ABSTRACT

The vascular endothelium came into view almost three decades ago following the introduction of the vascular hypothesis in scleroderma pathogenesis by Dr. LeRoy. Since that time, the endothelial cells, and other vascular cells, became the focus of investigations aimed at elucidating the etiology, pathogenesis and treatment of scleroderma. This review will summarize Dr. LeRoy's commitment to the disease, the relevant progress made since the introduction of the vascular hypothesis, and what we have learned since then about the vascular disease in scleroderma.

Introduction

In 1976 I joined Dr. LeRoy’s section as a first-year rheumatology fellow. A few months into my fellowship and upon his return from a Gordon conference on cell biology, Dr. Leroy suggested that I should explore techniques to isolate and investigate vascular endothelial cells in vitro. He affirmed that this cell type would be the cell type of the decade – and it has been the cell type of that decade and near the top of important cell types ever since. He talked about a presentation in that conference given by Dr. E Jaffe (1), who introduced the first technique to isolate endothelial cells from umbilical cord veins. This assignment represented an opportunity to break away from the hectic clinical work that involved patient’s interviews, physical examinations, data gathering, and the dreadful correspondence that must be sound scientifically, clinically and most importantly linguistically. Three decades later, I am still working on techniques to isolate endothelial cells, but now from skin biopsies, from peripheral blood, or from bone marrows. This early interaction impacted my professional career and is an illustration of the profound influence that Dr. LeRoy had on his associates.

Dr. LeRoy’s interest in the role of vascular endothelium in health and disease begun early in his career. Still, it was the introduction of the vascular hypothesis in SSc pathogenesis in 1975 that established him as the leader in the vascular aspects of this disease (2). Although many notable studies of vascular disease in SSc preceded the introduction of the hypothesis, particularly by Drs. Fleischmajer and Norton (3, 4), the hypothesis helped to focus research efforts on the biology and pathology of endothelial cells and vascular function in SSc. Since that time the endothelial cell has become the focus of numerous studies as the target of injury in the disease that initiates a cascade of events including platelet activation and vascular dysfunction. Mechanisms leading to endothelial dysfunction, intimal proliferation and vascular wall thickness, tissue hypoxia and the impact of hypoxia on organ fibrosis have been partially identified. Therapeutically, vascular studies established the rational basis for the use of angiotensin converting enzyme inhibitors for scleroderma renal crisis, calcium channel blockers for peripheral vascular ischemia, and the endothelin receptor antagonists for pulmonary hypertension and digital ischemia. These therapeutic breakthroughs have improved the plight of the SSc patient although, despite the symptomatic improvement, direct regression of the vascular lesions has not been attained.

Current thinking on SSc vascular disease suggests an autoimmune contribution, chemical modifications of the endothelium and a possible viral trigger as was proposed and passionately pursued by Dr. LeRoy later in his career (5). This paper will summarize some of what we have learned in the last three decades about scleroderma vascular disease.

The vascular problem in scleroderma

Prominent SSc vascular abnormalities
are noted in the capillaries and the small blood vessels (6). Swelling of the intima and intimal proliferation with mononuclear cell infiltration are seen in vessels of 150 to 500 μm diameter. In capillaries, the vascular disease is characterized by distorted and irregular capillary loops described in all involved organs and half or more of the expected capillaries are missing (by morphometric capillary density measurements), probably obliterated by endothelial apoptosis and removal. This underperfused state (ischemia, acidosis) should be fertile soil for neangiogenesis, but new capillaries are rare and broad avascular areas are common, suggesting defective pathways in angiogenesis (7). On the ultrastructural level, the earliest changes in the edematous stage of the disease consist of large gaps between the endothelial cells (EC), vacuolization of EC cytoplasm with an increase in the number of basal lamina-like layers and occasional entrapment of lymphocytes and cellular vesicles (8). Further signs of nuclear injury in association with cell membrane disruption occur in more advanced cases. Perivascular cellular infiltrates are prominent in the early stages and consist of infiltrating macrophages, T and B cells, with the predominance of CD4+ T cells and γδ receptor bearing T cells (9).

In the arteries, intimal proliferation of a uniform and symmetrical nature forms a neointima indistinguishable from that formed in other autoimmune diseases, in chronic homograft rejection, and in chronic atherosclerosis such as restenosis after coronary bypass (10). The principal cell forming the neointima is probably a smooth muscle cell from the vessel wall media which becomes activated, leaves its resting extracellular matrix (ECM) microenvironment, exhibits smooth muscle actin in its cytoskeleton, and is called a myointimal cell. In SSC, the neointima seems to form in waves, as seen in repeatedly reduplicated internal elastic membranes (11).

