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Endoglin in fibrosis and scleroderma

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Abstract

Fibrosis plays a role in many pathological conditions, among which is the autoimmune disease systemic sclerosis (SSc). SSc is characterized by fibrosis in the skin and internal organs, but the etiology remains to be elucidated. Transforming growth factor- (TGF-) is a key player in the fibrotic process, also in SSc. TGF- induces the production of several components of the extracellular matrix and induces differentiation of fibroblasts to myofibroblasts, which further worsens fibrosis. Although TGF- has been extensively investigated in fibrosis, the roles of several components of its signaling pathway are still unknown. Endoglin is a co-receptor for TGF- and is known to modulate TGF- signaling. Therefore, endoglin could enhance the effects of TGF- in fibrosis or act as an inhibitor. Multiple studies have been conducted that support either hypothesis. Elucidating the exact role of endoglin in TGF- signaling during fibrosis is important in understanding the process of fibrosis and could lead to the development of better treatment.

Keywords

Endoglin; systemic sclerosis; fibrosis; TGF-

1. Introduction

Fibrosis is characterized by excessive deposition of extracellular matrix (ECM) proteins, such as collagen and fibronectin, by fibroblasts. Fibrosis is important in wound healing, where remodeling of the ECM serves to preserve tissue integrity and provide cues for migrating cells (Mutsaers et al., 1997). This is evident after myocardial infarction, where fibrotic tissue provides strength to the weakened cardiac wall (Cleutjens et al., 1999). In the normal situation, remodeling of the ECM ends and the formed scar tissue is – partially – degraded, resulting in new, functioning tissue (Mutsaers et al., 1997). However, when fibrosis continues uncontrolled this process can be deleterious and disruptive for the affected tissue.

Fibrosis is known to play a role in many pathologic conditions such as Crohn's disease, pulmonary hypertension and diabetic nephropathy. In many cases of fibrosis, inflammation is the likely underlying cause (Pohlers et al., 2009). Products released by inflammatory cells and cell-cell interactions have pro-fibrotic effect on fibroblasts, inducing the fibrotic process

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Disclosure of potential conflicts of interest

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(Chizzolini et al., 2011). Systemic sclerosis (SSc) or scleroderma is an auto-immune disease in which fibrosis affects the skin and internal organs. Microvascular injury is seen as one of the earliest events in the pathology of SSc (Hunzelmann and Krieg, 2010). Endothelial cell death leads to the loss of capillaries and tissue hypoxia. Hypoxia induces pro-angiogenic signals, but angiogenesis is defective in SSc patients (Trojanowska, 2010; Wipff et al., 2008). Fibroblasts respond to hypoxia by producing proteins involved in remodeling of the ECM, such as transforming growth factor- β (TGF- β), thrombospondin and connective-tissue growth factor (CTGF). Hypoxia is therefore seen as a central event in SSc. Inflammatory cells are also recruited to the tissue, enhancing the pro-fibrotic effect of fibroblasts (Distler et al., 2007; Hong et al., 2006).

Because of the role of fibrosis in many diseases, it is important to understand the underlying regulators and signaling pathways. Among the many cytokines involved in fibrosis, TGF- β is seen as the key player in fibrosis. Therefore understanding the role of TGF- β and its signaling pathways in fibrosis could lead to better treatment of the disease. Endoglin, a TGF- β co-receptor, has been implied to be involved in the fibrotic response mediated by TGF- β . Here we will review current knowledge about the role of endoglin in fibrosis.

