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Chemokines: novel targets for breast cancer metastasis

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Abbreviations:
ECM: Extracellular matrix, ER: estrogen receptor, GAG: glycosaminoglycan, PR: progesterone receptor, TAM: tumor associated macrophage

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Abstract

Recent studies have highlighted the possible involvement of chemokines and their receptors in breast cancer progression and metastasis. Chemokines and their receptors constitute a superfamily of signalling factors whose prognosis value in breast cancer progression remains unclear. We will examine here the expression pattern of chemokines and their receptors in mammary gland physiology and carcinogenesis. The nature of the cells producing chemokines or harboring chemokine receptors appears to be crucial in certain conditions for example, the infiltration of the primary tumor by leukocytes and angiogenesis. In addition, chemokines, their receptors and the interaction with glycosaminoglycan (GAGs) are key players in the homing of cancer cells to distant metastasis sites. Several lines of evidence, including in vitro and in vivo models, suggest that the mechanism of action of chemokines in cancer development involves the modulation of proliferation, apoptosis, invasion, leukocyte recruitment or angiogenesis. Furthermore, we will discuss the regulation of chemokine network in tumor neovascularity by decoy receptors. The reasons accounting for the deregulation of chemokines and chemokine receptors expression in breast cancer are certainly crucial for the comprehension of chemokine role in breast cancer and are in several cases linked to estrogen receptor status. The targeting of chemokines and chemokine receptors by antibodies, small molecule antagonists, viral chemokine binding proteins and heparins appears as promising tracks to develop therapeutic strategies. Thus there is significant interest in developing strategies to antagonize the chemokine function, and an opportunity to interfere with metastasis, the leading cause of death in most patients.

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1. Introduction

1.1. Chemokines and chemokine receptors: a general view

Chemokines are members of a superfamily of *chemo*tactic cyto*kines* (Table 1) initially characterized because of their association with inflammatory responses, by stimulation of leukocyte chemotaxis during inflammation [1, 2]. However, it is now known that they also play roles in homeostasis, cell proliferation, haematopoiesis, viral/cell interactions, angiogenesis, neovascularization and cancer metastasis [3-7].

Chemokines are defined independently of their function, based on their amino acid composition, specifically on the presence of a conserved tetra-cysteine motif [8-12]. The relative position of the first two consensus cysteines (either separated by a non-conserved amino acid or next to each other) provides the basis for classification of chemokines into the two major subclasses, CXC (17 members) and CC (28 members) chemokines, respectively [8, 13, 14] (Figure 1). Three homologous molecules are also regarded as chemokines. These are CX3CL1, with three intervening amino acids between the first cysteines, and XCL1 and XCL2, which lack two out of four canonical cysteines. Interestingly, CX3CL1 is the only chemokine with a localization at the membrane. To date, the official nomenclature accounts for at least 48 human chemokines [11, 14, 15].

Chemokines were the first members of cytokine family that were shown to interact with G-protein-coupled receptors (GPCRs) with seven transmembrane (7TM) domains. Chemokine receptors comprise 10 CCR family members, 7 CXCR family members and other receptors (XCR1, CCRL1 and 2, and CX3CR1). The chemokine system also includes at least 3 “silent receptors. These receptors bind ligands with high affinity but do not elicit signal transduction. The D6, Duffy antigen receptor for chemokines (DARC) and CCX-CKR (ChemoCentryx, chemokine receptor) are specialized for chemokine sequestration acting to regulate chemokine bioavailability and therefore influence responses through signaling-competent chemokine receptors [16-18]. Chemokine receptors function as allosteric molecular relays

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where chemokine binding to the extracellular portions modifies the tertiary structure of the receptor, allowing the intracellular part to bind and activate heterotrimeric G-proteins [19] (Figure 2). Upon receptor binding, a cascade of downstream signals takes place, including calcium mobilization and the activation of extracellular signal-regulated kinases 1 and 2 (ERK1 and ERK2), p38 mitogen-activated protein kinase (p38 MAPK), phospholipase-Cβ, phosphatidylinositol 3-kinase (PI3K), RAS, the RHO family of GTPases, p21-activated kinase (PAK), and NF-κB [12, 20] .

There is a high redundancy in chemokine family as multiple chemokines bind to the same receptor [14] (Table 1). This feature might be essential for a fine tune of specific responses. In general the CC receptors are more promiscuous than the CXC receptors. Some chemokines bind to multiple receptors and some receptors in turn bind multiple chemokines, whereas certain chemokines interact with single receptor and some receptors bind only one chemokine.

1.2. The particular case of CXC chemokines

CXC family comprises 17 members and CC family has 28 members (Table 1). The CXC chemokines are further divided into ELR+ and ELR– chemokines based on the motif glutamate-leucine-arginine (E-L-R), which is located upstream from the CXC sequence. CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, CXCL7 and CXCL8 are all ELR-positive chemokines [21]. This appears important as the NH2-terminal motif has been shown to be responsible for the ability of chemokines to attract neutrophils as well as a necessary motif involved in angiogenic properties of these chemokines [7, 22]. All angiogenic ELR+ chemokines bind to CXCR2. In addition, CXCL6 and CXCL8 bind to CXCR1 [23]. CXCL12, which binds CXCR4 and CXCR7 [24, 25] is the only ELR-negative chemokine which possesses angiogenic properties [26-28]. CXCL4, CXCL4L1, CXCL9, CXCL10, CXCL11, CXCL14 are angiostatic and generally anti-invasive [29-32], although recent studies on colorectal cancer have shown that CXCL10 is able to promote invasion [33]. CXCL4 and its

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variant CXCL4L1, which differs from CXCL4 by 3 amino acids are potent inhibitors of angiogenesis [31, 32]. CXCL4L1 is even more angiostatic than CXCL4 [32]. The high affinity of these chemokines for heparin sulphate could account for their angiostatic properties [30]. CXCL9, CXCL10 and CXCL11 bind CXCR3, whereas it remains unclear whether CXCL4 and CXCL4L1 bind to CXCR3.

