AWARD NUMBER: W81XWH-12-1-0472

TITLE: “Aberrant Recapitulation of Developmental Program: Novel Target in Scleroderma”

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REPORT DATE: December 2015

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

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Aberrant Recapitulation of Developmental Program: Novel Target in Scleroderma

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Fibrosis in scleroderma is associated with altered Wnt/β-catenin signaling. This project seeks to define how Wnts activate fibrotic responses, to determine whether blocking Wnt/β-catenin signaling can prevent or attenuate fibrosis in scleroderma, and to ascertain whether markers of Wnt/β-catenin signaling can be used as biomarkers of disease activity, progression and severity, as well as tools to identify patient subsets that will respond to Wnt/β-catenin-targeted therapy in a “personalized” or precision medicine strategy. We have previously shown that in scleroderma, fibrosis is consistently accompanied by subdermal fat loss and is associated with the down-regulation of PPAR-γ (peroxisome proliferator activated receptor), a master regulator of adipogenesis that signals through the adipokine adiponectin (APN). Circulating and tissue levels of adiponectin are significantly reduced in scleroderma. Here we report that APN causes a time-dependent down-regulation of both Wnt3a-induced canonical signaling and fibrotic responses at the mRNA and protein levels. Mechanistically, these effects involve suppression of both the expression and activation of the Wnt co-receptor low density lipoprotein receptor-related protein-6 (LRP6). These results demonstrate that adiponectin inhibits short-term Wnt/β-catenin signaling as well as the Wnt3a-mediated fibrotic response, identifying adiponectin as a “natural” anti-fibrotic agent with tremendous therapeutic potential. Furthermore, these data suggest that adiponectin directly inhibits the Wnt/β-catenin pathway, identifying a novel mode of regulation of this key profibrotic pathway that has been shown to play a critical role in scleroderma. Restoring normal adiponectin signaling thus represents a promising therapeutic approach to prevent or attenuate fibrosis in scleroderma. We also show that mice lacking the Wnt co-receptor, Lrp5, are protected against bleomycin-induced pulmonary fibrosis, an effect that is phenocopied by direct inhibition of β-catenin/TCF signaling by the small molecular inhibitor, iCRT3. These results support the role of Wnt/β-catenin signaling in the pathogenesis of pulmonary fibrosis. Finally, we use lung fibroblasts explanted from Axin2 WT, +/-, and KO mice to demonstrate that loss of the Wnt inhibitor Axin2 is sufficient to stimulate Type I Collagen in a dose-dependent manner and to sensitize cells to TGF-β treatment. Taken together, these results provide further evidence of the importance of aberrant Wnt/β-catenin signaling in fibrosis in scleroderma. Additionally, these findings lay the groundwork for future experiments to examine the effect of pharmacological modulation of Wnt/β-catenin signaling as a means to treat systemic sclerosis.
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Task 7

Develop fibroblast-specific Wnt signature at the genome-wide level using explanted normal skin fibroblasts; evaluate the expression of the signature in a core dataset of SSc skin biopsy-derived microarrays (Aim 4d). We will treat human explant 3 SSc skin fibroblast cell lines for 24 h with Wnt 3a or vehicle, and prepare RNA for microarray analyses utilizing the BUMC microarray core. The resulting profile of genes found to be regulated by Wnt (Wnt-responsive signature) will be compared to gene expression from control and SSc patient samples already available in the partnering PIs database with the goal of defining a Wnt signature in SSc skin (months 1-12). Expression of individual genes in skin RNAs from SSc patients will be further characterized by RT-PCR and correlated with the MRSS in years 2-3. Genes whose expression is significantly correlated with MRSS will be further tested by multiple linear regression as possibly contributing to a refined 4-gene biomarker we recently described (47).

We have completed several experiments examining the effects of Wnts on SSc fibroblasts.

