Many Roles of CCL20: Emphasis on Breast Cancer

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Abstract
CCL20 or MIP3α is a small ~8 kDa protein primarily expressed in the liver, colon, prostate, cervix, and skin. The cellular receptor for CCL20 is CCR6. CCl20 unlike many other cytokines only binds CCR6, making the CCL20/CCR6 pathway an attractive drug target. Since the initial discovery of CCL20 in the early 1990's, there has been an increase in the evidence implicating the chemokine and its receptor in a number of diseases, including rheumatoid arthritis and human immunodeficiency virus infection. CCL20 has also been linked to malignancies such as ovarian, colorectal and pancreatic cancers. CCL20 can also attract tumor-promoting immune-suppressive cells to the tumor microenvironment, which may contribute to the immune evasive potential of the tumor and tumor progression.

Keywords: breast cancer, CCL20, CCR6, chemokines, MIP3α

Introduction

Advances in our basic scientific understanding of cancer have led to a clear link between the progression of cancer and the infiltration of immune cells. Understanding how immune response propagates tumor development has become a field of study all its own. Recent studies of the tumor microenvironment (TME) have expanded our knowledge about the involvement of the immune system in tumor progression [1, 2]. Many of these studies have shown a correlation between the infiltration of immune cells (primarily CD8+ and CD4+ T-lymphocytes and macrophages,), tumor burden and patient survival [3, 4].

In a number of cancers, cytokines are significantly upregulated [3, 5], adding to a complex communication network within the TME and systemic recruitment of immune responding cells. It seems apparent that tumor cells utilize autocrine/paracrine-signaling mechanisms to promote cell survival by inducing a shift in gene and protein expression among themselves and neighboring cells. This in turn, facilitates positive feedback loops, which enhance production and secretion of many pro-survival cytokines, proteases, and growth factors [6, 7].

Chemokines and their receptors are exciting targets for therapeutics with several drugs targeting chemokine pathways having reached clinical trials [8-10]. To date, more than 51 chemokines have been identified, and only a subset of these have been studied in depth. Chemokines in general are divided into 4 major groups based on N-terminal arrangement of conserved cysteine residues. The CC chemokines represent the largest of the four groups with 28 identified members. Receptors of the CC family of chemokines are G-protein coupled receptors expressed in a variety of inflammatory trafficking cells and some cancer cells [11]. In many cases, chemokine secreting cells also express the concomitant chemokine receptors and hence can function in a paracrine and/or autocrine manner [12-15].

CCL20

CCL20, also known as macrophage inflammatory protein 3 alpha (MIP3α) [16], Liver and Activation Regulated Chemokine (LARC) [17], , and Exodus-1 [18], was first discovered and characterized in hepatocytes [17] and later shown to be

expressed in the lung [19] and various connective and lymphatic tissues [20, 21]. The gene encoding CCL20 was mapped to chromosome 2q and contains 4 exons and 3 introns [17, 22]. This is a slight variation from most CC chemokine gene sequences that contain 3 exons and 2 introns. The gene contains many transcription factor-binding sites including NFkB, AP-1 and 2, C-EBP, ETS, and SP1 [23-27].

The full-length pro-CCL20 is 96 amino acids in length and contains a classical N-terminal signal sequence, which is cleaved to yield the mature peptide sequence of 70 amino acids (CCL20, 1-70). A single in-frame deletion of alanine at position 27 in the pro-peptide results in a fully functional variant of the full-length protein (CCL20, 2-70) [19, 22]. The mature translated protein contains 2 cysteine-based disulfide bridges that form a "Greek Key" motif in its tertiary structure (Fig.1).

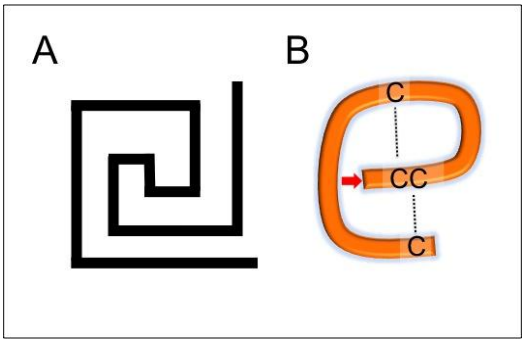

Figure 1. "Greek Key" motif. (A) A representative illustration of the "meandros" art pattern, commonly known as the Greek key (B) The antiparallel strands of the CCL20 protein loop to form a structure reminiscent of the artistic Greek key. The strands are bound together by cysteine disulfide bonds, and CC chemokines have a classical pair of cysteine residues near their amino terminus (red arrow).

