Small intestinal neuroendocrine tumours and fibrosis: an entangled conundrum

Anela Blažević, Johannes Hofland, Leo J Hofland, Richard A Feelders and Wouter W de Herder

Department of Internal Medicine, Sector Endocrinology, ENETS Centre of Excellence, Erasmus University Medical Center (Erasmus MC) and Erasmus MC Cancer Institute, Rotterdam, Netherlands

Correspondence should be addressed to A Blažević: a.blazevic.1@erasmusmc.nl

Abstract

Small intestinal neuroendocrine tumours (SI-NETs) are neoplasms characterized by their ability to secrete biogenic amines and peptides. These cause distinct clinical pathology including carcinoid syndrome, marked by diarrhoea and flushing, as well as fibrosis, notably mesenteric fibrosis. Mesenteric fibrosis often results in significant morbidity by causing intestinal obstruction, oedema and ischaemia. Although advancements have been made to alleviate symptoms of carcinoid syndrome and prolong the survival of patients with SI-NETs, therapeutic options for patients with mesenteric fibrosis are still limited. As improved insight in the complex pathogenesis of mesenteric fibrosis is key to the development of new therapies, we evaluated the literature for known and putative mediators of fibrosis in SI-NETs. In this review, we discuss the tumour microenvironment, growth factors and signalling pathways involved in the complex process of fibrosis development and tumour progression in SI-NETs, in order to elucidate potential new avenues for scientific research and therapies to improve the management of patients suffering from the complications of mesenteric fibrosis.

Introduction

Small intestinal neuroendocrine tumours (SI-NETs) are rare and mostly slow-growing neoplasms originating from the enterochromaffin (EC) cells of the intestine (de Herder 2005, Yao et al. 2008). EC cells are chemo- and mechanosensory cells that integrate signals from the bowel content and peristalsis and communicate with the neural sensory system in order to control gut motility, secretion and visceral sensation (Linan-Rico et al. 2016, Bellono et al. 2017). Retention of the ability of EC cells to secrete amines and peptides can cause distinct hormonal syndromes in SI-NETs (de Herder 2005, Yao et al. 2008). Carcinoid syndrome, marked by diarrhoea and flushing, is the most established of these hormonal syndromes and is caused by release of 5-hydroxytryptamine (serotonin), tachykinins and bradykinins and many other mediators (de Herder 2005, Halperin et al. 2017).

Another hallmark of SI-NETs is the ability to induce fibrosis. The fibrosis can occur around the tumour or at distant sites (Modlin et al. 2004, Niederle et al. 2016). Endocardial fibrosis of the right heart valves, known as carcinoid heart disease, is the most frequent distant fibrotic complication that occurs in 20–30% of SI-NET patients. Carcinoid heart disease is associated with metastatic disease and carcinoid syndrome, suggesting an etiological role for circulating tumour-secreted factors (Modlin et al. 2004, de Herder 2005, Niederle et al. 2016). However, in this review, we will focus on local fibrotic complications, of which mesenteric fibrosis (MF) is most notable and occurs in up to 50% of SI-NET patients (Rodriguez Laval et al. 2017). It is caused by a metastatic lesion circumscribed by an extensive fibrotic reaction in the mesentery.
Fibrosis and small intestinal neuroendocrine tumours. The search strategy is provided in the Supplementary Data (see section on supplementary data given at the end of this article). The reference lists of included studies and relevant reviews were assessed to identify additional articles.

**Results**

**Tumour microenvironment (TME)**

Tissue homeostasis is maintained by intricate interactions between cells and their microenvironment. Bidirectional communication between SI-NET cells and other components of the TME alters the composition of the microenvironment that can become profibrotic and tumourigenic. Therefore, understanding the TME is crucial in order to decipher how SI-NETs induce fibrosis (Quail & Joyce 2013). Over the last decades, cancer research has been increasingly focused on the TME and found many commonalities with chronic wound healing that results in fibrosis (Rybinski et al. 2014). The local microenvironment of cancer cells is commonly referred to as ‘reactive stroma’. This reactive tumour stroma consists of immune cells, fibroblasts, capillaries, basement membrane and extracellular matrix (ECM). The TME is crucial for tumour growth, invasion and metastasis, with both cancer-promoting as cancer-restraining actions of most components and is known to differ between cancer types (Quail & Joyce 2013). The tumour stroma of SI-NETs differs from other cancers with a characteristic desmoplastic reaction and limited leukocytic infiltration (Chaudhry et al. 1992, Pantongrag-Brown et al. 1995, Zhang et al. 2004). Therefore, the pathobiological processes in the SI-NET TME differ from other cancer types. Moreover, because of the commonalities between pathways involved in development of fibrosis and cancer progression, insight gained in the distinct effects of different TME components in SI-NETs can result both in effective anticancer as well as antifibrotic treatment.

Fibroblasts are the dominant cellular component of tumour stroma, next to tumour cells. The majority of these fibroblasts have a modified phenotype, similar to fibroblasts during wound healing. This activated phenotype of cancer-associated fibroblasts (CAFs) is identified by expression of α-smooth muscle actin (αSMA). In contrast to quiescent fibroblasts, CAFs are able to proliferate, produce growth factors and ECM (Kalluri 2016). Compared to other neuroendocrine tumours, SI-NETs have a high expression of αSMA in the fibroblast component of the TME both in primary
tumours and metastases (Facco et al. 1998, Kidd et al. 2007c, Cunningham et al. 2010). Further evidence on the presence of synthetic fibroblasts in SI-NETs was detected in primary cultures in which cells from the tumour stroma developed the typical stellate shape of CAFs and increased growth factor transcription after stimulation with transforming growth factor beta 1 (TGFβ1, see later) (Kidd et al. 2007c). This suggests that also in SI-NETs, CAFs are important regulators of fibrotic stromal programmes.

