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The Role of the Tumor Microenvironment in the Pathogenesis of Cholangiocarcinoma

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1. Introduction

Cholangiocarcinoma is a type of liver cancer arising from the neoplastic transformation of the epithelial cells that line the intra- and extrahepatic bile ducts. Symptoms are usually evident only after blockage of the bile duct by the tumor. This is an extremely aggressive tumor, which has very poor prognosis and limited treatment options. Cholangiocarcinoma is relatively resistant to chemotherapy and radiation therapy leaving conventional treatment like surgery as the only option. Therefore, further understanding into the factors that are involved in tumor initiation, promotion and progression is required for designing alternate therapies to combat this devastating disease.

The tumor microenvironment is one of the most important factors regulating tumor angiogenesis, tumor invasion and metastasis. The microenvironment is a well-recognized system that plays a key role in tumor progression. However, the mechanism through which tumor microenvironment regulates tumor progression and invasion is largely unknown. In this review, we discuss the current knowledge about the role of the tumor microenvironment in the pathogenesis of cholangiocarcinoma, the role of the tumor microenvironment in the classification of cholangiocarcinoma and efforts to develop treatments targeting the tumor microenvironment.

2. Background

Cholangiocarcinoma arises from the neoplastic transformation of cholangiocytes and can exist as either intrahepatic, perihilar or distal extrahepatic tumors (Alpini et al. 2001). Typically, cholangiocarcinomas are adenocarcinomas and have a poor prognosis and limited treatment options. This is due, at least in part, to the late presentation of symptoms and the relative resistance to current treatment options (Sirica 2005).

The incidence of both intra- and extra-hepatic cholangiocarcinoma is typically more prevalent in Asian countries (Patel 2002). The mortality rates for intrahepatic cholangiocarcinoma have increased since the 1970s, whereas deaths from extrahepatic cholangiocarcinoma have declined in most countries (Patel 2002). There is a slight

preponderance for cholangiocarcinoma in males (Tominaga and Kuroishi 1994) and the incidence in both sexes increases with age (Patel 2002).

2.1 Risk factors

Cholangiocarcinoma occurs with varying frequency in different regions of the world. This can be explained in part by the distribution of risk factors in geographic regions and ethnic groups (Ben-Menachem 2007). The common link between these regional risk factors seems to involve chronic inflammation and biliary irritation (Gores 2003).

The prevalence of cholangiocarcinoma in Asian countries shares a relationship with infections such as liver flukes, Hepatitis B and Hepatitis C (Ben-Menachem 2007). In contrast, approximately 90% of patients diagnosed with cholangiocarcinoma in Western countries do not have any recognized risk factors (Ben-Menachem 2007). However, the remaining 10% of cases are associated with certain risk factors. Apart from factors related to chronic inflammation, both intra- and extrahepatic cholangiocarcinomas are well-known complications of primary sclerosing cholangitis (de Groen et al. 1999). Other known risk factors include obesity, hepatolithiasis, bacterial infection and/or bile stasis-related chronic cholangitis (Chen 1999; de Groen et al. 1999; Catalano et al. 2009).

3. Tumor microenvironment

Neoplastic epithelial cells coexist with a biologically complex stroma composed of various types of stromal cells as well as the extracellular matrix, both of which create the complexity of the tumor microenvironment (Orimo and Weinberg 2006). Mouse models of tumorigenesis have revealed that stromal cells, in particular inflammatory cells, vascular endothelial cells and fibroblasts actively support tumor growth (Olumi et al. 1999; Tlsty 2001; Cunha et al. 2003; Bhowmick et al. 2004). In addition, the microenvironment is now well recognized as playing a role in neoplastic transformation, malignant progression and metastasis and invasion of cancer cells (Tlsty 2001; Bhowmick et al. 2004). Furthermore, the interaction between the cancer cells and the tumor microenvironment is a major factor influencing cancer treatment resistance to radiotherapy and chemotherapy (de Visser and Jonkers 2009; Shinohara and Maity 2009). Research indicates that the interplay between the cancer cells and the stromal cells of the microenvironment is bi-directional and dynamic. For example, neoplastic cells often secrete factors that work in a paracrine manner to recruit and activate a number of types of stromal cells into the tumor microenvironment as required (Rasanen and Vaheri 2010; Rojas et al. 2010; Onimaru and Yonemitsu 2011). Conversely, stromal cells, once recruited and activated, release factors into the extracellular milieu that can either stimulate or inhibit growth of the tumor (Rasanen and Vaheri 2010; Rojas et al. 2010; Onimaru and Yonemitsu 2011). The effects of the components of the tumor microenvironment on tumor growth are summarized in Figure 1. In particular, the proliferation and recruitment of vascular endothelial cells and subsequent formation of new blood vessels brings a nutrient supply thereby allowing growth and metastasis of the tumor. Cancer associated fibroblasts, on the other hand, can stimulate angiogenesis as well promote tumor growth and invasion. The presence of immune cells, in particular tumor-associated macrophages, in the microenvironment, confers resistance to toxic insults and also promotes growth. Lastly, proliferation of lymph endothelial cells and subsequent increase in lymphatic vessel density promotes tumor metastasis.

