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Effects of D-4F on vasodilation, oxidative stress, angiostatin, myocardial inflammation, and angiogenic potential in tight-skin mice

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Weihrauch D, Xu H, Shi Y, Wang J, Brien J, Jones DW, Kaul S, Komorowski RA, Csuka ME, Oldham KT, Pritchard KA. Effects of D-4F on vasodilation, oxidative stress, angiostatin, myocardial inflammation, and angiogenic potential in tight-skin mice. *Am J Physiol Heart Circ Physiol* 293: H1432–H1441, 2007. First published May 11, 2007; doi:10.1152/ajpheart.00038.2007.—Systemic sclerosis (scleroderma, SSc) is an autoimmune, connective tissue disorder that is characterized by impaired vascular function, increased oxidative stress, inflammation of internal organs, and impaired angiogenesis. Tight skin mice ($Tsk^{-/+}$) have a defect in fibrillin-1, resulting in replication of many of the myocardial and vascular features seen in humans with SSc. D-4F is an apolipoprotein A-I (apoA-I) mimetic that improves vascular function in diverse diseases such as hypercholesterolemia, influenza, and sickle cell disease. $Tsk^{-/+}$ mice were treated with either phosphate-buffered saline (PBS) or D-4F (1 $mg \cdot kg^{-1} \cdot day^{-1}$ for 6–8 wk). Acetylcholine and flow-induced vasodilation were examined in facialis arteries. Proinflammatory HDL (p-HDL) in murine and human plasma samples was determined by the cell-free assay. Angiostatin levels in murine and human plasma samples were determined by Western blot analysis. Hearts were examined for changes in angiostatin and autoantibodies against oxidized phosphotidylcholine (ox-PC). Angiogenic potential in thin sections of murine hearts was assessed by an in vitro vascular endothelial growth factor (VEGF)-induced endothelial cell (EC) tube formation assay. D-4F improved endothelium-, endothelial nitric oxide synthase-dependent, and flow-mediated vasodilation in $Tsk^{-/+}$ mice. $Tsk^{-/+}$ mice had higher plasma p-HDL and angiostatin levels than C57BL/6 mice, as did SSc patients compared with healthy control subjects. $Tsk^{-/+}$ mice also had higher triglycerides than C57BL/6 mice. D-4F reduced p-HDL, angiostatin, and triglycerides in the plasma of $Tsk^{-/+}$ mice. $Tsk^{-/+}$ hearts contained notably higher levels of angiostatin and autoantibodies against ox-PC than those of control hearts. D-4F ablated angiostatin in $Tsk^{-/+}$ hearts and reduced autoantibodies against ox-PC by >50% when compared with hearts from untreated $Tsk^{-/+}$ mice. Angiogenic potential in $Tsk^{-/+}$ hearts was increased only when the $Tsk^{-/+}$ mice were treated with D-4F (1 $mg \cdot kg^{-1} \cdot day^{-1}$, 6–8 wk), and cultured sections of hearts from the D-4F-treated $Tsk^{-/+}$ mice were incubated with D-4F (10 μg/ml, 5–7 days). Failure to treat the thin sections of hearts and $Tsk^{-/+}$ mice with D-4F resulted in loss of VEGF-induced EC tube formation. D-4F improves vascular function, decreases myocardial inflammation, and restores angiogenic potential in the hearts of $Tsk^{-/+}$ mice. As SSc patients have increased plasma p-HDL and angiostatin levels similar to the $Tsk^{-/+}$ mice, D-4F may be effective at treating vascular complications in patients with SSc.

systemic sclerosis; angiogenesis

SYSTEMIC SCLEROSIS (scleroderma, SSc) is an autoimmune, connective tissue disorder that causes marked increases in fibrosis of the skin and internal organs (11, 25). Vascular features are impaired vasodilation, mononuclear cell telomeric shortening, endothelial cell (EC) senescence, and impaired angiogenesis (3, 20, 40). Mechanistically, a perplexing feature of SSc is the fact that in the early stages, these vascular changes occur despite increased vascular endothelial growth factor (VEGF) (1, 10). Logically, this suggests angiostatic factors are offsetting the angiogenic effects of VEGF. Histology of hearts from people who died from complications of SSc reveal varying degrees of myocardial inflammation, with accompanying focal regions of necrosis, leukocyte invasion, scarring, and collagen deposition (2, 7). Taken together these reports suggest the balance of angiogenic and angiostatic factors in SSc is shifted toward a proinflammatory and angiostatic profile that likely impairs vascular function, increases inflammation, and attenuates angiogenic responses of the myocardium.

SSc is also characterized by chronic increases in oxidative stress (47, 49) and inflammation (13). Both are well recognized for impairing angiogenesis in diabetes and atherosclerosis (19, 51, 56). In hypercholesterolemia, autoantibodies against oxidized low-density lipoprotein (ox-LDL) have been found to correlate directly with increased risk of heart disease (44, 46). Likewise, autoantibodies against ox-LDL have been shown to directly correlate with the severity and progression of sclerosis in humans with SSc (17, 49).

Recent advances in atherosclerosis research have led to the discovery of 4F, an apoA-I mimetic of which its design evolved from the α-helical structures in apoA-I (8). 4F, which was originally designed to improve HDL function (30, 33), has been shown to protect vascular function in animal models of such diverse disease states as hypercholesterolemia (30, 33, 41), influenza infection (53, 54), and sickle cell disease (41). D-4F is believed to improve HDL function by decreasing proinflammatory lipids (32, 33) and increasing paraoxonase activity on the HDL particle (32). D-4F has also been shown to

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decrease monocyte-macrophage infiltration into the vessel wall during influenza infection in hypercholesterolemic mice (53, 54), a finding that is consistent with its ability to inhibit MCP-1 expression in vitro induced by proinflammatory lipids in LDL (30, 32).

Although the idea that angiostatic factors might play a role in SSc is attractive, support remains lacking. On the basis that SSc is a connective tissue disorder, one might predict that endostatin, which is released during matrix metalloprotease (MMP) degradation of collagen-18, is involved (39). Recent reports are mixed, however, with one indicating plasma endostatin is increased in SSc (15) and another indicating it is not (10). In contrast to angiostatic factors derived from matrix proteins, degradation of plasminogen generates angiostatin (38).

Previously, we (22) showed angiostatin was a negative regulator of the endothelium that inhibits endothelium-dependent vasodilation by uncoupling endothelial nitric oxide synthase (eNOS) activity. In addition, angiostatin was markedly increased in the hearts of diabetic or N^G-nitro-L-arginine methyl ester (L-NAME)-treated dogs, where it was credited with inhibiting collateral growth induced by repetitive occlusion (27, 56). From these reports, we reasoned that angiostatin might be increased in SSc and, if it was, then D-4F, which decreases inflammation and improves vasodilation in other disease states, may improve vascular function and decrease myocardial inflammation in $Tsk^{-/+}$ mice, an established murine model of SSc (6, 45).