Immune cells are another important constituent of the TME, and dysregulation of the local immune system and inflammatory response is implicated in both tumourigenesis and development of fibrosis (Wynn 2008, Rybinski et al. 2014). A major component of the leukocytic infiltrate in the TME is the tumour-associated macrophages (TAMs) (Quail & Joyce 2013, Rybinski et al. 2014). TAMs have in general a tumour-promoting role, suppress the adaptive immune system and stimulate fibrosis by secretion of profibrotic factors such as TGFβ (Mantovani et al. 2008, Quail & Joyce 2013). In SI-NETs, there is less leukocytic infiltration compared to other cancers (Funa et al. 1990). The sparsely found leukocytes are mostly macrophages, as identified by Leu M5 antibody staining (Funa et al. 1990, Chaudhry et al. 1992). These macrophages also stained strongly for TGFβ and platelet-derived growth factor (PDGF), suggesting a polarized, TAM-phenotype, which is associated with cancer-promoting effects (Chaudhry et al. 1992, Mantovani et al. 2008).

The final component of the TME to be discussed is the extracellular matrix (ECM). This tissue compartment provides structural support and provides biochemical and biomechanical cues necessary for tissue homeostasis. The ECM consists of a panoply of proteins and polysaccharides. The specific concentration of these different matrix components controls the biomechanical properties of the ECM. Remodelling and a shift in the composition of the ECM are shown both in fibrotic and neoplastic diseases (Cox & Erler 2011). As the EC cell and BON1 cells, a pancreatic NET cell line, are mechanosensitive and mechanical stress has been shown to induce release of signalling molecules such as serotonin in these cells, changes in ECM composition might influence tumour functionality in SI-NETs by biochemical and biomechanical signals (Linan-Rico et al. 2016). Unfortunately, little is known about the specific composition and changes in the ECM of SI-NETs. In fibrotic diseases, inappropriate activation of fibroblasts results in increased collagen production (Cox & Erler 2011). Kidd and colleagues have shown the presence of collagen III surrounding CAFs in SI-NETs, further confirming an activated, synthetic phenotype (Kidd et al. 2007c). Next to collagen, the ECM can contain various proteoglycans such as heparan and chondroitin sulfate (Cox & Erler 2011). Analysis of transcription levels of these proteoglycans in NETs showed changes during disease progression; however, their role in tumour progression and development of fibrosis in SI-NETs is still elusive (García-Suárez et al. 2014).

**Profibrotic growth factors**

As mentioned earlier, deregulation of signals changes the microenvironment resulting in tumour progression and fibrosis. Therefore, along with understanding the TME, it is important to investigate the signalling molecules that mediate these changes. These molecules, which regulate cell-fate processes such as proliferation, differentiation and migration, are commonly referred to as growth factors (Witsch et al. 2010). In this next part, growth factors with known profibrotic effects will be discussed, in order to further elucidate the pathobiology of fibrosis in SI-NETs (Fig. 2).

**Serotonin**

Serotonin is a biogenic amine that can act as a neurotransmitter, hormone or growth factor (Mohammad-Zadeh et al. 2008). As a high level of serotonin secretion characterizes functional SI-NETs, this was considered as the causal agent of fibrosis (Modlin et al. 2004). Serotonin controls various physiological functions and is known to have a mitogenic effect on a variety of cells (Mann & Oakley 2013). The ability of serotonin to have a wide array of effects is attributed to its diverse receptor system. It consists of seven families of 5-HT receptors of which six are G-protein coupled. As most families exhibit heterogeneity in form and function, they are further divided in subtypes as 5-HT\textsubscript{1A} and 5-HT\textsubscript{1B}, etc. The EC cells of the intestine are the main source of peripheral serotonin (Mohammad-Zadeh et al. 2008). As SI-NETs originated from these EC cells, serotonin immunohistochemistry (IHC) is positive in >85% of the SI-NETs and can be used as a marker for intestinal origin of neuroendocrine tumours (Yang et al. 1983, Krishnamurthy & Dayal 1997, Facco et al. 1998, Niederle et al. 2016). Furthermore, the majority of patients with SI-NET have increased urinary excretion of 5-hydroxyindoleacetic acid (5-HIAA), the main metabolite of serotonin (Niederle et al. 2016, Zandee et al. 2016, Rodriguez Laval et al. 2017). Also, increased 5-HIAA excretion is associated with the presence of MF (Rodriguez Laval et al. 2017). A causal link between serotonin and fibrosis is furthermore suggested by the association
of fibrotic complications with drugs targeting 5-HT2B receptor such as methysergide, an anti-migraine drug (Mann & Oakley 2013). In KRJ-I cells, a small intestinal EC cell-derived NET cell line, serotonin stimulation resulted in increased proliferation of tumour cells, which could be reversed by ketanserin, a 5-HT2A/C receptor antagonist (Kidd et al. 2007a, Drozdov et al. 2009). Also, a 5-HT1A receptor antagonist resulted in decreased viability of KRJ-I cells and reduced secretion of serotonin and the profibrotic growth factors TGFβ1, connective tissue factor (CTGF) and basic fibroblast growth factor (FGF2) by these cells (Svejda et al. 2010).

Furthermore, fibroblasts are shown to express 5-HT receptors and in other fibrotic diseases, such as pulmonary and liver fibrosis, the direct proliferative effect of serotonin on fibroblasts seems to be mediated via specific 5-HT receptors, namely the 5-HT1A/B, 5-HT2A and 5-HT2B receptors (Mann & Oakley 2013). These results point to serotonin as the main driver of fibrosis. However, a direct proliferative effect of serotonin on SI-NET CAFs has not been shown (Beauchamp et al. 1991, Svejda et al. 2010). Also, MF is not exclusively found in patients with elevated 5-HIAA excretion (Rodriguez Laval et al. 2017). In combination with increased knowledge of the profibrotic effects of growth factors that might be therapeutically targeted, this resulted in a shift of focus to possible other etiological agents of MF (Rybinski et al. 2014).

