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Abstract

Tenascin-C (TN-C) interacts with extracellular molecules and plays a role in cell adhesion, signaling, and differentiation. There is evidence that TN-C is regulated by mechanical forces and since the heart valve is a highly dynamic structure, it is possible that TN-C is expressed within specific sites within the aortic valve, as these sites are subject to unique mechanical forces. It is also reasonable to expect differences in TN-C expression between the pulmonary and the aortic valve because these two locations experience different levels of mechanical force. Here we compare TN-C expression levels in adult pulmonary versus aortic valves and determine if different cell types within aortic valves express different amounts of TNC. Our results indicate that TN-C is expressed in normal post-natal heart valves and expression is higher on the aortic versus ventricular side of the aortic valve. TN-C expression may be higher in aortic versus pulmonary valve. SMC, fibroblasts or myofibroblasts may produce TN-C, but endothelial cells appear not to produce TN-C. These studies will help us in determining the role TN-C plays in normal heart valve tissue homeostasis and how different cell types react to different mechanical forces.

Comments

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ABSTRACT

Tenascin-C (TN-C) interacts with extracellular molecules and plays a role in cell adhesion, signaling, and differentiation. There is evidence that TN-C is regulated by mechanical forces and since the heart valve is a highly dynamic structure, it is possible that TN-C is expressed within specific sites within the aortic valve, as these sites are subject to unique mechanical forces. It is also reasonable to expect differences in TN-C expression between the pulmonary and the aortic valve because these two locations experience different levels of mechanical force. Here we compare TN-C expression levels in adult pulmonary versus aortic valves and determine if different cell types within aortic valves express different amounts of TN-C. Our results indicate that TN-C is expressed in normal post-natal heart valves and expression is higher on the aortic versus ventricular side of the aortic valve. TN-C expression may be higher in aortic versus pulmonary valve. SMC, fibroblasts or myofibroblasts may produce TN-C, but endothelial cells appear not to produce TN-C. These studies will help us in determining the role TN-C plays in normal heart valve tissue homeostasis and how different cell types react to different mechanical forces.

INTRODUCTION

Heart valves help control the flow of blood through the heart's four chambers. The upper two are the small, round atria, and the lower two are large, cone-shaped ventricles. Each ventricle has an inlet valve and an outlet valve to ensure that blood flow is unidirectional. In the left ventricle, the inlet valve is the mitral valve, and the outlet valve is the aortic valve. In the right ventricle, the inlet valve is the tricuspid valve, and the outlet valve is the pulmonary (pulmonic) valve. Each valve consists of flaps, which function like a one-way swinging gate. There are a number of inherited and/or post-natal diseases that are associated with abnormal heart valve development or disease. Heart valves can malfunction either by leaking (regurgitation), or by not opening adequately and partially blocking the flow of blood through the valve (stenosis). Well-known diseases include aortic valve sclerosis (calcification) and aortic regurgitation.

Every heartbeat has two distinct components: diastole and systole. During diastole, the heart relaxes and ventricles fill with blood; then the atria contract and force more blood into the ventricles. During systole, the ventricles contract and pump blood to the arteries; then the atria relax and begin filling blood again. As the valve opens and closes 70 times per minute on average, heart valves undergo stress fluctuations repeatedly due to the blood flow. In addition to gross fluctuations in valve movement, it is likely that there are local differences in tissue biomechanics throughout the valve structure. For example, folding corrugations form on the aortic surface of the valve, and at the base of the valve where it connects to the heart wall. These supplemental biomechanical deformations are likely to impose additional forces and modes of behavior on the underlying cells.

Tenascin-C (TN-C) is a large, secreted glycoprotein that functions outside the cell within the extracellular matrix. The domain structure of TN-C allows for interaction with multiple extracellular molecules and for multiple roles in cell adhesion, signaling and differentiation. TN-C interacts with the cell surface through numerous receptors, including integrins, which are used to send signals to the cell interior. These signals can instruct the cell to grow, migrate, survive, die, or differentiate, depending upon the cellular and extracellular context (1, 2). TN-C is synthesized in an ordered fashion at particular sites during development: It is made in large amounts by certain tumors (at pathological sites), and is found in restricted tissue locations in the adult. TN-C is also expressed in normal processes, such as wound healing and nerve regeneration (1, 2). Usually, however, TN-C is suppressed in normal adult tissues. For example, TN-C is also expressed within the developing heart valve (3), and it is highly expressed in diseased valves where it is believed to promote sclerosis (hardening of the valve) (4). However, it is not known whether TN-C also plays a role in normal valve tissue homeostasis.

