Endoglin and the immune system: immunomodulation and therapeutic opportunities for cancer

Schoonderwoerd, M.J.A.

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General introduction

CANCER

Cancer results from the accumulation of genetic [1] and epigenetic changes [2] in cells over time, which converts healthy cells into cancerous cells. These changes enable the cells to grow out of control and become invasive. Cancer cells can ignore signals that are generally used to stop dividing or that initiate the process known as programmed cell death or apoptosis, which eventually results in the formation of the tumor [3]. Malignant cells can influence and transform surrounding normal cells like fibroblasts [4], immune cells, [5] and endothelial cells [6]. These cells combined form the tumor microenvironment (TME).

Colorectal cancer

Colorectal cancer (CRC) is also known as bowel cancer and refers to all malignancies in the colon or rectal area (large intestine) [7]. CRC is the 3rd most common cause of cancer in the Netherlands with a 5-year survival of only 65% [8]. CRC starts with the formation of benign polyps in the large intestine. Some of these polyps can grow out to form invasive cancer [9]. The process from polyp to invasive cancer is a process that can take up to 15 years due to the slow accumulation of mutations. Fearon and Vogelstein proposed a model known as the Vogelgram in which normal epithelium acquires mutations in the Adenomatous Polyposis Coli (APC), Kirsten (K)RAS, Deleted in Colorectal Carcinoma (DCC), and *P53* genes in sequential order leading to cancer progression [10]. However, they emphasized that the number of accumulated mutations rather than the order in which they are acquired is most important during carcinogenesis [11]. The most commonly (34-70%) mutated gene in all CRC is the *APC* gene, which produces the APC protein and is involved in Wnt signaling [12]. Beyond defects in Wnt signaling, other mutations must occur for the cell to become cancerous [13]. Approximately 30%-40% of CRC carry an activating mutation in the *KRAS* gene, driving cell proliferation. Patients with a *KRAS* mutation are unlikely to benefit from therapies that target the Epithelial Growth Factor (EGF) pathway since the mutation is associated with resistance to the EGFR tyrosine kinase inhibitors. Furthermore, other proteins responsible for programmed cell death and differentiation are commonly mutated in CRC like the P53 protein [14] and members of the transforming growth factor- ß (TGF-ß) pathway [15]. The TGF-ß pathway displays inactivating mutations in at least half of CRCs, mostly in a downstream protein called SMAD4. SMAD4 is the central mediator of the TGF-ß, Bone morphogenic protein (BMP) and Activin signaling pathways, by forming a heterotrimeric complex with receptor-regulated SMADs, enabling translocation to the nucleus, where the complex binds Deoxyribonucleic acid (DNA) and regulates gene expression. Metastasis is the major cause of death in CRC patients. The most common side of metastasis are the liver and peritoneum [16]. Once metastasized the life expectancy declines dramatically.

Pancreatic cancer

Pancreatic cancer has a five-year survival rate of only 4-7%, which makes it one of the deadliest types of cancer known in humans [17]. The most common mutations in pancreatic cancer are in *KRAS* (95%), *P53* (75%), and *SMAD4* (55%) [18]. Pancreatic cancers are characterized by a high proportion of non-epithelial, stromal cells [19]. These consist mainly of fibroblasts which are known as "cancer-associated fibroblasts (CAFs)". These are found in high numbers within the tumor, forming a barrier that prevents immune cells and therapeutic agents from entering the tumor [20]. Multiple cellular and molecular levels underlay the therapeutic resistance in pancreatic cancer, including stromal proliferation, reduced vascular density and immune suppression contributing to therapeutic resistance [21].