1.3 Decoy receptors

Three Decoy receptors, namely Duffy Antigen Receptor for Chemokines (DARC), D6 and CCX-CKR have been identified [16-18]. DARC binds various pro-inflammatory chemokines of both CC and CXC (containing ELR motif) subclasses. DARC is present on erythrocytes and vascular endothelial cells, where it is up-regulated during inflammation [34, 35]. DARC lacks canonical intracellular signaling motifs and does not support any ligand induced signaling and migration. Thus, DARC is thought to be involved in transcytosis or neutralization of chemokines at EC barriers and, on erythrocytes it may regulate plasma chemokine concentrations. Overexpression of DARC on endothelial cells of mice results in reduced angiogenic response to certain CXC chemokines [36]. Recent data suggests that DARC is key modulator of the progression of prostate cancer by clearing angiogenic chemokines from the tumor microenvironment and attenuating angiogenesis [37]. Furthermore in humans who lack erythroid DARC it may be an important contributing factor to the increased progression and mortality of prostate cancer. Additionally, DARC is also shown to interact with tumor metastases suppressor protein KAI1/CD82, which results in transmission of senescent signal to the cancer cell [38].

D6 receptor binds to almost all inflammatory CC chemokines (CCLs 2,3L1, 4,5,7,8,11-14 and 22) and weakly to CCL17 [39]. D6 undergoes rapid internalization and degradation of the ligands, while the receptor recyles. D6 knock out mice have provided useful insight into biological role of D6, with impaired chemokine clearance, excessive inflammation and diminished resolution efficacy being described [39].

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CCX-CKR binds constitutive CC chemokines CCLs19,21 and 25 and also weakly to the follicular CXC chemokine, CXCL13 [40, 41]. Similar to D6 and DARCs, no signal is detected in response to the chemokine binding. Though there is little specific information regarding the expression and function of CCX-CKR at present, its ligand binding profile provides a compelling case for its involvement in homeostatic lymphocyte trafficking and immune responses [40, 41]. Therefore further investigation is required to characterize the functional significance of the decoy receptors which might provide an opportunity for therapeutic intervention.

2. Chemokines and chemokine receptors: correlation with breast cancer disease progression

2.1. Levels of chemokines and chemokine receptors in normal and cancerous breast

The normal breast expresses a set of chemokines, even though generally at low levels. Indeed, CXCL1, 2, 3, 5, 6, 7 and 8 have been detected in human milk [42, 43]. Primary culture of normal breast epithitelial cells from healthy patients also secrete CXCL8 in addition to TNFα and IL-6 [43]. But in many cases, chemokines are found at higher levels in cancer tissues compared to normal tissues even though a global gene expression profiling comparing normal epithelial cells and in situ, invasive and metastatic breast carcinomas using SAGE analysis revealed that many chemokines are highly expressed in normal breast and lost in carcinoma, including CXCL1, CXCL2, CXCL5, CXCL6, CXCL8, CXCL20, CX3CL1, CCL2 and CCL7 [44].

Among the chemokines which have been analyzed in breast cancer, CXCL8, CCL2, CCL4 and CCL5 are certainly the ones which have been the most studied.

CXCL8 has been shown to play an important role in numerous pathologies and in many types of cancers [45]. CXCL8 is overexpressed in breast cancer compared to normal tissues both at the protein levels and the RNA levels in primary tumors [46-48] and in the sera of

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patients [49, 50]. To our knowledge, only one study performed on a limited number of samples did not report any differences in CXCL8 levels between infiltrating mammary carcinoma and normal breast [51]. We have also reported, using the novel Luminex technology, that multiple cytokines including chemokines such as CXCL8, CCL2, CCL4 were also present at high levels in cancer tissues compared to normal tissues [47].

CCL5 has been shown to be mainly expressed in tumor epithelial cells, but found only rarely in normal breast biopsies [52, 53]. Serum CCL5 levels are also higher in high grade breast tumors compared to low grade tumors [54]. In addition, CCL5 levels are elevated in primary tumors and metastatic sites [54].

Interesting studies have also shown that several receptors of chemokines are highly expressed in cancers. This is the case of CXCR4 and CCR7 in breast tumors, which bind CXCL12 and CCL19/CCL21 respectively [55, 56]. CCL21 is mainly expressed in lymph node, which could explain the migration of breast tumor cells to the lymph node [55]. On the other hand, CXCL12 is present at high levels in lymph node, bone marrow, liver and lung, which could also account for the migration of breast cancer cells to the sentinel lymph node and subsequently to distant metastasis organs [11, 55, 57, 58]. It is important to note that the levels of chemokine receptors might be not be sufficient to determine whether a particular cell will be responsive or not to its regular ligand. Indeed, Holland et al. have shown by analyzing a panel of breast cancer cell lines that CXCR4 expression was relatively uniform between the different cells at the protein levels (western blot, FACS, ligand binding) and RNA levels. But when looking at CXCR4 signalling, only metastatic cell lines displayed a functional receptor [59].

2.2 Correlation of Chemokines and Chemokine receptors expression with the progression of the disease

The systemic dissemination of tumor cells is thought to be an organized process as certain malignancies have a bias to metastasize to a specific distant organ, suggesting a crucial role

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of the organ microenvironment for the localization and growth of the metastatic tumor cells. For example breast cancer has a distinct metastatic pattern with metastases commonly localized to lung, liver, lymph nodes, brain and bone [60].

Multiple chemokines or chemokine receptors are also correlated to the grade of breast tumors, metastasis and to prognosis [61]. Using luminex technology, we we have observed that CXCL8 protein levels are correlated with the grade [47], which is in agreement with other studies [50]. CXCL8 expression is also linked to macrophage infiltration [47]. CXCL1, 2, 3, 4, 5, 6, 7 and 8 are localized in a narrow region of chromosome 4 (4q13 locus) [11, 48]. This feature of an "activated cluster" is important as we have reported that CXCL1, 3, 5 and 6 transcripts were also more abundant in CXCL8 – positive breast tumors [48]. CXCL8 gene promoter polymorphisms appears also important to predict the risk of breast carcinoma. Increased risk of breast cancer with heterozygous CXCL8 (-251) TA and homologous CXCL8 (-251) AA variants is observed [62]. Interestingly, the presence of high CXCL8 levels in metastatic breast cancer cells [48] could explain the effects of metastatic cells in bone metastasis, as CXCL8 has been shown to stimulate osteoclastogenesis and bone resorption, leading to an enhanced osteolysis [63].