There is significant evidence that TN-C is regulated by mechanical forces (5). Studies show that hemodynamic stress of increased pulmonary arterial flow induces TN-C (6). In keeping with this, removal of mechanical stress suppresses TN-C expression (7). Collectively, these and other studies indicate that TN-C expression is regulated by tissue biomechanics, and that this may account for its appearance in settings where it plays a regenerative role, such as healing wounds. Since the heart valve is a highly dynamic structure, it is possible that TN-C is expressed within specific sites within the aortic valve, because these sites are subject to unique and local mechanical forces. As well, since the adult pulmonary valve, located in a low pressure vascular bed, experiences a different level of me-

chanical force when compared to the adult aortic valve, which is subject to higher pressures, it is also reasonable to expect differences in TN-C expression at these two different locations. Thus, a primary goal of this project was to compare TN-C expression levels in adult pulmonary versus aortic valves.

Another aim of this research project was to determine whether different cell types within aortic valves express different amounts of TN-C. There are believed to be at least 3 different cell types in the aortic valve: The surface of the leaflet is covered with a layer of endothelial cells (ECs), while within the valve there are fibroblasts and smooth muscle cells (SMCs). Importantly, each of these distinct cell types can be identified using specific markers: ECs (von Williebrand factor), fibroblasts (fibroblast specific protein), and for SMCs (smooth muscle alpha–actin). By determining which cells express TN-C in normal heart valves, we may be able to find out more about the role TN-C plays in normal heart valve tissue homeostasis and how different cell types react to different mechanical forces.

Since heart valves are highly dynamic structures subjected to different levels of mechanical force, we hypothesized that (1) TN-C would be expressed in the post-natal heart valve; (2) TN-C would be preferentially expressed in aortic versus pulmonary valve; and (3) TN-C expression would be higher on the aortic versus ventricular side within the same valve. To address these hypotheses, we used an immunohistochemical approach using porcine heart valves and antibodies raised against TN-C, and cell type specific markers.

MATERIALS AND METHODS

As mentioned above, the primary experimental approach used throughout the semester was immunohistochemistry using a peroxidase-based approach. Immunohistochemistry is a method of detecting the presence of specific proteins in cells or tissues. By conducting immunostaining for TN-C and for markers that are specific for the 3 different cell types in the aortic valve (i.e. vWf, fibroblast specific protein, and smooth muscle alpha-actin), we were able to identify if the different cells in the valve are surrounded by TN-C. Upon retrieving normal adult porcine heart valves (Landrace X Yorkshire, ~ 250 lbs., castrated), longitudinal and transversal tissue sections were taken. Longitudinal sections are cut along the Arantii nodule, while transversal sections are along the closure line of the valve. To prepare for immunohistochemical analysis, each sample was then embedded in paraffin, and these were then sectioned to a thickness of approximately 7 μm. Swine are particularly convenient to use for research due to their similarities in body size and with the biology of the human, in particular with the heart and blood vessels.

The polyclonal primary antibodies used were anti-chicken TN-C antibody, anti-human von Williebrand factor (vWf) for ECs, and anti-human S100A4 for fibroblasts. Primary antibodies were applied in dilutions of 1:500 for TN-C, and 1:2000 for vWf. S100A4 was applied in dilutions of 1:100 and 1:200. Monoclonal mouse anti-smooth muscle actin antibody was used for immunostaining for smooth muscles cells. This was applied in 1:500 dilution. Biotin was then added to bind to the antibody-antigen complex using biotinylated secondary antibodies. The secondary antibodies (all from Vectastain) used were biotinylated anti-rabbit IgG (for TN-C, VWf, and fibroblasts), and biotinylated anti-mouse IgG (SMA). For negative controls, normal rabbit IgG or normal mouse IgG was used in place of the primary test antibodies.

Samples were prepared for the immunohistochemistry by first removing the paraffin from the sections and this was followed by rehydration of tissue samples. Sections were then subject to antigen retrieval, which is a method to enhance exposure of the protein of interest to the applied antibody that may have been masked by fixation. A pronase digestion solution was used for TN-C and S100A4 immunostaining, while proteinase-K digestion was used for the vWf immunostaining. For the SMA immunostaining, samples were subjected to antigen retrieval by placing them in citrate buffer, and then heating them on a hotplate after which they were allowed to stand at RT for 30 min. Endogenous peroxidase activity was blocked with methanol containing 0.3% hydrogen peroxide for 30 min at RT, followed by washing with phosphate-buffered saline solution (PBS). Nonspecific binding was inhibited with a solution of 1% BSA in PBS, 0.1% Tw20, and 5% normal goat serum for 30 min at RT. This is necessary to eliminate any non-specific adsorption of the primary antibody to tissue elements. Immunostaining was then carried out by applying the appropriate concentration of primary antibody to each sample and incubating overnight at 4°C. For negative controls, normal rabbit IgG or normal mouse IgG was used in place of the primary test antibodies. Following incubation, the samples were washed repeatedly in 1% BSA and .1 % BSA and then were incubated with their respective biotinylated secondary antibody for one hour at RT. The antibody-antigen complex is now bound by a secondary, enzyme-conjugated, antibody. The avidin-biotin-peroxidase complex (ABC) was then performed for 30 min at RT. This allows for a complex to form between the avidin and biotinylated enzymes, enhancing the signal as the avidin has four binding sites for biotin. The color reaction was developed with the use of diaminobenzidine (DAB), which is a peroxidase procedure for localizing markers. The DAB reaction is performed to deposit a brown substrate where there are peroxidase enzymes present. This reaction was done for the same amount of time on each sample so that the level of TN-C expression could be compared between them. Thus, an assessment of relative TN-C expression can be completed in qualitative terms according to the resulting staining intensity from the DAB reaction. Counter-staining was performed using hematoxylin to visualize and orient nuclei. Following washing and dehydration, a coverslip was applied to the section.