Transforming growth factor beta (TGFβ) family

The TGFβ family of cytokines is a pivotal regulator of proliferative and profibrotic processes. The family consists of three isoforms (TGFβ1, TGFβ2 and TGFβ3); however, the extended superfamily includes many more proteins such as bone morphogenetic proteins (BMPs). TGFβ binds exclusively to TGFβ type 1 receptor (TGFβR1) and type 2 receptor (TGFβR2). After binding to these receptors, the cellular effects are mediated via SMAD pathway (Witsch et al. 2010, Massague 2012). TGFβ signalling has a dual role with on the one hand antitumourigenic and antiproliferative

Figure 2
Development of mesenteric fibrosis in SI-NETs. Interactions of profibrotic growth factors within the tumour microenvironment, which consists among others of tumour cells, fibroblasts, immune cells and extracellular matrix (ECM). 5-HTR, serotonin receptor; CTGF, connective tissue growth factor; EGF, epidermal growth factor; EGRF, epidermal growth factor receptor; FGF2, basic fibroblast growth factor; FGRF, fibroblast growth factor receptor; PDGF, platelet-derived growth factor; PDGFRA, platelet-derived growth factor receptor; TGFα, transforming growth factor alpha; TGFβ, transforming growth factor beta; TGFβR, transforming growth factor beta receptor.
effects in physiological and early neoplastic conditions, and on the other hand, protumourigenic effects such as proliferation and invasion in later stages of malignant disease (Massague 2012). Also, it stimulates stromal cells to induce myofibroblastic differentiation and altered ECM production. Once differentiated to myofibroblast, these cells secrete TGFβ creating a self-sustained, profibrotic feedback loop (Witsch et al. 2010). Due to its profibrotic and protumourigenic effects, TGFβ is one of the most extensively studied growth factors in SI-NETs.

It has been shown that BON1 cells and tissue specimens of SI-NETs express the transcripts of all three TGFβs (β1, β2 and β3) and both receptors (TGFβR1 and TGFβR2) (Beauchamp et al. 1991, Chaudhry et al. 1994, Wulbrand et al. 1998, Wimmel et al. 2003). The expression of TGFβ isoforms differs between tissue components with all three isoforms being present in tumour tissue, but only TGFβ2 showed strong IHC staining in stroma (Chaudhry & Oberg 1993). However, no significant correlation was found with MF and expression levels of TGFβ1 and TGFβ3 in SI-NET tumour tissue (Zhang et al. 2004, Kidd et al. 2007). Also, in a small series only 2 out of 5 SI-NETs were positive for TGFβR1 and TGFβR2 in the tumour stroma, suggesting lacking expression in fibroblasts (Wimmel et al. 2003). As TGFβR2 deletion in fibroblasts seems to increase the oncogenic potential of selected epithelial cells, this finding emphasizes the importance of investigating TGFβ signalling embedded in a concept of TME (Bhowmick et al. 2004).

In vitro, it was shown that BON1 cell conditioned medium could induce TGFβ-mediated proliferation of AKR-2B cells, a mouse fibroblast cell line, suggesting that factors produced by NET cells influence fibroblast function. Furthermore, it was confirmed that TGFβ stimulation results in an increased production of TGFβ by these fibroblasts, confirming a positive autocrine feedback loop (Beauchamp et al. 1991). Moreover, CAFs isolated from a patient with a fibrotic SI-NET show increased transcription of CTGF after TGFβ1 stimulation (Kidd et al. 2007). However, the effect of TGFβ differs on the model used. TGFβ1 has an inhibitory effect on growth of normal EC and BON1 cells, while it has a proliferative effect on KRI-1 cells (Wimmel et al. 2003, Kidd et al. 2007). Exposure of KRI-1 cells to TGFβ1 resulted in phosphorylation of SMAD2, indicating an intact TGFβR2 signalling, even though KRI-1 cells have decreased TGFβR2 expression compared to normal EC cells. However, there was a decreased nuclear staining of phosphorylated SMAD2 in these cells, which is suggestive of a block at the level of SMAD nuclear translocation (Kidd et al. 2007).

Furthermore, in a series of 48 SI-NETs, 22 had mutations or deletions in genes (Banck et al. 2013). This underlines the importance of the TGFβ1 signalling pathway in SI-NETs. Tumours with inactivating mutations in TGFβ1 signalling pathway can paradoxically benefit from a TGFβ-rich microenvironment by the effects of intact signalling in the tumour stroma. These favour tumour progression, e.g. production of growth factors and stimulation of invasion and metastasis, while the suppressive effects of TGFβ signalling on tumour cells are avoided by inactivating mutations (Massague 2012).

**Platelet-derived growth factor (PDGF)**

PDGF is released in response to tissue injury, and it is shown to be involved in multiple fibrotic diseases such as scleroderma, intestinal fibrosis in Crohn’s disease and renal fibrosis (Bonner 2004). PDGF has a strong proliferative effect on fibroblasts and can induce proliferation of epithelial cancer cells (Rybinski et al. 2014). The profibrotic effects of PDGF are mediated by binding to the PDGF α- and β-receptors (PDGFRα and PDGFRβ). Expression of the receptors differs between cell types and can be induced by diverse factors, such as TGFβ. Furthermore, upregulation of both receptors is found in many fibrotic diseases, although it depends on the involved tissue which of the PDGF receptors is predominantly upregulated (Bonner 2004).