2. TGF- β /Eng signaling

TGF- β is part of the structurally related TGF- β superfamily of cytokines, which also includes BMPs and activins, and regulates many cellular responses, such as proliferation, migration and differentiation (Goumans et al., 2009; ten Dijke and Arthur, 2007). TGF- β signals via a specific transmembrane serine/threonine kinase complex, consisting of a type I and a type II TGF- β receptor. Two different TGF- β type I receptors have been identified, i.e. activin receptor-like kinase (ALK)1 and ALK5 (Franzen et al., 1993; ten Dijke et al., 1993). ALK5 is broadly expressed and the predominant TGF- β type I receptor on most cells, while ALK1 is mainly expressed on endothelial cells (Goumans et al., 2002). Upon TGF- β - induced heteromeric complex formation the type II receptor phosphorylates the type I receptor on serine and threonine residues in the juxtamembrane Glycine-Serine (GS)-rich domain. Subsequently, intracellular signaling is initiated by the phosphorylation of receptor regulated Smads (R-Smads). While ALK1 induces Smad1/5/8 phosphorylation, ALK5 mediates Smad 2/3 phosphorylation (Goumans et al., 2002; Goumans et al., 2009; Oh et al., 2000; ten Dijke and Arthur, 2007). The activated R-Smads form heteromeric complexes with the co-Smad, Smad4, which accumulate in the nucleus. As Smad complexes have low intrinsic affinity for DNA, the binding to promoters/enhancers of target genes occurs in cooperation with other transcription factors. Co-activators and co-repressors, such as CBP/p300 and Ski/Sno, respectively, are recruited to the Smad complexes and mediate the transcriptional activation or repression of target genes (Derynck and Zhang, 2003; ten Dijke and Hill, 2004). The TGF- β signaling pathway can be inhibited by inhibitory Smads (I-Smads), among other inhibitory factors. These I-Smads, Smad6 and 7, are able to bind to the type I receptor and prevent the recruitment and phosphorylation of the R-Smads, thus inhibiting further signaling (Derynck and Zhang, 2003). They can also inhibit the binding to Smad4 (Hata et al., 1998) or target the receptor for ubiquitination and subsequent proteasomal degradation (Ebisawa et al., 2001; Kavsak et al., 2000). An overview of TGF- β signaling can be found in Figure 1.

Endoglin is a 180 kD homodimeric covalently disulphide-linked transmembrane glycosylated protein that acts as a co-receptor for TGF- β (ten Dijke et al., 2008). A co-receptor is defined as a cell surface protein that is capable of interacting with ligand, but does not actively transmit signaling responses. Although originally thought to be a marker for activated endothelial cells, endoglin is also expressed on, for example, hematopoietic cells and syncytiotrophoblasts (Burrows et al., 1995; Cho et al., 2001; Gougos and Letarte,

1988; St-Jacques et al., 1994). Endoglin consists of an extracellular domain, a transmembrane region and a relative short intracellular region. While the intracellular region does not have any intrinsic enzymatic activity or motif (Gougos and Letarte, 1990), it contains many serine residues that can become phosphorylated by TGF- β type I and type II receptors. At its carboxy terminus, endoglin has a PDZ interaction motif (ten Dijke et al., 2008). The extracellular domain contains a ZP domain and an RGD sequence (Bork and Sander, 1992) (Gougos and Letarte, 1990). The ZP domain is involved in complex formation with the TGF- β type II and type I receptors (Guerrero-Esteo et al., 2002) while the RGD sequence is known to be involved in the interaction with integrins and other proteins (ten Dijke and Arthur, 2007). Endoglin binds TGF- β in association with the TGF- β type II receptor, but is unable to bind TGF- β on its own (Letamendia et al., 1998). The presence of endoglin enhances TGF- β signaling via ALK1 and inhibits signaling via ALK5. Knockdown of endoglin showed diminished ALK1 signaling and enhanced ALK5 signaling, implying an important role for endoglin in balancing the TGF- β signaling via the two different receptors (Goumans et al., 2002; Lebrin et al., 2004). While endoglin has a positive role in TGF- β / ALK1 signaling, it has a negative role in TGF- β /ALK5-induced Smad3 signaling (Lebrin et al., 2004). A further twist is that if the TGF- β /Smad3 pathway is inhibited, endoglin has been reported to have positive effect on TGF- β /Smad2 signaling (Guerrero-Esteo et al., 2002; Santibanez et al., 2007). Another complication is that there are two splice variants of endoglin, i.e. a short and long form, which differ in their intracellular region and have opposite functional effects (Perez-Gomez et al., 2005; Velasco et al., 2008). Lastly, endoglin is not only a receptor for TGF- β but also for the other TGF- β family ligands BMP9 and BMP10 (David et al., 2007). In contrast to TGF- β , BMP9 was shown to directly interact with endoglin (Scharpfenecker et al., 2007).

