Review

How does endothelial cell injury start? The role of endothelin in systemic sclerosis

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Abstract

A considerable amount of research time has been invested in studies aimed at elucidating pathogenic processes in systemic sclerosis (SSc). Despite this, major challenges for biomedical science remain, such as identification of the key factors that determine susceptibility to SSc, and elucidation of the precise nature of the initiating event that causes endothelial cell injury and ultimately brings about the biological cascade(s) that lead to the pathologic vascular changes. Involved factors are likely to include genetic perturbations, environmental cues, tissue injury, infection and hypoxia/oxidative stress. As important as determining the initiating events are the identification and characterization of key factors that are functionally important in driving vascular disease progression, because these factors are potential targets for therapeutic intervention. This article reviews the role of endothelin as an example of a pleiotropic mediator with effects on various aspects of SSc pathogenesis, such as inflammation, vasculopathy and tissue remodelling.

Introduction

In order to understand the initiators of endothelial injury and how the ensuing vascular dysfunction contributes to the development of systemic sclerosis (SSc), it is necessary to consider the normal biology of the endothelium and of the myriad of biological molecules and biological functions under its control, and to assess which specific processes are dysregulated in disease. In SSc, the vasculopathy is one of the earliest pathological events, characterized by endothelial cell activation and altered vascular tone. These pathological changes are accompanied by the presence of pro-inflammatory cytokines and angiogenic regulatory factors, and the loss of redox control, leading to oxidative stress and hypoxia. This complex array of molecular interactions involves a number of cell types including the endothelial cells and their attendant perivascular supporting cells (pericytes and vascular smooth muscle cells [vSMCs]), and inflammatory cells, and they are profoundly influenced by the presence of growth factors, cytokines, chemokines and potent vasoactive factors. Together, this diverse range of factors is believed to initiate and drive the vascular pathogenesis that leads to severe vessel disease and occlusion. Here we focus on one particularly attractive candidate, endothelin, and critically examine the cellular and molecular activities mediated by endothelin and its receptors that are intimately associated with endothelial cell injury and vascular dysfunction in SSc.

The endothelium

The vascular endothelium is a complex, highly specialized and metabolically active organ, which performs a number of essential biological functions. The endothelium provides a compatible interface to facilitate blood circulation, it inherently inhibits excessive platelet aggregation and leuco-cyte adhesion, and it produces a balance of vasoconstrictive and vasodilatory molecules that coordinate vascular tone and serve to inhibit extracellular matrix (ECM) deposition and prevent smooth muscle cell proliferation. The endothelium also serves as a multifunctional interface between blood and all internal organs, selectively determining the movement of macromolecules and governing the recruitment of circulating cells from the blood into the extravascular tissues. The prominent endocrine functions of the endothelium work to regulate vascular tone, through the production of vaso-constrictive (for instance, endothelin [ET]-1) and vasodilatory (for example, nitric oxide and prostaglandins) molecules; to maintain blood fluidity; to regulate platelet function; and to control inflammation. Healthy functioning of the endothelium is critical for remodelling of blood vessels, through angio-genesis and vasculogenesis, during times of tissue growth and repair. Thus, the endothelial cell phenotype must be regulated, and this is achieved by environmental cues and

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AECA = anti-endothelial cell autoantibody; ECM = extracellular matrix; ET = endothelin; ET_A/ET_B = endothelin receptor subtype A/B; ICAM = inter-cellular adhesion molecule; MMP = matrix metalloproteinase; NF-κB = nuclear factor-κB; PDGF = platelet-derived growth factor; SSc = systemic sclerosis; TGF = transforming growth factor; TIMP = tissue inhibitor of metalloproteinase; TNF = tumour necrosis factor; vSMC = vascular smooth muscle cell.

