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CC chemokine ligand 2 (CCL2) promotes prostate cancer tumorigenesis and metastasis

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Abstract

CCL2 is a chemokine known to recruit monocytes and macrophages to sites of inflammation. A growing body of research suggests CCL2 is progressively overexpressed in tumor beds and may play a role in the clinical progression of solid tumors. Cancer cells derived from several solid tumor types demonstrate functional receptors for CCL2, suggesting this chemokine may achieve tumorigenicity through direct effects on malignant cells; however, a variety of normal host cells that co-exist with cancer in the tumor microenvironment also respond to CCL2. These cells include macrophages, osteoclasts, endothelial cells, T-lymphocytes, and myeloid-derived immune suppressor cells (MDSCs). CCL2 mediated interactions between normal and malignant cells in the tumor microenvironment and plays a multi-faceted role in tumor progression.

Keywords

CCL2; CCR2; tumorigenesis; metastasis; prostate cancer

1. Introduction

Cancer cells coexist in the tumor microenvironment with normal host cells, including hematopoietic precursors. While some host cells inhibit cancer growth, many produce a multitude of soluble factors and nutrients that promote tumor progression and growth. In turn, cancer cells provide growth factors, cytokines, and chemokines that promote host cell proliferation and survival. Understanding the factors that contribute to this vicious cycle of cooperation is of primary importance to understanding malignant progression and has revealed new therapeutic targets for cancer treatment.

Altered regulation of chemokines and their receptors has been demonstrated to promote tumor development and progression through multiple mechanisms including enhanced proliferation, increased invasiveness, increased angiogenesis, and recruitment of immune cells [as reviewed in [1-3]]. Among the many chemokines and their receptor pairs, CC

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chemokine ligand 2 (CCL2) and its receptor CCR2 have recently been shown to play key roles in promoting tumorigenesis and metastasis [as reviewed in [4]].

CCL2 is a member of the CC chemokine family that regulates the recruitment of monocytes, macrophages, and other inflammatory cells to sites of inflammation via activation of CCR2 [5]. In addition to its role in inflammatory responses, CCL2 has been demonstrated to directly stimulate prostate cancer cell chemoattraction, proliferation, and survival [6-8]. CCL2 has also been shown to play a central role in the maturation of osteoclasts in the microenvironment of skeletal metastases [9]. The multiple roles of CCL2 in the promotion of tumorigenesis make the CCL2/CCR2 axis an attractive therapeutic target for cancer treatment (Figure 1 and Table 1).

2. CCL2 and its receptor CCR2

CCL2, also known as monocyte chemoattractant protein-1 (MCP-1), was first identified by its ability to attract monocytes *in vitro* [10-12]. CCL2 is a 76-amino acid polypeptide and is a member of the C-C chemokine family characterized by adjacent cysteine residues. It binds with high affinity to the seven transmembrane G-protein coupled receptor CCR2. The CCL2 gene is one of several cytokine genes clustered on the q-arm of chromosome 17 [13].

Investigations of *CCL2* and *CCR2* genotypic variants through single-nucleotide polymorphism (SNP) analysis suggest that this chemokine may play a role in host susceptibility to the development of cancer and/or cancer metastasis. For example, *CCL2* SNPs have been shown to associate with chronic myelogenous leukemia risk [14]. The -2518 G/A SNP occurs in the promoter region of the *CCL2* gene. Carriers of the G-allele demonstrate elevated *CCL2* transcription [15]. A study of 83 women from northern Italy undergoing surgery for breast cancer found G-allele carrier status strongly correlates with the presence of metastases at the time of operation [16]. Carriers of the A-allele were prone to developing distant metastasis following radiation therapy when compared to G/G heterozygotes in a study of 411 nasopharyngeal carcinoma patients [17]. Although no association was observed between prostate cancer risk and the *CCL2* 2518 G/A polymorphism [18], the role of this SNP in prostate cancer progression and metastases remains an open question.

