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A research on the pathological mechanisms of aortic dissection

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Abstract. In this paper, the main content is to investigate which experimental methods are needed in the study of the case mechanism of aortic dissection and how to analyze the results by analyzing the experimental methods. The researchers used RNA sequencing, ChIP-Seq technology, transcription factor prediction, the dual-luciferase reporter assay, immunohistochemical staining, immunofluorescent staining, matrix metalloproteinases (MMP) activity staining, western blot and statistical analysis. The researchers used quantitative RT-PCR, In-Gel zymography, oxidant measurement by dihydroethidium, and flow cytometric analysis of inflammatory cells.

Keywords: aortic dissection, SirT1, matrix metalloproteinases.

1. Introduction

Aortic dissection (AD) is a common cardiovascular condition characterized by a tear in the inner layer of the aortic wall [1], causing ongoing bleeding in the middle layer. The Stanford University classification identifies two types of aortic dissection: type A aortic dissection (TAAD), which involves a proximal dissection of the left subclavian artery, and type B aortic dissection, which is limited to the descending aorta without any proximal extension. AD has a yearly incidence rate of approximately 3 to 6 per 100,000 individuals, and its occurrence has dramatically increased in the past decade [2-4]. If left untreated, acute AD can result in a mortality rate of up to 90%. As a potentially life-threatening condition, understanding the etiology and pathogenesis of AD is crucial for guiding clinical diagnosis and treatment, thus improving clinical outcomes.

Vascular smooth muscle cells (VSMCs) are specialized and differentiated cells found on the walls of blood vessels. They serve multiple functions such as regulating vascular tone, maintaining blood pressure, redistributing blood flow, and contracting [5]. Vascular smooth muscle cells (VSMCs) exhibit a high degree of adaptability and can undergo a transformation from a contractile to a synthetic phenotype when exposed to different stimuli, including signals from their surrounding micro-environment [6]. The capacity for proliferation, migration, and promotion of extracellular matrix protein synthesis is heightened in synthetic VSMCs. On the other hand, contractile VSMCs exhibit elevated expression levels of alpha-smooth muscle actin (SMA or ACTA2), calponin (CNN1), smooth muscle myosin heavy chain (MYH11) and SM22a (TAGLN) [7]. Under certain pathological conditions, vascular smooth muscle cells (VSMCs) have the ability to transition from a contractile phenotype to a synthetic one. This shift can result in a pro-inflammatory response and heightened production of matrix metalloproteinases (MMPs) [8, 9]. This switch results in the degradation of the

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extracellular matrix and weakening of the aortic wall, ultimately making it susceptible to rupture and progression of aortic aneurysm dissection (AAD) [8].

Belonging to the annexin protein superfamily [10], Anxa1, also known as Annexin A1, is a molecule that provides cytoprotection and offers numerous advantages, such as anti-inflammatory and anti-apoptotic characteristics [11]. Inhibiting neutrophil infiltration, Anxa1 and its mimetics have been found to safeguard against myocardial infarction [12]. Additionally, in mouse models, Anxa1 has been shown to reduce atherosclerotic plaque buildup and restenosis following vascular damage. Furthermore, in a type 1 diabetes mouse model, Anxa1 has exhibited its potential to enhance microvascular complications.

SirT1, the mammalian equivalent of the silent information regulator in yeast, is a deacetylase that relies on nicotinamide adenine dinucleotide+ and is influenced by polyphenols and calorie restriction. Its association with favorable metabolic outcomes is due to its antioxidant and anti-inflammatory properties. SirT1 plays a crucial role in vascular homeostasis by deacetylating and modifying the function of critical molecules. Consequently, a reduction in SirT1 activity can be harmful to the vasculature. This paper explores and analyzes the methodology and results on the pathological mechanisms of AD.