The tumor microenvironment

Tumors consist not only of a heterogeneous population of cancer cells but also a variety of resident and infiltrating cells known as the tumor microenvironment (TME) [22]. The TME consists of extracellular matrix (ECM), stromal cells (including fibroblasts, pericytes, adipocytes, endothelial cells forming blood- and lymphatic vessels) and immune cells (such as T and B cells, natural killer cells and tumor-associated macrophages and neutrophils). Both CRC and pancreatic cancer are known for their high influx of stromal cells. High accumulation of stromal cells is a predictor for worse survival in both CRC and pancreatic cancer [23]. Furthermore, the TME can also shape therapeutic responses and resistance, justifying the recent interest in targeting components of the TME as a novel therapeutic strategy [24]. One of the best examples of successfully targeting the TME are the immune checkpoint inhibitors [25] of which inhibitors targeting programmed cell death protein 1 (PD-1) and its ligand, programmed cell death ligand 1 (PD-L1) are established examples. These inhibitors are widely used in the clinic and lead to lasting disease response in several cancer types [26]. Although this all sounds very promising, only a minority of patients currently respond to these immunomodulatory therapies. Therefore, multiple therapeutic combinations are being developed to target both the tumor cells and the TME to increase therapeutic responses.

Immune responses against cancer

The immune system consists of a network of multiple organs, tissues, and specialized cells that protect the body from infections and other conditions like cancer. Although these immune cells typically remove damaged or abnormal cells from the body, some cancer cells can evade the immune system [27]. Immune cells continuously scan the body for the occurrence of any molecules that are considered to be 'non-self'. Cancer cells acquire mutations that lead to antigen formation that is recognized as 'non-self', the so-called neo-antigens. Once the immune system recognizes these

cancer cells, a specific immune response is generated that results in the proliferation of antigen-specific lymphocytes. These T-cells can recognize the tumor cells by the binding of the T-cell receptor to the major histocompatibility complex (MHC)-1, presenting the antigen on the cell surface of the tumor. After recognition, the T-cell secrete cytotoxic granules which can kill the tumor cell. However, multiple escape mechanisms enable the tumor to evade the immune response against the tumor. Many of these escape mechanisms can be targeted by immunoregulatory antibodies. Currently, numerous different immunoregulatory antibodies are approved to treat multiple different cancers and many more are being tested pre-clinically or clinically [28]. These antibodies are directed against molecules on immune cells that inhibit or activate the immune system. One of these antibodies is directed against programmed cell death protein 1 (PD-1), which prevents the binding of PD-1 to its ligand PD-L1 [29]. PD-1 and PD-L1 regularly interact with each other preventing the overactivation of the immune system. However, in tumors, cancer cells can overexpress PD-L1, thereby inhibiting the T-cell responses against the tumor and preventing the killing of cancer cells.

Table 1. T-cell subsets

Cell type	Cytokines produced	Markers	Role
Cytotoxic T-cell	IL-2, INFγ	CD3, CD8	Kill virus-infected cells and tumor cells
T helper cell type 1	INFγ	CD3, CD4, CCR5, T-bet	Induce inflammatory response key for defense against viruses and cancer
T helper cell type 2	IL-4	CD3, CD4, CCR3, GATA-3	Induce differentiation and antibody production by B-cells
Regulatory T-cell	IL-10, TGF-ß	CD3, CD4, CD25, Foxp3	Inhibit T-cell mediated immunity

T-cells (characterized by CD3 expression) are usually grouped into subsets based on their function. These can be identified by their expression of various cell surface markers [30]. While T-cell subsets were initially defined by function, they can also be defined by their associated gene or protein expression patterns. Table 1 shows the subsets of T-cells that are described in this thesis. Besides, many more T-cell subsets have been described, which are not discussed and therefore not included here.

CD8 positive, cytotoxic T-cells can kill virus-infected cells and tumor cells [31]. They recognize their target by binding to short peptides presented on MHC class I molecules on the surface of all nucleated cells. Cytotoxic T-cells also produce key

cytokines like Interleukin (IL)-2 and INFγ, which influence the effector function of other immune cells, particularly macrophages and Natural Killer (NK) cells.
T helper cells (Th-cells) assist other lymphocytes, including stimulating the maturation of B-cells into plasma cells and memory B-cells, and the activation of cytotoxic T-cells and macrophages. These Th-cells express CD4 on their surface and s become activated once an antigen is presented by antigen-presenting cells (APCs), in association with MHC class II molecules. After activation, they divide rapidly and secrete cytokines that regulate or assist the immune response [32].
Regulatory T-cells (T-regs) are crucial for the maintenance of immunological tolerance. Their primary role is to shut down T-cell mediated immunity at the end of an immune reaction and suppress autoreactive T-cells that have escaped the process of negative selection in the thymus. T-regs can develop either during normal development in the thymus or can be induced peripherally and are called peripherally derived T-regs. Both subsets require the expression of transcription factor Foxp3, which can be used to identify these cells [33].