In this report, we determine the effects of D-4F on vasodilation of pressurized facialis arteries, myocardial inflammation, and angiogenic potential in hearts from $Tsk^{-/+}$ mice. Our findings indicate angiostatin is increased in $Tsk^{-/+}$ mice and that D-4F improves vascular function, reduces angiostatin, and decreases oxidative stress to improve angiogenic potential in the hearts of $Tsk^{-/+}$ mice.

METHODS AND MATERIALS

Mice. Female and male $Tsk^{-/+}$ mice (0305-B6.Cg-Fbn1$^{Tsk+/+}$ Pldn^{Pa}/J) were purchased from Jackson Laboratories (Bar Harbor, ME). All animal protocols were approved by the Institutional Animal Care and Use Committee of the Medical College of Wisconsin.

Vasodilation. Acetylcholine (ACh)-induced vasodilation was determined on isolated and pressurized facialis arteries as previously described (41). Flow-induced vasodilation was determined as described by Chilian and associates (29). Briefly, the facialis arteries were cannulated and pressurized using two reservoirs. To induce flow one reservoir was raised while the other reservoir was lowered by 5 mmHg to generate a difference in reservoir height of 10 mmHg. These steps were repeated until the total difference in reservoir height was raised to 40 mmHg. Vessel diameters were recorded 2 min later.

Total cholesterol, triglycerides, and HDL cholesterol. Total cholesterol was determined using Cholesterol E Kit from Wako Diagnostics (Richmond, VA). Triglycerides were determined using L-type TGH kits from Wako Diagnostics. HDL cholesterol was determined using HDL Cholesterol E kit from Wako Diagnostics except that apolipoprotein B containing lipoproteins were precipitated with dextran sulfate-$MgCl_2$ solution (10 g/l, 0.5 M) made with dextran sulfate purchased from G. Russell Warnick (Berkley HeartLab, Berlingame, CA) following the basic protocol outline.

Proinflammatory HDL. This protocol is based on a report showing that relative rates of DCF fluorescence provide an index of the levels of seeding molecules of lipid hydroperoxides (LOOH) in HDL (37). Briefly, 1 μg of HDL cholesterol was incubated with $CuCl_2$ (5 μM, final concentration) for 1 h in a 384-well microtiter plate from MJ Research (Waltham, MA). After this preincubation, 10 μl of DCF (0.2 mg/ml) was added to the HDL-Cu^{2+} mixture in a total volume of 30 μl. DCF fluorescence (Excitation 488 nm; Emission 530 nm) was measured at 30-min intervals over the next 2 h on a LJL Biosystem Analyst HT (Molecular Devices, Sunnyvale, CA). Relative rates of DCF fluorescence were calculated and expressed as a function of the rate of fluorescence in control samples.

Histology. Hearts were isolated from anesthetized mice, placed in MOPS buffer, and snap frozen until analysis. Murine hearts were mounted in OCT and 10-μm sections cut. Angiostatin was visualized with a rabbit anti-angiostatin antibody (no. ab2904, Abcam, Cambridge, MA). T15-type autoantibodies were detected with a murine anti-T15 idiotype antibody (5, 48). Secondary biotinylated antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Images were captured using a confocal microscope (Ex 488nm for FITC and 633 for TO-PRO-3 and Em >580 nm and 661 nm, respectively). Nuclei were counterstained for 2 min at room temperature using To-Pro3 (no. 23605, Molecular Probes, Eugene OR).

Western blot analysis. Angiostatin levels in plasma and hearts of $Tsk^{-/+}$ mice were examined by Western blot analysis. Briefly, 25 μg of plasma proteins or heart homogenates were separated by SDS-PAGE (12% or 10%). Nylon membranes containing the separated proteins were blocked in Tris-buffered saline (TBS, pH 7.4) containing nonfat dry milk. The blot was then incubated with the primary antibody against angiostatin (ab2904, Abcam) in TBS containing nonfat dry milk. The blot was washed three times and incubated with the appropriate secondary antibody, and bands were visualized with ECL plus reagent (no. RPN2132) from GE Biomedical Sciences. Relative densities of the bands were determined using Scion Image software. All protein samples were analyzed in triplicate.

Angiogenic potential: EC tube formation. Murine hearts were isolated and stored in sterile 4°C MOPS buffer. The hearts were placed in a Krumdieck tissue slicer (Alabama Research and Development, Mumford, AL) in sterile 4°C MOPS buffer and cut into thin slices (100–160 μm). The heart sections were placed in fibrin-coated 24-well cluster plates, allowed to adhere, and incubated in 10% FBS DMEM media containing 2× antibiotics-mycotics. Culture media were supplemented with either nothing or VEGF (50 ng/ml, final concentration) and/or D-4F (10 μg/ml, final concentration) and then incubated for 5–7 days. EC tube formation was quantified after *day 5*, as described (56). Hearts from EC-specific green fluorescent protein (GFP) transgenic mice [Tg(TIE2GFP)287Sato/J, stock no. 003658] were used to confirm that tubes emanating from the thin sections were of EC origin.

Statistical analysis. Statistical differences in vasodilation were determined by two-way ANOVA using Prism 4 software from GraphPad Software. Differences between test groups were examined either by the Student's *t*-test or one-way ANOVA with Bonferroni correction for selected pairs of test groups. A P value <0.05 was accepted as being significant.