In stromal cells of SI-NETs, the PDGFRβ was detected by IHC in 66–85%. The IHC staining was mostly found adjacent to tumours cells, without staining of the tumours cells themselves. The positive cells showed frequently a fibroblastic morphology with muscle actin antigen positivity, suggesting an activated phenotype characteristic of CAFs (Funa et al. 1990, Chaudhry et al. 1992, Kalluri 2016). Furthermore, PDGFRβ immunoreactivity was more prevalent in metastases and associated with the presence of macrophages. There was no correlation with urinary S-HIAA excretion (Funa et al. 1990). On the other hand, the majority of tumour cells are positive for PDGFRα and PDGF with limited focal staining in the stroma surrounding positive tumour cells (Chaudhry et al. 1993). These results suggest that components of the TME (e.g. tumour cells, macrophages) induce PDGFRβ expression on adjacent fibroblasts and upregulate PDGFRβ activity in a paracrine fashion. Moreover, the increased expression of PDGFRβ in the stromal cells of metastases links it to tumour proliferation and metastatic potential. Together with the finding that 20% of SI-NET show copy number gains of PDGFR, suggesting augmented activation of this...
pathway in a subset of these tumours, inhibition of PDGF signalling is potentially an attractive therapeutic target (Banck et al. 2013).

Basic fibroblast growth factor (FGF2)

FGF2 is an important regulator of wound healing and is known to have a strong mitogenic effect on fibroblasts. It can be induced by TGFβ and is linked to several fibrotic disorders (Rybinski et al. 2014). Its role in cancer is less obvious. FGF2 is suggested to have anti-apoptotic, proliferative effects on tumour cells and to stimulate angiogenesis. Conversely, other studies have shown that in some conditions, FGF2 has a tumour-suppressive role, making it a complex signalling factor to investigate (Turner & Grose 2010).

Studies performed on SI-NETs showed positive IHC staining for FGF2 in 60–100% of tumour cells and adjacent stroma (Chaudhry et al. 1993, Zhang et al. 2004). Neither the prevalence nor the intensity of FGF2 staining was correlated to MF (Zhang et al. 2004). In vitro, FGF2 is shown to be expressed by BON1 cells and to stimulate proliferation of these cells (Beauchamp et al. 1991). FGF receptors are found in most SI-NETs, both in tumour and stromal cells (Facco et al. 1998, Wulbrand et al. 1998). Although the presence of FGF2 in tumour cells and stroma suggests involvement in cellular processes of the TME, the effect of FGF2 on tumour development remains uncertain. Therefore, FGF2-targeted therapy remains to be considered with caution, until the effects of FGF2 signalling are better understood (Akl et al. 2016).

Transforming growth factor alpha (TGFα) and epidermal growth factor receptor (EGFR)

TGFα and epidermal growth factor (EGF) are both ligands of EGFR, a receptor tyrosine kinase (Jorissen et al. 2003). After binding of the ligand, the EGFR activates multiple signalling pathways via its tyrosine kinase activity (Jorissen et al. 2003). Like FGF2, EGFR signalling is involved in wound healing, stimulates proliferation of mesenchymal cells and correlates with fibrotic and malignant diseases (Rybinski et al. 2014).

The expression of EGFR detected in SI-NETs differs vastly between studies, ranging from 33 to 100% (Nilsson et al. 1995, Krishnamurthy & Dayal 1997, Wulbrand et al. 1998, Shah et al. 2006). In 2006, Shah and colleagues analysed 98 NETs, of which 42 were from midgut origin, for the presence of EGFR. In 96% of the samples, there was positive IHC staining for EGFR, predominantly cytoplasmic in tumour cells and absent in the surrounding stroma (Shah et al. 2006).

Of all EGFR ligands, TGFα is most extensively investigated in SI-NETS. It was found to be present in all specimens in a series of 20 SI-NET metastases as well as in BON1 cells (Beauchamp et al. 1991, Nilsson et al. 1995). The expression of TGFα is co-localized with EGFR on cell membranes and cytoplasm of tumour cells, suggesting effective ligand binding and intracellular signalling (Nilsson et al. 1993). Also, TGFα is secreted by primary SI-NET cultures, which is not inhibited by targeting of the somatostatin receptor 2 (SSTR2) by octreotide, a somatostatin analogue (Nilsson et al. 1995). Furthermore, TGFα stimulates proliferation in SI-NET primary cell cultures and cell lines (e.g. BON1 and KRI-1) (Nilsson et al. 1995, Siddique et al. 2009). This effect can be inhibited by blocking the EGFR, suggesting an autocrine regulatory loop (Nilsson et al. 1995). However, these studies focused mostly on the proliferative effect on tumour cells, leaving the role of EGFR signalling in SI-NET-associated fibrosis elusive. Therefore, possible effectiveness of targeting EGFR signalling by tyrosine kinase inhibitors for the treatment of MF remains uncertain (Bergsland et al. 2012).

Connective tissue growth factor (CTGF, also known as CCN2)

CTGF is a member of the CCN family of growth factors, which are induced by cytokines such as TGFβ and importantly also by serotonin (Leask & Abraham 2006, Jacobson & Cunningham 2012). Although CTGF can influence cell processes independently, it acts mainly by modifying signalling of other molecules. CTGF is involved in tissue repair and pathologic tissue fibrosis. CTGF enhances profibrotic actions of TGFβ, EGF and FGF by increasing collagen synthesis, fibroblast proliferation and differentiation into myofibroblasts. As CTGF enhances the effect of tumourgenic and profibrotic growth factors, it is a potential target for therapy (Leask & Abraham 2006, Adler et al. 2010). Treatment with CTGF-neutralizing antibodies has already been shown to decrease tumour growth and metastasis in pancreatic cancer models (Leask & Abraham 2006).