In addition to CXCL8, other CXC chemokines of 4q13 region have an enhanced expression in metastatic sites. This is the case of CXCL1, 2, 3, 5, and 6 [48]. In the same line, CXCL12 is more abundant in lymph node- positive tumors than in lymph node- negative tumors, in tumors from patients who relapsed, who metastasized or did not survive than in patients that were disease free [64, 65].

Concerning CC chemokines, CCL2 and CCL4 levels are also correlated to the grade of breast tumors [47]. High levels of CCL2 or CCL4 trigger macrophage, B and T lymphocytes recruitment to the tumor [47, 66], which is correlated with a poor prognosis [67]. We did not report an association with lymph node status, although another study showed that CCL2 was correlated to lymph node status [66]. CCL2 -2518A/G promoter polymorphism has been shown to be correlated with staging and metastasis of breast cancer patients [68].

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CCL5 is also highly expressed in high grade tumors [52, 54] and is a predictor of worse disease progression of stage II breast cancer patients [53, 69]. Serum CCL5 levels are also more abundant in high grade breast tumors compared to low grade tumors [54].

The expression of chemokine receptors is also altered in breast cancer. Müller et al. first demonstrated a potential mechanism for site-specific metastasis which is related to expression of the CXC chemokine, CXCL12 [55]. The CXCR4 receptor is upregulated in primary breast cancers compared to normal breast tissue, whilst the CXCL12 ligand shows peak levels of mRNA expression in the common metastatic sites of breast cancer. There is compelling evidence that CXCR4 is a key mediator of metastatic breast cancer. Immunohistochemical staining of primary breast tumors has revealed that normal breast epithelial cells do not express CXCR4 whilst between 5 and 73% of cancers do express this receptor [55, 70, 71]. CXCR4 expression in the primary tumor has also been positively correlated with the degree of lymph node metastasis [65, 70, 71], bone metastasis [72], poor patient overall survival [73] and tumor grade [74]. Although the expression pattern of CXCR4 does not show a significant correlation with haematogenous metastasis in a study by Kato et al. [71], CXCR4 expression has been linked to the ability of breast cancer cells to metastasize to the lungs [75].

CCR7 is expressed in several tumor including upregulation in primary tumors of human invasive lobular and ductal carcinoma compared with normal mammary tissue [70, 76]. CCL21 the ligand for CCR7 is strongly expressed in lymph nodes, and it's over expression in murine melanoma cell line resulted in increased metastasis to lymph nodes in vivo [77]. Furthermore, CCR7 expression correlates with and can be used to predict, metastasis in both breast and colorectal cancer [78].

Other reports have shown that the levels of CX3CR1, CXCR4, CCR6 and CCR7 are not correlated to overall survival or disease-free survival [76]. On the other hand, CX3CR1 expression is associated with an increased risk of relapse to the brain of breast cancer patients, CXCR4 is associated with metastasis to the liver and CCR6 to the pleura and

CCR7 to the skin [76]. DARC is inversely correlated to microvessel density, lymph node status and distant metastasis [79].

3. Chemokine producing cells and chemokine receptor expressing cells in human breast cancer: modulation of angiogenesis and infiltration

3.1 Angiogenesis and leukocyte infiltration

Vessel density has been shown to be an independent predictor of pathological stage in many solid cancers. New vasculature is essential for tumor growth beyond 1-2 mm. In the formation of new blood vessels the important balance between angiogenic factors and angiostatic factors is disrupted and shifts towards angiogenesis. Angiogenesis is a complex process which appears critical for cancer development [80, 81]. Blood vessel density is correlated with a higher incidence of metastasis and a shorter relapse-free survival [82-84]. Tumor associated macrophages are also important in breast cancer development. High levels of macrophages in tumors are frequently correlated with a poor prognosis [85]. By analogy with the Th1/Th2 dichotomy in T cell responses, macrophages have also been classified as M1 and M2. Indeed, IFNγ activated macrophages correspond to the M1 population, whereas, whereas M2 macrophages are those which have been exposed to IL-4 [86]. M1 macrophages are potent effector cells which kill microorganisms and tumor cells and produce pro-inflammatory cytokines. On the other hand, M2 macrophages tune inflammatory responses and adaptive Th1 immunity and promote angiogenesis [86]. Interestingly, TAM have the properties of polarized M2 macrophages, which promote tumor progression and angiogenesis [87]. TAM are immobilized in low vascularized [88] and necrotic hypoxic regions of tumors [89]. In hypoxia conditions, TAM cooperate with tumor cells to promote angiogenesis [90]. The “macrophage balance” hypothesis [91] emphasises the dual potential of TAMs, which can both increase tumor growth but also induce an immune response and tumor regression. Recent advances also suggest that

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immunosurveillance as part of immunoediting may be responsible for regression/elimination of some tumors and for shaping the phenotype of other tumors that survive [92, 93].

Alterations in expression patterns of adhesion molecules such as integrins, selectins and cadherins on tumor cells can affect interactions with ECM components such as laminins, collagens and fibronectin [94]. This alteration increases the ability of tumor cells to bind to these components once the basement membrane is breached [95], thereby contributing to invasion and metastasis.

The production of proteases by malignant cells that are usually involved in ECM remodelling has been implicated in allowing migration of the cells through the ECM [96]. Matrix metalloproteinases (MMPs) are a family of highly conserved zinc dependent endopeptidases, capable of degrading the majority of the components of the basement membrane and the ECM, but also a number of chemokines [97]. Within the tumor environment there is a mixture of cell types providing agonistic, antagonistic and synergistic molecules which modify tumor cell behavior. The ECM surrounding ductal carcinoma cells contains active cells, including fibroblasts and TAMs [98, 99]. Tumor cells, fibroblasts and TAM all contribute to a rich milieu of cytokines. The interplay of all these cytokines on tumor cell behavior is highly complex, some cytokine effects are advantageous and some are disadvantageous to tumor growth. Such cytokines include the interleukins, e.g. IL-6, growth factors e.g. EGFs (epidermal growth factors), TGFs (transforming growth factors), interferons, TNF (tumor necrosis factor), and VEGFs [91].