RESULTS

Effects of D-4F on vasodilation. Acetylcholine-induced vasodilation was impaired in female $Tsk^{-/+}$ mice compared with female C57BL/6 mice (Fig. 1*A*). D-4F improved acetylcholine-induced vasodilation to approximately half of that achieved by control C57BL/6 mice. Such differences were exaggerated in male $Tsk^{-/+}$ mice. Acetylcholine-induced vasodilation in male $Tsk^{-/+}$ mice was significantly attenuated compared with male C57BL/6 mice (Fig. 1*B*). D-4F treatments significantly improved acetylcholine-induced vasodilation in male $Tsk^{-/+}$ mice. When compared with C57BL/6 female mice, flow-induced vasodilation was impaired in female $Tsk^{-/+}$ mice, which was improved by D-4F treatments (Fig. 1*C*). In contrast, flow-induced vasodilation in male $Tsk^{-/+}$ mice was not sig-

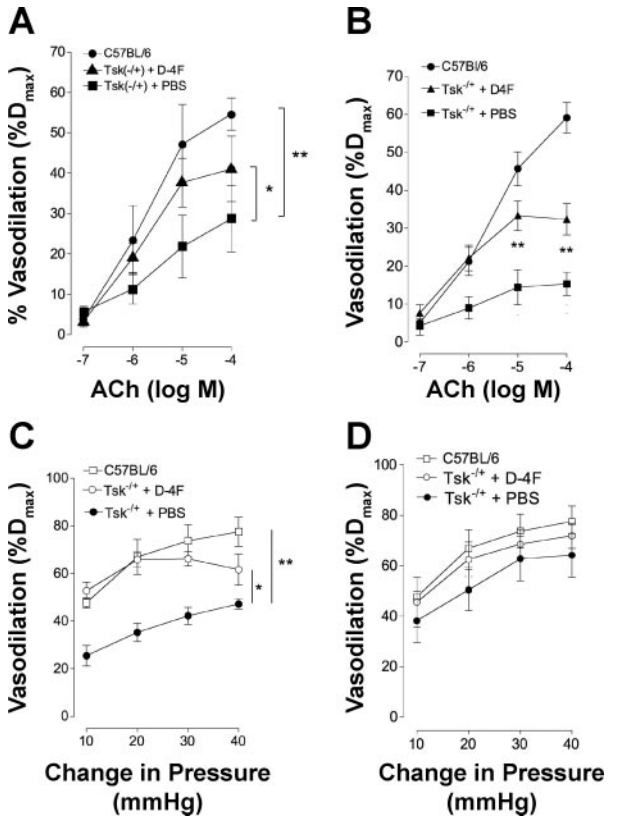

Fig. 1. D-4F improves ACh-induced and flow-induced vasodilation in $Tsk^{-/+}$ mice. Isolated and pressurized (60 mmHg) facialis arteries were stimulated with increasing concentrations of ACh (*A*: female, *B*: male) or increasing differences in pressure to induce flow (*C*: female, *D*: male). D-4F treatments (1 $mg \cdot kg^{-1} \cdot day^{-1}$, 6–8 wk) improved ACh-induced vasodilation in female and male $Tsk^{-/+}$ mice and increased flow-induced vasodilation in female $Tsk^{-/+}$ mice. Although a tendency to increase flow-induced vasodilation in male $Tsk^{-/+}$ mice was observed, the increase did not achieve statistical significance. $n = 4$–5 mice. $^*P < 0.05$, $^{**}P < 0.025$.

nificantly impaired compared with C57BL/6 male mice, and accordingly, D-4F treatments promoted minimal increases in vasodilation (Fig. 1*D*). Thus D-4F has the ability to improve and normalize vasodilation in an established murine model of SSc.

Effects of D-4F on proinflammatory HDL and angiostatin. Proinflammatory HDL levels in $Tsk^{-/+}$ mice were increased compared with levels in C57BL/6 mice (Fig. 2*A*). D-4F treatments reduced p-HDL in $Tsk^{-/+}$ mice to the levels observed in control C57BL/6 mice. Western blot analysis revealed angiostatin was increased in $Tsk^{-/+}$ mice compared with levels in C57BL/6 mice (Fig. 2*B*). D-4F treatments reduced angiostatin in $Tsk^{-/+}$ mice by about 50% compared with the increased levels in PBS-treated $Tsk^{-/+}$ mice. $Tsk^{-/+}$ mice treated with PBS had higher triglyceride than C57BL/6 and $Tsk^{-/+}$ mice treated with D-4F. The decrease in angiostatin occurred even though HDL levels were essentially the same in all three test groups (Fig. 2*C*). No differences in the total cholesterol or HDL cholesterol were observed. Findings here support the notion that $Tsk^{-/+}$ mice experience chronic states of increased oxidative stress and generation of angiostatin and that both can be effectively reduced by D-4F.

Plasma proinflammatory HDL and angiostatin in SSc patients. Proinflammatory HDL and angiostatin in plasma from SSc patients were increased compared with levels in plasma from control subjects (Fig. 3, *A* and *B*, respectively). Cholesterol, triglyceride, and HDL analysis in control and SSc patients reveals no differences in total cholesterol or triglycerides but a slight yet significant decrease in HDL cholesterol (Fig. 3*C*). Thus SSc patients experience changes in p-HDL and angiostatin similar to that which is observed in $Tsk^{-/+}$ mice.

Effects of D-4F on myocardial angiostatin and autoantibodies against Ox-PC in $Tsk^{-/+}$ mice. Immunofluorescent histology studies reveal angiostatin (bright green) was markedly increased in the interstitium of hearts from $Tsk^{-/+}$ mice compared with hearts from C57BL/6 mice (Fig. 4*A*, *left*, *middle* vs. *top*). Autoantibodies (T15-type) against ox-PC were markedly increased in hearts of PBS-treated $Tsk^{-/+}$ mice compared with the levels detected in hearts from C57BL/6 mice (Fig. 4*A*, *right*, *middle* image vs. *top* image). Blue dots in these images are To-Pro-3 staining of nuclei in the myocardium. Where the pattern of angiostatin immunostaining was localized to the interstitium, the distribution of autoantibodies against ox-PC was diffuse and occurred throughout the myocardium of $Tsk^{-/+}$ mice. Image analysis revealed D-4F essentially ablated formation of angiostatin and reduced autoantibodies against ox-PC by more than half (Fig. 4*A*, *left* and *right*, respectively, *bottom* images vs. *middle* images). Western blot analysis was also used to assess angiostatin levels in hearts of the C57BL/6, $Tsk^{-/+}$ mice treated with D-4F and $Tsk^{-/+}$ mice teated with PBS (Fig. 4*B*). The levels of angiostatin in heart lysates of $Tsk^{-/+}$ mice treated with D-4F were notably less than the levels in lysates of hearts from $Tsk^{-/+}$ mice treated with PBS (Fig. 4*B*). Findings here clearly demonstrate that $Tsk^{-/+}$ mice experience increases in myocardial inflammation and generation of angiostatic factors. More importantly, however, is the fact that D-4F treatments essentially prevent such changes from taking place in the myocardium demonstrating its effectiveness as a potential therapeutic agent in SSc.