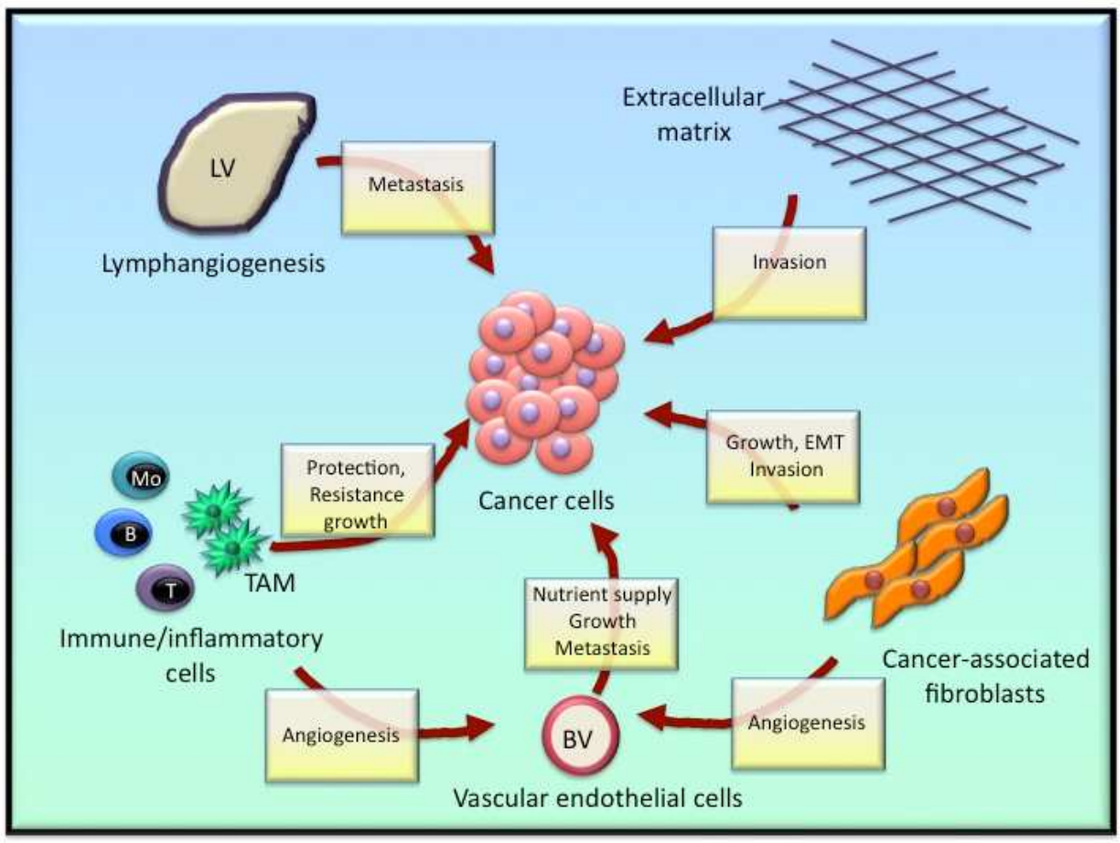

Fig. 1. Schematic representation of the effects of stromal support cells on tumor growth and metastasis. Abbreviations: B, B cell; BV, Blood vessel; EMT, Epithelial-mesenchymal transition; LV, lymph vessel; M, monocyte; T, T cell; TAM, Tumor associated macrophage.