Figure 1: Gene regulation by different Wnts. SSc fibroblasts were treated with Wnt2, Wnt3, Wnt 5 or Wnt 10 for 24 hours and cells analyzed by microarray. Shown are the most differentially expressed genes showing detectable levels (defined by baseline expression greater than 50).

These initially showed intriguing results (Fig. 1), indicating that some genes are regulated similarly by different Wnts (FDX1, EGR2), while and others appear regulated by one Wnt but not other Wnts (HMOX1). We were anxious to repeat these results to verify this interesting difference in regulation, which might be related to canonical and non-canonical signaling, differences in receptor utilization, or other poorly defined differences in signaling.

On the basis of these studies we constructed a nanostring to permit us to confirm the differential regulation of genes by different Wnt 2, 3a, 5a and 10 (Figure 2). We then stimulated several cell lines with these Wnts and tested expression of the genes found regulated by microarray. Surprisingly we were not able to confirm any of the original results on the microarray regulation of specific Wnts. The reason for this remains uncertain but most likely is related to noise in the microarray. We were able to determine, also surprisingly, that regulation of classical Wnt-induced genes such as axin1 does not occur in dermal fibroblast cultures in the absence of serum, the conditions we were using for our microarray experiments.
Task 8

Determine Wnt signaling in SSc patients using assays to measure Wnt activity in serum from a well-characterized cohort of patients (Aim 4a). Dr. Lafyatis’s group already has collected sera from 80 limited cutaneous SSc (lcSSc) patients, 40 diffuse cutaneous SSc (dcSSc) patients and 20 control sera as part of an NIH-sponsored biomarker study. These samples currently are part of a larger repository. Associated with each of these serum samples are clinical data, including the modified Rodnan skin score (MRSS), pulmonary function tests (forced vital capacity (FVC) and diffusion capacity (DLCO), and pulmonary artery pressure (PAP determined by echocardiogram or right heart catheterization). Serum Wnt activity will be measured in these samples using LSL reporter cells by measuring cell β-galactosidase activity controlled for luciferase activity from a stably co-transfected CMV luciferase plasmid (44, 46). Assays will be performed in the absence or presence of the Wnt inhibitors WIF-1 (R&D) or XAV-939 (Tocris). Wnt activity will be calculated from a standard curve generated by incubating LSL cells with Wnt3a. The relationship between Wnt activity and skin score and disease subtype, FVC, DLCO and PAP will be analyzed by linear regression. (Months 1-12)

We have successfully measured Wnt activity using LSL cells in several patients with SSc and shown that the addition of the Wnt inhibitor, WIF-1, partially blocks this activity. Thus LSL cell activation detected in our previous work appears to be indeed related to circulating Wnts. However, the magnitude of this effect and WIF-1 inhibition is very small. Likely this system is tightly regulated by not only Wnts but also Wnt inhibitors, as we show in work from Task 9, several DKK proteins appear to be downregulated in SSc sera. Given the small differences between SSc and healthy controls sera in this system and the modest effect of blocking Wnts with WIF-1, we did not carry out further experiments using this approach, instead focusing on direct measures of Wnts in sera as in Task 9.

Task 9

Identify and quantitate Wnts and endogenous Wnt inhibitors in SSc sera (Aim 4b). We will determine relative levels of Wnts, modulators and inhibitors, including Wnt2, WIF-1 (R&D Systems), and DKK-1 and SFRP4 (USCN Life Sci) using sandwich ELISAs. We will develop a sandwich ELISA for Wnt 2 and Wnt 10 using commercially available antibodies (R&D Systems), and measure these serum analytes in a discovery cohort of 20 dcSSc, 20 lcSSc and 20 healthy control sera. Analytes showing differences significant will be confirmed in a validation cohort of the same size. (Years 2-3)