Research has shown that endoglin is particularly important in angiogenesis, since knockout mice for endoglin do not survive beyond E10.5 and have reduced blood vessel formation (Arthur et al., 2000; Bourdeau et al., 1999). Heterozygous mutations in the endoglin and ALK1 genes are responsible for hereditary hemorrhagic telangiectasia in humans, a dominant genetic disease (Johnson et al., 1995; McAllister et al., 1994). Patients with HHT suffer from malformation in the blood vessels such as telangiectasis, nose bleeds and potentially life-threatening arteriovenous malformations (Abdalla and Letarte, 2006). Although the role of endoglin is best understood in endothelial cells, endoglin is still likely to play a role in other diseases associated with TGF- β signaling as well. In patients with pre-eclampsia soluble endoglin levels were found to be very high and contribute to the pathology of vascular dysfunction (Levine et al., 2006; Venkatesha et al., 2006). Soluble endoglin may exert its anti-angiogenic function by sequestering BMP9 and BMP10 and prevent binding to its receptors (Castonguay et al., 2011).

3. TGF- β signaling in fibrosis/scleroderma

TGF- β is a key regulator in the fibrotic process. CTGF, a mitogen capable of inducing the production of several ECM proteins, is a target gene of TGF- β signaling. This pathway is mainly mediated via Smad3 signaling (Flanders et al., 2002; Verrecchia and Mauviel, 2007). Furthermore, CTGF induces the differentiation of fibroblasts into myofibroblasts, which are more specialized in the production of ECM (Santibanez et al., 2011). CTGF positively affects TGF- β signaling by enhancing binding of TGF- β to the type I and type II receptors (Abreu et al., 2002). TGF- β signaling via Smad3 has a direct pro-fibrotic effect by inducing the production of collagen. Like CTGF, TGF- β can induce the differentiation of fibroblasts into myofibroblasts (Santibanez et al., 2011).

The main effects of TGF- β in fibrosis are mediated by ALK5, via Smad3 signaling. However, ALK1 has also been implicated in fibrosis. Hepatic stellate cells express ALK1

and ALK5 and TGF- β induces both the Smad1/5/8 and the Smad2/3 pathway. Downstream of Smad1, Id1 is upregulated and induces the differentiation to myofibroblasts, leading to enhanced fibrosis (Wiercinska et al., 2006). Smad1 is also more activated in SSc fibroblasts, where it binds to the CTGF promoter. Here it was found that TGF- β /ALK1-induced fibrosis is mediated by Smad1 and ERK activation (Pannu et al., 2008). *In vivo* data revealed that mice heterozygous for ALK1 have less radiation-induced fibrosis than wildtype mice, together with a reduction in macrophage invasion. ALK1 heterozygous mice showed an overall delayed fibrotic response, both in gene expression as in tissue damage, all indicating to the involvement of ALK1 in fibrosis (Scharpfenecker et al., 2011).

Non-canonical TGF- β signaling is also involved in fibrosis. Activation of JunD by Smad3 is increased in SSc. JunD knockout cells are less responsive to TGF- β and expression of CTGF, collagen and PAI-1 is decreased. JunD knockout mice were protected from bleomycin induced fibrosis. The skin of JunD^{-/-} mice had lower collagen content and reduced numbers of myofibroblasts, suggesting that JunD is involved in the pro-fibrotic response (Palumbo et al., 2011). Also p38 signaling is involved in fibrotic TGF- β signaling. Inhibition of p38 in rat myofibroblasts inhibited TGF- β induced collagen production in cultured cells (Rodriguez-Barbero et al., 2006). An overview of TGF- β signaling in fibrosis can be seen in figure 2.