The *CCR2* gene, residing in the chemokine receptor gene cluster region, encodes two isoforms. *CCR2* mRNA and protein are differentially expressed by various cancer cell lines including prostate, lung, breast cancer, and myeloma [as reviewed in [4]]. Certain prostate cancer cell lines were responsive to CCL2 in proliferation and *in vitro* cell invasion assays [6,8], indicating the presence of functional CCR2 receptors. Furthermore, CCR2 antagonist only partially inhibited CCL2-induced proliferation and invasion, suggesting the presence of other functional CCL2 receptors with functional redundancy [8]. Although all prostate cancer cells express CCR2 mRNA and protein, more aggressive cancer cells express higher levels of CCR2 when compared to less aggressive or benign prostatic cells. Diminished CCL2-induced prostate cancer cell invasion and proliferation demonstrated by short-hairpin RNA (shRNA) knockdowns of CCR2 in the prostate cancer cell lines C4-2B and PC3 [19] reiterates the connection between activation of the CCL2/CCR2 axis and the phenotype of aggressive prostate cancer. Interestingly, the I-allele of the *CCR2*-V64I polymorphism studied extensively as a source of genetic variation in clinical HIV progression [20] may protect against sporadic breast cancer [21] and invasive cervical carcinoma development [22].

3. CCL2 is present in the tumor microenvironment

CCL2 expression has been demonstrated in many cancer types including prostate, breast, lung, melanoma, ovary, gastric, renal, colorectal, esophageal and multiple myeloma [as reviewed in [4]]. Prostate cancer in particular demonstrated elevated CCL2 expression in malignancies when compared to benign prostatic tissues [8]. One study identified 4-fold upregulation of CCL2 as determined by cytokine antibody arrays comparing the tumor-bone microenvironment to non-neoplastic bone [6]. CCL2 is expressed not only in tumor cells, but also in osteoblasts and bone marrow endothelial cells [23,24]. It was also reported that parathyroid hormone-related peptide (PTHrP) can upregulate CCL2 in both osteoblasts and bone marrow endothelial cells [9]. Although the primary source of CCL2 in bones still needs to be identified, it has been demonstrated that CCL2 contributes to prostate cancer skeletal metastasis through both direct effects on prostate cancer cells and indirect effects on osteoclast activity in tumor-induced bone lesions [25].

4. The effect of CCL2 on cancer cells

4.1. CCL2 and prostate cancer cell proliferation and survival

Recombinant human CCL2 induces dose-dependent prostate cancer cell proliferation *in vitro* through the activation of the phosphatidylinositol 3-kinase (PI3K)/AKT pathway [6,8]. In addition, this activation of the AKT pathway also provides cancer cells with a survival advantage through up-regulation of survivin [26]. Specifically, PI3K/Akt activation by CCL2 mediates mTORC1 activation, survivin up-regulation, and subsequent down-regulation of autophagosome formation. Autophagy protects cells from nutrient depletion stress; however, excessive autophagy will result in cell death [27]. As survivin may be a key molecule protecting prostate cancer cells from autophagic death (Figure 2), combination therapy targeting both CCL2 and the PI3K/Akt/mTOR/survivin pathways may become a clinical strategy to overcome therapeutic resistance.

4.2. CCL2 and prostate cancer cell migration and metastasis

CCL2 is chemotactic for prostate cancer cells in both autocrine and paracrine manners [6,8]. CCL2 induces actin rearrangement through phosphorylation of p70-S6 kinase (a downstream target of Akt) and activates the small GTPase Rac through the actin-associated protein PCNT1 [28]. This activation of Rac allows the cells to undergo diapedesis through the bone marrow endothelium. This migration is mediated in part by thrombin/thrombin receptor facilitated positioning of cancer cells in a “ready state” for migration in response to a chemotactic signal (23). Taken together, these data suggest that CCL2 has a significant role in promoting prostate cancer cell extravasation into the bone microenvironment.

5. CCL2 and tumor angiogenesis

Angiogenesis correlates with cancer progression and/or poor prognosis in a variety of solid tumors [29]. The process can be activated at different stages of tumor development, depending on the cancer types and the tumor microenvironment. Identifying critical angiogenic factors and mediators in the tumor microenvironment remains an important challenge.

Previous findings suggest that CCL2 can directly mediate angiogenesis in endothelial cells, which express CCR2 [30]. Human brain endothelial cells, human umbilical cord vein endothelial cells (HUVEC), and human dermal microvascular endothelial cells (HDMVEC) were reported to express CCR2 and respond to CCL2 by forming more vessel sprouts *in vitro* [30,31]. It was reported in a study of prostate cancer angiogenesis that CCL2 stimulated tumor cells to produce proangiogenic factor(s) instead of directly stimulating bone marrow

endothelial cells (HBMEs) [32]. Conditioned media from CCL2-treated PC-3 cells significantly increased sprout formation in HBMEs, whereas pretreating PC-3 cells with anti-CCL2 antibody abolished this effect. CCL2 itself failed to directly stimulate capillary tube formation by HBMEs. The antibody against human CCL2 CNTO 888 also had no effect as a monotherapy on capillary tube formation; however, CCL2 increased VEGF-A mRNA expression levels in PC-3 cells after 4-6 hours of treatment. The induction of VEGF-A mRNA expression in PC-3 cells was blocked by pretreatment with a neutralizing antibody, indicating this induction was mediated by CCL2 (Figure 3).