**Vascular dysfunction in SSC**

The earliest signs of vascular dysfunction in SSC include enhanced vascular permeability and dysregulated control of vascular tone; progressive reduction of organ blood flow occurs in well-established disease. The cellular bases for these abnormalities are related to a pathologic shift in the EC functional profile from an anticoagulant, anti-inflammatory and vasorelaxant cell type to a procoagulant, vasospastic and pro-inflammatory phenotype. In SSC, an imbalance in endothelial signals (increased endothelin release and impaired endothelial dependent vasodilatory mechanism, EDRF), enhanced platelet aggregation and deficient sensory neu-ropetides lead to the well-recognized vasospastic propensity in the disease. Defective compliance of the vessel wall due to vessel wall fibrosis is also involved, particularly in the advanced stages of the disease. The impact of imbalanced signals is best illustrated by the imbalance between the production of endothelin and nitric oxide (NO) produced by eNOS.

**Endothelin**

The potent vasoconstrictor peptide endothelin-1 (ET-1) is a true pleiotropic cytokine that mediates vasospastic, mitogenic, profibrotic and inflammatory effects. Increased plasma ET levels have been associated with a number of vascular disorders, including Raynaud’s phenomenon and SSC – particularly diffuse SSC, SSC lung disease, pulmonary hypertension and renal crisis (12, 13). Increased ET-1 expression in microvascular endothelial cells (MVEC) of the upper dermis in association with an increased number of ET-1 binding sites (particularly type B receptors) is reported in SSC skin and lungs (14). Due to the wide-ranging biologic effects of ET it was proposed as the link between the vascular and fibrotic processes in SSC. The recent therapeutic successes of ET receptor antagonists in pulmonary hypertension and digital ischemia support the important role of ET in vascular disease; however, the link with fibrosis still need to be examined by direct evaluation of the antagonists on the fibrotic burden in SSC.

A positive effect on fibrosis will validate the vascular hypothesis as envisioned by Dr. LeRoy. Another area that still needs examination is the nature of ET stimulus in SSC. The enhanced production may be related to central alteration in endothelial cells leading to defects in the feedback loops that is normally seen in the healthy state.

**Endothelial Dependent Relaxation Factor and nitric oxide**

Deficient endothelial dependent relaxation in SSC is suggested by impaired maximal responses to endothelial dependent vasodilators with normal responses to endothelial independent dilators (15, 16) and by reduced expression of eNOS in the skin and microvascular EC in vitro.

The consequences of impaired endothelial nitric oxide (NO) release in SSC are not only related to defects in vascular tone control but may also influence other pathological events that can promote and augment disease pathogenesis. NO is known to inhibit platelet aggregation, and adhesion molecule expression (17-19). Furthermore, NO is a powerful inhibitor of smooth muscle cell proliferation and acts as a potent chemical barrier to oxidation injury (20). Thus, a reduction in steady state endothelial NO production may promote vascular wall inflammation, intimal proliferation and platelet aggregation, and augment oxidation injury. A fine balance between NO and ET exists in the healthy state where ET triggers NO release upon binding to the type B-ET receptors on the endothelium. Released NO in turn suppresses ET release, restoring the vasodilatory status of the blood vessels. Several experimental data suggest a disruption of this feedback loop, resulting in sustained ET production in SSC. Further understanding of this process and pondered therapeutic intervention may help in eliminating the progressive oblitative vascular disease in SSC and allow the ultimate testing of the vascular hypothesis.

**The nature of endothelial injury**

Recent studies identified EC apoptosis as an early event in SSC pathogenesis. EC apoptosis was first noted in chickens from University of California at Davis lines 200/206 that develop he-
Circulating anuclear endothelial cells in 8 SSc patients and 7 matched control subjects. The mean number of CEC in the controls was 3.01 ± 0.7 and in SSc patients 5.5.1 ± 1.6 (mean ± SD, P < 0.002).

Fig. 1. Circulating anuclear endothelial cells in 8 SSc patients and 7 matched control subjects. The mean number of CEC in the controls was 3.01 ± 0.7 and in SSc patients 5.5.1 ± 1.6 (mean ± SD, P < 0.002).

Possible microbial triggers
A persuasive and credible argument supporting a viral trigger of SSc was enthusiastically presented by Dr. LeRoy because of his observation of increased levels of antibodies to CMV, a large DNA herpes virus in SSc patients, and because accelerated neointima formation had already been indirectly associated with CMV in graft rejection and arterial restenosis (27). Moreover, what is known about CMV infection fits well into the scenario of SSc vascular pathogenesis. CMV-infected endothelial cells round up, detach, and circulate to distant capillary beds, which serve to disseminate the virus and infect distant EC, leading to diffuse systemic vascular disease. This process could also lead to endothelial cell apoptosis, as well as subsequent autoimmunity, including anti-endothelial cell and antiphospholipid antibodies. Dr. LeRoy theorized the mechanisms of a latent CMV infection in which immediate early (IE1 and 2) CMV genes drive host gene expression. SSc skin lesions have been shown to contain mononuclear cells which express TGFβ1 alongside interstitial fibroblasts which express α1(I) collagen mRNA. These cells would also be expected to overexpress ICAM-1 and IL-6, characteristic abnormalities of the activated scleroderma fibroblast (a myofibroblast), which are also induced by CMV IE1-IE2. Therefore many of the pathological features of SSc are consistent with a latent CMV infection where CMV promoter genes drive host cytokine and matrix gene expression leading to neointima, capillary obliteration and interstitial fibrosis. In vitro CMV induces TGFβ1 (and thereby several collagen genes) and adhesion molecules, among its many other attributes. For SSc pathogenesis a latent CMV infection is a very interesting hypothesis that should be vigorously examined.