4. Endoglin in fibrosis

Endoglin was found to be upregulated in endothelial cells and fibroblasts from patients suffering from fibrosis or scleroderma (Burke et al., 2010; Clemente et al., 2006; Dharmapatni et al., 2001; Holmes et al., 2011; Leask et al., 2002; Morris et al., 2011). Soluble endoglin levels, which are known to be involved in the pathogenesis of pre-eclampsia, were reported to be increased in fibrosis patients, SSc patients and SSc patients with pulmonary arterial hypertension (Clemente et al., 2006; Coral-Alvarado et al., 2009; Fujimoto et al., 2006). Animal models of fibrosis also show a significant increase in endoglin expression compared to control conditions (Docherty et al., 2006; Prieto et al., 2005a; Prieto et al., 2005b; Rodriguez-Pena et al., 2001; Rodriguez-Pena et al., 2002). Above evidence suggests that endoglin is involved in the formation of fibrosis, and with endoglin being a co-receptor for TGF- β (family) members the question arises whether endoglin stimulates or inhibits TGF- β -induced fibrosis. Although multiple studies have been done regarding this subject, no definitive or simple answer has emerged.

4.1. Endoglin as a negative regulator of fibrosis

Endoglin has been reported to inhibit fibrosis in different *in vitro* studies, using both human and rat cells. Expression of endoglin in rat myoblasts and myofibroblasts reduces the responsiveness to TGF- β and subsequently leads to a decrease in collagen expression (Obreo et al., 2004; Rodriguez-Barbero et al., 2006). Endoglin expression also leads to a reduction in CTGF expression after TGF- β stimulation. In fibrosis, there is a positive feedback loop in which TGF- β induces its own expression and secretion. Presence of endoglin reduces the TGF- β stimulated TGF- β secretion, thereby inhibiting the feedback loop (Obreo et al., 2004). Endoglin can exert its effects on TGF- β signaling via ERK activation. It has been shown that endoglin shifts the balance between activated ERK1 and ERK2. Inhibition of the ERK pathway completely abolished the endoglin mediated effects (Rodriguez-Barbero et al., 2006). In human mesangial cells, involved in kidney fibrosis, TGF- β also induces increased expression of fibronectin and collagen I and II. Overexpression of endoglin reduced basal and TGF- β -induced expression levels of collagen (Diez-Marques et al., 2002).

In vitro research with human fibroblasts from healthy donors and patients with fibrosis or SSc suggest that endoglin might inhibit fibrosis. These fibroblasts have higher basal

expression levels of CTGF and collagen then fibroblasts from healthy controls. Stimulation of control and Ssc/fibrosis fibroblasts with TGF- β causes an increase in the expression of CTGF and collagen. Overexpression of endoglin in control fibroblasts abrogated the TGF- β -induced promoter activity of the CTGF gene as well as the production of CTGF. It also inhibited the accumulation of phosphorylated Smad3 in the cells, the key pathway in TGF- β -induced fibrosis. When Smad3 and -4 were co-overexpressed in combination with endoglin, the inhibitory effect of endoglin on CTGF promoter activity was abolished. This suggests that endoglin may exert its effects upstream of the R-Smads. Knockdown of endoglin had the opposite effect and caused an upregulation of CTGF and an increase in activated Smad3 levels. Also contraction of fibroblasts in collagen, both basal contraction and contraction in response to TGF- β , was increased after endoglin knockdown (Burke et al., 2010; Holmes et al., 2011; Leask et al., 2002). However, because some results have been obtained with promoter assays and overexpression experiments, these results should be carefully interpreted since they do not represent the physiological situation.