Multiple chemokines have shown to be involved in angiogenesis [22, 29, 100, 101]. CXC chemokines are the main group of chemokines involved in this process and affect the process depending on the presence or absence of the ELR motif on the amino terminus [8, 13, 14]. Angiogenic chemokines include (CXCL1, 2,3,5,6 and 8), only exception is CXCL12 that lacks the ELR motif but can exert angiogenic activity . The angiostatic CXC chemokines are mainly CXCL9, 10 and 11 [8, 13, 14]. They do not only inhibit the neovascularisation effects of the angiogenic chemokines but also inhibit more classical angiogenic factors such as bFGF and VEGF. Increased angiogenesis in breast cancer cells has been shown to

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correlate with increased macrophage infiltration and decreased survival [85, 102, 103]. The proangiogenic activity of most CXC chemokines appears to be mediated through CXCR1 and CXCR2 receptors, whereas CXCR3 appears to bind the angiostatic CXC chemokines [8, 13, 14]. Studies in vivo have demonstrated that CXCL8 regulates tumor development, angiogenesis and metastasis in breast cancer [47, 48, 104, 105] and many other solid tumors including prostate cancer, transitional cell carcinoma, ovarian cancer [45, 106, 107].

3.2 Cancer cells and tumor microenvironment contribute to chemokine action

Chemokines can also influence angiogenesis indirectly by modulating leukocyte recruitment. In breast cancer, epithelial cancer cells produce soluble factors that induce infiltration of cells of monocytic lineage and produce a repertoire of CXC chemokines (CXCL1,2,3,5,7 and 8) [98, 108]. CXC chemokines frequently recruit neutrophils and lymphocytes, whereas CC chemokines attract essentially lymphocytes and monocytes [98]. Chemokine recruited TAM also produce a rich milieu of cytokines/chemokines. In addition, there is evidence that chemokines direct endothelial progenitor cells in to tumor vasculature [109]. Many factors associated with both the tumor cells and the surrounding tumor microenvironment can affect the process of metastasis. An understanding of the microenvironment is essential to appreciate the role of chemokines in this complex cascade. As with many complex biological processes, several models have been put forward to explain the pathways involved. In the counter-current model of invasion and metastasis, tumor cells produce chemoattractants for mononuclear cells and granulocytes, which attract tumor-associated macrophages (TAMs) and tumor associated neutrophils (figure 3). These cells are a source of growth factors, cytokines, enzymes and chemokines associated with angiogenesis, growth, migration and invasion. Invasion and metastasis of cancer cells then occurs along channels created by chemo-attracted leukocytes, in a counter-current direction [110].

On the other hand, the tumor microenvironment, which includes cancer associated fibroblasts (CAFs), endothelial cells and leukocytes, is also a source of chemokines and

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appears to be a major factor in breast carcinogenesis through paracrine action (Figure 3). CAFs, which are suspected to promote carcinogenesis, secrete different types of chemokines including in particular CXCL12, which in turn acts on cancer cells by promoting their proliferation [27, 65, 111, 112]. In addition, CAFs induce angiogenesis, by recruiting endothelial cells into carcinomas [27]. More precisely, CXCL12 has been shown to be overexpressed by myofibroblasts , whereas CXCL14 is overexpressed by myoepithelial cells in cancer tissues compared to normal breast [113].

In addition to epithelial cells, CXCL8 is also produced by endothelial cells, although at lower levels [48], which may induce chemotaxis and transendothelial migration for cancer cells [114]. Several studies have reported a positive correlation between CXCL8 levels and Tumor associated macrophages in breast [47, 115]. Other CXC chemokines are also either mainly produced by epithelial cancer cells (CXCL5 and CXCL6), or expressed in blood cells (CXCL2, 3, 4, 7) [48]. In addition, endothelial cells and CAFs produce non negligible levels of CXCL8, 1, 2 and 3 [48].

CCL2, which is present both in epithelial cancer cells and stromal cells such as macrophages [115, 116], is associated with increased TAM recruitment and neovascularization in breast cancers [47, 116, 117], although this was not reported by another group [118]. CCL2 has been shown to be expressed by TAMs and endothelial cells [119]. We have shown that breast tumors expressing high levels of CCL2 and CCL4 exhibited a higher content in B lymphocytes, T lymphocytes and macrophages than tumors expressing low level of these chemokines [47]. CCL2 possesses chemotactic activity for monocytes and T lymphocytes [120, 121].

CCL5 has been shown to be mainly expressed in tumor epithelial cells, but found only rarely in normal breast biopsies [52, 53]. CCL5 expression in breast cancer is also correlated with monocyte infiltration, which leads to the enhanced expression of MMP-9 by breast cancer cells and possibly to an increase metastasis [69].

Combining these data, we propose a general model of action of chemokines in breast cancer in which both epithelial cancer cells and cells within the microenvironment secrete

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chemokines (Figure 3). In addition, cells present in the breast harbor various levels of chemokine receptors. This complex interaction between cells appears crucial for angiogenesis, and metastasis and homing of cancer cells to metastatic sites.

3.3 Chemokines and Glycosaminoglycans (GAG)

Several lines of evidence suggest that the interaction of chemokines with glycosaminoglycans (GAGs) is crucial for their action. Heparan sulphate (HS) proteoglycans are cell-surface and ECM macromolecules that comprise a core protein covalently linked to one or more HS glycosaminoglycan (GAG) chains [122, 123]. HS proteoglycans are classified into several families based upon their core protein structure. Glypicans and syndecans are the two major cell-surface HS proteoglycans, and are linked to the plasma membrane by a glycosylphosphatidylinositol (GPI) linkage or a transmembrane domain respectively. Perlecans are secreted HS proteoglycans that are mainly distributed throughout the ECM [124]. GAGs are unbranched complex acidic polysaccharide chains consisting of a backbone of repeating disaccharide units. These GAG chains are chemically defined as heparan sulphate, chondroitin sulphate, dermatan sulphate and keratin sulphate. HS is synthesised as a repeating disaccharide of glucosamine and glucuronic acid and is produced by virtually all cells from simple invertebrates to humans [122, 123].

GAGs, in particular HS are important in promoting chemokine activity in vivo. Evidence of chemokine involvement with GAGs is abundant but the diversity of chemokine/GAG interactions studied thus far has provided conflicting evidence of their role. In vitro almost all chemokines studied to date, including CXCL12, bind to HS, suggesting that this represents an important feature of these proteins [125]. Evidence that chemokine/GAG interactions are important in vivo is suggested by the finding that cytotoxic T-cells secrete CCL3, CCL4 and CCL5 complexed with sulphated proteoglycans [126]. Chemokines are known to bind GAGs both in vitro [127] and in vivo [128], however, it has only recently been demonstrated that this interaction is required for their function in vivo [129-131].