Figure 1
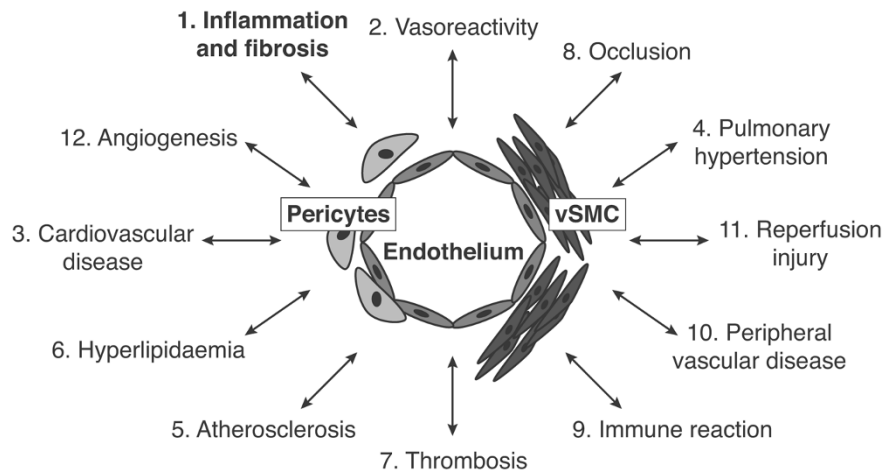
The myriad of potential consequences of endothelial dysfunction. Many human diseases, and in particular some of the most common diseases, are characterized by abnormal changes that take place within the blood vessel and alter vascular responses and function. vSMC, vascular smooth muscle cells.

mechanical forces such as shear stress, as well as inflammatory and angiogenic stimuli [1].

Triggers of endothelial cell injury and dysfunction

Endothelial dysfunction is an important component of a number of common human diseases, including those characterized by inflammation and fibrosis (Figure 1). Our particular interest is in the contribution of endothelial dysfunction to the pathophysiology of inflammatory and fibrotic connective tissue diseases and, in particular, SSc. Endothelial dysfunction is likely to result from endothelial cell injury triggered via a number of different mechanisms, including the following [2]: bacterial or viral infection; oxidative stress through abnormal regulation of reactive oxygen species, hypoxia, turbulent blood flow and shear stress; environmental irritants such as tobacco; and hyperlipidaemia. These factors all lead to the generation of an inflammatory process and endothelial cell activation.

The endothelial response to injury can be divided into two 'levels' of response: an initial rapid response and a slower, phenotypic response. The initial rapid response involves (among other factors) changes in levels of nitric oxide, prostaglandins, ET-1, von Willebrand factor and tissue plasminogen activator. The slower response depends on fundamental changes in cell surface characteristics, and alterations in the underlying basement membrane and the smooth muscle cells that surround the endothelium. These changes are brought about by molecules that are particularly potent growth factors involved in the deposition of ECM, and activation and proliferation of the vSMCs, pericytes and other mesenchymal cell types associated with the blood vessel

wall. This ultimately results in vessel remodelling, with profound changes in cellular architecture [3].

Pericytes are associated with microvessels and capillaries (about 30 μm diameter) and smooth muscle cells are associated with intermediate (>50 μm) and large (1 to 5 mm) vessels and muscular arteries [4]. In terms of endothelial cells and blood vessels, one must consider these as functional units, which also involve both pericytes and vSMCs. In SSc there are a number of important endothelium-derived mediators that have been shown to be important in the early disease process. It is important to determine which of these link vascular damage to the ensuing fibrogenic or pathogenic tissue remodelling process and the characteristic signs and symptoms of SSc.

Pathogenesis of systemic sclerosis

Over the past two decades considerable research effort has been directed toward investigating and elucidating the pathogenic processes of connective tissue diseases such as SSc. Factors that determine susceptibility to these diseases, and the precise nature of the initiating event, remain largely unknown. However, they are likely to involve host genetics, the impact of environmental cues, tissue injury, infection, hypoxia and inability to control oxidative stress adequately [5] (Figure 2).

