflammatory agent and others suggest that it has a reparative role. Here, we demonstrate that in initial stages of cardiac dysfunction we observed the reparative mechanism and, in advanced stages, its expression results in developing inflammatory responses (Figure 3).

Conclusions

Overall our results confirm the aberrant expression of Smad6 and TGF-β in obesity-linked cardiac disease which is confirmed by investigating in different pathological stages of cardiac dysfunction.

Acknowledgement

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Conflicts of interest

The authors declare that no conflicts of interest exist.

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