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Immobilisation of chemokines on GAGs has long been thought to facilitate the retention of chemokines on cell surfaces and to enable localised high concentrations of chemokines to provide a directional signal for cells [132]. Chemokine/GAG interactions are not the only factor important for the migration of cells in vivo, as demonstrated by the importance of shear forces in promoting lymphocyte migration across a vascular endothelium bearing apical chemokines [133] (Figure 4). However, recent work in our group has demonstrated that a non-GAG binding mutant CCL7 maintains normal affinity for its receptor and can induce intracellular calcium flux, however, although the mutant CCL7 can cause chemotaxis in vitro, it cannot induce transendothelial migration or migration of leukocytes in vivo [129].

Oligomerization of chemokines is required for the activity of some chemokines, and chemokine/GAG interactions may be important in the oligomerization of chemokines for in vivo activity. GAGs have been shown to induce some chemokines to oligomerize [131, 134, 135]. Higher order oligomers of certain chemokines are required for in vivo but not in vitro activity [131] and mutant CCL5 chemokine which is unable to form an oligomer can activate receptors in vitro but is non-functional in vivo. In addition to these functions [130], variation of GAG chains may contribute to the selective presentation of chemokines to their receptors [136, 137]. GAG interactions may also protect chemokines including CXCL12 from enzyme degradation [138, 139].

4. In vitro and animal models evaluating chemokine roles in cancer

Recent work has implicated chemokines in tumor growth and metastasis. Work on chemokines has largely been focused on their involvement in regulating immune cell localization as either part of normal physiological cell trafficking or within an immune response [4, 140].

In normal leukocyte migration, leukocytes travelling under flow conditions have to undergo several steps in order to leave the blood stream and reach their target site within body tissues. These steps include slowing down by rolling along the activated endothelial surface and firmly adhering to the endothelium through interactions with upregulated integrins

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present on both the endothelial cells and the leukocytes [141]. After binding to the endothelium, the leukocyte extravasates into the surrounding tissue by diapedesis between endothelial cells; this process may be controlled by chemotactic signals and/or mechanical shear forces [133]. Subsequent breakdown of the ECM allows the cells to reach the target site of injury. A primary tumor is believed to act in a similar way to a site of injury, producing multiple cytokines, leading to an influx of TAMs.

Chemokines such as CXCL12 [142] play a fundamental role in orchestrating the integrin-mediated arrest of leukocytes on endothelial cells (triggering spatial and conformational change in integrins). Diapedesis then follows as the endothelial cells separate, allowing the leukocyte to move between the cells into the subendothelial space. Leukocyte migration is promoted in vitro by soluble chemoattractant gradients that lie beneath the endothelial cell barrier as shown in Boyden chamber assays [143]. Recent in vitro evidence suggests that apical chemokines even in the absence of a chemotactic gradient across the endothelial cells promoted lymphocyte trans-endothelial migration. In this study the continuous application of a shear force to the adherent lymphocytes was mandatory for lymphocyte migration [133]. Overall, this suggests that both apical and subluminal chemokines may be involved along with mechanical shear forces.

We and others have shown that human breast cancer cells secrete a number of chemokines, including CXCL1, 2, 3, 5, 6, 8 [48, 104, 105, 144, 145]. In addition, murine mammary tumor cells also express a number of chemokines, including in particular CCL2, CCL5 and CXCL1 [146].

In vitro studies have been performed to analyze the effects of different chemokines on breast cancer cell migration. Youngs et al. have reported using a chemotaxis assay that the migration of MCF-7 and ZR-75 cell lines respond chemotactically to CCL2, CCL4, CXCL1 and CXCL8, whereas T47D is unresponsive to the chemokines tested [147]. Interestingly, this is not due to a defect in chemokine receptor expression as T47D cells bound radiolabelled ligands with binding constants similar to the one of MCF-7 [147]. We have also shown that ZR-75 cells migrate in response to CXCL8 [104]. Other groups have shown that

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breast cancer cell lines can migrate in transwell assays in response to chemokines such as CCL3, CCL4 nor CCL5 [148]. CXCL8 promotes breast cancer angiogenesis and invasion in vitro, but not in vitro proliferation [104, 105]. In athymic mice models, CXCL8 levels are higher in metastatic sites such as lung compared to the primary tumor [144].

The effect of CXCL12 on the proliferation of CXCR4-expressing breast cancer cells has not been fully defined, although a small number of studies have demonstrated a proliferative effect in vitro and in vivo [149-151]. Importantly, a recent study using a CXCR7 antagonist has suggested that CXCL12 can enhance cancer cells growth and survival through stimulation of CXCR7 [25], suggesting that both CXCR4 and CXCR7 can play roles in breast cancer development. Overexpression of CXCL12 in breast cancer cells increases their invasiveness in vitro [65]. On the other hand, the down regulation of CXCR4 expression by RNA interference is able to inhibit breast cancer cell invasiveness in vitro [152]. In addition, CXCR4 knock-down in murine breast cancer cells is sufficient to decrease in vivo tumor growth and metastasis [151].

Other chemokines and chemokine receptors are also important in vivo for breast cancer development. CCL2 neutralization using antibodies has been reported in immunodeficient mice bearing human breast carcinoma cells to decrease lung micromestatases and enhance mouse survival [153]. CCR5 blockade with a dominant-negative form of CCR5 also potently inhibits in vivo tumor growth of p53-positive breast cancer cells but not the of p53-negative breast cancer cells [154]. In the same line, the stable expression of Duffy antigen receptor for chemokines (DARC) in metastatic breast cancer cells is able to reduce tumor growth, metastasis to the lung and angiogenesis [155].

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5. Mechanisms of control of expression of chemokines and chemokine receptors

5.1 A variety of signals regulate chemokine and chemokine receptor expression

Indirect evidence of correlation of chemokine expression with other factors suggest that chemokines are either regulated by these factors or share common pathways of regulation with these protein. In support of this, CCL2 expression has been shown to be correlated with VEGF, TNFα and CXCL8 expression in breast cancer samples [115]. The expression of CXCL8 is also correlated to one of the Eukaryotic initiation factor 4E (eIF4E) [156].

