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Chapter 2

Role of TGF- β in the tumor stroma

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Abstract

Recent findings have demonstrated that the tumor stroma actively contributes to tumorigenesis. The communication of malignant cells and tumor stromal components is orchestrated in part by a network of growth factors. One of these growth factors is transforming growth factor- β (TGF- β), a secreted multifunctional protein that acts in a highly cellular contextual manner. TGF- β can either stimulate or inhibit the tumor-promoting effects of the different components of the tumor stroma. In this review, we discuss our current understanding on how TGF- β influences different stromal compartments.

Introduction

For many years, cancer research focused on the genetic and phenotypic changes in malignant cells in order to understand progression of tumor growth and metastasis. However, it is evident today that the tumor stroma is more than an innocent bystander. The involvement of stroma may already occur as early as during transformation of the epithelial cells. This was demonstrated in animal studies in which either irradiation or treatment with carcinogenic chemicals of stromal cells, but not of epithelial cells, is sufficient to give rise to tumors from normal epithelial cells [1,2]. Moreover, genetic studies in breast tumors revealed that mutations also occur in the stromal compartment [3-5].

The stroma in the vicinity of the tumor consists of various cell types such as fibroblasts, immune cells, cells lining the blood and the lymphatic vessels, which are embedded in extracellular matrix (ECM) and produce soluble factors. The relative size and composition of the tumor stroma differs between tumors, and is distinct from normal tissue stroma. Malignant cells produce growth factors which can induce a 'reactive' stroma that will favor tumor cell proliferation, migration and invasion and angiogenesis [6]. This 'reactive stroma' has also been described as desmoplasia [7]. Tumor stroma resembles the stroma usually found in injured tissues. However, wound 'reactive' stroma is resolved after the tissue is repaired, whereas tumor stroma is not. Therefore, tumors have been described as 'wounds that do not heal' [8]. TGF- β is an important regulator of wound healing [9].

TGF- β is well-known for its dual role in malignant cancer epithelial cells: in early stages it blocks tumor growth, whereas in later stages it stimulates invasion and metastasis of tumor progression (reviewed in [10]). In this review, we will focus on the effects of TGF- β on the different components of the tumor stroma.

Cancer Associated Fibroblasts

Fibroblasts are the predominant cells in tumor stroma of almost all primary carcinomas [11], suggesting that fibroblasts may support or induce tumor progression. Moreover, progression to invasive carcinoma is accompanied by the differentiation of fibroblast to myofibroblasts or so-called cancer-associated fibroblasts (CAF) [6,12]. CAFs are an important source of growth factors, such as insulin growth factor (IGF), vascular endothelial growth factor (VEGF), epithelial growth factor (EGF), hepatocyte growth factor (HGF) and TGF- β [13-16], as well as matrix metalloproteases (MMPs) [17] and ECM components [18].

The origin of CAFs still remains controversial. CAFs can be derived *in situ* by transdifferentiation of cells of the connective tissue surrounding the carcinoma. Immunohistochemical analysis of primary tumors revealed that fibroblasts are the main source of myofibroblasts in breast cancer [19]. Interestingly, it has been shown that TGF- β can transdifferentiate fibroblasts into myofibroblasts *in vitro* [20,21]. However, TGF- β may not be the essential factor in CAF differentiation, since TGF- β receptor type II (T β RII) knockout fibroblasts are still able to promote tumorigenesis (see below).

There is also evidence that malignant cells attract mesenchymal stem cells (MSC) to differentiate into CAFs. Studies exploring the potential of MSC to deliver anticancer agents showed that these cells are incorporated in tumors [22-26]. Co-transplantation of MSCs with breast tumor cells enhances metastasis to the lung [27]. Moreover, transplantation of eGFP+ hematopoietic stem cells demonstrated that stem cells can give rise to fibroblasts as well as perivascular cells [28]. TGF- β can induce fibroblast migration [29], but whether TGF- β is involved in MSC migration and their transformation into CAFs remains to be determined.