Effects of D-4F on angiogenic potential in $Tsk^{-/+}$ mice. Phase contrast and fluorescent microscopy images show GFP-expressing EC growing out of organ cultures of thin sections of heart from a transgenic TIE2-GFP mouse (Fig. 5). These images demonstrate that the cells emanating from the organ cultures of thin sections of hearts were EC. EC tubes from the organ cultures of sections of hearts in media containing VEGF and D-4F isolated from $Tsk^{-/+}$ mice treated with D-4F were structurally different (Fig. 6*A*, *center*) from the EC tubes emanating from the organ cultures of sections of hearts from C57BL/6 mice (Fig. 6*A*, *left*). EC tubes emanating from the thin sections of $Tsk^{-/+}$ hearts were thin and spindly, with little body to them. In contrast, EC tubes emanating from the thin sections of C57BL/6 hearts were thick, with multiple layers and lattice patterns emanating from the edge. These images reveal EC tube formation from cultured sections of $Tsk^{-/+}$ hearts (Fig. 6*A*, *right*) was dramatically impaired compared with EC tube formation from cultured sections of hearts from D-4F-treated $Tsk^{-/+}$ mice or from sections of hearts from C57BL/6 mice (Fig. 6*A*, *middle* and *left*, respectively). Quantitative studies reveal that the only time EC tube formation occurred in organ cultures of sections from $Tsk^{-/+}$ hearts was when the $Tsk^{-/+}$ mice were previously treated with D-4F and when D-4F was added to the culture media containing VEGF

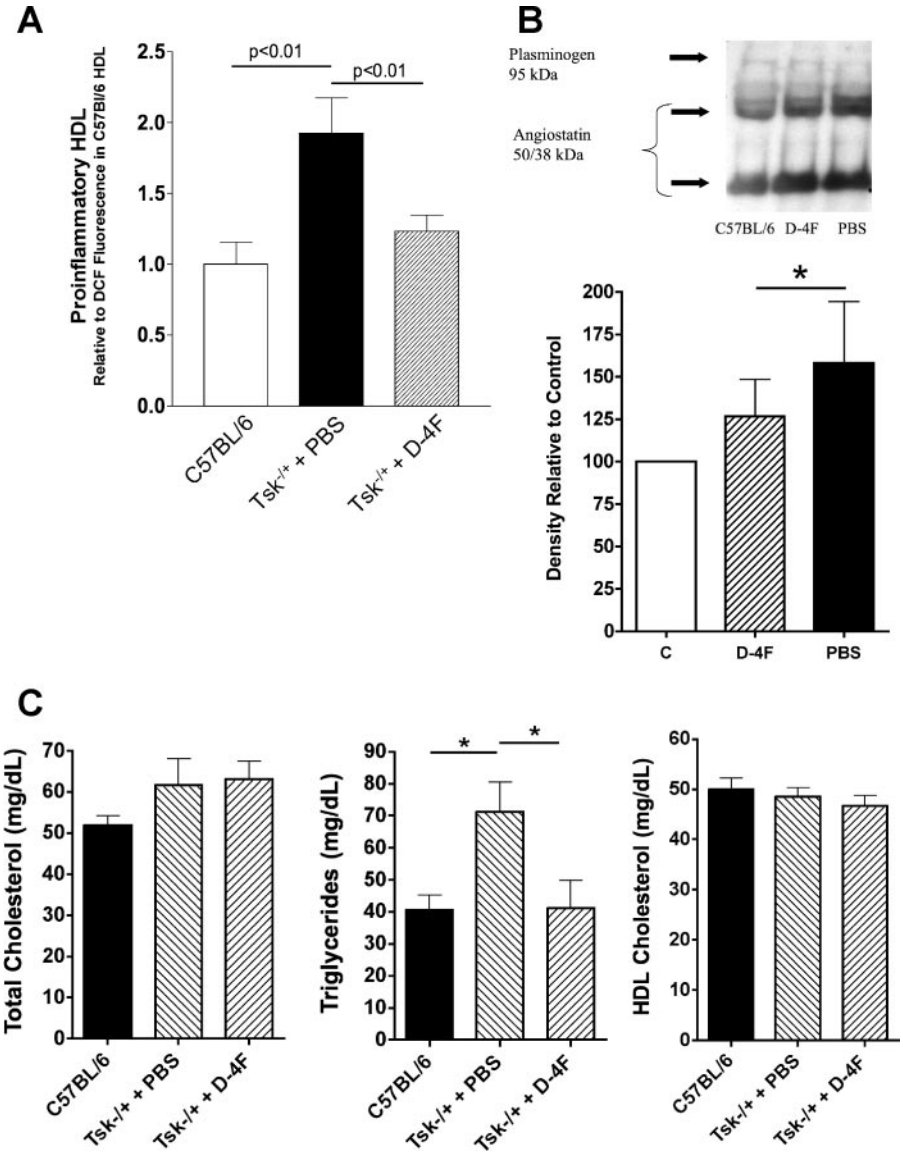

Fig. 2. D-4F decreases plasma levels of proinflammatory-HDL and angiostatin in $Tsk^{-/+}$ mice. *A*: p-HDL in plasma of $Tsk^{-/+}$ mice has higher levels of p-HDL than C57BL/6 mice. D-4F treatments (1 $mg \cdot kg^{-1} \cdot day^{-1}$, 6–8 wk) decreased p-HDL in $Tsk^{-/+}$ mice to nearly the same levels as the p-HDL levels in C57BL/6 mice. ($n = 7$) *B*: *Top* equals representative Western blot analysis of plasma of C57BL/6 mice, $Tsk^{-/+}$ mice treated with D-4F (1 $mg \cdot kg^{-1} \cdot day^{-1}$ 6–8 wk), and $Tsk^{-/+}$ mice treated with PBS (6–8 wk). Image analysis of bands of identity of angiostatin (50 and 38 kDa) images reveal D-4F treatments decreased angiostatin densities relative to densities in plasma from C57BL/6 mice ($n = 6$, $^*P < 0.05$). *C*: total cholesterol, triglycerides, and HDL cholesterol levels in C57BL/6 mice, $Tsk^{-/+}$ mice treated with PBS and $Tsk^{-/+}$ mice treated with D-4F. $^*P < 0.05$, $n =$ 10–12 C57BL/6; 7–8 for $Tsk^{-/+}$ + PBS; and 7–8 for $Tsk^{-/+}$ + D-4F.

(Fig. 6*B*). These findings clearly indicate angiogenic responses to VEGF stimulation are impaired in $Tsk^{-/+}$ mice and that even in organ culture, D-4F is required to improve angiogenic responses in the D-4F-treated $Tsk^{-/+}$ mice. We interpret these findings to mean that regardless of the mechanism(s) impairing angiogenesis in the $Tsk^{-/+}$ mice, D-4F is sufficient to restore angiogenic responses.