More direct proof of chemokine regulation by numerous factors have been brought in the context of breast cancer, in particular in the case of CXCL8. CCL2 and CXCL8 gene are regulated by IL-1β and TNFα in breast cancer cells [157, 158]. In the case of IL-1β, the regulation of CXCL8 level occurs at the post-transcriptional level by stabilization of CXCL8 mRNA through 3'UTR sequences [159]. CXCL8 gene expression has been shown to be regulated negatively by tumor suppressor Tid-1 through the NF-κB site of CXCL8 promoter and appears to be associated to the anti-invasive properties of Tid-1 [160]. COX2 overexpression increases CXCL8 levels in ER-negative breast cancer cell lines, but not in ER-positive breast cancer cell lines [161]. Interestingly, CXCL8 gene is also regulated by other chemokines. Indeed, CCL2 and CCL5 up-regulate the secretion of CXCL8 in monocytic cells, but not in breast tumor cells [162]. EGF potently up-regulated CXCL8 secretion by breast tumor cells, and its effect was promoted by a consecutive treatment of the cells by estrogen and progesterone [162]. In the same line, CXCR3 expression is increased when breast cancer cells are serum starved, or upon exposure to its ligand CXCL10 [163]. CXCL10 levels are down-regulated by IL-6 [163]. CXCR4 levels may also be regulated by the hypoxia-induced Hif-1α pathway [28, 164]. Wild-type but not cancer specific mutants (R175H or R280K) of P53 down-regulate CXCR4 expression through a CRE/AP-1 element present in CXCR4 promoter [165].

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5.2 Estrogen receptors, Progesterone receptors and chemokines

Growth of human breast cancers is closely regulated by estrogens [166], that interact with estrogen receptors alpha (ERα), the main estrogen receptor in breast tumors [167, 168]. ERα expression increases in about two third of breast cancers. Patients with ERα-positive breast tumors can generally benefit from anti-estrogen therapy. Despite treatment, almost all patients with metastatic disease and about 40% of patients receiving adjuvant tamoxifen eventually relapse [169]. It is also essential to note that ERα-negative breast tumors are more aggressive than their ERα-positive counterparts, which is essentially due to their higher ability to develop metastases. In addition, some ERα-positive breast cancer cells will anyway metastasize for unknown reasons. For these reasons, estrogen receptors and progesterone receptors (whose expression is tightly regulated by estrogens) appear to be critical markers for the classification of breast cancer.

Interestingly, several reports show that chemokines (e.g. CCL2, CCL3, CXCL8, CXCL12) or chemokine receptors (CCR2, CXCR3) have an expression dependent of estrogen or progesterone receptors. In rodents, estrogen have been shown to decrease significantly the levels of the chemokines CCL2 and CCL3 in murine mammary tissue, and this regulation is partially abrogated by the co-treatment with 4-hydroxytamoxifen [170]. In addition, transfection of in estrogen receptor beta (ERβ) in SKBR3 cells also down-regulate CCL2 promoter activity in the presence of estrogens. This does not require the DNA-binding domain of the receptor, suggesting that this effect is not mediated by a direct binding of the receptor to CCL2 promoter [171]. We have also demonstrated that CCL2 and CCL4 displayed an expression inversely correlated to ER and to PR in breast cancer biopsies [47]. In the same line, CCL2 is also down-regulated by progesterone receptor in the presence of progestins in breast cancer cells [172]. This might be the result of a negative interaction between the RelA(p65) subunit of NF-κB and the progesterone receptor [173].

CXCL12 has been shown to be regulated positively by estrogens in breast cancer cells [149]. This response is blocked by the pure ER antagonist ICI 182,780. More importantly, CXCL12

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antibody is able to abrogate E2-induced proliferation of breast cancer cells [149]. To date, the mechanisms responsible for E2-regulation of CXCL12 gene remain unclear. In addition, Chemokine receptors are also regulated by estrogens. This is the case for CCR2 and CXCR3 which are up-regulated by estrogen and tamoxifen in murine monocytes [174].

We and others have reported that CXCL8 is overexpressed in ER-negative breast cancer cell lines [48, 104, 105]. There is also an inverse correlation between CXCL8 and ER or PR in breast cancer biopsies [47, 48, 175]. Serum levels of CXCL8 are higher in patients with PR-negative breast tumors who are older than 50 [176]. In addition, we have shown that ER could down-regulate CXCL8 promoter [157], by inhibiting in particular NF-κB activity. Reintroduction of ER into ER-negative cancer cells is known to inhibit their proliferation and their invasion potential [168, 177-180]. This reduced invasion is linked to a down-regulation of CXCL8 expression [104].

5.3 NF-κB and AP-1 pathways are important regulators of chemokines

Multiple proofs of evidence suggest that NF-κB might be involved in tumor progression by regulating steps such as angiogenesis and invasion [181]. NF-κB promotes cell survival through inhibition of apoptosis [182]. Constitutive

NF-κB activity is associated with aggressive forms of breast cancer [183, 184] and is also involved in cancer growth and development [185, 186]. In addition, NFκB activity has been shown to be suppressed by cotransfection of ER [157, 183, 187].

A number of chemokines are regulated by NF-κB, frequently through the presence of NF-κB response elements in their promoter region. This is the case in particular of CXCL8 gene which displays a high expression in breast cancer cell lines exhibiting important NF-κB activity [157] (Figure 5). In addition, inhibition of NF-κB pathway with a dominant negative form of Iκ-B is sufficient to abolish CXCL8 expression [157]. NF-k up-regulation of CXCL8 occurs at the transcriptional level through a NF-κB site present in CXCL8 promoter [157].

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CXCL1 promoter displays many features common to CXCL8, particularly a crucial NF-κB site involved in constitutive expression of CXCL1 gene in melanoma [188, 189]. CXCL2 is also regulated by NF-κB pathway. Indeed, Muellerian-inhibiting substance (MIS) induces CXCL2 expression in breast cancer cells through NF-κB and Smad1 networks, and phospho-Smad1 and p65 are recruited to CXCL2 promoter as shown by Chips experiments [190].