Anti-tumor responses mostly rely on adaptive immunity, as described above. However, innate immune cells are also present in the TME [34]. Neutrophils are one of the most abundant cells within the circulation and also found in the tumor. Neutrophils have been described as having both pro-tumor and anti-tumor effects [35]. Two distinct subsets are found within the tumor, the N1 and N2 neutrophils. N2 neutrophils induce cancer growth, metastasis, and immune suppression, whereas N1 neutrophils can induce a cytotoxic response, induce T-cell activation, and antibody-dependent cellular cytotoxicity (ADCC).

ADCC can be induced by cells that express the Fcγ Receptor (FcγR) like macrophages, monocytes, neutrophils, and NK-cells [36]. These cells express FcγRIIA and FcγRIIIA, which are the activating receptors. However, FcγRIIB is an inhibitory FcγR expressed by B-cells, macrophages, monocytes, neutrophils, eosinophils, and basophils. This inhibitory receptor reduces ADCC activity. FcγRs can bind the Fc tail of an antibody, and in this way induce an ADCC response. Many subclasses of Fc tails are known in humans. For example, the IgG1 Fc tail is known for its high-affinity binding to FcγRIIA. Once an antibody has bound its target via its antigen-binding variable region, the effector cell expressing the FcγR can bind the Fc tail of the antibody and thus induce lysis or phagocytosis of the cell.

Tumor vascular system

Blood vessel formation is vital for tumor development and metastasis. Once the tumor grows beyond 2-3mm^3, the lack of nutrients and oxygen promotes the generation of tumor-associated neovasculature [37]. This process is known as the

angiogenic switch and is regulated directly and indirectly by the tumor using pro- and anti-angiogenic signaling molecules, including vascular endothelial growth factor (VEGF) [38], platelet-derived growth factor (PDGF) [39], TGF-ß [40] and BMP9 [41], among others. These newly formed blood vessels are characterized by their immature phenotype. The first cancer therapy that specifically targeted blood vessels was FDA approved in 2004 (bevacizumab), and neutralizes vascular endothelial growth factor (VEGF) [42]. However, therapy resistance has been an enormous setback in targeting the tumor vasculature, and many mechanisms have been described in which both tumor and stromal cells induce resistance [43]. These mechanisms include the activation of alternative angiogenic signaling pathways [44]. Furthermore, host-derived cells such as myeloid cells, pericytes, and CAFs can contribute to therapy resistance by various mechanisms. Myeloid cells can secrete angiogenic and lymphangiogenic cytokines [45], pericytes can increase vessel stabilization [46], which mediates resistance to VEGF(R) therapy and CAFs can secrete proangiogenic cytokines [47].

Cancer-associated fibroblasts

CAFs provide the structural framework of the tumor [4]. They form a vital component of the tumor microenvironment in multiple solid tumors. CAFs have diverse functions, including matrix deposition and remodeling, extensive reciprocal signaling interactions with cancer cells, and crosstalk with infiltrating leukocytes [48]. The precise origin of CAFs is still under debate, but the consensus is that most CAFs likely result from the activation of local fibroblasts or recruitment of precursor cells, although alternative sources have been proposed [49]. Previously CAFs were seen as one group, however, it is becoming increasingly clear that multiple subtypes of CAFs exist. These include myCAFs, with a high TGF-ß driven α-Smooth Muscle Actin (SMA) expression and contractile phenotype, and iCAFs which are known for their high secretion of IL-6. In the future, multiple subtypes will probably be defined, since the function of CAFs ranges from matrix remodeling and the secretion of growth factors to metabolic functions and immune crosstalk. In **Chapter 3** of this thesis, we describe a subset of Endoglin-expressing CAFs responsible for the migration and metastasis of CRC tumors [50].