DISCUSSION

The essential features of this study are that $Tsk^{-/+}$ mice have impaired vasodilation, increased plasma levels of p-HDL and angiostatin, and impaired angiogenic responses to VEGF stimulation. When $Tsk^{-/+}$ mice were treated with D-4F, vasodilation improved while the concentrations of p-HDL and angiostatin decreased. Hearts from $Tsk^{-/+}$ mice contained high levels of angiostatin and autoantibodies against ox-PC. As it did in the plasma, D-4F decreased angiostatin and autoantibodies against ox-PC in the hearts of the $Tsk^{-/+}$ mice. The only time sections of hearts from $Tsk^{-/+}$ mice formed and grew EC tubes was when the $Tsk^{-/+}$ mice were treated with D-4F and the cultured heart sections were stimulated with VEGF in the presence of D-4F. Thus D-4F improves vasodilation, decreases oxidative stress, and inhibits the production of angiostatin. Such decreases in angiostatin and oxidative stress begin to explain how D-4F improves vascular function to increase vasodilation and the angiogenic potential of EC in the organ cultures of sections of hearts isolated from $Tsk^{-/+}$ mice. Our data indicate D-4F is required in vivo and in vitro to increase the angiogenic potential of organ cultures of sections of $Tsk^{-/+}$ hearts.

D-4F is a novel apo A-I mimetic that is in Phase 1 clinical trials (4). Previous studies have shown that D-4F synergizes with statins to inhibit atherosclerosis and decrease p-HDL levels (31). Recently several laboratories have proposed using therapies that target vascular EC function to treat patients with SSc (12, 23, 24). These studies show that statins increase the levels of circulating endothelial progenitor cells in SSc patients (23), improve vascular EC function (12), and inhibit collagen deposition by fibroblasts from SSc patients (24). As D-4F has been shown to synergize with statins to inhibit atherosclerosis

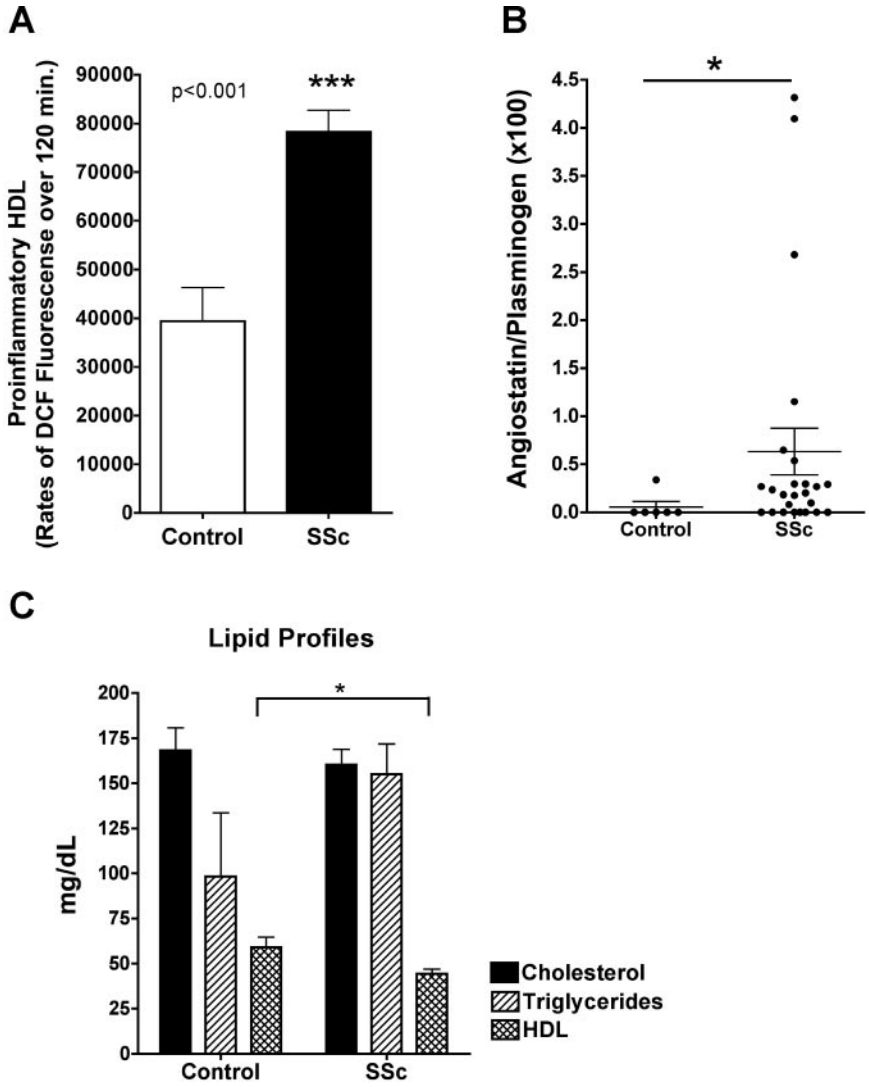

Fig. 3. Proinflammatory HDL, angiostatin, and lipid profiles in plasma from controls and SSc patients. *A*: p-HDL levels in control subjects and patients with SSc. Control, 6; SSc, 27. ***$P < 0.001$. *B*: individual data points for the ratio of angiostatin to plasminogen via Western blot analysis are increased in plasma of SSc patients compared with control subjects. Control, 6; SSc patients, 27. *$P < 0.05$. *C*: lipid profiles of control and SSc patients. No differences in total cholesterol and triglycerides between control subjects ($n = 6$) and SSc patients ($n = 27$) were observed. SSc patients ($n = 27$) did, however, have less HDL than the control subjects ($n = 6$, *$P < 0.05$).

(31), such combined therapy may also be advantageous in treating vascular disease in patients with SSc.

Previously, we reported that angiostatin impaired endothelium- and eNOS-dependent vasodilation by uncoupling eNOS activity (22). The present study is a logical extension of our previous report (22) in that this murine model of SSc had notable increases in circulating levels of angiostatin and impaired vasodilation. Exactly how chronic inflammation and increased angiostatin production in the $Tsk^{-/+}$ mice impairs vascular function is unclear. However, data here show that in female $Tsk^{-/+}$ mice, flow-mediated vasodilation was impaired to a greater extent than ACh-mediated vasodilation. In contrast, in male $Tsk^{-/+}$ mice, ACh-mediated vasodilation was impaired to a greater extent than flow-mediated vasodilation. Such observations are reminiscent of findings in humans with sickle cell disease, where forearm blood flow in women is more sensitive to ACh stimulation than in men (14). These data suggest that male arteries in disease states are more sensitive to loss of nitric oxide (·NO)-mediated vasodilation than female arteries. Likewise in rats, male rat arteries were found to be more sensitive to L-NMMA inhibition than female rat arteries (43). Together these reports suggest that impaired ·NO activity will likely have a greater negative impact on vascular physiology in males more than females. Such conclusions are consistent with our data showing that ACh-mediated vasodilation is impaired to a greater extent in male $Tsk^{-/+}$ arteries than female $Tsk^{-/+}$ arteries. More importantly, D-4F treatments seemed to normalize male and female responses to ACh- and flow-dependent vasodilation.