AP-1 activity has also been associated with more aggressive forms of breast cancers. Members of AP-1 family such as Fra-1 are overexpressed in aggressive tumors such as ER-negative breast cancer cells [157, 191-193]. We have shown that CXCL8 levels were correlated to AP-1 expression in breast cancer biopsies [47, 48]. In addition, AP-1 and in particular Fra-1, Fra-2 and c-jun directly regulate CXCL8 promoter activity in synergism with NF-κB transcription factors p50 and p65 [157] (Figure 5).

5.4 Phosphorylation pathways and chemokines

Chemokines trigger a cascade of phosphorylation through different pathways. On the other hand, chemokines are also regulated by several types of phosphorylation and in particular by Heregulin-beta 2 (HER2/ c-erb-B2/ neu). Indeed, CXCL8 and CCL4 are linked to HER2 status [47]. This is in agreement with studies demonstrating that CXCL8 and CXCL1 are regulated by HER2 in breast cancer cell lines [194, 195]. In the same line, CXCR4 is also correlated to HER2 expression in breast cancer patients [70, 73]. ER-negative breast cancer cells stably transfected with HER-2 display an up-regulation of CXCR4, which appears essential for lung metastasis [73]. On the other hand, CXCL12 also transactivates HER-2 in breast cancer cells through Src kinase activation [196].

Ha-Ras (12V), the activated form of Ras is also able to enhance CXCL10 promoter activity, through RAF and PI3K signaling. The levels of CXCR3B, receptor of CXCL10, are also down-regulated by Ras [197]. Finally, CXCL1 and CXCL3 expression is also down-regulated by the Syk tyrosine-kinase, whereas CXCL2 levels are unaffected [198].

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6. Targeting chemokines for therapy

Chemokines and their receptors have multifunctional purposes and may well be very important in the pathogenesis of cancer progression. Current therapies such as surgery, radiotherapy and chemotherapy are primarily concerned with destruction of cancer. Targeting chemokines and chemokine receptors will allow limiting angiogenesis or metastasis and may enable such therapies to act as chemotherapeutic agents alone or in synergism with conventional agents. The up-regulation of certain chemokine molecules in tumor as compared with normal cells offers a potential avenue-where cancer cells and their metastases can be specifically targeted. This selective destruction of cells is also pre-requisite of non toxic treatment regimens.

6.1. Chemokines as therapy:

Manipulation of the tumor microenvironment by treatment with chemokines can be used to recruit either immature dendritic cells for the initiation of anti-tumor responses or effector cells for cytotoxic responses. Intratumoral delivery of CCL21 using pox virus vaccine into established tumors derived from murine colon cancer line, CT26 results in enhanced infiltration of CD4 T cells which correlated with inhibition of tumor growth [199].

Various murine tumors have been engineered to over express chemokines in order to stimulate increased immune cell infiltration for generation of anti-tumor response. Furthermore, non-immunogenic murine breast carcinoma is rejected after transducing cells with CCL19. The rejection of tumor was mediated by activated NK and CD4+ cells [200]. Adenoviral delivery of the CCL16 is able to inhibit growth of mammary tumors and prevent metastatic growth. Similarly, intratumoral injections of adenoviral vectors expressing CCL17, CCl22 or CCL27 suppress growth and attracted activated T cells [201].

In nude mice models, labeled recombinant CXCL4 injected intravenously or intra-arterially preferentially target the endothelium of the breast cancer, induce neovasculature, which

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suggests that CXCL4 could have some interesting applications in anti-tumoral strategies [202].

In treatment involving delivery of chemokines to the tumor environment, there is a major problem of heterogeneity of the tumor cells. Chemokines which may be beneficial to one patient might be harmful to another. However, this problem can be circumvented by chemokine typing every tumor prior to deciding on an appropriate therapy regime. They may be used as an adjunct to increase the efficacy of currently available therapies.

6.2. Blocking chemokines and chemokine receptors as therapy

Tumor infiltrating leukocytes or angiogenesis can be modulated by targeting specific chemokines. High CXCL8 expression levels render tumor cells highly tumorigenic, angiogenic and invasive [47, 48, 104]. A humanized anti-CXCL8 antibody ABX-IL8 has been developed by Abgenix. In preclinical models, this antibody inhibits angiogenesis, tumor growth and metastasis of human melanoma and tumor growth and MMP activity in orthotopic bladder cancer xenografts [203, 204]. The therapeutic value of blocking CXCL8 has yet to be assessed in cancer patients.

Given the role of CCL2 in endothelial and fibroblast cell activity it is reasonable to speculate that targeting CCL2/CCR2 axis will also affect tumor vascular and stromal components to provide benefits. Furthermore, in a murine model of breast cancer treatment with Met-CCL5, an antagonist of CCR1 and CCR5 led to a reduction in the total number of infiltrating inflammatory cells, in particular a decrease in macrophage infiltration and reduced growth of tumors [205].

The 7-transmembrane structure of chemokine receptors makes them attractive targets for small molecule inhibitors. Small molecule inhibitors are relatively easy to produce and efficacious, therefore they make up a substantial proportion of world wide sale.

There is compelling evidence that CXCR4 is a key mediator of metastatic breast cancer [55, 65, 70, 71]. Furthermore, blocking the CXCL12/CXCR4 axis by targeting either the ligand or

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the receptor inhibits cancer metastasis. Silencing CXCR4 expression using small interference RNA has also been a successful approach for decreasing both breast cancer growth and metastasis [150, 152]. The CXCR4 antagonists TN14003 and AMD3100 have both shown some potential for reducing breast cancer metastases [151, 206]. Other CXCR4 antagonists include antibodies to CXCR4, which is planned to enter phase I trial (Northwest Biotherapeutics Inc, USA) and small molecule antagonists being developed by ChemoCentryx (Mountain View, CA, USA).

6.3. The Virus encoded chemokine binding proteins:

Large DNA viruses in particular herpes- and poxviruses, have evolved proteins that serve as mimics or decoys for endogenous proteins in the host. The virus encoded chemokine-binding proteins (vCKPBs) are encoded by member of pox viruses and Herpesvirus (MHV68). They function as chemokine scavengers and represent a unique class of chemokine modulators. The vCKBPs are divided into different classes based on structural differences. For example M-T7-encoded by Myxoma virus binds with low affinity to a broad range of XC-CC and CXC chemokines at the heparin binding site and therefore inhibit the interaction of these chemokines with glycosaminoglycans. On the other hand M3 is unique as it interferes at two distinct levels with both the receptor binding and the GAG binding site within all four classes of chemokines, thus offering an opportunity of broad spectrum chemokine inhibition.