Other studies have suggested that epithelial cells can transdifferentiate into myofibroblasts via TGF- β dependent epithelial mesenchymal transition (EMT) [30,31]. EMT occurs normally during embryonic development, but has also been observed in the progression of some tumors. Thus, malignant epithelial cells could transdifferentiate into fibroblasts themselves. The observation that stromal and breast cancer cells share genetic alterations supports the idea that tumor cells undergo EMT and differentiate to CAFs [5]. Moreover, a fibroblast cell line established from breast tumor stroma carried the same X-chromosome inactivation pattern as the malignant epithelial cells and expressed certain epithelial markers, suggesting that these fibroblasts are of epithelial origin [32]. A process similar to EMT is the so-called endothelial to mesenchymal transition. Recent evidence suggests that endothelial cells can give rise to CAFs through this process, which can be induced by TGF- β *in vitro* [33].

TGF- β signaling

TGF- β family members are multifunctional cytokines with pivotal roles in several cellular functions including cell growth, differentiation, extracellular matrix production, motility and regulation of the immune system. Three TGF- β isoforms (1, 2 and 3) have been identified [34]. TGF- β is initially secreted as a latent complex that is activated by components of the extracellular matrix. Activated TGF- β can bind to its cell surface receptors. There are three types of transmembrane TGF- β receptors, namely type I, II and III (T β RI, T β RII and T β RIII). T β RI and T β RII are serine/threonine kinase receptors, whereas T β RIII are accessory receptors with short intracellular domain that lacks catalytic activity. Mature TGF- β 1 and 3 bind directly to T β RII, whereas TGF- β 2 binds to T β RII only in the presence of betaglycan, a T β RIII. Once TGF- β binds to T β RII, the T β RI receptor is recruited into the complex and is phosphorylated by T β RII on serine and threonine residues in a glycine-serine rich juxtamembrane intracellular domain [35]. T β RI, in turn, phosphorylates specific members of the Smad family, i.e. the receptor-regulated (R)-Smads Smad2 and Smad3 at two carboxy terminal serine residues. Receptor phosphorylated Smad2 and 3