3.1 Angiogenesis

The physiological process of the formation of new blood vessels from pre-existing blood vessels is termed angiogenesis. Tumors require the formation of new blood vessels to supply oxygen and other essential nutrients, without which their growth would be severely restricted (McDougall et al. 2006). Generally, the process of angiogenesis involves a sequence of co-ordinated events that is initiated with the expression and release of various angiogenic factors from the tumor cells, such as vascular endothelial growth factor (VEGF), epidermal growth factor (EGF) and fibroblast growth factor (FGF). Once these angiogenic factors bind to their corresponding receptors on the cell surface of the endothelial cells, there is an increase in vascular permeability, leading to extravasation of plasma proteins and dissociation of pericyte coverage (Roberts and Palade 1997; Dvorak 2005). This is followed by proliferation and migration of the endothelial cells to initiate new vessel formation (Ausprunk and Folkman 1977). For new vessel formation to occur, there also needs to be localized degradation of the extracellular matrix, which is performed by the matrix metalloproteinases, cathepsin B and other degradation enzymes, as well as the expression of matrix proteins such as fibronectin and laminin (Mikkelsen et al. 1995; Gladson 1999; Ljubimova et al. 2006). The expression of these essential extracellular matrix proteins largely

occurs in the tumor cells or cancer associated fibroblasts (Rasanen and Vaheri 2010), which then secrete them into the extracellular milieu.

3.1.1 Angiogenesis in cholangiocarcinoma

A recent immunohistochemical study of microvessel density and lymphatic microvessel density revealed that intrahepatic cholangiocarcinoma tumors demonstrated tumor-associated angiogenesis (Thelen et al. 2009). Tumors with increased microvessel density were correlated with a higher recurrence rate, lower 5-year survival rates and increased nodal spread which in turn influences patient survival (Thelen et al. 2009). Recent studies have also shown that the overexpression of the angiogenic factors nerve growth factor-β (NGF-β) and vascular endothelial growth factor-C (VEGF-C) occurred in approximately 57.1% and 46.4% of cholangiocarcinoma samples, respectively (Xu et al. 2010). A number of human cholangiocarcinoma cell lines and samples have also been shown to overexpress VEGF-A and VEGF receptors (VEGFRs), the angiogenic factors angiopoietin-1, -2, and thrombospondin-1, as well as EGF, EGF receptors (EGFR) and basic fibroblast growth factor (Ogasawara et al. 2001; Alvaro et al. 2006; Tang et al. 2006; Yoshikawa et al. 2008; Harder et al. 2009). Secretion of these factors may individually or co-ordinately bring about increased angiogenesis as demonstrated by increased microvessel density. For example, VEGF-A has been shown to play a role in the neovascularization of extrahepatic cholangiocarcinoma (Mobius et al. 2007).

The factors that drive angiogenesis have also been shown to have distinct effects on cholangiocyte and cholangiocarcinoma growth in an autocrine manner (Gaudio et al. 2006; Tang et al. 2006; Mobius et al. 2007; Yabuuchi et al. 2009; Yoshikawa et al. 2009; Glaser et al. 2010). Indeed, the proliferative effects of estrogen on cholangiocarcinoma cell lines have been attributed to a mechanism involving the upregulation of VEGF expression, as blocking VEGF ameliorates the estrogenic effects on proliferation (Mancino et al. 2009).

Taken together, these data suggest that agents that block angiogenesis (by blocking VEGF expression, for example) may also have a direct effect on cholangiocarcinoma cell proliferation in addition to their anti-angiogenic effects. In support of this notion, inhibition of VEGFR and EGFR signaling with vandetanib (ZD6474, a tyrosine kinase inhibitor) can be an important approach for the management of the subset of cholangiocarcinoma that lack KRAS mutations and/or have EGFR amplification (Yoshikawa et al. 2009). Furthermore, ZD1839 (IRESSA), an orally active, selective inhibitor of EGFR tyrosine kinase has clinical activity against cholangiocarcinoma by stabilizing the cell cycle inhibitor, p27Kip1 and enhancing radiosensitivity in cholangiocarcinoma cell lines (Yabuuchi et al. 2009). Curcumin, a natural phenol found in tumeric has recently been shown to suppress the expression of VEGF and decrease the microvessel density in a hamster model of cholangiocarcinoma (Prakobwong et al. 2011a). In parallel, curcumin also exerts antiproliferative and proapoptotic effects on cholangiocarcinoma cells independent of the effects on angiogenesis (Prakobwong et al. 2011a; Prakobwong et al. 2011b). Similar effects have been shown with inhibitors of histamine synthesis (Francis et al. 2011), H3 histamine receptor agonists (Francis et al. 2009), and Endothelin-1 (Fava et al. 2009) just to name a few. The interaction between angiogenesis, angiogenic factors and cholangiocarcinoma growth and progression is summarized in Figure 2.