Hoover et al. resolved the X-ray crystal structure of human CCL20 [28], and Perez-Canadillas and Chan defined the nuclear magnetic resonance structure [29, 30]. These studies revealed the underlying structural scheme of CCL20, which confers the chemokine innate antimicrobial activity. The groups showed that CCL20 contains a carboxy-terminal alpha helix loop structure consisting of several positively charged residues and yielding a highly cationic region of the peptide. Such cationic properties are commonly associated with naturally occurring antimicrobial peptides such as human lactoferrin [31, 32] and the defensins [28, 33, 34]. CCL20 likely plays an important role in innate immune defense as its expression is induced at common sites of infection, including gastric mucosa and respiratory epithelium [35, 36].

Chan et al. showed that CCL20 existed as a monomeric protein in solutions of pH 3.5 and 4.6, and as a homodimer in solutions of pH 7.0 and 7.5. These data suggest that pH may regulate the structure and bioactivity of CCL20 [30]. This would allow for alternate modes of action for CCL20 based on local pH although no data are yet available to support such functions [37, 38].

CCL20 Expression

As a chemoattractant, CCL20 plays a crucial role in the recruitment of CD34+ derived dendritic cells and T cells [36, 39-41]. Accordingly, upregulation of CCL20 mRNA and protein upon pro-inflammatory stimuli has been shown in various mouse and human tissues [27, 42, 43]. Many pro-inflammatory chemokines and small molecules mediate downstream signaling through activation of the NFkB pathway. This is also true for CCL20, as studies have shown that its expression can be altered by effectors of NFkB signaling namely: TNF, IL-1α and beta, IL6, and IL-17 [24, 42, 44-46].

CCL20 Receptor

The receptor for CCL20 is a 7-transmembrane G-protein coupled receptor of the beta chemokine family [47]. Initially acknowledged as an orphan receptor and given several different names including: GPR-CY4, STRL22 [48], and CKRL-3 [49]. The receptor was designated CCR6 in 1997

by Baba et al. [21]. CCR6 is one of 11 identified receptors belonging to the CC family of chemokine receptors. The only ligands known to bind to CCR6 are β-defensins 1 and 2 and CCL20 [50]. CCR6 shows low-level mRNA and protein expression in most tissues under non-pathological conditions; intestinal mucosa, lung mucosa and lymphoid tissues have the highest levels of expression [51-53]. At the cellular level CCR6 is primarily expressed in Th17 [54], T-reg cells [47], immature dendritic cells [19, 39, 55], subsets of CD8+ cytotoxic T cells [56], memory and effector T cells , and B cells [57]. Basal expression of CCR6 on immune cells is utilized for cellular homing to sites of ligand secretion [47, 56, 58]. In pathological autoimmune diseases, such as inflammatory bowel disease and rheumatoid arthritis, CCR6 is significantly upregulated [59-61]. Interestingly, some epithelial tumors have also been found to express CCR6 [62].

CCL20: Rheumatoid Arthritis and HIV

CCL20 is a key player in the recruitment of inflammatory cells, implicating the chemokine in a variety of inflammatory diseases. Matsui et al. showed that synovial fluid from rheumatoid arthritis (RA) patients had increased concentrations of CCL20 compared to synovial fluid from patients with osteoarthritis [63]. To date multiple studies have established a link between CCL20 and the infiltration of CCR6+ dendritic cells, macrophages and CD4+ T cells to synovial joints [43, 45]. Murakami et al. have shown that recruitment of CD4+ cells to synovial joints in a mouse model of rheumatoid arthritis is a key step in the development of this autoimmune disease [64]. The mechanism of recruitment was identified as an IL-6 and IL-17 mediated expression of chemokines, in particular of CCL20.