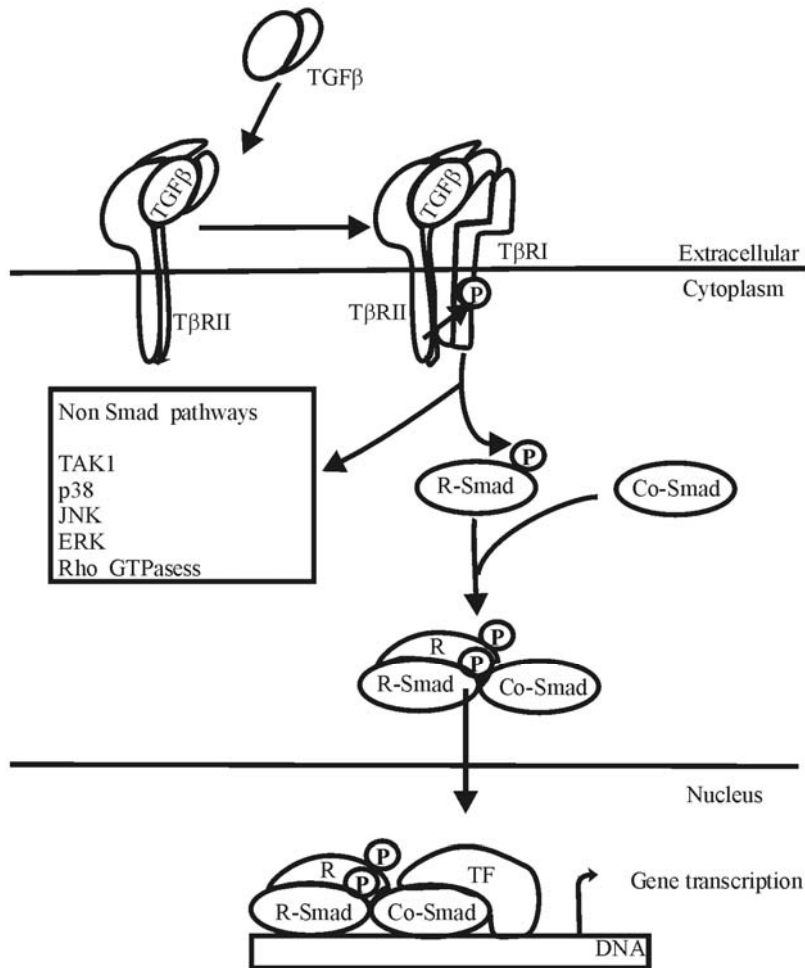


Figure 1. Overview of the TGF-β signaling. TGF-β homodimer binds to TβRII receptor resulting in recruitment and phosphorylation of TβRI receptor. Subsequently, TβRI phosphorylates receptor regulated Smads (R-Smads), which then can associate with the common Smad (Co-Smad) in a heterotrimeric complex. This complex translocates to the nucleus, where it affects gene transcription in cooperation with other transcription factors (TF). In addition to the Smad-dependent pathways, non-Smad pathways can be activated by TGF-β.

form heterotrimeric complexes with the common mediator Smad (Co-Smad) Smad4.

These complexes then translocate to the nucleus, where they regulate transcription of TGF-β target genes in collaboration with other transcription factors (TF) (Figure 1) [34].

In most cells types activin receptor-like kinase 5 (ALK5) is the predominant TβRI receptor. In certain cell types TGF-β can signal via another TβRI. In endothelial cells besides

ALK5, ALK1 can also be activated in response to TGF- β . ALK5 signaling results in the activation of Smad2 and Smad3 and ALK1 activation results induces Smad1, Smad5 and Smad8 phosphorylation [36,37] (reviewed in [38]).

In addition to the previously described TGF- β Smad pathway, TGF- β receptor activation results in activation of several other non-Smad signaling pathways. Non-Smad signaling pathways can involve TGF- β -activated kinase-1 (TAK-1), extracellular regulated kinase (ERK), Jun kinase (JNK), p38 and Rho GTPases, which can cross-talk with the Smad pathways [34,39].

Role of TGF- β in tumor stroma interactions

Studies on the role of TGF- β signaling in stromal-epithelial interactions in mammary gland development suggested that stromal fibroblasts can regulate epithelial cell morphogenesis and development. Specific overexpression of a dominant negative TGF- β type II receptor in mouse mammary stromal fibroblasts resulted in an increase in lateral branching of the ductal epithelium that was correlated with increased HGF mRNA expression [40]. In tumors, TGF- β is also important for the communication between epithelial malignant cells and stromal cells. For instance, inhibition of TGF- β signaling by a soluble extracellular domain of T β RII, a latency-associated peptide (LAP) or TGF- β 1 neutralizing antibody resulted in inhibition of tumor growth and angiogenesis in a reactive stroma xenograft model [41]. Moreover, transplantation of TGF- β 1 overexpressing fibroblasts with normal human epithelial cells gave rise to breast tumors [42].

Effects of TGF- β on CAFs

One of the first studies on the role of TGF- β in CAFs was done in a tissue specific T β RII knockout mouse model using the fibroblast specific protein-1 (FSP1, S100A4) promoter to drive expression of Cre recombinase *in vivo* [43]. The endogenous FSP1 promoter is active in a subset of the stromal fibroblasts that reside in tissues including prostate, forestomach, and skin. FSP1-Cre inducible T β RII knock out mice developed squamous cell carcinoma of the forestomach and prostatic intraepithelial neoplasia, accompanied by an

increased abundance of fibroblasts. Disruption of TGF- β signaling appears to result in tissue-specific fibroblast activation, with increased proliferation and upregulation of growth factor expression such as HGF, with parallel increased levels of phosphorylated c-Met, the HGF receptor [43].

In order to investigate the role of TGF- β signaling in the reactive stroma in breast cancer Cheng and colleagues used the FSP1 Cre lox system to conditionally delete the T β RII in mouse mammary fibroblasts [44]. Co-implantation of mouse mammary carcinoma cells with T β RII-FSP knockout fibroblasts under the renal capsule resulted in increased tumor growth and invasion compared to tumors derived from implantation of the mammary carcinoma cells with T β RII-FSP wild type cells. Moreover, the T β RII-FSP knockout tumors displayed increased tumor cell survival, proliferation, and tumor angiogenesis. This phenotype was correlated with increased expression of macrophage-stimulating protein (MSP), HGF and TGF- α [44]. The effects of T β RII downregulation on tumor progression could be blocked with a c-Met pharmacologic inhibitor (EXEL-7592) and with siRNA targeting c-Met [45].