4.2. Endoglin as a positive regulator of fibrosis

Besides reports stating that endoglin has an inhibitory on fibrosis, there are also studies that have shown that endoglin can promote fibrosis. In cardiac fibroblasts, endoglin is upregulated by TGF- β and angiotensin II, another known pro-fibrotic factor. Treatment of the fibroblasts with angiotensin II caused an increase in collagen I expression and a decreased matrix metalloproteinase (MMP)-1 expression. Addition of an endoglin blocking antibody abolished this effect, implying that endoglin has a pro-fibrotic function (Chen et al., 2004; Shyu et al., 2010). In liver damage, endoglin expression is upregulated and hepatic stellate cells (HSCs) that trans-differentiate into myofibroblasts. The increase in endoglin is induced by TGF- β , since treatment of HSC with an ALK5 inhibitor diminished endoglin upregulation. The inhibitor also blocked both the Smad2/3 pathway and the Smad1/5/8 pathway. Overexpression of endoglin resulted in increased Smad1/5/8 activation and the production of α -smooth muscle actin, a hallmark of HSC activation (Meurer et al., 2011). In fibroblasts from SSc patients, the increased levels of endoglin correlated with an increase in Smad1 activation, CTGF and collagen I expression. Conversely, knockdown of endoglin caused reduced protein levels of activated Smad1, CTGF and collagen I. ALK1 knockdown had similar effects in the fibroblasts, suggesting effects of endoglin are mediated by affecting the ALK1 pathway (Morris et al., 2011).

The effect of reduced endoglin levels during fibrosis have been studied *in vivo* in mice heterozygous for endoglin. A fibrotic response can be induced by radiation or ischemia/reperfusion damage in the kidney. Comparison of renal structures in wildtype and endoglin heterozygous mice after I/R damage shows that wildtype mice have more abnormal renal structures while the kidney histology of endoglin heterozygous mice is almost normal. Kidneys of endoglin heterozygous mice also have reduced inflammatory cell infiltration. Similar results were seen after radiation of the kidneys, where endoglin heterozygous mice have less fibrosis than wildtype mice. Kidney function was preserved in endoglin heterozygous mice after I/R damage, as seen by lower plasma creatinine levels, while function was reduced in wildtype mice. Markers of fibrosis, such as CTGF and collagen were induced to a greater extent in wildtype mice, confirming a greater fibrotic response. Reduced TGF- β signaling is the most likely cause, since TGF- β and a downstream target of TGF- β , PAI-1 were reduced in endoglin heterozygous mice (Docherty et al., 2006; Scharpfenecker et al., 2009).

5. Conclusions

Endoglin, as a co-receptor for TGF- β family members, can mediate or inhibit TGF- β family signaling and thereby enhance or repress fibrotic signaling. However, its exact role still

remains to be elucidated. Endoglin can inhibit the classical TGF- β pathway, which induces the fibrosis via Smad3, and enhance the Smad1 pathway. Several studies support this hypothesis that endoglin is a negative regulator of fibrosis. Yet other studies have shown that the ALK1/Smad1 pathway is also involved in the formation of fibrosis and endoglin is a positive regulator of fibrosis, both *in vitro* and *in vivo*. The role of endoglin remains disputed and more evidence is needed to conclude definitely how endoglin regulates fibrosis.

The answer to the disparate role of endoglin in fibrosis will likely lie in various factors that influence the function of endoglin: endoglin functions as co-receptor for different TGF-family members, of which individual members have been shown to have opposite functions in fibrosis (Hawinkels and ten Dijke, 2011; Weiskirchen et al., 2009); contribution of different splice variants and soluble endoglin (Castonguay et al., 2011; Perez-Gomez et al., 2005; Velasco et al., 2008), which mediate different (even opposite) biological responses; and of course the context in which endoglin operates in different cell types, such as extracellular and intracellular interaction partners, post-translational modification, cellular location, which influences distinct cellular responses. More mechanistic studies on different cultured cells complemented with animal models using conditional endoglin knock outs, soluble endoglin and neutralizing endoglin antibodies as tools that have recently become available (Castonguay et al., 2011; Mahmoud et al., 2010; Seon et al., 2011), and validation of findings in human clinical samples are needed. They will clarify the role of endoglin in fibrosis, and may ultimately lead to new therapies for fibrosis and scleroderma.

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Figure 1.

Schematic overview of TGF- β signaling. TGF- β can signal via two distinct receptors, ALK1 and ALK5. The signal is propagated intracellular via Smad1/5/8 and Smad2/3 for ALK1 and ALK5, respectively. Smad6/7 can inhibit these signaling pathways. Besides TGF- β , BMP9 and BMP10 can also signal via ALK1. Endoglin is a co-receptor for ALK1 and enhances its signaling upon binding of TGF- β . Soluble endoglin is able to inhibit signaling of BMP9 and BMP10.