The CCL20/CCR6 axis has also been associated with human immunodeficiency virus (HIV) infection. A report by Gosselin et al. suggests that CCR6 is involved in the HIV infection process as CCR6+ T cells showed a significantly higher rate of HIV infectivity vs. CCR6- T cells [65]. Another group has shown that CCL20, CCL19, and CXCL10 can induce latent infection of resting activated CD4+ T cells. This provided a mechanism for a marked reinfection, as these chemokines can recruit CD4+ cells to the blood stream where they target HIV virus laden lymphoid tissues [66].

In contrast to the cooperative function of CCR6 in HIV infection, CCL20 acts as an antiviral agent against HIV [67]. Ghosh et.al discovered that CCL20 directly inhibited HIV infection of TZM-bl cells when recombinant CCL20 was pre-incubated with the virus before first round infection of cell cultures [67]. A study by Fontaine et al. revealed elevated steady state levels of CCL20, CCL2, and CCL19 in blood samples from HIV+ patients [68]. These data support a role for CCL20 in innate immunity. Additionally, many studies have shown that CCL20 is secreted by neutrophils as a first response to infection and that increased CCL20 recruits dendritic cells, thereby implicating CCL20 in the initiation of acquired immunity [69, 70].

CCL20 and Breast Cancer

In recent years, the immune response associated with developing tumors has become a hot topic of study. Chemokines being primary effectors of inflammation correspondingly play a role in immune response. Chemokines have been linked to several types of malignancies, including prostate [71], colorectal [72, 73], ovarian [74, 75], and breast cancer [3, 76]. This is an expected linkage since many tumors generate an inflammatory response [77, 78].

<u>Dendritic cells</u>

CCL20 and its receptor CCR6 may have a significant functional role in the progression and invasion of breast carcinomas. Although there is a limited understanding of the specific contribution of CCL20 in breast cancer development, CCL20 can recruit CCR6-expressing dendritic cells into epithelial tissues [40] and the infiltration of dendritic cells into tumors or

surrounding stroma has been associated with poor prognosis [79]. Bonnotte et al. showed that infiltrating immune cells of colon adenocarcinoma in female rats did not develop an antitumor phenotype as these cells failed to acquire the ability to activate T cells [80]. The mechanism by which dendritic cells retain an immature phenotype in a tumor antigen-rich environment is unknown; however, upregulation of tumor promoting genes may play a role [81]. This phenomenon has also been observed systemically in cancer patients [82], suggesting a significant tumor-mediated reprogramming of dendritic cell function. Le Mercier et al., showed that the pro-tumorigenic/immature phenotype of tumor-associated dendritic cells might be a result of the loss of Toll-like receptor 7 (TLR7) activation. Intra-tumoral injection of TLR7 ligand in an orthotopic mammary tumor model has been shown to be sufficient to induce a shift in the cytokine signature of the tumors and their regression [83]. These data provide a possible mechanism by which dendritic cells are reprogrammed to support tumor immune evasion and tumor progression.

Recently, Marsigliante et al. reported that CCL20 induces migration of human breast cancer cells through activation of both Akt and mitogen-activated protein kinase pathways [84]. Both pathways have been shown to promote tumor growth and metastasis [85, 86]. In addition, CCL20 induces the expression of MMP-9 via NFκB activation [84]. These data are consistent with CCL20 playing a role in both tumor cell survival and metastasis.