The above mentioned studies clearly indicate a suppressive role for TGF- β in the tumor-promoting activities of CAFs. However, there are studies suggesting that TGF- β promotes the tumor-supportive effects of CAFs. Verona et al examined the role of TGF- β in a xenograft co-transplantation model of fibroblasts and prostate tumor cells [46]. In their study, tumor latency is prolonged when fibroblasts expressing dominant negative T β RII are cotransplanted, suggesting that intact TGF- β signaling is needed for tumor progression. Micro-array analysis revealed that TGF- β 3 induced expression of genes involved in ECM remodeling as well as growth factors and factors typical for myofibroblasts [46]. In a similar prostate tumor mouse model, it was found that co-transplantation of T β RII knockout or dominant negative Smad3 CAFs resulted in decreased tumor growth and microvessel density. This was accompanied by a decrease in stromal fibroblast growth factor 2 (FGF-2) staining. Re-expression of FGF-2 in the CAFs rescued the effect of impaired TGF- β signaling [47]. The discrepancy between these results and the results discussed before can be explained by the different models used in

these studies, i.e. syngeneic and spontaneous tumor models versus human xenograft models. Moreover, the level of downregulation of TGF- β pathway in the different models might be different. For example, overexpression of dominant negative T β RII or Smad3 might not result in complete abrogation of TGF- β signaling or the non-Smad signaling pathways might be perturbed. Further investigation is needed to elucidate the mechanisms behind this dual effect of TGF- β .

TGF- β and the local road: the ECM

One of the main characteristics of reactive stroma is remodeling and changes in the composition of ECM. Cancer cells and CAFs secrete ECM proteins, such as collagen, fibronectin, as well as ECM-degrading enzymes, such as MMPs. ECM remodeling results in changes in the microenvironment that facilitate local proliferation, invasion and metastasis of cancer cells. Collagen fibers serve as tracts along which cancer cells can migrate [48]. In addition, the ECM network accommodates transport of nutrients and waste products [49].

Several studies have shown an important role of TGF- β in ECM synthesis. TGF- β induces expression of collagens, fibronectin, tenascin and basement membrane components such as laminin. Moreover, TGF- β regulates also expression of MMPs, and induces expression of protease inhibitors such as tissue inhibitors of metalloproteases (TIMPs) and plasminogen activator inhibitor-1 (PAI-1) [50-52]. Changes in the ECM are sensed by integrins. This allows the cells to organize the cytoskeleton and activate intracellular signaling pathways. Cancer cells tend to express integrins that activate signaling pathways that promote proliferation, survival and migration [53]. TGF- β has been shown to induce expression of the latter integrins, thus contributing to invasion [54,55].

TGF- β and tumor angiogenesis

Tumor growth over a certain size requires formation of new blood vessels, to provide nutrients and oxygen and remove waste and toxins, a process referred to as tumor

angiogenesis. Several *in vitro* and *in vivo* studies have revealed the importance of TGF- β signaling in physiological and tumor angiogenesis, since TGF- β regulates both endothelial cells and the surrounding vascular smooth muscle cells and pericytes in a context-dependent manner (reviewed in [56]). Several studies have provided evidence for the role of TGF- β on tumor angiogenesis. High TGF- β mRNA expression in breast tumors or high levels of circulating TGF- β in patients with carcinoma correlate with increased angiogenesis and poor patient prognosis [57]. Prostate carcinoma cells overexpressing TGF- β showed enhanced tumor angiogenesis in tumor xenografts, while local administration of neutralizing antibodies to TGF- β 1 strongly reduced tumor angiogenesis. Moreover, intraperitoneal injection of TGF- β 1 antibodies reduced angiogenesis and tumorigenicity of a renal carcinoma cell line *in vivo* [58].