Patients with SSc often exhibit early signs of vasculopathy, with many experiencing Raynaud's phenomenon, often for many years before developing overt signs of skin fibrosis. Consistent with this, morphological changes in capillaries are detectable before or at disease onset, which can be used for early diagnosis using nail-fold capillaroscopy. Changes in the

Figure 2
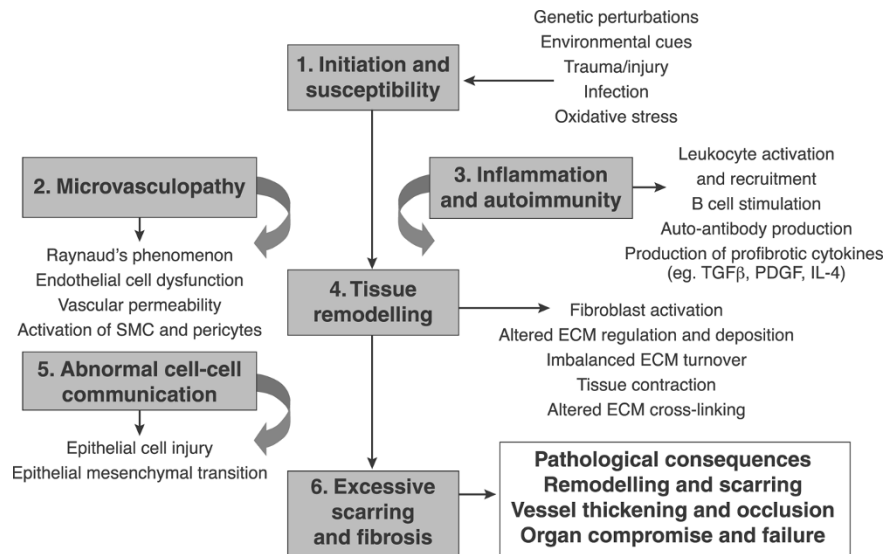
Sequence of pathological events leading to the development of SSc. ECM, extracellular matrix; IL, interleukin; PDGF, platelet-derived growth factor; SMC, smooth muscle cell; SSc, systemic sclerosis; TGF, transforming growth factor.

blood vessels of the SSc patient are frequently associated with stimulation of both the innate and adaptive immune responses, resulting in B-cell and T-cell activation and, in many cases, autoantibody production. Inflammation is believed to be a primary driver that leads to tissue remodelling and gives rise to the characteristic features of excess matrix production, caused by an imbalance in turnover and increased deposition of various extracellular proteins [6]. The impact of this inflammation probably depends on the organs involved, with evidence for a stronger association of inflammation and fibrosis, for example, in the lungs, whereas significant inflammatory infiltrates in the skin are limited to perivascular areas in early-stage disease [7]. However, the expression of profibrotic mediators is not limited to inflammatory cells in the skin, and resident cells, such as dermal fibroblasts, express increased levels of these mediators in later stage disease [8].

These changes result in a sequence of pathological events that includes impaired cell-cell communication between epithelial cells and fibroblasts, and development of fibrotic lesions that disrupt the normal tissue architecture and lead to impaired expression of the correct molecular cues and cellular mediators that maintain normal tissue structure and function. Ultimately, these abnormalities lead to vascular and interstitial fibrogenic processes. This so-called 'replacement fibrosis' replaces normal tissue architecture and often leads to organ compromise and failure [9].