SI-NETs have a high expression of CTGF compared to other neuroendocrine tumours (Kidd et al. 2007c, Cunningham et al. 2010). Immunoreactivity was strongest in SI-NET cells adjacent to fibrovascular stroma, suggesting a local effect on the tumour stroma interaction at the invasion border. Furthermore, CTGF co-localized with serotonin in 93% of tumour cells and with TGFβ...
in 80%. The tumour stroma, however, shows little CTGF immunoreactivity (Cunningham et al. 2010). High expression of CTGF in tumour tissue of NETs was found to be correlated with SI-NETs, MF and serum levels of CTGF (Kidd et al. 2007c). However to date, no association has been found in SI-NETs between serum levels of CTGF and the presence of MF.

Other growth factors
Next to the previously discussed factors, many more mediators have been found to be present in SI-NETs. However, their effects on fibrosis and MF are much less well studied. In the next section, putative profibrotic molecules will be discussed. However, further research is needed to determine their importance in the deregulation of the TME and development of MF.

Nerve growth factor (NGF) was originally characterized by its effect on the development of peripheral sensory and sympathetic neurons. However, NGF signalling has protumourigeneic, angiogenic and profibrotic effects also and is known to induce TGFβ expression (Vizza et al. 2015, Boilly et al. 2017). In SI-NETs, NGF expression is present on tumours cells and decreased in patients with mesenteric angiopathy and extensive fibrosis as compared to patients with limited MF (Nilsson et al. 1993, Zhang et al. 2004).

Substance P (SP) is a member of the tachykinin family and regulates biological functions mainly by binding to neurokinin-1 receptor (NK1R). SP is expressed by SI-NETs and is one of the kinins potentially involved in flushing and carcinoid heart disease (Vinik et al. 1990, Facco et al. 1998, Niederle et al. 2016). Furthermore, SP acts as a profibrotic cytokine in inflammatory fibrotic diseases such as intestinal and liver fibrosis and NK1R antagonist have been found to counteract the SP-induced fibrosis and secretion of profibrotic growth factors such as TGFβ (Koon et al. 2010, Wan et al. 2017). Also, SP has a proliferative and promigratory effect in cancer (Esteban et al. 2006). NK1R antagonists are already in clinical use as antiemetic drugs and the recent research done in SP/NK1R pathway in fibrosis and mitogenesis generated new interest in NK1R antagonist as an antitumour and antifibrotic treatment option (Esteban et al. 2006, Wan et al. 2017). However, the contribution of the SP/NK1R pathway to development MF is unknown, and further research is essential before use of NK1R antagonist as antiproliferative and antifibrotic therapy can be considered.

The insulin-like growth factor (IGF) system is an important regulator of cell proliferation and ageing processes. The most important molecules in this signalling network are the ligands IGF1 and IGF2, the receptors IGF1R and IGF2R and the IGF-binding proteins (IGFBPs). Increased IGF signalling by increased levels of ligands or receptors is associated with increased risk of cancer. IGFBPs modulate the bioavailability of IGFs and their role in cancer development is complex and bimodal (Pollak et al. 2004). The majority of SI-NETs expresses IGF1, IGF1R and IGFBPs (Nilsson et al. 1993, Wulbrand et al. 2000). Media from primary SI-NETs cultures also contained IGF1 and incubation with IGF1 induced proliferation in BON1 cells and IGF1R-positive primary cultures (Nilsson et al. 1993, von Wichert et al. 2000). Conversely, only 1 out of 9 SI-NET specimens had expression of IGF2 or IGF2R (Wulbrand et al. 2000). As regulation of IGF signalling is complex and its function during SI-NET-associated fibrogenesis is unknown, further studies are needed to determine the role of the IGF system in MF (Pollak et al. 2004, Feghali-Bostwick 2005, Nishizawa et al. 2016).

Developmental signalling pathways
Embryonic development is tightly regulated by a limited number of signalling pathways. In adulthood, these pathways regulate tissue homeostasis and are known to be deregulated in aberrant wound healing, chronic fibrosis and cancer (Rybinski et al. 2014). The research performed in SI-NETs on three well-known pathways (Hedgehog, Notch and Wnt), which are all implicated both in fibrotic diseases and cancer, will be discussed below (Hoeft & Kramann 2017).

Sonic Hedgehog (SHh) is the most studied Hedgehog ligand. Binding of SHh to Patched, its receptor, promotes expression of Gli transcription factors (Rybinski et al. 2014). Gli1 is the most abundant transcription factor and is shown to induce the expression of Snail. Subsequently, upregulation of Snail results in loss of E-cadherin and induction of epithelial–mesenchymal transition (EMT) (Rybinski et al. 2014). In models of lung, kidney, heart and liver fibrosis, SHh is upregulated after tissue injury. Also, SHh stimulation induced matrix production and myofibroblastic differentiation in vitro (Hoeft & Kramann 2017). In a series of 37 SI-NETs, the Hedgehog signalling pathway was assessed by IHC. SHh expression was found in 73% of tumours and 59% stained positive for Snail. Snail-positive cells were mostly identified at the invasive front of the tumours and had suppressed E-cadherin expression (Fendrich et al. 2007). Also, it was found that Snail1 expression is associated with worse prognosis and metastatic spread in NETs (Galvan et al. 2013). However,
the role of Hedgehog signalling in the development of MF is still undetermined.