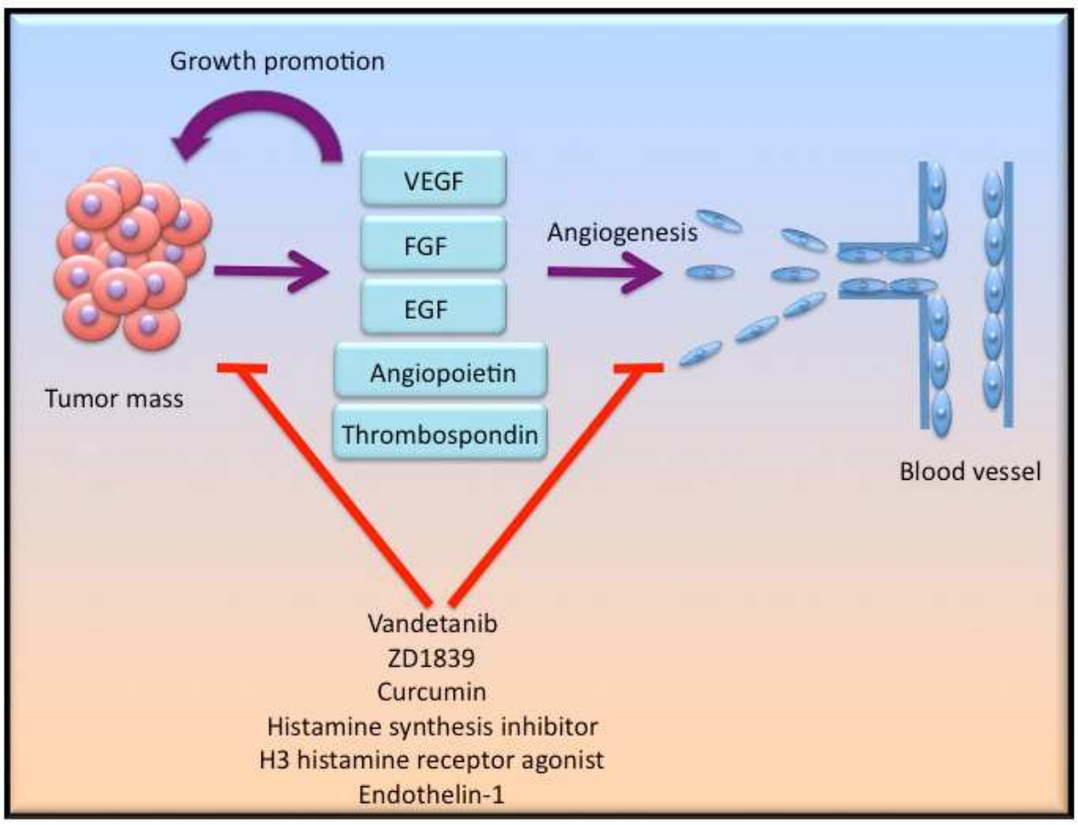

Fig. 2. Schematic representation of the interactions between cholangiocarcinoma and angiogenic factors regulating cell proliferation and angiogenesis

3.2 Cancer associated fibroblasts

Under normal physiological conditions, fibroblasts have a low proliferative index and only secrete factors needed to maintain normal tissue homeostasis (Tuxhorn et al. 2001; Beacham and Cukierman 2005). Indeed, normal fibroblasts provide biochemical cues that constrain epithelial tumor cells within their basement membrane (Tuxhorn et al. 2001; Beacham and Cukierman 2005). In contrast, when homeostasis is disrupted during tissue injury, stromal cells rapidly and reversibly alter their phenotype and proliferation rate (Tuxhorn et al. 2001). However, during tumorigenesis, the fibroblastic wound healing machinery lacks the regulatory mechanisms to revert to normal homeostasis (Tuxhorn et al. 2001). The inability to down-regulate the wound healing response affects stromal dynamics. Tumor-dependent changes in signaling and plasticity of the stroma trigger a continuum of alterations yielding a 'primed' stroma that can support and incite tumor initiation or progression (Tuxhorn et al. 2001).