In endothelial cells, TGF- β can signal through both ALK5 and ALK1 pathways, which have opposing effects on angiogenesis. Signaling through ALK1 stimulates endothelial cell proliferation and migration, whereas signaling through ALK5 inhibits these processes [36]. The balance between these two pathways is skewed towards an ALK1 response by endoglin, another T_{RIII} [59]. TGF- β plays also important role in the differentiation of MSC into vascular smooth muscle cells and pericytes [56].

Besides the direct effects of TGF- β on the vasculature, TGF- β can indirectly induce tumor angiogenesis by inducing production of angiogenic factors such as VEGF, FGF, connective tissue growth factor (CTGF) and platelet derived growth factor (PDGF) from different cell types in the tumor microenvironment [60,61]. In addition, TGF- β may regulate angiogenesis through induction of pro-angiogenic proteases such as urokinase Plasminogen Activator (uPA) [62] and MMPs [50,63,64]. Degradation of ECM components by MMPs may release certain angiogenic growth factors and specific cleavage of angiogenic growth factors may activate latent growth factors [65].

TGF- β and the immune system

Another important component of reactive stroma is the immune system. Infiltrations of cells of both the innate and the adaptive immune system have been found to affect both tumor initiation and progression [66]. TGF- β plays an important role in regulation of immune responses [67]. Numerous studies have demonstrated the importance of tumor associated macrophages (TAM) in tumor progression [68,69]. TAMs are an important source of pro-angiogenic factors [70-72] and have been implicated in tumor invasion [73]. TGF- β stimulates chemotaxis of monocytes, the precursors of macrophages, in head-and-neck squamous-cell carcinomas [74]. The phenotype of TAMs is similar to that of a subset of macrophages, which are poor stimulators of anti-tumor immunity. Differentiation of monocytes towards this phenotype is possibly regulated by TGF- β [75].

Other innate immune cells that have been found in tumors are neutrophils and mast cells. Depletion of mast cells ablated tumor progression in a mouse model of skin tumors. Mast cells may contribute to progression by secretion of pro-angiogenic proteases [76]. Neutrophils can promote tumor destruction, but also tumor growth by release of VEGF [77] and MMP9 [78]. As has been observed during wound healing, TGF- β might recruit mast cells and neutrophils in tumors [67].

Tumor infiltrating T cells have a T-helper cell 2 (Th2) phenotype which skews the immune response towards activation of B cells [66]. Although the antibodies produced by B cells can have anti-tumorigenic activity, there is also data indicating that B cells play a role in the recruitment of innate immune cells to tumors [79]. Kim and colleagues showed that T cell specific deletion of Smad4 in mice, results in aberrant expansion of the gastrointestinal stromal compartment, increased infiltration of antibody-producing B cells and T cell maturation towards the Th2 phenotype [80]. Interestingly, T cell specific deletion of Smad4 resulted in spontaneous development of gastrointestinal carcinomas and oral squamous cell carcinomas. These tumors are similar to tumors observed in Familial Juvenile Polyposis (FJP), in which germline mutations in Smad4 are frequently found [80]. Additionally, disruption of TGF- β signaling in T cells by transgenic expression of a dominant negative T β RII, has been shown to increase azoxymethane-induced colon carcinogenesis [81]. The above

studies demonstrate that deregulation of TGF- β signaling in the immune cells can initiate and promote carcinomas *in vivo*, independently of epithelial cell defects.

Concluding remarks

Recent research has aroused the view that cancer cells do not solely direct cancer progression. Instead, the tumor stroma actively regulates tumor growth, invasion and metastasis. One of the critical factors of tumor progression is TGF- β . TGF- β plays an important role in regulating the balance between cancer cells and tumor microenvironment (summarized in Figure 2).

Several therapeutic strategies targeting TGF- β signaling have been developed for treatment of cancer patients (reviewed in [9]). However, TGF- β can have tumor-suppressive and tumor-promoting effects on cancer cells and stimulate as well as inhibit the tumor-supporting role of the stromal cells. Thus, it is important to specifically tackle the pro-tumorigenic action of TGF- β , while keeping the tumor-suppressive actions of TGF- β intact. This could be achieved by specifically targeting certain cells in the stroma.