2. Method and result analysis

2.1. Vitro and vivo experiments

The scientists utilized a blend of in vitro and in vivo testing methods to investigate the involvement of Anxa1 in the development of AAD [11]. In vitro, they isolated primary VSMCs from mouse aortas using collagenase digestion and established a culture medium for cell culture. Next, the researchers used RNA sequencing to study the effect of Anxa1 on VSMC gene expression. Specifically, RNA was isolated, libraries were constructed, and RNA sequencing was performed using VSMCs from Anxa1 knockout mice and wild-type mice. Then, the researchers used ChIP-Seq technology to study the binding sites of Anxa1 in VSMCs. To create a sequencing library, precisely 5 ng of refined ChIP DNA was utilized, and sequencing was performed using the Illumina NovaSeq platform. These steps identified the binding sites of Anxa1 in VSMCs and further studied their effects on VSMC phenotypic transformation and atherosclerosis. The researchers used transcription factor prediction to determine the transcription factors that may bind to the MYL9 promoter. The position of the MYL9 promoter was determined, and it was predicted that 59 transcription factors could potentially bind to it. According to the prediction, five transcription factors have the potential to interact with both MYL9 and Anxa1. JunB ranked first among these five transcription factors. Based on these analyses, the researchers speculated that Anxa1 may activate MYL9 through JunB. Therefore, transcription factor prediction played a role in determining the potential mechanism by which Anxa1 regulates MYL9 expression in this study. The researchers used the dual-luciferase reporter assay to study the effect of Anxa1 on JunB-luc promoter activity. To be specific, lipofectamine 2000 was used to transfect VSMCs obtained from both wild-type mice and anxa1 knockout mice with pRL-SV40-N (20 ng). We employed the Dual-Luciferase Assay Kit to gauge luciferase activity in cell lysates obtained 24 hours following treatment. By contrasting the variance in luciferase activity between the two sets of cells, we determined the impact of Anxa1 on JunB-luc promoter activity. Therefore, the dual-luciferase reporter gene detection method played a role in determining the effect of Anxa1 on JunB-luc promoter activity in this study. Researchers used Immunohistochemical staining to study the expression of MMP1, Anxa1, MMP9, TNFa and MMP2 in frozen sections of the aorta. The stained samples underwent two different staining procedures: Hematoxylin and Eosin staining or antibody incubation targeting MMP1, Anxa1, MMP9, TNFa, and MMP2. Afterward, the samples were stained with HRP-conjugated goat anti-rabbit IgG polymer. This study utilized representative histological images to assess protein expression in IHC. The average density of protein expression was quantified in each image using Image-Pro Plus 6.0 and analyzed with consistent parameters. Thus, immunohistochemical staining was instrumental in determining the expression levels of MMP1, Anxa1, MMP9, TNFa and MMP2 in the

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aorta. Researchers used Immunofluorescent staining to study the expression of Anxa1, SM22a, and CD31 in the aorta. To be more specific, the aortic sections underwent incubation with antibodies that were tailored for Anxa1, SM22a, and CD31. Subsequently, they were subjected to staining with secondary incubation. The expression of Anxa1, SM22a, and CD31 in the aorta was determined by observing the fluorescence signal. Therefore, immunofluorescent staining played a role in determining the expression of Anxa1, CD31 and SM22a in the aorta in this study. The matrix metalloproteinases (MMPs) activity in the aorta was assessed using MMP activity staining. In order to accomplish this task, we employed the EnzChek Gelatinase/Collagenase assay kit according to the provided protocol. In addition, we assessed the protein expression levels in the aorta by means of Western blotting. To do so, they prepared whole-cell protein samples. In order to aid in the separation of proteins by electrophoresis, the SDS-PAGE gels were loaded with an equivalent quantity of total protein, and their concentration was determined through the use of the Bradford protein concentration detection kit. Thus, the use of Western blotting was instrumental in evaluating the expression levels of specific proteins in the aorta in this study.