Because early disease is initiated in the vasculature, and many of the manifestations of SSc are vascular in nature (capillary abnormalities, vessel occlusion and fibrosis, and digital ulceration), identification of the components of the pathway from the initial vascular insult to the downstream fibrotic phenotype is critical. However, characterization of the early vascular events is challenging in SSc, because patients are usually available only in later stages of disease when the disease is already established and because animal models only incompletely reflect the human condition [10,11]. Regardless of the precise mechanisms that underlie the initial vascular injury, it might be even more important to identify and characterize the array of mediators driving the vascular remodelling that leads to the vascular features typical of SSc. Those mediators that are over-produced (beyond normal levels of production) by endothelial cells of SSc patients include growth factors, cytokines, and other mediators of hyperplasia and hypertrophy. These include ET-1, connective tissue growth factor and transforming growth factor (TGF)-β, and matrix-modulating proteins such as matrix metalloprotein-ases (MMPs) and natural tissue inhibitors of metalloproteinases (TIMPs) [3,12]. They are also important regulators of the activation and function of inflammatory mediators, including tumour necrosis factor (TNF), interleukins and some chemo-kines. These mediators in turn have a significant impact on the progression of connective tissue diseases.

Anti-endothelial cell antibodies and oxidative stress

Another important pathological feature of SSc, and one which is probably highly relevant to the vasculopathy, is the presence of specific anti-endothelial cell autoantibodies (AECAs) [13,14]. These antibodies are a heterogeneous

group of autoantibodies that specifically recognize endo-thelial cell proteins and molecules present on the endothelial cell surface. Studies of AECAs have shown that these antibodies can also activate endothelial cells to express cell adhesion molecules, thereby altering leucocyte attachment, and can also lead to endothelial cell damage and apoptosis. Several studies have also suggested that AECAs may play a prominent role in disease progression. A recent study [15] yielded the exciting finding that all SSc sera examined expressed autoantibodies directed against platelet-derived growth factor (PDGF) receptors. These autoantibodies were found to stimulate PDGF receptor signalling, which led to the generation of reactive oxygen species. This increase in oxidative stress (a hallmark feature of SSc) strongly suggests a pathogenic role for these autoantibodies in disease, and that they are involved in autocrine and paracrine activities, and the cascade of events that leads to enhanced ECM synthesis.

Thus, the identification of functional key players in the processes that link vascular dysfunction to fibrogenesis will assist in defining potential molecular targets for therapeutic intervention. Along this line, processes that impact on both the vascular and fibrotic pathogenic processes are of particular interest. Although several molecular mediators are involved in these processes, one of the key contributors is ET-1. This article focuses on the biological function(s) of this molecule, and the pivotal cell and molecular mechanism(s) regulated by ET-1 in health and pathological activities in connective tissue diseases.

The endothelin axis: role in the control of vascular tone

The endothelins are a family of three vasoactive peptides (ET-1, ET-2 and ET-3). The three 21-amino-acid endothelin isoforms bind to two endothelin receptors (endothelin receptor subtype A [ET_A] and ET_B), with equal affinities for ET_B but with a hierarchy of affinities for the ET_A receptor. ET-1 has the highest affinity for ET_A, followed by ET-2 and ET-3 [16]. The roles of the ET_A and ET_B receptors are becoming less clearly distinguished as the literature expands. Indeed, both have been implicated as mediators of the deleterious effects of ET-1. The receptors exhibit a wide tissue distribution and are expressed in kidney, liver, lung and skin; they are known to be abnormally expressed in various diseases characterized by defective vascular control. Abnormalities observed in the endothelin system have been associated with vasoconstriction, vasospasm and vascular hypertrophy.

Endothelin-1 and receptor interaction

ET-1 is the major and most studied endothelin isoform. ET-1 is produced by endothelial and mesenchymal cell types, such as fibroblasts and smooth muscle cells, and acts in a paracrine and autocrine manner. ET-1 production is controlled by a number of soluble factors, including TGF-β, which is a well recognized major profibrotic factor, and it plays a major role in constriction and proliferation.