RESULTS AND DISCUSSION

To begin to determine whether TN-C is expressed in the biomechanically active environment of normal adult heart valves, and whether valves in different vascular beds express more or less TN-C than one another, we first compared TN-C expression in post-natal porcine aortic and pulmonary heart valves (Figure 1). Although TN-C expression (in brown) is usually suppressed in normal cells, our study showed that TN-C is expressed in the normal post-natal adult aortic porcine valve and it is expressed predominantly on the aortic side. This is interesting for several reasons: First, most cells are constantly exposed to mechanical forces, but not all of them express TN-C. The heart is a very dynamic structure, and cells in the heart experience a significant amount of mechanical stress, and so this may be part of the reason that TN-C expression does not turn off in normal cells. In addition, cells on one surface of the valve leaflet experience a very different hemodynamic environment than those on the opposite surface. The aortic side may experience more disruptions than the ventricular

side due to turbulent blood flow. In fact, this may account for the fact that calcification and sclerosis occur predominantly on the aortic side of the valve leaflet, especially since TN-C has been shown to participate in this process (Refs). It is also worthy to note that TN-C is not located on the very edge of the valve but, instead, there is a gap where TN-C is absent right along the surface of the valve. This indicates that there are site-specific differences and TN-C seems to be in discrete sections within the valve. There is also considerable variation of TN-C within the same valve of the same individual. This can be due to varied mechanics or to different cell types that are in these positions.

It is useful to compare TN-C expression in aortic valve with the pulmonary valve for several reasons. Structurally,
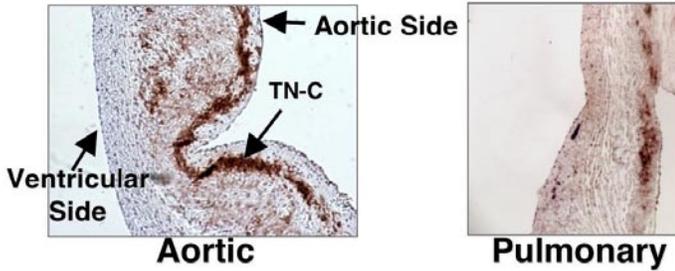

Figure 1: TN-C in Ao vs Pulmonary.
Ao Valve: TN-C is strongly expressed towards the Ao side, and more diffusely within the valve.
Pu. valve: TN-C is moderately expressed towards the outflow side & less is evident in the interior.

the aorta is thicker than the pulmonary artery. The pulmonary valve experiences different types of mechanical force than the aortic valve. Since TN-C has been shown to be controlled by biomechanical forces, it would reasonable to see differences in TN-C expression because the pulmonary valve is in a lower pressure vascular bed. Thus, the aortic valve should express more TN-C if this gene is mechanosensitive. From examining TN-C expression in pulmonary valves, however, the results are not conclusive. TN-C levels may be less in the pulmonary valve than in aortic valves (Figure 1). In the aortic valve, TN-C is strongly expressed toward the aortic side, as well as more diffusely within the valve. On the other hand, in the pulmonary valve TN-C is moderately expressed toward the outflow side, and is less evident in the interior of the valve. This suggests that the different types of pressures and hemodynamic forces that aortic and pulmonary valves experience may account for differences in TN-C expression. Regardless of the mechanism, it is clear that in both valves, more TN-C is expressed on the aortic versus ventricular side. These results indicate that there are site-specific differences in TN-C expression, and this could mean either that different cells are making TN-C at different sites, and/or that external mechanical influences on the valve differentially regulate TN-C within the same cell type.