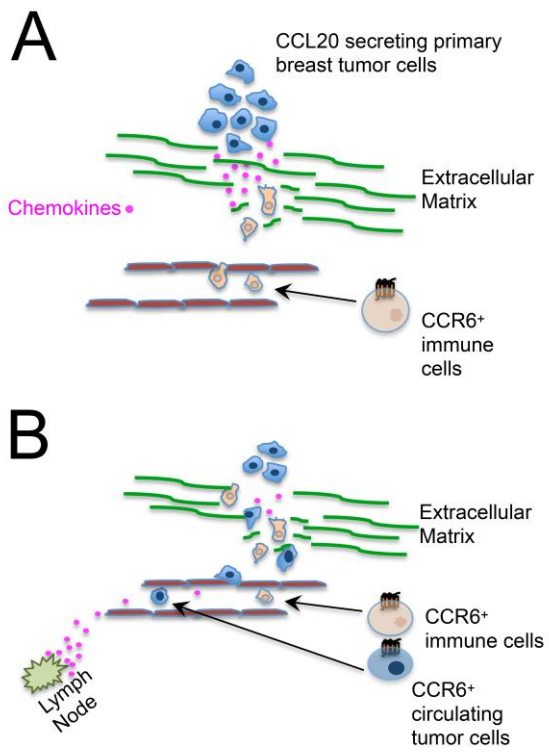

Figure 2. Chemoattractant capability of CCL20. (A) Breast tumor cells expressing CCL20 can attract CCR6 expressing immune cells to the tumor microenvironment, therefore facilitating breakdown of the extracellular matrix. (B) Breast tumor cells that break through the extracellular matrix can migrate to and invade nearby blood vessels. Once in the blood stream tumor cells expressing CCR6 can home to lymph nodes or other organ sites that secrete CCL20.

Tumor-Associated Macrophages

Tumor-associated macrophages (TAMs) are directly involved in cancer progression [87]. TAMs are pro-neoplastic and have a pro-inflammatory gene signature [88], which result in an upregulation of cytokines and the recruitment of immune cells to the TME (Fig. 2). To date a substantial body of evidence supports the involvement of TAMs in the progression and metastasis of breast cancer [89-91]. Bierie et al., showed data that altered TGFβ signaling in the breast tumor microenvironment can upregulate CCL20 in breast tumor cells and that secretion of proinflammatory cytokines may promote early tumor progression [92].

TAMs overexpress and secrete proteases, such as those of the matrix metalloproteinase family, cysteine cathepsins, and serine proteases [90, 93, 94], into the TME. The cysteine cathepsin B and the aspartic cathepsin D have been shown to differentially cleave the monomeric peptide of CCL20 in vitro yielding either a fully functional

CCL20 or a chemoattractant null peptide, respectively [95]. Both of these lysosomal proteases are frequently upregulated in pathological conditions including breast cancer [96-99]. The altered expression of cathepsin B occurs at the message and protein level and results in an increase in cell surface and soluble secreted cathepsin B [100]. As CCL20 is a secreted chemokine there may be an extracellular interface of protease and chemokine. The distinct in vivo relationship between cathepsin B and CCL20 has yet to be studied.

Micro RNAs

The study of micro RNAs (miRNAs) has redefined our understanding of gene regulation and revealed novel regulatory functions for small non-translated RNAs [101]. The introduction of next generation sequencing modalities has rapidly identified several miRNAs, including miR-205, miR-125b, miR-145, and miR-21, which are differentially expressed in breast carcinomas vs. normal breast tissue [102-104]. Interestingly, miR-21 binds to the 3' untranslated region of CCL20 mRNA initiating its degradation [105]. Terao et al. found miR-21 to be differentially expressed in the MCF-7 human breast cancer cell line, and to have different downstream effects in estrogen receptor positive versus estrogen receptor negative breast cancer cell lines [106].

In summation a specific role for CCL20 in breast cancer has not yet been clearly established. Many studies discussed here indicate that CCL20 is deregulated in breast cancer, but to what extent and in what capacity CCL20 may contribute to tumorigenicity is not known [107, 108]. Moving forward, we should evaluate the functional role of CCL20 and how signaling through CCR6 affects gene transcription. These studies should be followed with assays that can monitor functional changes in cell behavior as a result of CCL20 mediated CCR6 activation. Further studies should examine the effects of up-regulation of CCL20 in an in vivo context where an immune cell response can be monitored. This would require the use of animal models with an intact immune system or a humanized immune system. Additionally, examining the effects of CCL20 on immune cell receptor presentation will aid in our understanding of how CCL20 might alter immune cell function. Results from such studies will be invaluable for understanding the role of CCL20 in breast cancer and given the selectivity of CCL20 for its receptor CCR6, therapeutics targeting the ligand/receptor interaction will be relatively easy to test.

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