3.2.1 Cancer-associated fibroblasts in cholangiocarcinoma

Cancer-associated fibroblasts are the predominant cell type in the stroma of cholangiocarcinoma tumors (Sirica et al. 2009). Increased α-smooth muscle actin-positive fibroblasts were correlated with shorter survival times and larger tumor sizes in resected

cholangiocarcinoma tissue (Chuaysri et al. 2009; Okabe et al. 2009). The origin of these cancer-associated fibroblasts is unknown, although a number of possibilities have been suggested, including hepatic stellate cells (Okabe et al. 2009), portal fibroblasts (Dranoff and Wells 2010) or circulating bone marrow-derived precursor cells (Shimoda et al. 2010). Given the apparent heterogeneous population of cancer-associated fibroblasts observed in cholangiocarcinoma tumors, it is highly likely that these fibroblasts are derived from more than one source. Recently, researchers have performed genetic screening to determine the differences in gene expression between cholangiocarcinoma-derived cancer-associated fibroblasts and non-malignant liver fibroblasts and showed a number of genes associated with angiogenesis, cell proliferation and motility (Utispan et al. 2010). In particular, periostin, a cell adhesion molecule, was shown to be significantly upregulated correlating with shorter survival time in patients and increased cell proliferation and invasive properties *in vitro* (Utispan et al. 2010). Another gene specifically expressed by cholangiocarcinoma-derived cancer-associated fibroblasts is the extracellular matrix protein tenascin-C (Aishima et al. 2003; Iguchi et al. 2009). This gene was expressed predominantly in the stroma near the invasion front of the tumor (Aishima et al. 2003) and was associated with poor prognosis in intrahepatic cholangiocarcinoma (Aishima et al. 2003; Iguchi et al. 2009). Furthermore, the expression of thrombospondin-1 by cancer-associated fibroblasts correlated with increased metastatsis (Kawahara et al. 1998; Tang et al. 2006).

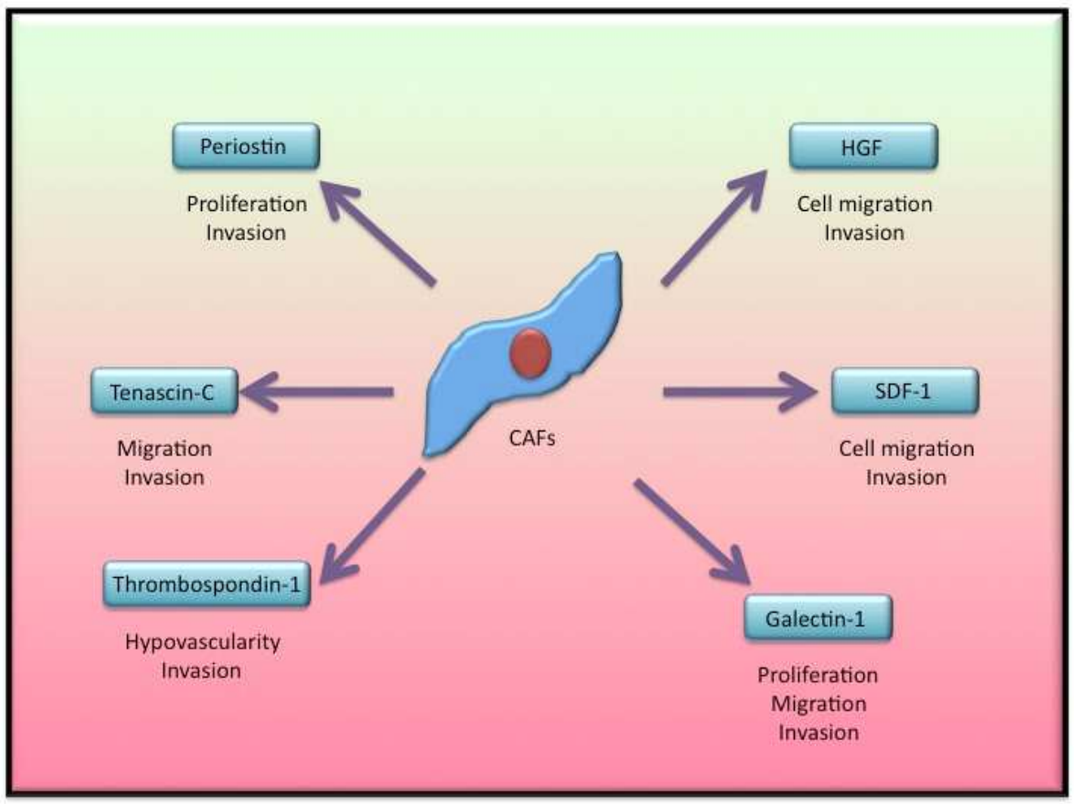

Fig. 3. Summary of the signalling molecules released by cholangiocarcinoma-derived cancer-associated fibroblasts and their known effects on cholangiocarcinoma progression. CAFs; cancer-associated fibroblasts, HGF; hepatocyte growth factor, SDF-1; stromal derived factor-1.