The ET_A and ET_B receptors have seven transmembrane domains and are coupled to G proteins. ET_A is expressed in mesenchymal cells and ET_B is expressed on endothelial cells. ET-1 is produced as a pre-pro transcript that is translated into a pre-pro polypeptide of 212 amino acids. The pre-pro polypeptide is then processed by a series of converting enzymes to proET-1 (or 'big ET-1'), which is a large, relatively inactive endothelin molecule that exhibits approximately 1% of the activity of the fully functional 21-amino-acid peptide. Big ET-1 is then converted by the membrane-bound enzyme endothelin converting enzyme-1 into its functional, 21-amino-acid form (Figure 3) [17-20]. It is then released as an active form and binds to the specific endothelin receptors that are expressed on a number of different cell types. ET-1 activity can be inhibited with a number of classes of inhibitors that act at different points along the endothelin cascade. Inhibitors are available that selectively block endothelin converting enzyme-1 to prevent production of the active peptide from big ET-1; single or dual endothelin receptor antagonists block the effects of ET-1 on target cells; cascade inhibitors disrupt the intracellular signalling processes that are induced by ET_A/ET_B binding; and inhibitors of transcription prevent transcriptional activation and the downstream production of proteins that are elevated by ET-1 and associated with the SSc phenotype. Many of these inhibitors have been shown to interrupt the effects of ET-1 effectively and have been the subject of a plethora of basic science studies.

The widespread distribution of endothelin receptors on many cell types suggests that they have important and wide-ranging biological activities *in vivo*. In the context of our understanding of the processes involved in SSc and other connective tissue diseases, the primary 'target' cells for the activity of ET-1 include smooth muscle cells, endothelial cells, fibroblasts (and their differentiation to myofibroblasts, which is the characteristic cell type of SSc and other connective tissue diseases) and macrophages, in which activation by ET-1 elicits a number of cell-type specific responses. In smooth muscle cells ET-1 promotes vasoconstriction and results in proliferation and elevated production of the key profibrotic factors TGF-β and PDGF. In fibroblasts ET-1 increases ECM production and increases adhesion molecule expression, thereby facilitating leucocyte-fibroblast inter-actions. This is also observed in endothelial cells [21]. ET_B receptors are expressed by macrophages, and in these cells ET-1 modulates inflammatory responses such as nuclear factor-κB (NF-κB) activation, release of free radicals, and increased levels of interleukin-8, monocyte chemoattractant protein-1 [22] and TGF-β.

