SUPPRESSION OF ATHEROGENESIS BY DELIVERY OF TGFβ1\textsuperscript{ACT} USING ADENO-ASSOCIATED VIRUS TYPE 2 IN LDL KNOCKOUT MICE

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Atherosclerosis and its manifestations, such as myocardial infarction and stroke, are the most common causes of morbidity and mortality in the U.S. There have been several attempts to identify steps leading to atherosclerosis. Current attempts at prevention of atherosclerosis and related events are focused at lifestyle modification and control of risk factors, such as diabetes, hypertension, dyslipidemia, and smoking.

Recently there has been much emphasis on the inflammatory basis of atherosclerosis, and a number of approaches directed at limitation of inflammation have shown some success, at least in animal studies.

Oxidative stress is also present in all stages of atherosclerosis. Inflammation and oxidative stress generally coexist. Therapies directed at limitation of oxidative stress, such as use of antioxidant vitamins, have shown promise in animal studies. However, a large majority of trials in humans have been negative.

Over the last several years, it has become evident that growth factors play a significant role in protecting tissues from ischemic injury. This is contrary to the earlier thought that growth factors, such as fibroblast growth factor, platelet-derived growth factor, and transforming growth factor β\textsubscript{1} (TGFβ\textsubscript{1}), may be detrimental by causing hypertrophy and growth of smooth muscle cells in the cardiovascular system. Recently, it has been suggested that growth factors such as TGFβ\textsubscript{1} may also exert anti-atherosclerotic effects [1].

TGFβ superfamily consists of a large number of structurally related cytokines that participate in a vast range of biological processes and the development of cardiovascular tissues and the maintenance of normal vessel wall structure. TGFβ\textsubscript{1} is present in high levels in healthy blood vessel wall, whereas other members of the superfamily are either absent or present at low levels. TGFβ\textsubscript{1} is typically produced as dimeric latent precursor with no biological activity, and is subsequently activated in the extracellular environment to release active moiety which binds to its receptors. Conversion of TGFβ\textsubscript{1} from latent to active form is reduced in ischemia, inflammation, and other stressful events [2]. Loss of active TGFβ\textsubscript{1} results in the accumulation of inflammatory cells in the vessel wall, enhances monocyte adhesion to endothelium, and exacerbates lipid accumulation in the adult apoE knockout mice. These observations support the concept that TGFβ\textsubscript{1} may protect against vascular injury and formation of atherosclerotic lesions. However, until now there have been no studies that show atherosclerosis can be reduced by enhancement of TGFβ\textsubscript{1}.

We conducted a study to determine the role of TGFβ\textsubscript{1} in the pathogenesis of atherosclerosis. We cloned a bioactive mutant of TGFβ\textsubscript{1} (TGFβ\textsubscript{1}\textsuperscript{ACT}) and incorporated it into AAV which is capable of efficient and stable gene delivery over time in the LDLR knockout mice. We used AAV because of its ease in manipulation, its efficiency, its safety, and its ability to achieve long-term transgene expression as the gene therapy vector. We also used gene array technology to study the expression of pro-inflammatory genes.
The technology for generating TGFβ1 cDNA has been described earlier [3]. The cysteines in positions 223 and 225 of TGFβ1 mRNA were substituted with serines. This mutation results in TGFβ1 protein in its biologically active form upon secretion. The mutant rat TGFβ1 cDNA was inserted into AAV type 2 vector as described for other AAV vectors.

Homozygous LDLR knockout mice on C57BL/6J background were divided into four groups. Group 1-C57BL/6J mice were fed regular diet for the entire study. This served as the negative control group. Group 2 consisted of LDLR knockout mice that were injected 100 μl of saline via the tail vein. Group 3 consisted of LDLR knockout mice injected with 100 μl of AAV/GM-CSF (as an additional control group). Group 4 consisted of LDLR knockout mice injected with 100 μl of AAV/TGFβ1ACT. All groups 2 to 4 were animals fed high cholesterol diet for 18 weeks.

At the end of the study the animals were sacrificed and transgene vector DNA, mRNA, and protein were assessed by Southern blot analysis, RT-PCR, and immunohistochemistry, respectively. We also performed gene array to detect gene expression in the aortic tissues using GE Array Q Series Mouse Cardiovascular Disease Gene Array I.