One last cancer-associated fibroblast gene of note is the expression of the chemokine, stromal-derived factor 1, which is released from stromal fibroblasts and stimulates the invasion and migration of cholangiocarcinoma cells via interaction with the chemokine receptor, CXCR4 (Ohira et al. 2006). A summary of these and other cholangiocarcinoma-derived cancer-associated fibroblasts can be found in Figure 3.

The preponderance of data demonstrating a role for cancer-associated fibroblasts in the growth and invasion of cholangiocarcinoma suggest that targeting molecular signals released from cancer-associated fibroblasts may be a viable option, in addition to strategies for suppressing cholangiocarcinoma cell proliferation, for the treatment of cholangiocarcinoma.

3.3 Tumor-associated macrophages

Inflammation and the immune system share a long-standing relationship with tumor initiation and progression. Indeed, the primary risk factor for the development of a number of different tumor types is chronic inflammation of the target organ (Sica 2010). Once a tumor is initiated, tumor-associated macrophages (TAMs) are the major immune cell found within tumors. Macrophages generally have the potential to express and secrete pro- and anti-inflammatory molecules, and as such, may have pro- and anti-tumor activities depending upon the activation stimulus (Sica 2010). For example, macrophages activated with tumor necrosis factor α, (considered M1 activation), have anti-tumor activity and signal tissue destruction (Mantovani et al. 2002). Alternatively, in response to interleukin-4, macrophages undergo M2 activation and are involved in tissue repair, remodelling and tumor promotion (Mantovani et al. 2002).

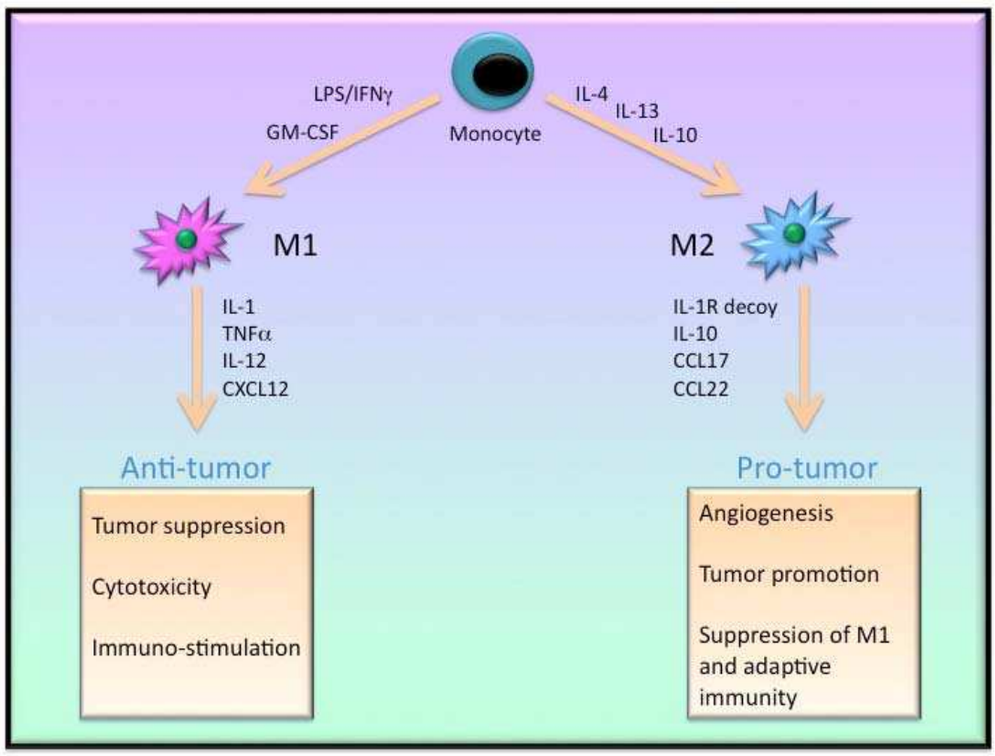

Fig. 4. Schematic representation of the pro- (M1) and anti-(M2) inflammatory activation of macrophages and their effect on tumor growth.