Endoglin

Endoglin (CD105) is a homodimeric transmembrane protein with a short cytoplasmic domain, which reflects its co-receptor function for the ligands of the transforming growth factor (TGF-ß) superfamily. Endoglin is predominantly expressed by activated endothelial cells and plays a crucial role in angiogenesis. Endoglin expression is regulated by TGF-ß, bone morphogenic protein (BMP)-9, and hypoxia. Since Endoglin is highly expressed by newly formed endothelial cells, therapies targeting Endoglin

have been evaluated as potential new anti-angiogenic therapies [51]. TRC105 is one of these therapies, targeting Endoglin with a Human IgG1 antibody capable of inducing ADCC and successfully passed multiple phase 1 and 2 clinical studies [52-59]. However, TRC105 showed no additional clinical effects over the standard of care in a phase 3 study at the interim analysis, eventually resulting in discontinuation of its clinical development for oncology. However, more evidence is arising that TRC105 targets not only endothelial cells but also CAFs, T-regs and other cells in the TME.

THESIS AIM AND OUTLINE

The TME has increasingly been recognized as an important player in tumor progression and metastasis and a possible target for therapy. The TME consists of multiple cell types secreting growth factors and cytokines that exert either pro- or anti-tumor effects. This thesis mainly focusses on studies of the TME, especially the effects of Endoglin, on several cell types within the TME, including endothelial cells, fibroblasts, and immune cells.

This thesis **aims** to unravel the role of Endoglin as a possible target on various cell types within the TME of solid tumors. Endoglin is known for its role during angiogenesis, however, an increasing number of studies have shown the importance of Endoglin expression on several other cell types (e.g., immune cells, CAFs, tumor cells). Therefore, in **Chapter 2,** the studies on Endoglin beyond endothelial cells are summarized and discussed. CAFs are a major component of the TME and causally involved in tumor progression and metastasis. Multiple subsets of CAFs, with either pro- or anti-tumor effects, are being identified in different tumor types. In **Chapter 3**, we report the presence of an Endoglin-expressing subset of CAFs, localized at the invasive borders of CRC. The presence of these cells is associated with the formation of metastases in stage-II CRC patients. This chapter furthermore shows that Endoglin plays a role in CAF invasion *in-vitro* and appears to be involved in CRC metastasis *in-vivo*. To further investigate fibroblast-specific Endoglin expression and especially in early stages of carcinogenesis, we generated a fibroblast-specific Endoglin knockout mouse in **Chapter 4**. Fibroblast-specific Endoglin deletion resulted in enhanced tumorigenesis in a model for colitis-associated cancer, accompanied by an expansion of stromal cells, with a possible role for myeloid cells. To further investigate the effects of Endoglin targeting in a model that is characterized by a high influx of CAFs, we employed a murine pancreatic cancer model in **Chapter 5**. Although increased immune activation was observed in both fibroblast-specific Endoglin knockout mice and mice treated with an Endoglin neutralizing antibody, no effect on tumor growth was seen. Since increased immune activation was observed, we combined anti-Endoglin therapy with anti-PD-1 treatment to enhance

these effects in multiple colorectal cancer models, as described in **Chapter 6**. Here we describe that anti-Endoglin therapy is effective in reducing tumor volume/progression and reducing the percentage of T-regs within these tumors. Furthermore, we show a subset of Endoglin expressing T-regs in both mouse and human CRC samples. Since the immune system plays a vital role during immunotherapy and therapeutic responses to both TRC105 and PD-1, we were curious to explore the extent to which tumor-draining lymph nodes are involved. In **Chapter 7** we have investigated the effects of the tumor-draining lymph nodes during PD-1/PD-L1 checkpoint therapy. We show that removal of these tumor-draining lymph nodes resulted in a dramatic decline in therapeutic responses, suggesting a pivotal role of local draining lymph nodes during PD-1/PD-L1 checkpoint therapy. In **Chapter 8** the data from the various studies are summarized and discussed.

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