A recent study using low doses of a T β RI kinase inhibitor shows promising results. Low doses only affected pericytes in the vasculature, thereby enhancing the delivery of nanodrugs [82]. Moreover, endoglin, a T β RIII, is strongly upregulated in the tumor-associated angiogenic vasculature compared to nonmalignant tissue. Systemic administration of endoglin antibodies conjugated with immunotoxins and immunoradioisotopes efficiently suppressed tumor growth in murine models of breast and colon carcinoma [83]. Thus inhibition of the endoglin pathway may be another target for therapeutic strategies.

TAM is another stromal cell type in which inhibition of TGF- β signaling may result in inhibition of tumor growth. Further studies for the elucidation of the role of TGF- β in tumor microenvironment will give more insights in the complex tumor-stroma interactions and may reveal new potential targets for therapeutic strategies.

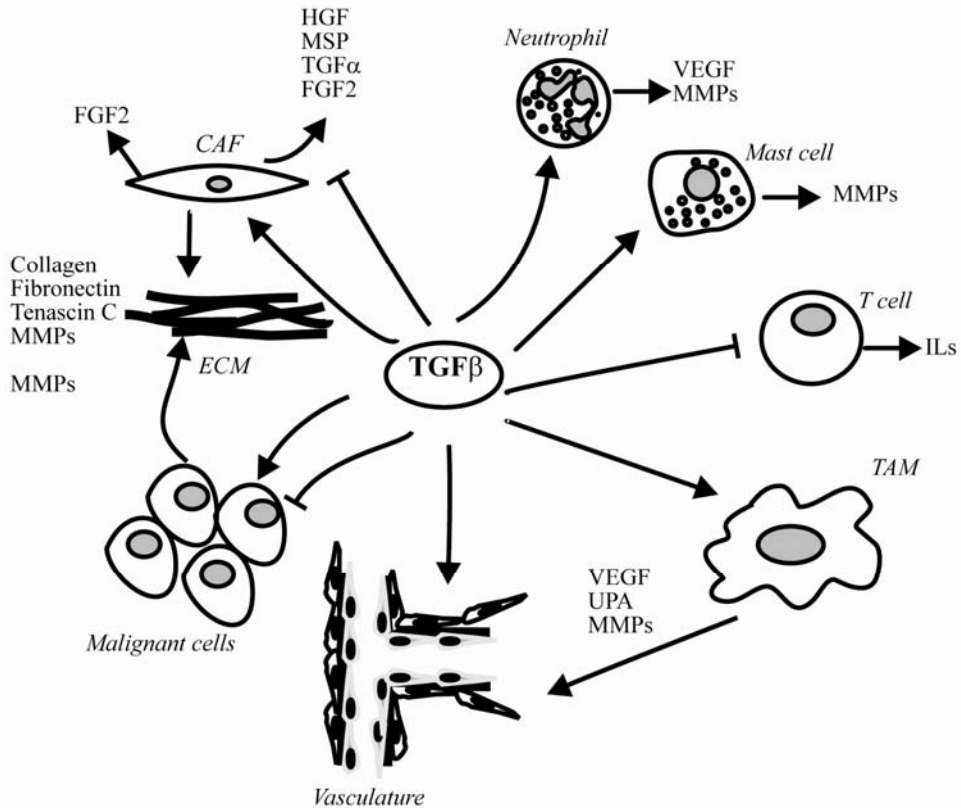


Figure 2. Effect of TGF- β on the different tumor compartments. TGF- β plays an integral role in cancer by regulating tumour initiation, progression and metastasis. TGF- β can act both as a tumor suppressor or a tumor promoter depending on the cellular context. The role of TGF- β in cancer is not limited to autonomous signaling of the malignant epithelial cell, but also on the tumor stroma. TGF- β contributes to generation of CAFs, ECM remodeling and expression of growth factors, from various cells in the tumor microenvironment, that can affect tumor growth and tumor angiogenesis. TGF- β can also recruit and regulate immune cell function in the tumor stroma.

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