Logically, most TAMs have an M2-like phenotype (Mantovani and Sica 2010) thought to be brought about by various signals expressed within the tumor microenvironment, including interleukin-10, transforming growth factor-β and colony stimulating factor-1 (Sica 2010). These signals responsible for the M2-activation of macrophages have been reported to come from myeloid-derived suppressor cells, IL-10+ B lymphocytes, Th2 subtype of T helper cells and the tumor cells themselves (Sica 2010). Once alternatively activated, TAMs exhibit reduced anti-tumor activities, while increasing the production of mediators of angiogenesis such as VEGF and IL-10 (Mantovani and Sica 2010), as well as M2-specific genes that are known to be involved in promoting cell proliferation (Mantovani and Sica 2010). These events are summarized in Figure 4.

It has been proposed that strategies to inhibit the M2- and activation of the M1-inducing signals may lead to the restoration of the anti-tumor functions of TAM and help to remove the protective signals originating from the M2 TAM (Sica and Bronte 2007), which may trigger an innate immune response, thereby reducing tumor size (Sica 2010).

3.3.1 Tumor-associated macrophages in cholangiocarcinoma

As mentioned previously, cholangiocarcinoma shares a long-standing relationship with chronic inflammation (Gores 2003). Indeed, cholangiocarcinoma cells are known to over-produce many inflammatory cytokines, with IL-6 being the most studied (Isomoto et al. 2007). The role of TAMs in the development and progression of cholangiocarcinoma is poorly understood. However, recent studies have demonstrated that the density of infiltrating macrophages (as demonstrated using the MAC387 antibody to specifically stain macrophages) was high in over half of the tumor samples studied and that a high density of MAC387-positive cells correlated to a poor survival rate although conclusive proof that these cells were of the M2-phenotype is lacking (Subimerb et al. 2010a). Similarly, a subset of monocytes ($CD14^+CD16^+$) thought to be the precursors of tissue-resident macrophages are increased in the blood from cholangiocarcinoma patients, the levels of which were correlated with the density of MAC387-positive infiltrating macrophages (Subimerb et al. 2010b). The circulating $CD14^+CD16^+$ monocytes also expressed higher levels of angiogenic factors such as VEGF and the chemokine CXCL3 (Subimerb et al. 2010b). Lastly, Hasita et al. demonstrated that the macrophages infiltrating intrahepatic cholangiocarcinoma are mainly of the M2 phenotype (using CD163 as a marker of M2-type macrophages); their number correlates closely with neovascularization and infiltration of FOXP3+ regulatory T cells (Hasita et al. 2010). Furthermore, treatment of macrophages in culture with the supernatant from a number of CCA cells induced macrophage polarization toward the M2 phenotype and induced the macrophage-derived expression and secretion of VEGF-A, IL-10 and TGFβ (Hasita et al. 2010). Taken together, these data suggest that TAMs may play a role in cholangiocarcinoma progression. However, the molecules regulating the crosstalk between M2-type TAMs and cholangiocarcinoma cells needs to be further clarified.

3.4 Lymphangiogenesis in cancer

Tumor metastasis is the most lethal aspect of cancer. The spread of tumor cells is often via the lymphatic vasculature and the presence of tumor foci in lymph nodes is considered an adverse prognostic factor in most carcinomas (Achen and Stacker 2008). Metastatic spread of tumor cells via the lymphatic system was previously thought to be via a passive process by

which detached tumor cells enter pre-existing lymphatic vessels in the vicinity of the tumor (Achen and Stacker 2008). However, recent studies suggest that the formation of new lymphatic vessels in the tumor microenvironment correlates with lymphatic metastasis (Achen et al. 2005).

To date, the growth factors recognized to be associated with the control of lymphangiogenesis are similar to those that control angiogenesis. That is, the most characterized factors are VEGF-C and VEGF-D, which are secreted from the tumors, and then activate VEGFR-3 expressed on lymphatic endothelium (Lymboussaki et al. 1998). Activation of VEGFR-3 induces the proliferation of lymphatic endothelial cells *in vitro* (Makinen et al. 2001) and the formation of new lymphatic vessels *in vivo* (Veikkola et al. 2001). Other identified lymphangiogenic factors include VEGF-A (Nagy et al. 2002), fibroblast growth factor-2 (Kubo et al. 2002), angiopoietin-2 (Gale et al. 2002) and platelet-derived growth factor-BB (Cao et al. 2004).