To determine which cell types express TN-C, or are in contact with TN-C, we performed additional immunohisto-chemical studies. Initially we stained for smooth muscle cells (SMCs) using α-actin as a marker. Figure 2 shows that SMCs are more heavily concentrated on the aortic side (AS) as opposed to the ventricular side (VS). Examining this against the TN-C immunostaining, TN-C is expressed at the border of the SM alpha-actin positive layer. However, both the TN-C and the SM α-actin layer do not match perfectly. Instead, there seems to be a sub-set of SMCs that make TN-C. In addition, unlike TN-C expression, SMCs are present on the ventricular

side. It is important to note that SMA α-actin is a marker for smooth muscle cells, as well as myofibroblasts. Thus, it would be useful to find out if these cells that seem to be producing TN-C are SMCs or myofibroblasts. To distinguish between the two types of cells, future immunostaining studies will be performed using myosin heavy chain (MHC) that is frequently used as an additional marker for smooth muscle.

We also wished to determine whether endothelial cells (ECs) are in contact with TN-C. To achieve this vWF was
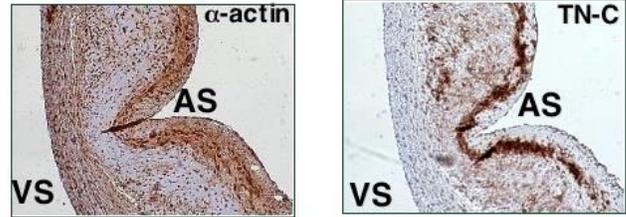

Figure 2: Site-specific differences in SM α-actin in the aortic valve.
TN-C is expressed at the border of the SM α-actin positive layer.
There appears to be a sub-set of SMCs that make TN-C. Are these really SMCs or are they myofibroblasts?

used as a marker of ECs. As expected, ECs are located on both surfaces of the valve (Figure 3). Although ECs are expressed on both sides, it was also clear that vWF staining was more prominent on the aortic side. This may indicate that the ECs on each side of the valve are behaving differently due to differences in tissue biomechanics. Regardless of this issue, our data show that TN-C is not expressed within the endothelial layer.

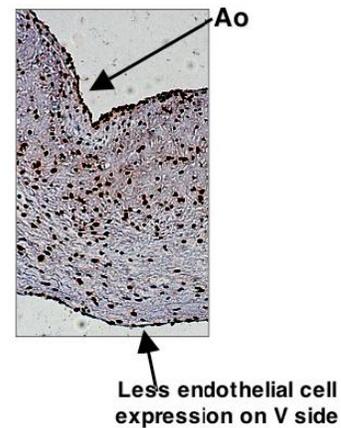Finally, to determine whether fibroblasts express TN-C, we examined expression of S100A4. Of note, these experi-

Figure 3: Site-specific differences in vWF in the aortic valve.

ments showed that the aortic side contains more fibroblasts (Figure 4). Although it resembles and overlaps with both TN-C and SMCs, the band of fibroblasts does not extend as far into the valve and is thinner. The fibroblast cells do not match up precisely with TN-C either. In some cases, fibroblasts are expressed right against the edge of the endothelial layer and this is where TN-C is absent. Fibroblasts may also be expressed on the endothelial layer on the surface of the valve. Alternatively, it is possible that S100A4 also marks ECs.

In summary, TN-C is expressed in normal post-natal heart valves and expression is higher on the aortic versus ventricular side of the aortic valve. In addition, TN-C expression may be higher in aortic versus pulmonary valve. SMC, fibroblasts,

or myofibroblasts may produce TN-C, but endothelial cells appear not to produce TN-C. However, these EC studies were

Figure 4: S100A4 immunostaining in the Aortic Valve.

of interest, because vWf expression was found to be greater on the aortic side.

Future Directions

To continue this project, it would be interesting to map biomechanical forces throughout the valve and determine how this relates to TN-C expression. By measuring forces throughout the valve, a comparison could be made between TN-C expression and different mechanical forces.

Additionally, immunostaining of TN-C in the aortic versus the pulmonary position will be conducted to begin to determine whether hemodynamic factors differentially regulate TN-C. Accordingly, expression patterns of TN-C will then be compared in the aortic and pulmonary position in the heart valve to see if TN-C is expressed within specific sites.

Finally, it would be interesting to find out what exactly controls TN-C expression in the valve. Bone morphogenetic protein-4 (BMP-4) is a growth factor that is expressed on the aortic side endothelium, and it has been proven that BMP-4 suppresses TN-C expression (Ihida-Stansbury and Jones, unpublished). Given that TN-C is expressed on aortic side but it stops short of the endothelium layer, BMP-4 may negatively regulate TN-C within the interior of the valve. There may be a gradient of BMP-4 that decreases from the endothelium layer toward the interior of the valve. As BMP-4 levels decrease, this may correlate with an increasing gradient of TN-C.

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