In addition, it has been shown that systemic administration of CCL2 neutralizing antibodies significantly decrease PC3 tumor burden *in vivo* and dramatically reduce tumor blood vessel density [32]. Similar findings were reported in a subcutaneous VCaP xenograft model [33]. In that study, VCaP cells were implanted in SCID mice and monitored for tumor growth and microvascular density. Systemic administration of CCL2 neutralizing antibodies significantly reduced VCaP tumor growth and decreased tumor microvascular density. Therefore, these results support that CCL2 promotes the tumor angiogenesis both *in vitro* and *in vivo*.

In other tumor types such as breast cancer and multiple myeloma, CCL2 expression has been reported to associate with tumor progression and angiogenesis [as reviewed in [34,35]]. CCL2 was suggested to induce angiogenesis through multiple mechanisms including direct induction of VEGF-A [36] and hypoxia inducible factor-1 (HIF-1) [37]. CCL2 can also stimulate angiogenesis through the attraction of tumor associated macrophages (TAMs) which are known to secrete VEGF [38]. TAMs are a major component in the inflammatory response and produce a variety of growth factors for epithelial and endothelial cells, as well as inflammatory cytokines and chemokines that contribute to the tumor cell survival, proliferation, and invasion.

6. CCL2 and TAMs in the tumor progression

Many solid tumors contain an inflammatory infiltrate that often comprises a majority of the cells in the tumor's mass [39]. In many tumors, the majority of the infiltrating cells are TAMs derived from myeloid progenitor cells in the bone marrow compartment [40]. Myeloid progenitor cells develop into pro-monocytes in the bone marrow prior to being released into the circulation where they undergo differentiation into monocytes. Monocytes then migrate into tissues where they differentiate into resident macrophages, protect against infection, and respond to tissue injury. In cancer these cells differentiate into TAMs; however, it is not clear whether TAMs are derived from peripheral blood monocytes recruited into tumors from the circulation or from resident macrophages present in the otherwise healthy tissue where a tumor develops [41].

Macrophages demonstrate functional "polarization" toward one of two phenotypically distinct macrophage subsets: T_H1 (also known as M1 macrophages) or T_H2 (also known as M2 macrophages). M1 macrophages are known to produce pro-inflammatory cytokines and play an active role in cell destruction, whereas M2 macrophages primarily scavenge debris, promote angiogenesis, and facilitate wound repair [42]. The M2 macrophage population is thought to be phenotypically similar to the TAM population [40].

TAMs have been demonstrated to play a direct role in the regulation of tumor growth and angiogenesis in prostatic malignancies [33]. Elevated expression by both cancer and stromal cells of a number of monocyte chemoattractants including CCL2, CCL3, CCL4, CCL5 (RANTES), and CCL8 has been shown to positively correlate with increased TAM numbers in several human tumors [43,44]. Specifically, CCL2 has been demonstrated to regulate

monocyte/macrophage trafficking and has been reported to be present in prostatic tumor beds (Figure 4), suggesting a role for this chemokine in TAM recruitment [45].

7. The effect of CCL2 on osteoclasts

Prostate cancer preferentially metastasizes to bone, resulting in high mortality. Histopathological examination of tumor-induced bone lesions reveals a mixture of pre-requisite osteolytic bone resorption and a predominantly bone forming osteoblastic reaction. The tumor-induced osteoclast activity is essential for prostate cancer establishment in the bone microenvironment.