Aberrant Notch signalling is found in cancer and fibrosis (Hoeft & Kramann 2017, Nowell & Radtke 2017). Increased Notch signalling has a profibrotic effect, as is demonstrated in renal and hepatic fibrosis (Hoeft & Kramann 2017). On the other hand, Notch signalling in cancer is more complex with pro- and antitumourigenic effects depending on the context (Nowell & Radtke 2017). Notch canonical signalling is induced by ligand binding to one of the four Notch receptors. This ligand binding promotes the expression of target genes of which hairy/enhancer of split (HES) and hairy/enhancer of split related with YRPW motif (HEY) are best characterized. Also, canonical Notch signalling represses expression of transcriptional factor achaete-scute complex-like 1 (ASCL1) (Nowell & Radtke 2017). The role of ASCL1 in tumourigenesis is complex and not well studied, but in transfected BON1 cells, Notch signalling mediated loss of ASCL1 and decreased serotonin production (Kunnimalaiyaan et al. 2005, Nakakura et al. 2005). However, the findings on Notch signalling in SI-NETS in vivo are conflicting. In 2005, Nakakura and colleagues demonstrated the transcription of NOTCH1, NOTCH2, NOTCH3, HES1, HEY1 and HEY2 in a series of 8 SI-NETS to be comparable to normal tissue, but upregulated ASCL1 transcription was found in 50% of the tumours (Nakakura et al. 2005). However, later research showed no NOTCH1 and HES1 IHC positivity on a tissue micro-array of 31 SI-NETs, underscoring the need for further research to unravel the role of this signalling pathway in SI-NETs (Wang et al. 2013).

The canonical Wnt/β-catenin signalling controls gene expression by regulating the amount of β-catenin. In the absence of a Wnt signal, β-catenin cytoplasmic levels are maintained low by binding to a destruction complex consisting of multiple proteins (Hoeft & Kramann 2017). One of these proteins is the adenomatous polyposis coli protein (APC), which associated with familial adenomatous polyposis and sporadic colon cancer (Bottarelli et al. 2013, Hoeft & Kramann 2017). In the presence of a Wnt signal, β-catenin dissociates and the destruction complex is inactivated. This results in an accumulation of β-catenin that consequently can translocate to the nucleus where it activates target gene expression. Although mutations in the APC gene have been found in 23% of SI-NET cases, only membranous expression of β-catenin has been found in SI-NETs (Fendrich et al. 2007, Bottarelli et al. 2013). Furthermore, the severity of MF was not correlated with β-catenin expression (Zhang et al. 2004). These findings suggest a minor role for Wnt/β-catenin signalling in SI-NET development and fibrosis.

Learning from other diseases
In the previous part, we have discussed a variety of known and putative mediators of fibrosis in SI-NETS. However, research done both in malignant and benign diseases with profound fibrosis suggest an even wider array of factors that influence tissue homeostasis and when deregulated can induce fibrosis. Since the microenvironment in wound healing shares many commonalities with the stroma in chronic fibrosis, the role of inflammation and the adaptive immune response seems important also for the development of fibrosis. Secretion of specific profibrotic cytokines and chemokines within the tissue microenvironment is found to be largely homologous in different fibrotic diseases. Next to immune system, tissue hypoxia and activation of the renin–angiotensin system (RAS) are also shown to be present in many fibrotic diseases such as cardiac and hepatic fibrosis (Wynn 2008). Angiotensin II is able to induce fibrosis via the AT1 receptor and increased expression of profibrotic growth factors such as TGFβ, FGE2 and PDGF (Ager et al. 2008, Murphy et al. 2015). Furthermore, overexpression of AT1 receptor is found in many malignancies, linking RAS to tumour and fibrosis development (Ager et al. 2008). Finally, recent insights into microRNAs show an even more intricate network of regulatory mechanisms of fibrogenesis, especially since numerous common microRNA alternations are found over a wide array of different fibrotic diseases, such as renal, hepatic and pulmonary fibrosis (Vettori et al. 2012). As there is a paucity of research on the precise role of these mediators in SI-NET-associated fibrosis, we have not elaborated on these subjects. However, focusing future research on these mediators could illuminate new therapeutic targets.

Treatment of mesenteric fibrosis
MF can cause severe abdominal complications such as intestinal obstruction, oedema or ischaemia (Makridis et al. 1990). To date, treatment of MF is limited to symptomatic relief by bowel resection or bypass (Makridis et al. 1990, Modlin et al. 2004). However, abdominal surgery in patients with MF is challenging and can lead to complications such as short-bowel syndrome and bile-salt diarrhoea (Makridis et al. 1990). Furthermore, resection is not always possible due to the patient’s clinical condition or disease characteristics such as the location of the mesenteric mass and encasement of the superior mesenteric artery.
mesenteric vein (SMV) or superior mesenteric artery (SMA). Consequently, vascular stenosis and the following ischaemia or bowel wall oedema is an important cause of abdominal complaints in patients with MF. Therefore, stenting of the SMV in case of stenosis and encasement has been suggested for symptom relief. In a small series of seven patients with SI-NET and proven SMV obstruction, stenting resulted in four patients in improvement of their symptoms (Hellman et al. 2010). However, these results have not been replicated in larger studies, and in our experience, SMV stenting is a complex procedure with often disappointing results. However, in the era of targeted therapy and with increasing knowledge of the pathobiological processes, possible new avenues for treating and preventing MF can be developed. In the next section, we discuss possible antifibrotic agents by linking the known mediators of fibrosis in SI-NETs with drugs currently used to target these mediators, both in SI-NETs and other diseases (Table 1).