The study revealed that the level of Anxa1 expression is elevated in the aortic tissue of humans and mice with AAD, in contrast to healthy aortic tissue. Anxa1-/- mice were created, and it was discovered that they exhibited a greater false lumen area and volume in comparison to WT mice. Furthermore, the severity of AAD was more pronounced in the Anxa1-/- mice. These findings imply that the presence of Anxa1 is significant in reducing the onset and severity of AAD in mice, while its absence may hasten the disease's progression. By examining the transcriptome, it was found that Anxa1 plays a critical function in preserving the balance of vascular smooth muscle cells (VSMCs). By utilizing a hierarchical clustering heatmap, they detected the MYL9 gene, which is a well-established regulator and preserver of the VSMC contractile phenotype, as being differentially expressed. To assess the impact of Anxa1 deficiency on VSMC homeostasis and its contribution to AAD, an reverse transcription PCR analysis was conducted to examine the expression of crucial genes linked to VSMC contractile phenotype. Results indicated that the mRNA level of MYL9 in VSMCs from Anxa1-/- mice was 80.8% lower than that of VSMCs from WT mice. Additionally, the mRNA expression of characteristic genes associated with VSMC contractile phenotype was significantly reduced in Anxa1-/- mice compared to WT mice. Specifically, the mRNA levels of ACTA2 decreased by 47.6%, CNN1 by 28.8%, and TAGLN by 39.1%. These findings provide evidence to support the theory that the absence of Anxa1 leads to a decline in the contractile phenotype of VSMCs. In isolated VSMCs from both Anxa1-/- mice and WT, the levels of MYL9 and various contractile proteins were assessed. The results revealed a significant decrease of 76.7% in MYL9 expression in Anxa1-/- mice VSMCs compared to WT mice VSMCs. Additionally, SMA expression was decreased by 48.4%, calponin expression by 33.1%, and SM22a expression by 74.2% in Anxa1-/- mice VSMCs compared to WT mice VSMCs. These data also confirmed that the lack of Anxa1 triggers the synthetic phenotype of VSMCs. They concluded that Anxa1 is crucial for VSMC homeostasis. To investigate whether Anxa1 plays a protective role in AAD, they manipulated its expression levels in primary VSMCs using siRNA knockdown or overexpression techniques. They found that Anxa1 knockdown promoted the transition of VSMC phenotype to a synthetic state. The researchers discovered that a lack of Anxa1 in the body worsened the progression of AAD. The researchers also investigated the mechanisms by which Anxa1 plays a protective role in VSMC phenotype transition and AAD pathogenesis. They found that the lack of Anxa1 led to the downregulation of the JunB/MYL9 pathway, which is involved in regulating the VSMC contractile phenotype. This downregulation promoted the transition of VSMC phenotype to a synthetic state, characterized by increased inflammation and MMP secretion, and led to elastic fiber degradation. The researchers proposed that Anxa1 mimetic peptides, such as Ac2-26, could be developed as a new therapy for AAD. However, more research is needed to determine the safety and efficacy of these peptides in humans.

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2.2. Vascular smooth muscle SirT1

The researchers measured the thickness and diameter of the medial layer of the mouse aortic arch [12]. The aortic arch was incubated in a nuclear stain and automatic fluorescence of the elastic fiber layer was captured using fluorescence microscopy. The aim of the study was to assess how SirT1 in VSMC affects the response of the aortic wall to angiotensin II. To accomplish this, the investigators utilized a method which employs the inherent two-photon fluorescence of elastin to visualize elastic fibers in tissue. This method does not require the use of markers and allows direct imaging of elastic fibers in tissue. In this study, the researchers used this method to evaluate the morphology and quantity of elastic fibers in the mouse aortic wall and compared the results between different experimental groups. Several genes' expression levels were evaluated using qPCR, namely AT1R, exon 4, MYH11, SirT1, MMP9, MMP19, MMP3, and MMP2, in both mouse aorta and vascular smooth muscle cells. By utilizing this approach, the researchers could examine the variations in gene expression among diverse experimental sets and gain a deeper comprehension of the impact of vascular smooth muscle SirT1 on hypertension and aortic dissection. The VSM Cell, Western Blot technique and Aortic Lysate Immunoprecipitation was employed to identify protein-protein interactions and measure protein expression levels. This study used this method to detect the interaction between SirT1 and AT1R in mouse aortic VSMCs and evaluate their expression level differences between different experimental groups. Using this method, the researchers were able to further understand the effect of VSMC SirT1 on hypertension and aortic dissection. In-Gel Zymography is a method for detecting enzyme activity. In this method, the researchers first separate protein samples by polyacrylamide gel electrophoresis and then transfer them onto a gel containing substrates. Next, a reaction is carried out in the gel, causing the substrate to be hydrolyzed by the enzyme and forming a visible transparent band. Using In-Gel Zymography technology, the researchers can detect differences in enzyme activity between different experimental groups and further understand their role in physiological and disease processes. In this study, the researchers used In-Gel Zymography technology to evaluate the metalloproteinase activity of genes encoded by MMP2 and MMP9 in mouse aorta and vascular smooth muscle cells. Oxidant Measurement by Dihydroethidine is a method for detecting oxidative stress levels. In this method, the researchers used Dihydroethidine (DHE) to evaluate oxidative stress levels in vascular smooth muscle cells and aortic tissue. When exposed to oxidants, particularly superoxide anions, dihydroethidium (DHE) is oxidized and binds to nuclear DNA, resulting in a red fluorescence signal. A 10 mmol/L DHE solution was made fresh using dimethyl sulfoxide (DMSO), and then diluted to a working solution of 10l mol/L in phosphate-buffered saline (PBS). The Flow Cytometry method for Inflammatory Cell Quantification is utilized to determine the quantity of inflammatory cells present in the aorta. In this study, the inflammatory cells were analyzed quantitatively. Specifically, the aorta was cut into small pieces and digested with trypsin, and then single-cell suspensions were obtained by filtering through a mesh screen. Subsequently, flow cytometry was employed to examine individual cells, enabling the identification of various immune cell subtypes based on their respective cell counts.