It has been reported that blocking Receptor Activator of Nuclear Factor KappaB Ligand (RANKL) in murine models partially inhibits the establishment and progression of prostatic tumors in bone [46]; however, the cell line used in these studies produced very low levels of RANKL, suggesting other soluble factors may mediate prostate cancer-induced osteoclast activity. Recently, it was reported that CCL2 significantly induces osteoclast fusion both *in vitro* [25] and *in vivo* [32]. Human bone marrow mononuclear cells (HBMC) cultured *in vitro* using conditioned media collected from prostate cancer PC3 cell cultures differentiated into bone resorbing osteoclast-like cells (Figure 5). Both recombinant human CCL2 and IL-8 directly stimulated HBMC differentiation into osteoclast-like cells, but only IL-8 induced bone resorption on dentin slices following 21 days of culture in the absence of RANKL. Nevertheless, conditioned media-induced bone resorption was inhibited by a CCL2 neutralizing antibody. The inhibition was enhanced by combining CCL2 neutralizing antibody with an IL-8 neutralizing antibody, indicating that CCL2 and IL-8 cooperatively mediate tumor-induced osteoclastogenesis and bone resorption. In support of this findings, another report showed CD11b+ cells isolated from human peripheral blood differentiate into functional osteoclasts when grown in PC3 conditioned media [47].

The effects of CCL2 on osteoclastogenesis were further demonstrated *in vivo* using intratibial-injections of prostate cancer cells in mice as a preclinical mouse model of prostate cancer bone metastasis [32]. Systemic treatment with CCL2 antibody not only decreased tumor burden, but also decreased osteoclast numbers at the tumor–bone interface in this model. These observations were accompanied by a decrease in the serum marker of bone resorption TRAP5b [32]. These results demonstrate an active role for CCL2 in tumor-induced osteoclastogenesis.

8. The effects of CCL2 on T lymphocytes in the tumor microenvironment

Several studies have demonstrated CCL2 overexpression at sites of inflammation and the contribution of CCL2 to the inflammatory components of such diseases as atherosclerosis, multiple sclerosis, and rheumatoid arthritis [5,48]. In addition, growing evidence has implicated chemokines as a key component of cancer-related inflammation due to the role of chemokines in inflammatory cell recruitment and function in the tumor microenvironment [36]. Although, CCL2 is chemoattractant not only for inflammatory monocytes, but also for memory T-lymphocytes, natural killer cells, and perhaps dendritic cells [49]. Several studies indicate that CCL2 may also influence T-cell differentiation and function.

T-lymphocytes express the CCR2 and may react to CCL2 to differentiate into Th2 “helper” T-cells [50]. Th2 cells are a subset of CD4+ T-helper cells that stimulate B-lymphocytes to produce antibody and suppress the action of cytotoxic CD8+ T-cells [51]. The Karpus group showed that naïve T-lymphocytes from TCR transgenic mice differentiate toward a Th1 phenotype *in vitro* when exposed to antigen in the presence of macrophage inflammatory protein-1a [52]. In contrast, the same cells became Th2-like in the presence of CCL2. CCL2-induced Th2 differentiation was blocked by a neutralizing antibody against IL-4, suggesting

CCL2 induced Th2 differentiation by enhancing IL-4 expression. It has also been reported that IL-4 induces CCL2 expression in endothelial cells [53], suggesting a self-amplifying loop whereby CCL2 and IL-4 mutually enhance their expression, resulting in T-cell differentiation toward a Th2 phenotype in the tumor microenvironment [54].

9. The effects of CCL2 on immune suppressor cells

Failure of host anti-tumor immunity may be caused by exaggerated suppression of tumor-associated antigen-reactive lymphocytes. Suppression of reactive lymphocytes is mediated by regulatory T-cells (Tregs). Tumor derived TGF β can convert CD4⁺ CD25⁻ T cells into CD4⁺ CD25⁺ T cells, which is one immune evasion mechanism available to tumor cells [55]. Other mechanisms include production of the immunosuppressive cytokine IL-10 [56], generation of immunosuppressive DCs [57], and induction of T-cell anergy [58]. Abrogating immune evasion by eliminating Treg cells appears to be an effective immunotherapy approach against both murine and human melanoma [59,60]. Thus, identifying and targeting chemokines facilitating Tregs-mediated immune evasion remains a noteworthy goal.

An analysis of malignant human glioma cell lines and tumor cells from patients with Glioblastoma multiforme (GBM) detected intracellular expression of chemokines CCL22 and CCL2; however, only CCL2 was detected by ELISA. Supernatant collected from the glioma cell lines in this study induced Treg cell migration, as did recombinant human CCL2 and CCL22. The induced migration was successfully inhibited by neutralizing antibodies [61]. Taken together, these findings suggest targeting CCL2 as a strategy to inhibit Treg recruitment by tumors.