**Somatostatin analogues (SSAs)**

SSAs are first-line therapy with proven efficacy on tumour growth control in SI-NETs (Pavel et al. 2016). Two commercially available agents, octreotide and lanreotide, also effectively reduce carcinoid syndrome symptoms (Modlin et al. 2010, Pavel et al. 2016). Moreover, SSAs are known to attenuate fibrosis in animal models of peritoneal sclerosis, pulmonary and liver fibrosis by among others reducing the secretion of profibrotic growth factors such as TGFβ (Lang et al. 2005, Borie et al. 2008, Ertilav et al. 2011). However, the effect of SSAs on MF in SI-NETs has not been examined. Also, while approximately half of the patients have a reduction of bioactive peptide secretion of >50%, complete biochemical response in carcinoid syndrome occurs only in a minority of patients (Modlin et al. 2010). Since increased urinary 5-HIAA excretion is associated with MF, effective inhibition of serotonin production could reduce the risk of development and growth of MF in patients with SI-NET. It can therefore be hypothesized that SSA therapy should aim to fully normalize serotonin production in order to minimize development of fibrosis, although this should be evaluated in prospective studies.

**Serotonin synthesis inhibitors**

Serotonin is synthesized in two enzymatic steps from the essential amino acid tryptophan. First, tryptophan is hydroxylated by tryptophan hydroxylase (THP) to 5-hydroxytryptophan (5-HTP) and in a second step, 5-HTP is decarboxylated to form serotonin (Mohammad-Zadeh et al. 2008). Almost sixty years ago, the first attempts to block peripheral serotonin synthesis were undertaken. These first attempts aimed to inhibit 5-HTP decarboxylation by agents such as phenylacetic acid and α-methyl-dopa. These drugs had a moderate effect on decreasing serotonin production and side effects that limited their clinical use (Sandler & Close 1959, Sjoerdsma et al. 1960).

The next step was to inhibit THP. Using para-chlorophenylalanine (PCPA), serotonin production and carcinoid syndrome symptoms could be reduced. However, the psychiatric side effects precluded therapeutic use of the compound (Engelman et al. 1967). The search for THP inhibitors that primarily inhibit peripheral serotonin synthesis resulted in the development of telotristat. This year, a phase III study was published comparing the effect of telotristat with placebo on carcinoid syndrome symptoms. Compared to placebo, the majority of patient had >30% reduction of 5-HIAA excretion (Kulke et al. 2017). However, also this study did not aim to completely normalize serotonin production nor was the effect on fibrosis assessed.

**5-HT receptor antagonist**

Next to inhibition of serotonin synthesis, targeting 5-HT receptors can modify serotonin signalling. As the profibrotic effects of serotonin seem to be mainly mediated via the 5-HT1A/B and 5-HT2A/B receptors, drugs targeting these receptors should be considered for antifibrotic treatment (Svejda et al. 2010, Mann & Oakley 2013). Non-selective 5-HT2 receptor antagonists such as cyproheptadine and ketanserin were found to be able to reduce diarrhoea in the context of carcinoid syndrome (Robertson 1990, Moertel et al. 1991). However, due to the modest effects compared to SSAs and serious adverse effect of ketanserin, the clinical utility of these drugs is limited (Robertson 1990, Moertel et al. 1991).

However, advancements have been made with new potential antifibrotic agents. Terguride, a 5-HT2A/B receptor antagonist, is proven to reduce the profibrotic effects of serotonin in animals (Hauso et al. 2007). Furthermore, in a phase II study in scleroderma patients it was well tolerated and resulted in amelioration of the skin fibrosis (Distler et al. 2016). Even though more research is needed to establish the effect of terguride on SI-NET-associated fibrosis, it sparks hope for a potent, well-tolerated antifibrotic therapy.
Tyrosine kinase inhibitors (TKIs)

Tyrosine kinases consist of a large family of enzymes that are important mediators of cellular signal transduction. Receptor tyrosine kinases are activated by diverse extracellular signal molecules such as EGF, TGFα, PDGF and FGF (Rybinski et al. 2014). Furthermore, the TGFβ signalling pathway is also influenced by downstream effects of receptor tyrosine kinase signalling (Massague 2012). As tyrosine kinase signalling pathways are involved in both cancer and fibrosis development, they are an interesting therapeutic target in SI-NETs (Rybinski et al. 2014).

Unfortunately, the efficacy of TKIs in SI-NETs on tumour growth suppression seems limited and accompanied by significant toxicity (Hobday et al. 2007, Kulke et al. 2008, Phan et al. 2010, Chan et al. 2013). Furthermore, the studies examining the effect of TKIs in SI-NETs focused neither on reduction of fibrosis nor...
Studies | Effect in SI-NETs | Adverse effect | Reference
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First line of treatment for functional or progressive SI-NET | Half of patients have reduction of bioactive peptide secretion of >50% | GI complaints, cholelithiasis | Modlin et al. (2010), Pavel et al. (2016)
Phase III study on carcinoid syndrome symptoms | Reduction of stool movements | Nausea, asymptomatic increased gamma-glutamyl transferase | Kulke et al. (2017)
No study in fibrotic diseases | N/A | Nausea | Distler et al. (2016)
Phase II scleroderma patients | Reduction of peritoneal sclerosis, pulmonary and liver fibrosis in animal models | GI complaints, cholelithiasis | Lang et al. (2005), Borie et al. (2008), Ertl et al. (2011)
No study in SI-NETs | No data in SI-NETs. Overall are ACE-inhibitors well tolerated | No toxicity reported | Fendrich et al. (2014)
Preclinical studies on fibrogenesis | Sirolimus; reduction of KRJ-I cell proliferation | Infections, GI complaints and myelosuppression | Kidd et al. (2007a), Akl et al. (2016), Pavel et al. (2016)
Preclinical studies on cancer proliferation in SI-NETs | Enrolment SI-NET stopped due to insufficient efficacy | Increased toxicity compared to everolimus monotherapy | Bergsland et al. (2012)
Preclinical studies on fibrogenesis in pulmonary and liver fibrosis | Reduction of KRJ-I cell proliferation | Stomatitis, GI-disorders | Kidd et al. (2007a)
Preclinical studies on SI-NETs | N/A | Well tolerated | Adler et al. (2010)
Phase I study in patients with diabetes and microalbuminuria | N/A | | |
No study in SI-NETs

S-HT, serotonin; ACE, angiotensin-converting enzyme; CAFs, cancer-associated fibroblasts; CTGF, connective tissue growth factor; EGFR, epidermal growth factor receptor; FGF, fibroblast growth factor; FGF2, basic fibroblast growth factor; GI, gastrointestinal; MF, mesenteric fibrosis; PDGF, platelet-derived growth factor; PDGFR, platelet-derived growth factor receptor; SI-NET, small intestinal neuroendocrine tumour; SSAs, somatostatin analogues; TGF, transforming growth factor alpha; TGFβ, transforming growth factor beta; THP, tryptophan hydroxylase.