Based on technological advances, we have utilized a multi-analyte proteomic platform to analyze the spectrum of proteins encoded by a Wnt gene (Wnt7a), a Wnt-responsive gene (WISP-1) and a series of Wnt regulators (DKK-1, DKK-3, DKK-4 and WIF-1 and SFRP3). We initially analyzed 4...
healthy controls and 14 dSSc patients. These results show a strong trend toward increased levels of a series of proteins known to block Wnts, WIF-1, DKK-1 and DKK-4. This elevation was a bit counterintuitive as a recent report has suggested that DKK-1 is downregulated in SSc skin (recently described hypermethylation of the DKK1 promoter may explain its decreased expression (3)). Despite the elevation of DKKs in SSc skin, DKK-1 and DKK-4 also showed a trend toward a negative correlation with the MRSS, meaning they were lower in patients with the highest level of disease. We also examined Wnt 2 and Wnt 10 levels directly in sera. However, the levels of these Wnts in SSc were the same as seen in healthy controls (not shown).

Figure 3. Wnt and Wnt inhibitors in SSc sera

In order to clarify the significance of the above results, we examined DKK1-4 and WIF1 in a larger series of SSc patients with a variety of disease complications, including diffuse skin disease, SSc-ILD and SSc-PAH (Figure 4). Strikingly WIF-1 levels were elevated even more highly in patients with limited SSc and PAH, suggesting that this may be a strong marker for vascular disease. In contrast DDK1-4 did not show any differences between the groups suggesting that the trends seen in our initial studies are not significant (Figure 5).
Figure 4. WIF1 is elevated in SSc patients with limited cutaneous disease complicated by PAH more than diffuse SSc
Figure 5. DDK1-4 do not show altered protein levels in SSc sera
Task 10

Examine selected Wnt and Wnt inhibitors in SSc skin (Aim 4c). We will examine Wnt proteins showing altered mRNA expression in other aims in SSc and control skin biopsies by immunohistochemistry. We anticipate examining expression of WNT2 (anti-Wnt2, R&D Systems) as well as Frizzled 2 (anti-Fzd2, Abcam). We will also examine the source of WIF-1 and DKK protein expression in and the extent of their down-regulation in SSc skin. 20 dcSSc biopsies and 10 healthy control samples will be stained for each of these and other Wnt related antigens found important in above Tasks. These slides will be obtained from a repository of SSc samples under the direction of the partnering PI. Slides will be stained using standard protocols for immuno-alkaline phosphatase staining after development of proper conditions and any required antigen retrieval. The slides will be scored by two blinded observers on a 0-10 analog scale, and scores tested for differences between dcSSc and controls and for correlations with the MRSS. (Years 2-3).

We have succeeded staining Wnt 2, and WIF-1 (our targets of highest interest) in 15 SSc and 5 healthy controls skin sections. Wnt2 stains in a very distinct adventitial pattern around blood vessels in the skin. It also stains fibroblasts in both superficial and in fibrotic regions of the skin (Figure 6). These results suggest that it is indeed upregulated in SSc skin at the protein level as we have found previously looking at mRNA expression. WIF-1 stains diffusely without a distinct pattern and downregulation of its expression cannot be readily detected by IHC (data not shown).

Key Research Accomplishments

We have excluded regulation of several of the Wnt mediators (Wnt2 and DKK1-4) as the soluble factors leading to increased Wnt activity in sera. Most importantly we have identified WIF1 as a strong biomarker of vascular disease. IHC staining of this protein in sections is as of to date not sufficient quality to clearly indicate its cellular source. However we have indications from other funded work that it orginates from the macrophage. This is intriguing as we know these cells localize around blood vessels and the importance of these cells in pathogenesis.

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**Figure 6: Wnt2 stains in perivascular regions of SSc skin.**

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We have succeeded in staining Wnt2 by IHC, it is expressed primarily in a perivascular location. This positive Wnt signal may in some manner be balanced by a negative signal from WiF1.

**Reportable Outcomes**

We expect with further studies to prepare a report on WIF1 and Wnt2 in SSc and the relationship of these mediators SSc vascular disease.

**Conclusion**

WIF1 and Wnt2 are important mediators of Wnt activity in SSc.

**References**