With respect to other models of chronic inflammation and oxidative stress, it should be noted that the degree to which vasodilation was impaired in the $Tsk^{-/+}$ mice is not as great as in hypercholesterolemic or sickle cell mice (41). None the less, the findings are consistent with the idea that increased circulating levels of angiostatin negatively regulate EC function to impair vasodilation as we proposed earlier (22).

The $Tsk^{-/+}$ mice also had marked increases in p-HDL that were reduced by D-4F treatments. During chronic states of oxidative stress and inflammation, HDL is believed to accumulate seeding molecules of lipid hydroperoxides (LOOH), which in turn, inhibit paraoxonase activity, thereby decreasing the ability of HDL to participate in reverse cholesterol transport (32). Whether p-HDL is a cause or merely a marker of vascular disease continues to be debated (16). Previous studies

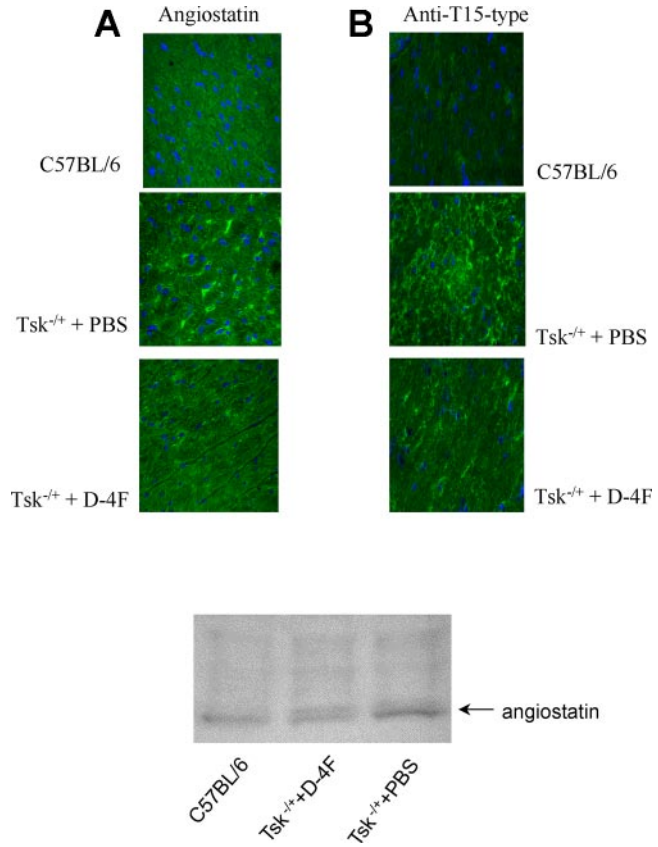

Fig. 4. *A*: angiostatin and autoantibodies against ox-PC in hearts of C57BL/6 and $Tsk^{-/+}$ mice. Immunofluorescence studies of sections of hearts from C57BL/6 mice and $Tsk^{-/+}$ mice treated with either PBS or D-4F (1 $mg \cdot kg^{-1} \cdot day^{-1}$, 6–8 wk). Hearts from C57BL/6 mice (*left*, *top* image) contain little to no angiostatin. Hearts from $Tsk^{-/+}$ mice (*left*, *middle* image) contain marked increases in angiostatin compared with hearts from C57BL/6 control mice. D-4F treatments essentially ablated angiostatin in the hearts of the $Tsk^{-/+}$ mice (*left*, *bottom* image). Hearts from C57BL/6 mice (*right*, *top* image) contain no autoantibodies against ox-PC. Hearts from $Tsk^{-/+}$ mice (*right*, *middle* image) contain marked increases in autoantibodies against ox-PC, which are reduced by >50% when the $Tsk^{-/+}$ mice are treated with D-4F (*right*, *bottom* image). *B*: representative Western blot analysis of angiostatin in hearts of C57BL/6 mice and $Tsk^{-/+}$ mice treated with D-4F and PBS, respectively.

find p-HDL increased in several murine models: hypercholesterolemia (42), sickle cell disease (unpublished observations), and influenza infection (54, 55). Studies here show for the first time, that increases in p-HDL correlate with impaired vasodilation. Taken together, these reports and data raise the possibility that it is the failure of HDL to perform its anti-atherogenic and anti-inflammatory functions that allows vascular disease to develop and progress unimpeded. Regardless, the fact that D-4F decreased p-HDL and improved vasodilation in $Tsk^{-/+}$ mice supports the concept that targeting oxidative stress improves vascular EC function in a murine model of SSc.

Loss of myocardial ·NO bioactivity, whether by chronic treatments with L-NAME or by experimentally-induced diabetes, profoundly impairs development of collateral vessels in response to repetitive occlusion (28, 56). Although the exact mechanism by which loss of ·NO bioactivity impairs coronary angiogenesis remains unclear, the fact that angiostatin is increased in the myocardial interstitial fluid to concentrations that are sufficient to inhibit EC tube formation in culture provides strong support for the idea that myocardial production of angiostatin impairs angiogenesis in the heart (28). More recently, pericardial fluid from coronary artery disease (CAD) patients with no evidence of collateral development was observed to contain ~35% more angiostatin than pericardial fluid from CAD patients who had well-developed collaterals (26). Such translational observations support the notion that chronic increases in oxidative stress inhibit vasculogenesis by increasing angiostatin.

In this context, it is important to note that sections of hearts from $Tsk^{-/+}$ mice bound more anti-T15 antibodies than sections from control hearts, a clear sign of oxidative stress. Moreover, it was these same sections that exhibited severely attenuated angiogenic responses to VEGF. D-4F treatments decreased anti-T15 antibody binding to sections of $Tsk^{-/+}$ hearts, indicating that D-4F therapy reduced oxidative stress in the myocardium just as it did in the plasma when it decreased p-HDL levels. Concomitantly, angiostatin deposition decreased in the hearts of $Tsk^{-/+}$ mice treated with D-4F. Thus the effects of D-4F on oxidative stress and angiostatin in the hearts parallel the effects of D-4F on p-HDL and angiostatin in the plasma of $Tsk^{-/+}$ mice. Taken together these observations strengthen the idea that oxidative stress increases myocardial angiostatin production.