The AAV/TGFβ1ACT mice displayed no adverse effects. After 18 weeks of high cholesterol diet, the animals were sacrificed. The transgene was expressed systemically in the lung, liver, kidney, heart, and the blood vessels. Notably the plasma lipid concentrations were markedly elevated in all animals given high cholesterol diet. There was no effect of AAV/TGFβ1ACT or AAV/GM-CSF administration on the elevated plasma lipid concentrations.

Most importantly, AAV/TGFβ1ACT-treated animals display dramatically smaller areas of sudanophilia than the saline-treated LDLR knockout mice or AAV/GM-CSF-treated LDLR knockout mice (Figure 1). These data strongly indicate that AAV/TGFβ1ACT injection and subsequent TGFβ1ACT upregulation results in an improved outcome in the form of less lipid accumulation in the subintima.

We analyzed the aortas for CD68 expression, a general marker of inflammation. There was a marked increase in CD68 positivity in the saline-treated or AAV/GM-CSF-treated LDLR knockout mice. In sharp contrast, mice injected with AAV/TGFβ1ACT demonstrated very low levels of CD68 staining. We analyzed aortic sections for oxidative stress by immunohistochemistry using anti-nitrotyrosine antibody. There was significant increase in nitrotyrosine immunopositivity in the LDLR knockout mice injected with saline or AAV/GM-CSF. Again in sharp contrast, mice injected with AAV/ TGFβ1ACT demonstrated a very low level of nitrotyrosine staining.

Next we examined the alteration of gene expression in the LDLR knockout mice and found that the expression of adhesion molecules (chemokine receptor-2, ICAM-1, VCAM-1, PECAM-1, and E-/P-/L-selectin), inflammatory cytokine TNFα, MMP3, and ox-LDL receptor LOX-1 all were increased in the saline-treated LDLR knockout mice. Importantly, expression of these genes was markedly inhibited in the LDLR knockout mice given AAV/ TGFβ1ACT.

This study for the first time demonstrates that a tail vein injection of AAV/ TGFβ1ACT provides long-term transgene expression. The sustained TGFβ1ACT expression was found to have a significant effect on atherogenesis. The administration of TGFβ1ACT in this process was associated with a reduction in pro-inflammatory genes, macrophage retention, ox-LDL receptor LOX-1, and oxidative stress product nitrotyrosine. The TGFβ1ACT administration also reduced gene expression of MMP3 which may contribute to plaque stabilization. There was no significant effect of TGFβ1ACT on lipid levels and liver and renal functions. In parallel experiments, therapy with GM-CSF had no effect on the development of disease; importantly the last observation
strongly indicates that the inhibition of atherosclerosis by TGFβ1\textsuperscript{ACT} was not just an effect of AAV administration.

\textbf{TGFβ1\textsuperscript{ACT}, Modification of Gene Expression, and Accumulation of Inflammatory Cells}

Over the last decade, a wide array of tissue-protective effects of TGFβ1 has been identified. These include suppression of myocardial ischemia-reperfusion injury, apoptosis of cardiomyocytes, pro-inflammatory adhesion molecule expression on the vascular endothelial cells, and foam cell formation in cultured macrophages. Our studies have shown that TGFβ1 can inhibit oxidized LDL-induced expression of adhesion molecules and monocyte adhesion to coronary artery endothelial cells. Other studies have suggested immunomodulatory role of TGFβ1 during atherogenesis. These observations collectively have critical implications for the chronic inflammatory component of atherogenesis.

\textbf{TGFβ1\textsuperscript{ACT} and Formation of Atherosclerotic Lesion}

Genetic manipulation including down- or up-regulation of genes has become a standard method for determining the importance of gene products in disease processes. Studies have shown that TGFβ1 null mouse dies soon after birth from severe multifocal inflammation in vessels and heart. Reduced TGFβ1 production in adult TGFβ1\textsuperscript{+/−} mice renders the vessel wall susceptible to endothelial activation and development of early stage lipid lesions when subjected to a pro-atherogenic challenge. Recent studies have used \textit{in vivo} neutralization approaches to show that the depletion of TGFβ1 in the blood vessel wall of adult apoE-deficient mice was sufficient to exacerbate lipid lesion development.

In summary, this study suggests that TGFβ1\textsuperscript{ACT} suppresses the changes in vessel wall architecture that are associated with deposition of lipid-laden cells.

\textbf{References}

3. Li D, et al. Suppression of atherogenesis by delivery of TGFβ1\textsuperscript{ACT} using adeno-associated virus Type 2 in LDLR knockout mice. Atherosclerosis 2006
Figure 1. Extent of atherosclerosis in different groups of animals. Treatment is mentioned on the left side of the figure.