In addition to Treg cells, recent reports demonstrate the role of another cell population in immune suppression - immature myeloid-derived suppressor cells (MDSCs) [62,63]. These cells are identified by a GR-1+CD11b+ F4/80- surface antigen profile [62]. It has been reported that MDSC accumulation associates with the inflammatory milieu of the tumor microenvironment. In addition to suppressing T cells, MDSCs also demonstrate a role in repressing the cytotoxicity of natural killer (NK) cells in tumor-bearing hosts [64]. More recently, Sinha reported that immature myeloid cells may also induce macrophage differentiation from an M1 type to an M2 phenotype [65]. Several tumor-derived cytokines and chemokines have been shown to influence MDSC function [66,67]. In particular, the CCL2/CCR2 pathway has been demonstrated to play a critical role in the migration of MDSCs to tumors [68]. This suggests CCL2 facilitates cancer's evasion of an anti-tumor immune response by recruiting immune suppressor cells into the tumor microenvironment.

10. Conclusion

CCL2 influences cancer tumorigenesis and metastasis via distinct mechanisms:

1. A direct promotional effect on tumor cell growth, survival and function
2. A modulatory effect on the tumor microenvironment by promoting macrophage mobilization and infiltration into the tumor bed
3. Promotion of osteoclast maturation in the bone tumor microenvironment
4. Suppression of cytotoxic lymphocytes.

Direct CCL2 stimulation regulates the growth, survival, invasiveness, and migration of tumor cells. This ability to stimulate cancer epithelial cells directly has implicated CCL2 in the progression and metastasis of several human carcinomas including breast, pancreas, and prostate. An equally influential role of CCL2 in the development and progression of tumors is its interaction with a variety of normal host cells in the tumor microenvironment. Through

its interaction with vascular endothelial cells, CCL2 promotes the development of tumor vasculature critical to sustained neoplastic growth. As a chemokine for monocytes and macrophages, CCL2 recruits inflammatory infiltrates that often constitute a significant portion of the tumor volume, contribute to tumor growth, and appear to promote tumor angiogenesis. In the bone microenvironment CCL2 induces osteoclast formation and activation, a critical step in the seeding of bone tumors. Finally, CCL2 acts as a chemokine attracting immunosuppressive Tregs and MDSCs into the tumor microenvironment, which contributes to cancers' evasion of an anti-tumor immune response. Taken together, these findings reveal CCL2 as an attractive, multifaceted target for cancer therapy.

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Figure 1.

CCL2 is tumorigenic through its direct effects on tumor cells and its influence on normal host cells.

Figure 2.
Proposed mechanism by which CCL2 controls autophagic death in prostate cancer cells.
Roca H et al., Autophagy.2008, 4(7):969-71.

Figure 3.
CCL2 has pro-angiogenic effects on endothelial cells through elevated tumor-derived VEGF-A. Li X et al., Cancer Res. 2009, 69(4):1685-92.

Figure 4.
Inhibition of CCL2 reduced the number of infiltrating macrophages in VCaP xenografts.
Loberg RD et al., Neoplasia. 2007, 9(7):556-62.

Figure 5.
CCL2 neutralizing antibodies diminish osteoclast formation induced by prostate cancer conditioned media. Lu Yet al., Cancer Res. 2007, 67(8):3646-53.

Table 1

CCL2 promotes prostate cancer tumorigenesis and metastasis

Source of CCL2	CCL2 functions	Potential mechanisms	References
LNCaP, PC3, VCaP	Promotes cancer cell proliferation; Protects autophagic cell death	PI3K/AKT-dependent; autocrine and paracrine manners; PI3K/Akt-dependent survivin up-regulation	[6-8,26]
LNCaP, PC3, VCaP	Induces cancer cell migration and invasion	PAR-1 mediates Rho activation; Differential regulation of PKC and MMP-9 activation	[6-8,69]
HBME	Promotes PC3 cells transendothelial cell migration	Small GTPase Rac activation	[28]
HBME, VCaP, PC3, LNCaP	Indirectly induces tumor angiogenesis	VEGF-A induction from TAMs; FGF-8b production	[32,33,70]
Osteoblast	PTHrP induces CCL2 production	NFκB and C/EBPβ activation	[9,32]
PC3, C4-2B, Osteoclast, hFOB, HBME	Mediates osteoclastogenesis	RANKL-dependent and -independent pathway	[9,19,25,47]
VCaP, PC3	Promotes tumor growth <i>in vivo</i>	Regulation of TAMs infiltration	[33]