As one of the features in SSc patients is dysregulation of angiogenesis (21) and in CAD patients with impaired collateral development is increased pericardial angiostatin levels (26), we reasoned that angiogenic responses in hearts of $Tsk^{-/+}$ mice might also be impaired. Furthermore, if angiogenesis was impaired, then D-4F, because of its ability to decrease oxidative stress and angiostatin should improve angiogenic responses to VEGF stimulation. To test this hypothesis, we cultured thin sections of hearts isolated from control and $Tsk^{-/+}$ mice in media containing VEGF without and with D-4F and quantified EC tube formation. VEGF stimulation of sections of control hearts increased EC tube formation, confirming that VEGF is angiogenic in healthy animals. In contrast, VEGF failed to induce EC tube formation in sections of hearts from untreated $Tsk^{-/+}$ mice. These in vitro findings are consistent with the observation that angiostatin is increased in the myocardium of untreated $Tsk^{-/+}$ mice. Interestingly, the only time EC tubes emanated from sections of $Tsk^{-/+}$ hearts was when the $Tsk^{-/+}$ mice were treated with D-4F and the thin sections were cultured in the presence of D-4F. Failure to include D-4F in the culture media resulted in EC sprouts that would not or could not form EC tubes (Fig. 6). We interpret these data to mean that the hearts from D-4F-treated $Tsk^{-/+}$ mice contain or generate angiostatic factors during culture that are sufficient to impair EC tube formation.

One of the questions that remains unanswered is why D-4F improves vascular function in a murine model that is recognized more for autoimmunity and fibrosis rather than hyperlipidemia and atherosclerosis. Our p-HDL data indicate that $Tsk^{-/+}$ mice likely experience chronic states of increased oxidative stress. One of the major consequences of oxidative stress is loss of HDL function (30, 52, 53). Normal HDL and its components remove and/or inhibit the pro-oxidant activity of proinflammatory lipids that are required for LDL oxidation by human artery wall cells (35, 36). HDL and its major

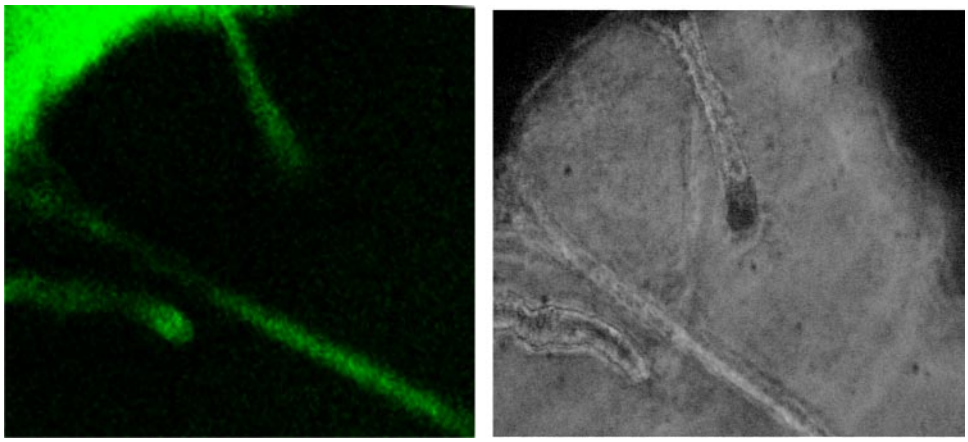

Fig. 5. Vascular endothelial growth factor (VEGF)-stimulated endothelial (EC) tube formation from hearts of TIE2-GFP mice. Phase-contrast image of EC tubes emanating from thin sections of a heart from TIE2GFP mouse (*right*). Fluorescent image of the same EC tubes in the phase contrast image (*left*).

atheroprotective apolipoprotein A-I are well recognized for inhibiting LDL oxidation and attenuating LDL-induced increases in monocyte chemotaxis (35, 36). HDL is an antiatherogenic lipoprotein that is responsible for transporting cholesterol from the vessel wall back to the liver for disposal (reverse cholesterol transport hypothesis) (50). HDL also plays a major role in inhibiting inflammation during a variety of diseases, even those mediated by T-cell activation (18, 34). Indeed, the chronic states of oxidative stress and inflammation that develop in sickle cell disease, hypercholesterolemia, and influenza infection have all been shown to impair HDL function (30, 33, 34, 52, 53). Our studies are the first to show that