6.4. Potential role of Heparinoids:

Heparan sulphate and heparin sulphate are chemically similar glycosaminoglycan species, with both macromolecules containing a repeating sequence of variably sulphated disaccharide units composed of glucosamine and glucuronic acid, heparin differs from heparan sulphate by being more heavily sulphated. Heparin sulphate seems to have a

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multitude of effects on chemokine function, including the inhibition of CXCL8 induced calcium flux in neutrophils [127]. Importantly, heparinoids have been shown to inhibit CXCL12-induced T cell migration and adhesion to extracellular matrix [207]. In a recent study we have shown that unfractionated heparin (UFH) and the low molecular weight heparin tinzaparin (clinically relevant dose regimens) reduced hematogenous metastatic spread of human breast cancer cells to the lung in a murine model [208].

UFH is readily available drug and many clinical experimental studies have suggested that it may effect malignancy progression. One multi- centre randomized trials in to effects of 5 week UFH treatment as additive to chemotherapy in small cell lung carcinoma showed an odds ratio of total 3-year mortality of 0.64 in favour of the UFH treated patients. Three clinical trials have recently been reported unpon the possible role of low molecular weight heparin in cancer treatment. The Famous (Fragmin Advanced Malignancy Outcome Study) trial randomized patients with solid cancers (breast, colorectal, ovarian, pancreatic and others) to a year of therapy with once daily of LMWH Dalteparin or placebo. Survival estimates at three years after randomization could only suggest a benefit (p=0.19) for patients receiving Dalteparin. However, a subgroup of patients with better prognosis was analysed and found to show a significant survival advantage with dalteparin (p=0.03) [209].

7. Conclusion

Depending on their nature, chemokines can be present at high levels either in the primary breast tumor or at distance, in metastatic sites such as bone, lung or lymph nodes. Chemokines are also produced either by epithelial cells, or by cells from the microenvironement, including in particular by cancer associated fibroblasts, endothelial cells and leukocytes (Figure 3). In addition, chemokine receptors are expressed by both breast epithelial cancer cells but also by cells present in the stroma. This complex interplay between chemokines and chemokines receptors produced in different sites and by various cell types dictates the outcome of the disease and in particular the development of metastases or not.

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In particular, there is clearly a balance between angiostatic and angiostatic chemokines. Chemokines will also affect not only angiogenesis but also other process such as proliferation, leukocyte infiltration and maybe apoptosis. In the different steps leading to metastasis, the roles of GAGs begins also to investigated and could be the source of other therapies. Finally, the study of the mechanisms responsible for chemokine and chemokine receptor expression in cancer remain poorly investigated.

8. Key unanswered questions

Current findings have focus on a limited number of chemokines and chemokine receptors to elucidate the role of chemokines in breast cancer metastasis, such as CXCL8, CXCL12, CCL2, CCL4, CCL5, CXCR2 and CXCR4. It is likely that these studies have mainly been dictated by the fact that these chemokines were present at relatively high levels in tumors or metastatic sites. The next step will be definitely to analyze the expression patterns of other chemokines and chemokine receptors, which are maybe less abundant, but could also play important roles in breast cancer metastasis. The expression of a number of chemokines and chemokine receptors is clearly deregulated in cancer. This could be helpful to design novel screening assays including luminex technology which would enable to detect in the sera of the patients early stages of the disease, and hopefully before metastasis onset. To date, only pioneer work has demonstrated that estrogen receptors, phosphorylation as well as NF-κB and AP-1 transduction pathways were involved in this process. A major challenge of the future will be to understand precisely to which extent these pathways or other routes of regulation control the abnormal expression of chemokines and chemokines receptors in breast cancer. This will serve as a basis for prospecting novel therapeutical approaches to inhibit metastasis. It is puzzling to observe that the promoter regions of most of chemokines and chemokine receptors have been poorly studied. Following the identification of the crucial chemokines and chemokine receptors involved in breast cancer metastasis, the development of novel drugs to target these molecules will require the use of preclinical models and the launch of clinical trials which could be bring new hope for cancer treatment.

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Figure Legends

Figure 1: Schematic representation of the four different classes of chemokines.

The position of the two first cysteines in the N-terminal part of the chemokine defines the class to which each chemokine belongs.

Figure 2: Chemokine receptors are seven-transmembrane receptors coupled to G-proteins

Figure 3: Putative model for chemokine action in breast cancer metastasis

The metastatic potential of breast cancer epithelial cells is mediated by two mechanisms. The first one involves the high secretion of chemokines such as CXCL8 by invasive cancer epithelial cells, which in turn can recruit leukocytes to the tumors and at a lesser extent act on endothelial cells and fibroblasts, both types of cells harboring specific receptors (e.g. CXCR1 and CXCR2). In addition, endothelial cells are also producing CXCL8. CXCL8 production on site by the tumor will enhance angiogenesis and basal membrane disruption leading to metastasis. In the breast, Cancer associated fibroblasts (CAFs) secrete CXCL12 which will affect mainly epithelial cancer cells (harboring high levels of CXCR4) and at lower degree, endothelial and leukocytes. These interactions will favor also angiogenesis, degradation of extracellular matrix and invasion of metastatic cancer cells. When reaching the circulation, cancer cells will be attracted by metastatic organs producing high levels of CXCL12, including lymph nodes (LN), bone and lungs. In the bone, cancer cells will then secrete CXCL8 which will in particular enhance osteolysis.

Figure 4: GAG and metastasis spread

Metastatic spread is a multiple step process which involved the interaction of chemokines with glycosaminoglycans (GAG) of endothelial cells. These steps require selectin-mediated

rolling, activation by chemokines, integrin-mediated adhesion, extravasation leading to angiogenesis and to metastasis.

Figure 5: Model of regulation of CXC8 gene in breast cancer cells

CXCL8 gene expression is higher in ER-negative breast cancer cells compared to ER-positive breast cancer cells. This difference of expression arises from a higher transcriptional activity of CXCL8 gene in ER-negative breast cancer cells involving the synergistic activation of the gene by NF-κB and AP-1 pathways and at a lower degree by C/EBP factors. NF-κB and AP-1 transcription factors are present at higher levels in ER-negative breast cancer cells.

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