Multiple actions of endothelin

The important actions of ET-1 from the perspective of connective tissue remodelling include induction of prolifer-ation, chemotaxis of macrophages and smooth muscle cells, activation of smooth muscle cells, and differentiation of fibroblasts to myofibroblast phenotypes.
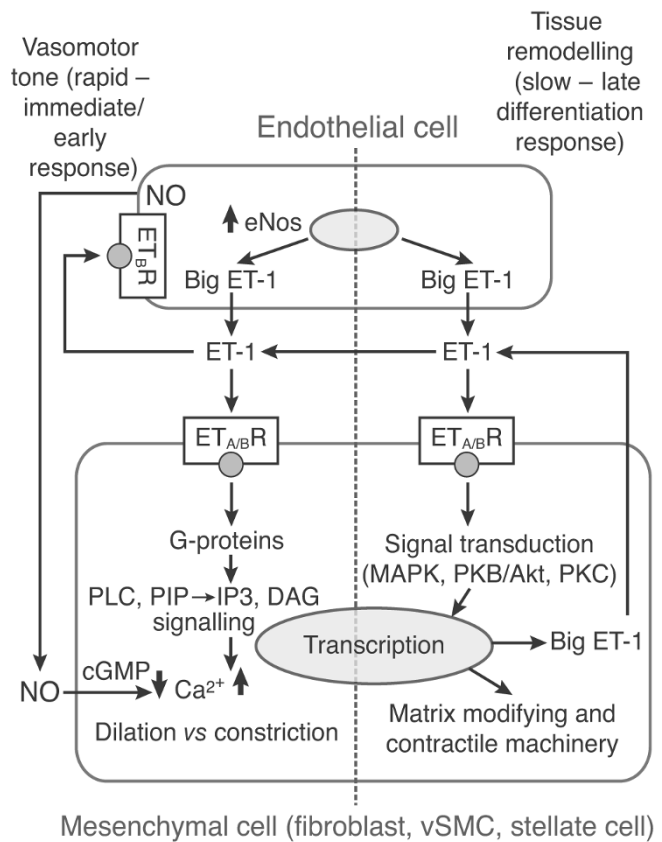
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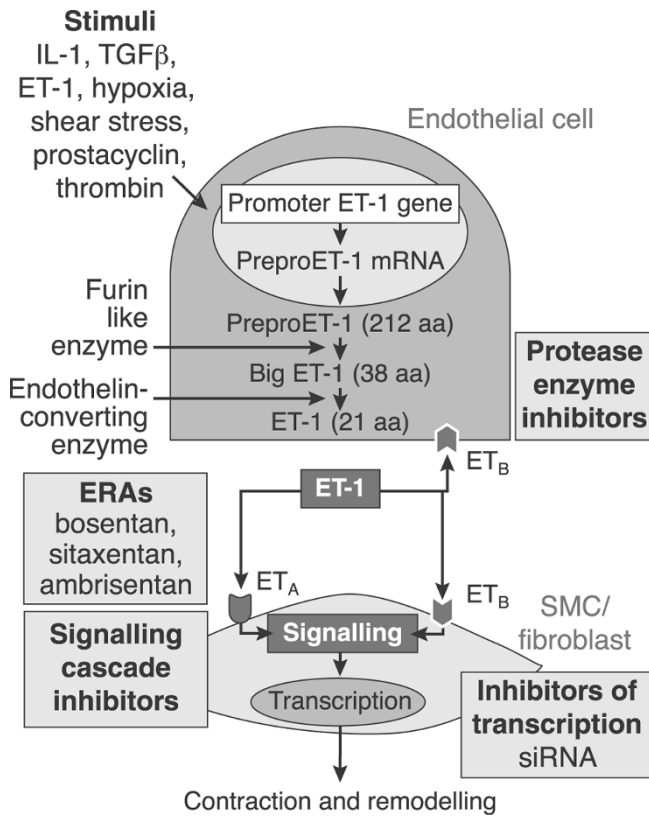
Overview of ET-1 biosynthesis and function. Endothelial cell stimulation promotes the synthesis of pre-pro endothelin (ET)-1 polypeptides, the activation of processes that result in production of ET-1, and local release of mature ET-1. ET-1 interacts with specific endothelin receptors (endothelin receptor subtype A [ET_A] and ET_B) expressed on the surface of vascular smooth muscle cells and adventitial fibroblasts, and with ET_B receptors in endothelial cells. Recognition of ET-1 by these receptors activates intracellular signalling pathways and cascades that result in rapid alterations in cell activity and function, and initiates transcriptional programmes. Several points along the pathways of production, release, receptor ligation and signalling, as well as transcriptional activation, are potential targets for intervention. aa, amino acids; ERA, endothelin receptor antagonist; IL, interleukin; siRNA, small interfering RNA; SMC, smooth muscle cell; TGF, transforming growth factor.

Figure 4

Dual action of ET-1 on mesenchymal cell types. Shown are key activities of endothelin (ET)-1 on endothelial cells, vascular smooth muscle cells (vSMCs) and fibroblasts. Rapid endothelin-mediated responses include the modulation of calcium concentration and influx, levels of nitric oxide (NO) and cGMP, and the associated contractile response in order to regulate vascular tone. Slower more adaptive responses include the activation of transcriptional programmes to effect mesenchymal cell transition and differentiation, and extracellular matrix (ECM) production resulting in tissue remodelling. DAG, diacylglycerol; $ET_{A/B}R$, endothelin receptor subtype A/B receptor; IP_3, inositol trisphosphate; MAPK, mitogen-activated protein kinase; PIP, phosphatidylinositol phosphate; PKB, protein kinase B; PKC, protein kinase C; PLC, phospholipase C.