Researchers used the SMKO mouse model to study the role of SirT1 in vascular smooth muscle cells and found that SMKO mice were more susceptible to aortic dissection after receiving AngII treatment. They created a mouse model by removing exon 4 of the VSM SirT1 gene in SMKO mice and validated the impairment of SirT1's function. Results showed that SirT1 mRNA in the aortic tissue of SMKO mice was reduced by approximately 99% compared to wild-type mice. After receiving AngII treatment, truncated SirT1 protein lacking functional deacetylase domain was produced in the aortic tissue of SMKO mice, which led to a higher incidence of aortic dissection. Therefore, this study suggests that SirT1 plays an important role in preventing aortic dissection in vascular smooth muscle cells.

In the SMKO aorta, the administration of AngII led to a significant increase in treatment. This was consistent with ex vivo measurements. Furthermore, mice that were anesthetized and treated with AngII showed a notable rise in the pulse wave velocity index, a metric used to evaluate arterial stiffness, after a duration of 5 days. The researchers noted that the rise in MMPs (enzymes responsible for breaking down elastic fibers) activity, a crucial factor contributing to aortic dissection, was

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observed in SMKO mice who lacked SirT1 in their vascular smooth muscle cells, and it occurred within the same range of mean arterial pressure as the AngII-treated SMKO mice. Specifically, they found that at baseline, MMP9 and MMP2 mRNA expression levels in the aorta of SMKO mice were significantly higher than those in wild-type mice. In vitro experiments showed that after stimulating vascular smooth muscle cells with AngII, an upregulation of MMP9 and MMP2 mRNA expression levels was observed in the aorta of both mice, but the increase was more significant in SMKO mice. Based on these findings, it can be inferred that the absence of SirT1 in VSMCs contributes to a rise in MMPs activity. This could be a crucial factor in the development of aortic dissection induced by AngII. Compared to AngII-treated WT mice, the production of oxidants in the aorta of AngII-treated SMKO mice was greater. Using flow cytometry analysis, it was discovered that the absence of SirT1 in VSMCs increased the generation of oxidants induced by Angiotensin II (AngII) in cultured VSM cells. Upon further examination, it was discovered that when exposed to AngII, inflammatory cells residing in or attracted to the aortic wall may trigger the generation of oxidants and MMP activity. This, in turn, could result in aortic dissection. It is worth noting that, following a five-day period of AngII stimulation, the levels of CD45+ CD11b+ cells, galectin-3 (a marker for macrophages), and VCAM-1 (an adhesion molecule) in the aortic wall of SMKO mice were similar to those observed in wild-type mice. These results suggest that the lack of SirT1 in vascular smooth muscle cells in SMKO mice does not affect the recruitment and activation of inflammatory cells in the aortic wall but may lead to aortic dissection through other mechanisms.

3. Conclusion

The researchers used RNA sequencing, ChIP-Seq technology, transcription factor prediction, the dual-luciferase reporter assay, immunohistochemical staining, immunofluorescent staining, matrix metalloproteinases (MMP) activity staining, western blot, and statistical analysis. The scientists employed various techniques in their study, including quantitative RT-PCR, In-Gel zymography, dihydroethidine for oxidant measurement, and flow cytometry for inflammatory cell quantification. In general, although the two techniques use slightly different experimental methods, the core idea is still the same, and the experiments focus on the expression of the subject, which can be used as both a variable and a dependent variable. The variables are changed by specific methods to explore the relationship between the experimental subject and the case mechanism and finally analyzed on the basis of the existing signaling pathways to give a new view and reflection of the experimental subject.

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