Circulating markers of the vascular disease
One of the intermediate goals of the vascular hypothesis was to identify markers of vascular dysfunction. Monitoring these markers would help in short-term trials to evaluate the effectiveness of therapies on the vascular disorder. Many markers were identified over the years including von Willebrand Factor (vWF), angiotensin converting enzyme, β-thromboglobulin and platelet factor VI, soluble adhesion molecules, thrombomodulin and many others. Many of these markers positively correlated with the extent of visceral involvement, disease prognosis, active phase of the disease and certain serologies. One of the markers that was examined early but not reported was circulating endothelial cells.

Circulating apoptotic endothelial cells (CAEC) in SSc
Damaged endothelial cells may slough
off the vascular wall and circulate as the anuclear remains of the cell skeleton that are increased in number in vascular diseases. The method to evaluate CAEC is based on collecting plateau-rich plasma, aggregating the platelets with thrombin, and counting the cell carcasses in the platelet-poor plasma following centrifugation (28). We evaluated the CAEC in 8 SSc and 7 matched control subjects. The number of circulating cells was higher in SSc patients (Fig. 1). Moreover, the circulating numbers increased dramatically in patients undergoing interferon-γ therapy in association with digital infarcts. These observations were not published because of the uncertainty of the origin of the cells. Still, the presence of non-hematological cells in the peripheral circulation in patients with cancer was reported in 1934 (29). Recent advances in cell isolation and immunohistochemical examination revealed that some of the circulating cells are endothelial in origin (30). Increased numbers of circulating endothelial cells (CEC) have been described in several pathologic disorders that have in common the presence of vascular injury. Thus, increased CEC numbers were described in sickle cell anemia (31), rickettsial (32) and cytomegalovirus (CMV) infections (33), thrombotic thrombocytopenic purpura (TTP, 34), acute coronary syndromes (35), Behçet’s disease (36), vasculitis (37) and in patients with active SLE (38). Normal adults have 2.6±1.6 CEC per milliliter of peripheral blood (31). This low number reflects the exceptionally slow turnover rate of vessel wall endothelium in the healthy endothelium (39). At least half of the CEC are derived from the microvasculature as defined by CD36 positivity (31). The phenotype of these cells is postulated to reflect the status of the endothelium in situ (40, 41), but whether the CEC themselves are derived from vessel walls is not known. Nonetheless, the measurement of CEC provides direct evidence of endothelial injury and should be examined in SSc.

Until recently, it was generally thought that the formation of new vessels in adults occurred exclusively through the extension of mature existing blood vessels and the associated vascular endothelium. A growing body of evidence now suggests that bone marrow-derived endothelial progenitor cells (EPCs) circulate in the blood and, at least in animal models, can play an important part in the formation of new blood vessels in these pathologic conditions (42, 43). Laboratory evidence suggests that these cells express a number of endothelial-specific cell surface markers and exhibit numerous endothelial properties (42, 43). In addition, when these cells are injected into ischemic animal models, they are apically incorporated into sites of neovascularization (44-46). These studies suggest a potential use of progenitor cells for improvement of therapeutic vasculogenesis in patients with a variety of vascular diseases. Attempts to characterize and propagate the progenitors cells in SSc are ongoing.

Conclusion

The etiology and pathogenesis of SSc remain unknown. Nonetheless, signs of vascular injury and devascularization of involved organs in association with evidence of profound endothelial dysfunction are well documented. Fibroblast activation leading to tissue fibrosis and immune involvement constitute the two other fundamental pathologic processes in the disease in addition to the vascular disorder. Countless central issues in the pathogenic process of SSc remain poorly understood. Issues related to the initial trigger in the disease, the nature of immune activation, mechanisms of intimal proliferation and the relationship of vascular injury to tissue fibrosis are some of the unresolved essential questions. The fact that the vascular tree, particularly the microcirculation, is the target tissue in this disease is now well established. The destruction of microvessels leading to the clinically recognized state of chronic organ ischemia and tissue underperfusion in SSc is fundamental to its pathogenesis. Chemical modification of the endothelium, reperfusion injury by mechanisms of RP, or viral infection of EC are some of the proposed triggers. The microvascular damage in the disease, which includes EC apoptosis, may not be related to the direct effect of virus replication but may be linked to immune mediated endothelial injury triggered by viral infection. Knowledge of the immune mechanisms in disease pathogenesis may offer an opportunity to develop a multiple step strategy for therapeutic intervention. The introduction of the vascular hypothesis by Dr. LeRoy ushered in a most exciting era in SSc investigation, the fruits of which we are already enjoying now in the care of SSc patients. The determination to pursue the vascular hypothesis will undoubtedly be a productive pathway of research in SSc for some time to come.

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