As introduced above, the biological activity of ET-1 with respect to specific cell types can broadly be divided into two types of functional response. The 'early responses' involve cell signalling and calcium metabolism, are rapid and transient, and are important with respect to cell contraction. The slower, so-called 'phenotypic responses' culminate in the characteristic remodelling phenotype of SSc. These latter responses, which occur within hours and days rather than minutes, involve transcriptional activation and stimulation of modifying genes that control matrix (such as those encoding MMPs and TIMPs), that potentiate the contractile machinery (such as the actin and paxillin genes) and that alter tissue architecture (for instance, genes encoding ECM proteins, including collagen; Figure 4).

Endothelin-1 in the pathogenesis of systemic sclerosis

Elevated levels of endothelin-1 are present in patients with systemic sclerosis

There is now a wealth of evidence that ET-1 is expressed in both early-stage and late-stage SSc. The protein has been shown to be over-expressed in biological fluids and tissue sections in patients with SSc, and increased expression of ET-1 and increased receptor binding sites are found in presclerotic and early diffuse skin lesions [23]. Elevated levels of ET-1 have been observed in multiple organs that are affected by SSc, notably blood vessels, lung, kidney and skin [24].

Endothelin-1 is implicated in the pathological sequence of events that is characteristic of systemic sclerosis

Within the context of tissue remodelling and fibrosis in SSc, it is the vSMCs, fibroblasts and myofibroblasts that are the cells of particular interest. Laboratory experiments have focused on evaluating the characteristics of these cell types and extrapolating the findings to what may occur *in vivo*, and on determining how this information can be used effectively in the clinical setting. Thus, we are interested in examining cell contractility and migration, cell migration, inflammation, and matrix deposition and remodelling.

ET-1 activates the surface expression of intercellular adhesion molecule (ICAM)-1 on fibroblasts, and collagen production by both dermal and pulmonary fibroblasts. It can also stimulate fibroblasts to exhibit potent procontractile properties and promote the formation of highly contractive myofibroblasts [25,26]. In SSc it is important to note that the MMP/TIMP cascade is dysregulated, with an increase in many MMPs. Particularly intriguing is the elevation in MMP-2 (gelatinase A), which is capable of processing endothelin precursors to active endothelin, thereby potentially exacerbating the disease process. Classical and MMP-activated endothelin can induce gene transcription and result in the familiar cellular changes of fibrosis, matrix deposition and tissue remodelling that are observed in SSc.

As well as matrix production and remodelling, SSc is characterized by an early inflammatory response that involves increased leucocyte infiltration to affected tissues and elevated leucocyte-fibroblast interactions. Examining biopsies from patients with early disease, Koch and coworkers [27] found a number of cell adhesion molecules known to mediate leucocyte interactions to be over-expressed. The nature of these interactions has been addressed in a number of studies, and the importance of the leucocyte function-associated antigen-1/ICAM-1 pathway in T-cell/fibroblast interaction has been noted [28]. In the laboratory, T cells attach and bind more readily to SSc fibroblasts than to control fibroblasts [29], and by using antibody blockade it is possible to inhibit specifically the binding of these T cells to SSc fibroblasts *in vitro*. Interestingly, ICAM-1 expression on both normal and SSc fibroblasts is activated by ET-1. By adding ET-1 and an antagonist, this increased attachment could be inhibited. ET-1 therefore appears to increase ICAM-1, which increases T-cell interaction with fibroblasts. It might thus be an important factor in leucocyte recruitment into extravascular tissues in the early inflammatory stages of SSc.