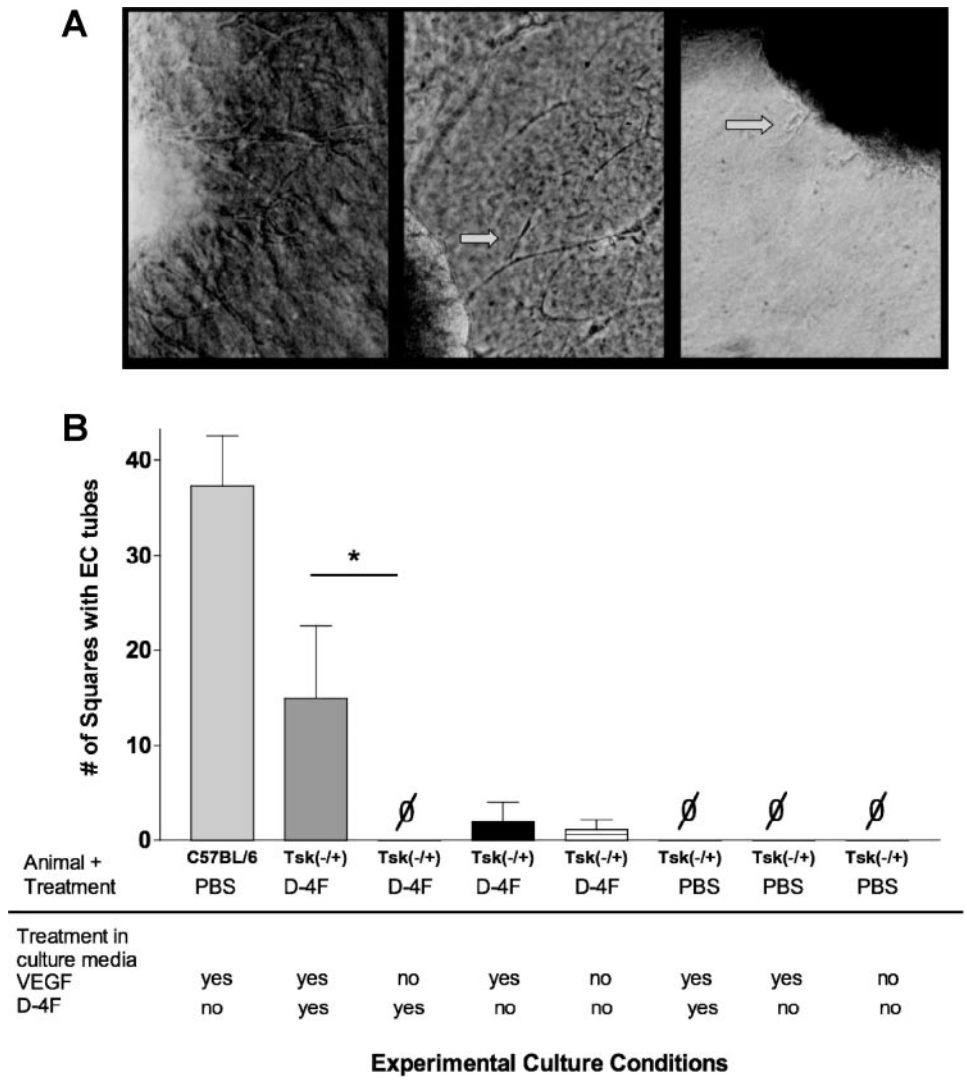

Fig. 6. D-4F increases angiogenic potential in hearts of $Tsk^{-/+}$ mice. *A*: organ culture of thin sections of hearts were used to assess angiogenic potential. *Left*, EC tube formation from cultured organ sections of hearts from C57BL/6 mice stimulated with VEGF. *Middle*, EC tube formation from cultured organ sections of hearts from $Tsk^{-/+}$ mice treated with D-4F (1 $mg \cdot kg^{-1} \cdot day^{-1}$ for 6–8 wk) and stimulated with VEGF in 10% FBS DMEM media containing D-4F (10 μg/ml). VEGF and D-4F stimulated EC tube formation from cultured organ sections of hearts from D-4F-treated $Tsk^{-/+}$ mice are thin and spindly (*right*) compared with EC tube formation from cultured organ sections of hearts from C57BL/6 mice (*left*). EC budding from hearts of $Tsk^{-/+}$ mice treated with PBS (*right*). *B*: quantitative data for EC tube formation under the various experimental conditions. The only time EC tube formation occurred in cultured sections of hearts from $Tsk^{-/+}$ mice is when the $Tsk^{-/+}$ mice were treated with D-4F and sections of hearts were maintained in media containing VEGF and D-4F (ø, zero EC tubes crossing squares in counting grid). $*P < 0.05$.

p-HDL is increased in SSc. The fact that D-4F lowered p-HDL levels and decreased angiostatin in the plasma and hearts of the $Tsk^{-/+}$ mice underscores the importance of losing this universal anti-inflammatory pathway in the development of vascular disease in SSc.

D-4F was designed to improve HDL function. When added to plasma, D-4F causes a remodeling of α-HDL to form a new pre-β HDL-like particle (32). Pre-β-HDL is a lipid-poor, apolipoprotein A-I particle that interacts with ABCA-1. The formation of pre-β-HDL and its binding to ABCA-1 is considered the first step in reverse cholesterol transport. Such remodeling of HDL may explain, in part, anti-atherogenic and anti-inflammatory properties of D-4F (32). In addition to remodeling HDL, D-4F possesses unique biophysical and antioxidant properties. Whereas vitamins E and C scavenge the LOOH and other radical species, D-4F not only scavenges LOOH (9) but also binds them, i.e., 15-HETE (32). This suggests D-4F has the potential to remove proinflammatory lipids not only from HDL but from the vessel wall to decrease inflammation. Our observation that D-4F reduced autoantibodies against ox-PC in hearts of $Tsk^{-/+}$ is consistent with this idea. Removing proinflammatory lipids from sites of inflammation has two advantages over traditional antioxidants. First, it decreases the negative influence of proinflammatory lipids on vascular function. Second, by decreasing ox-PC antigens, it reduces immune reactions mediated by autoantibodies against proinflammatory lipids. On the basis of these findings, we hypothesize that D-4F breaks the cycle between inflammation and innate immunity, which in turn decreases myocardial inflammation and vascular disease in SSc.

If D-4F is effective in breaking the cycle between inflammation and vascular dysfunction in $Tsk^{-/+}$ mice, then it might be useful in treating patients with SSc. Analysis of plasma from control and SSc patients revealed that SSc patients had higher levels of p-HDL and angiostatin just as the $Tsk^{-/+}$ mice. In this small study, we also noted that SSc patients had lower HDL cholesterol levels than our healthy controls. Such observations indicate that patients with SSc are at a disadvantage when comes to HDL because not only is p-HDL increased but also the concentration of this anti-inflammatory lipoprotein is reduced. This initial translational study reveals parallels between the $Tsk^{-/+}$ mice and the SSc patients. As D-4F has recently been shown to decrease p-HDL in human subjects with established coronary heart disease (4), findings here provide a new strategy for targeting growth inhibitors and amplifying the effects of growth factors in SSc. Finally, our findings indicate D-4F may be useful for promoting collateral development in myocardial ischemia as well as restoring angiogenesis in patients with SSc.

The clinical and pharmacological implications of our work is that targeting HDL function appears to be an effective means of improving vascular function in SSc just as it is in murine models of atherosclerosis. Where hypercholesterolemia increases foam cell formation and fatty streaks to increase atherosclerosis, chronic increases in oxidative stress and inflammation in SSc induce intimal hyperplasia, impair vascular function, and increase myocardial inflammation. However, in both diseases, D-4F decreases oxidative stress and inflammation to improve vascular function. In this report, we suggest that mechanisms by which D-4F improves vascular function are also responsible for improving angiogenic responses to VEGF stimulation in the $Tsk^{-/+}$ mice. The ability of D-4F to decrease oxidative stress and inflammation appears to play a central role in improving vascular function and increasing angiogenesis. Thus D-4F improves at least four key hallmarks of SSc. Additional research is required to determine whether D-4F can improve other hallmarks of SSc.

In conclusion, impaired vascular function and myocardial inflammation in SSc are driven by chronic states of oxidative stress that in turn increase production of angiostatin. D-4F reduces oxidative stress, which decreases angiostatin in the hearts of $Tsk^{-/+}$ mice. Thus D-4F targets the production of angiostatic factors and enhances the effects of angiogenic factors in hearts from $Tsk^{-/+}$ mice. This establishes a new environment that increases angiogenic potential of the myocardium and improves vascular function. Such changes may be critical for alleviating the pathophysiology that develops in SSc in humans.

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