Because of the overlap in angiogenic and lymphangiogenic activity of the above-mentioned factors, agents designed to block angiogenesis may also be effective in blocking lymphangiogenesis. For example, inhibitors that block the VEGF-C/VEGF-D/VEGFR3 signalling mechanism might have the potential to not only block angiogenesis, but to also block lymphangiogenesis and hence to block lymphogenous metastatic spread (Baldwin et al. 2002; Stacker et al. 2002a; Stacker et al. 2002b). Indeed, a neutralizing VEGF-D monoclonal antibody designed to block the interaction between VEGF-D and its receptors, inhibited angiogenesis, lymphangiogenesis and metastatic spread via the lymphatics in a mouse tumor model (Stacker et al. 2001). Further studies into therapeutic strategies designed to block lymphangiogenesis are required in an attempt to stop the metastatic spread of tumors.

3.4.1 Lymphangiogenesis in cholangiocarcinoma

The role of lymphangiogenesis in cholangiocarcinoma metastasis and progression is largely unknown and controversial. However, recent studies suggest that there is a correlation between lymphangiogenesis and lymph node metastases and prognosis; patients diagnosed with cholangiocarcinoma tumors exhibiting low lymphatic vessel density have a longer survival rate than those with higher lymphatic vessel density (Thelen et al. 2008). In addition, in intrahepatic cholangiocarcinoma tumors, high lymphatic vessel density correlated with increased nodal spread and higher recurrence rate (Thelen et al. 2009). Conversely, other researchers demonstrated that in intrahepatic cholangiocarcinoma tumors, lymph node metastasis did not correlate with lymphangiogenesis, but did correlate with VEGF-C expression and the presence of a subset of myofibroblasts expressing the same markers as lymphendothelial cells (Aishima et al. 2008), which may explain the discrepancy in conclusions.

NGF has previously been linked to tumor progression and growth (Sortino et al. 2000; Descamps et al. 2001a; Descamps et al. 2001b) as well as VEGF expression (Lazarovici et al. 2006a; Lazarovici et al. 2006b) in a number of other cell types. Therefore, Xu et al. assessed the correlation of NGF-β expression with lymphangiogenesis, lymph node metastasis or VEGF-C expression in hilar cholangiocarcinoma tissue (Xu et al. 2010). Indeed, high NGF expression was correlated with VEGF-C overexpression, lymphatic vessel density, and lymph node metastasis suggesting that NGF may also be responsible for stimulating lymphangiogenesis in cholangiocarcinoma tumors.

4. Conclusions

The work highlighted in this review clearly demonstrates a role for the tumor microenvironment in the growth, progression and metastatic invasion of cholangiocarcinoma. There is obviously a strong interplay between the cells found in the stroma and cholangiocarcinoma cells with signaling molecules passing back and forth between the cell types to co-ordinately support an environment that nurtures tumor growth and suppresses innate immunity while conferring resistance to cytotoxic insults (both endogenous and chemotherapeutic). The mechanism by which each of the support cells found in the stroma of cholangiocarcinoma tumors are recruited and activated is still largely unknown. Therapeutic strategies designed to target the microenvironment rather than specifically targeting the cholangiocarcinoma cells might prove fruitful in the quest to combat this devastating cancer.

5. Acknowledgements

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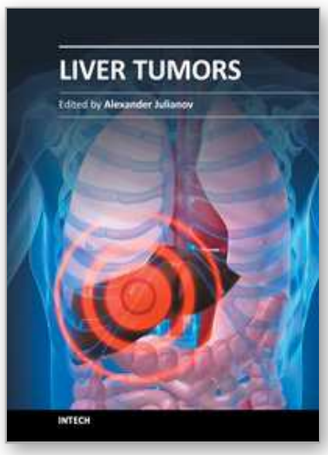

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This book is oriented towards clinicians and scientists in the field of the management of patients with liver tumors. As many unresolved problems regarding primary and metastatic liver cancer still await investigation, I hope this book can serve as a tiny step on a long way that we need to run on the battlefield of liver tumors.

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