While the focus in SI-NETs has been on inhibition of vascular endothelial growth factor (VEGF) signalling by TKIs and tumour growth, research on fibrotic diseases such as scleroderma focuses on TKIs targeting c-abl kinases and PDGF receptors. By blocking these kinases, important downstream signalling molecules such as TGFβ are reduced (Distler & Distler 2010). As these signalling molecules are also involved in tumourigenic processes, TKIs such as imatinib, which targets c-abl kinases and PDGFRs, could result in antitumourigenic changes in the TME (Pietras & Hanahan 2005). Furthermore, clinical studies with imatinib show decreased organ fibrosis in patients with scleroderma and pulmonary fibrosis (Distler & Distler 2010). Since the signalling pathways involved in the development of fibrosis in SI-NETs are similar to those fibrotic diseases, the use of TKIs should be extended beyond tumour growth control and also be evaluated as antifibrotic therapy.
Other antifibrotic agents

As fibrotic diseases share many commonalities with cellular processes in SI-NETs, potential effective antifibrotic agents can be identified by assessing drugs used in fibrotic diseases such as retroperitoneal fibrosis, idiopathic pulmonary fibrosis and scleroderma, but also more common disorders such as cardiac and renal fibrosis (Rybinski et al. 2014). Often fibrosis occurs after tissue injury, and hypertension is a frequent cause of heart and kidney injury. As RAS is an important regulator of cardiovascular homeostasis, inhibition of RAS by angiotensin-converting enzyme (ACE) inhibitors and angiotensin II receptor blockers (ARBs) forms one of the cornerstones of hypertension treatment (Murphy et al. 2015). However, the functions of RAS signalling extend beyond cardiovascular homeostasis. As discussed earlier, RAS activation is also involved in tumour and fibrosis development (Ager et al. 2008, Murphy et al. 2015). Epidemiological studies provide evidence that RAS inhibition may reduce tumour development and progression (Ager et al. 2008). In addition, in vitro ACE inhibition is shown to reduce BON1 cell proliferation (Fendrich et al. 2014). Since ACE inhibitors and ARBs are commonly used as antihypertensive treatment with few side effects, they might be considered as coadjuvant therapy in SI-NET treatment, but further studies are needed to confirm their clinical efficacy in this setting.

Another antifibrotic agent used in fibrotic diseases such as desmoid tumours and retroperitoneal fibrosis is tamoxifen (van Bommel et al. 2006). Tamoxifen is a synthetic nonsteroidal selective oestrogen receptor modulator (SERM), developed for the treatment of breast cancer. The antifibrotic effect seems to be mediated by an inhibitory effect of tamoxifen on TGFβ secretion by fibroblasts (Mikulec et al. 2001). Moreover, tamoxifen has been used in SI-NETs for tumour growth control and amelioration of carcinoid syndrome symptoms with varying success (Statopoulos et al. 1981, Myers et al. 1982, Moertel et al. 1984, Arganini et al. 1989). However, better patient selection and focus on the antifibrotic effects might establish tamoxifen as a treatment option for fibrotic complications of SI-NETs.

Conclusion

Since the development of a variety of palliative treatments, the survival of patients with metastasized SI-NETs has improved. As a result, morbidity caused by MF and the lack of therapeutic options have become major issues. In order to elucidate potential new roads for scientific research and therapies to improve the management of MF, we first discussed the pathobiology of MF. A deregulation of the cellular processes in the TME is at the core of fibrosis development. However, research on fibrosis in SI-NETs has just started to focus on the TME and should be extended beyond the interaction between tumour cells and fibroblasts. Especially knowledge about the changes in the composition and biomechanical properties of the ECM during tumourigenesis and fibrosis development in SI-NETs is lacking. In order to gain more insight in cellular processes in the TME, in vitro research should shift from 2D monocultures to more intricate systems such as cocultures and 3D cultures and the possibility of in vivo models should be explored. Also, the use of the BON1 cell line as an in vitro model for SI-NETs is not optimal as it is of pancreatic origin and known to differ substantially in gene level transcripts from EC cell-derived NET cell lines (Grozinsky-Glasberg et al. 2012). Since tumour cells and other components of the TME communicate via a multitude of hormones, growth factors and signalling pathways, we have discussed the putative profibrotic factors in SI-NETs. However, the exact effects and modulation of many of the discussed signalling molecules remain incompletely mapped. Therefore, targeted inhibition of these pathways should first be investigated in experimental models. Also, insights gained in other fibrotic diseases point to an even more intricate network of regulators of tissue homeostasis and fibrosis development with factors such as cytokines, microRNAs and hypoxia and RAS signalling. To date, therapeutic options are mainly limited to surgery. However, due to patient or disease characteristics, not all patients can undergo surgery, and intestinal resection can lead to complications with significant morbidity. Therefore, we discussed possible alternatives for patients with SI-NETs suffering from the complications of MF. However, in order to be able to determine the efficacy of these treatments, it is important to gain insight into the natural development of MF and design clinical trials focused on assessing drug effects on MF.
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