Further evidence for the role of ICAM-1 was recently reported [30]. This study detailed the intracellular signalling pathways that lead to the increased expression of ICAM-1 elicited by ET-1 by using inhibitors of signalling pathways, transfections with ICAM-1 promoter-reporter constructs, and electrophoretic mobility shift assays. The authors demonstrated that the regulation of fibroblast ICAM-1 expression by ET-1 was mediated by NF-κB. It was apparent that NF-κB is activated by ET-1, which activates the ICAM-1 gene in a manner similar to that with interleukin-1. ET-1 therefore can also play a proinflammatory role, and is able to turn on ICAM-1 gene expression and cause an increase in leucocyte-fibroblast interaction. This upregulation of fibroblast ICAM-1 was found to be potently inhibited by the dual endothelin receptor antagonist bosentan and specific inhibitors of ET-1-dependent signalling pathways [31].

Impact of endothelin receptor antagonists on the pathophysiology of systemic sclerosis

Evidence for the involvement of ET-1 in the pathophysiology of SSc also comes from receptor blockade studies. For example, ET-1 has been shown to induce an expression pattern in cultured lung fibroblasts similar to that in SSc fibroblasts, and bosentan was shown to attenuate this response and partially 'normalize' the scleroderma phenotype [26]. These studies have been extended using Affymetrix gene expression profiling of control fibroblasts and those from SSc patients. These studies have shown that bosentan reduces the differences observed between the gene expression patterns in SSc and control lung fibroblasts. The findings of experiments like these demonstrate that approximately one-third of genes that define the SSc signature are modulated toward a more normal profile in the presence of bosentan. However, it must be emphasized that the clinical setting is extremely complex, and it is therefore difficult to unravel fully the pathophysiological scenarios in the laboratory using cell culture studies and animal models. There is clearly significant crosstalk between different cell types in the disease process as well as between different factors. The temporal interaction(s) and links between these biological processes are also important, as are the levels and expression patterns of the critical mediators and responding cell types. Data obtained thus far in the context of SSc support the notion that ET-1 can play an important role in a number of relevant pathological processes, including inflammation, vasculopathy, tissue remodelling and fibrosis. This places ET-1 as a key mediator in this disease.

Returning to the initial question of how endothelial cell injury starts, the answer is that this remains elusive. There are some excellent data from the UCD200 chicken scleroderma model that suggest that endothelial cell apoptosis, promoted by AECAs, is a key early event in endothelial injury [32], but the human correlates have not been as convincing. However, is it absolutely necessary to know the exact nature of the initiating event(s), or is it equally important to understand the factors that subsequently drive the disease process? One example that supports the latter is the role played by TNF-α in rheumatoid arthritis. Although the role, if any, that TNF-α plays in the initiating events of rheumatoid arthritis is unclear, it is very important in driving inflammation and subsequent disease progression. This in turn defines TNF-α as an optimal target for therapeutic intervention in rheumatoid arthritis.

Indeed, TNF-α inhibitors are now successfully used to treat rheumatoid arthritis. Likewise, blocking critical mediators such as ET-1, TGF-β and certain chemokines such as monocyte chemoattractant protein-1 may likewise be effective in preventing the progression of fibrotic diseases such as SSc.

Conclusion

Significant evidence suggests that ET-1 is important in a number of aspects of fibrotic diseases such as SSc. In inflammation, through induction of ICAM-1, ET-1 activates adhesion molecule expression, which may be important in regulating the inflammatory response that occurs in the early stages of SSc. It is well accepted that endothelin plays a major role in vasoconstriction, and it is an important mediator in ECM remodelling and deposition. ET-1 can activate fibroblasts and other mesenchymal cells along the differentiation pathway toward the myofibroblast, which is the principal culprit in tissue remodelling, repair and ultimately fibrosis. ET-1 activates adhesion molecule expression, which may be important in regulating the inflammatory response. ET-1 receptor antagonists, such as bosentan, have been shown to block early and late-stage manifestations of disease and may thus help to prevent the progression of fibrotic diseases such as SSc.

Competing interests

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