The emerging role of CXC chemokines in epithelial ovarian cancer

Adam Rainczuk, Jyothsna Rao, Jessica Gathercole and Andrew N Stephens

Prince Henry’s Institute, Monash Medical Centre, Clayton, Victoria 3168, Australia

Correspondence should be addressed to A N Stephens; Email: andrew.stephens@princehenrys.org

A Rainczuk and J Rao contributed equally to this work

Abstract

In recent years, chemokines have generated intense investigations due to their involvement in both physiological and pathological processes of inflammation, particularly in ovarian biology. The physiological process of ovulation in the normal ovary involves various chemokines that mediate the healing of the ruptured endometrium. It is now being reported that many of these chemokines are also associated with the cancer of the ovary. Chronic inflammation underlies the progression of ovarian cancer; therefore, it raises the possibility that chemokines are involved in the inflammatory process and mediate immune responses that may favour or inhibit tumour progression. Ovarian cancer is a gynaecological cancer responsible for highest rate of mortality in women. Although there have been several investigations and advances in surgery and chemotherapy, the survival rate for this disease remains low. This is mainly because of a lack of specific symptoms and biomarkers for detection. In this review, we have discussed the emerging role of the CXC chemokines in epithelial ovarian cancer (EOC). The CXC group of chemokines is gaining importance in the field of ovarian cancer for being angiostatic and angiogenic in function. While there have been several studies on the angiogenesis function, emerging research shows that ELR K CXC chemokines, CXCL9 and CXCL10, are angiostatic. Importantly, the angiostatic chemokines can inhibit the progression of EOC. Given that there are currently no biomarkers or specific therapeutic targets for the disease, these chemokines are emerging as promising targets for therapy.

Introduction

As key mediators of the inflammatory response, chemokines represent an area of intense interest and study. Their influence on the chemotactic recruitment of leukocytes towards sites of inflammation is well established and involves multiple cell and tissue types (Charo & Ransohoff 2006). Apart from their established roles in chemotactic cell recruitment, however, there is a growing body of evidence suggesting that interference with their established functions may promote tumour development and metastasis (Mukaida & Baba 2012). In particular, a complex chemokine-signalling network may influence the development and progression of epithelial ovarian cancers (EOCs). The study of chemokines is therefore becoming important in furthering our understanding of this disease.

An overview of the CXC chemokines and their functions

Chemokines, comprising pairs of ligands and their associated receptors, are a superfamily of heparin-binding cytokine molecules with molecular weights between 8 and 10 kDa (Mukaida & Baba 2012). They are important mediators of the formation of new blood vessels by neovascularisation, involved in the angiogenic sprouting of vessels in both normal (e.g. embryogenesis and menstruation) and pathological states (Keeley et al. 2008). This role is achieved not solely through the promotion of angiogenesis but via a complex interplay between angiogenic and angiostatic signalling to influence the behaviour of the vascular endothelium (Kiefer & Siekmann 2011). Chemokine ligands exert their effects through their cognate G-protein-coupled receptors, a group of seven transmembrane-spanning proteins expressed on the cell surface and belonging to the class A rhodopsin-like family (Kiefer & Siekmann 2011; Fig. 1).

On the basis of their primary structure, chemokines are classified as either C, CC, CXC or CX3C where ‘X’ represents a non-conserved amino acid substitution (Fig. 1). With the exception of the ‘C’ subgroup, all chemokines contain a common four-cysteine residue motif linked by disulphide bonds in conserved positions, one between the first and the third and one between the second and the fourth cysteine, to form triple-stranded β-sheet structures (Fernandez & Lolis 2002). The
nomenclature for chemokines is extended by the addition of receptor ‘R’ (e.g. CXCR1) and respective ligand ‘L’ (e.g. CXCL8). CXC chemokines are further subgrouped according to the presence or absence of a three amino acid ‘ELR’ Glu-Leu-Arg motif preceding the CXC sequence. Thus, the CXC chemokines are often referred to as ELR$^+$ or ELR$^-$ (Strieter et al. 2004).

Over the past two decades, many studies have examined the dual roles of CXC chemokines in both the promotion and the inhibition of angiogenesis. The angiogenic sprouting of new, thin-walled capillary networks from pre-existing ones is a critical factor in tumour growth and spread (Keeley et al. 2008, Kiefer & Siekmann 2011). As a general rule, ELR$^+$ CXC chemokines mediate angiogenesis, typically via interaction with the CXCR2 receptor (see Table 1 for a listing of CXC chemokines and their cognate receptors) and are also involved in other diverse functions such as stem cell migration, lymphocyte migration and lymphoid tissue neogenesis (Romagnani et al. 2004, Kiefer & Siekmann 2011). CXCR2 is typically expressed on endothelial cells but can be constitutively expressed by specific tissues and cells (Table 1; Mukaida & Baba 2012). By contrast, the ELR$^-$ CXC chemokines typically promote angiostasis (rather than angiogenesis), through their common receptor CXCR3 (Mukaida & Baba 2012).

Chemokine ligands lacking the ELR motif can promote anti-tumour effects by recruiting both innate and adaptive immune effector cells (in the case of CXCL9 and CXCL10 that can be secreted from tumour cells for example) and can also inhibit endothelial cell migration and proliferation (Keeley et al. 2008, Kiefer & Siekmann 2011, Whitworth & Alvarez 2011).

There are, however, two exceptions to the rule. CXCL4 is an ELR$^+$ chemokine, which also binds to CXCR3. Unlike the other ELR$^+$ members, CXCR4 promotes angiostasis similar to the ELR$^-$ chemokines. Contrastingly, the ELR$^-$ chemokine CXCL12 binds to the CXCR4 receptor to promote angiogenesis (Kiefer & Siekmann 2011). Therefore, both the ELR status and the cognate receptor for each chemokine must be considered when evaluating the function (either angiogenic or angiostatic) of the CXC chemokine family. The functions of the CXC chemokines are also extensively regulated through post-translational modification. This is a well-established mechanism for regulating their bioactivity and function and has been reviewed elsewhere (Mortier et al. 2011).

It is now well established that besides leukocytes, fibroblasts and endothelial cells, tumour cells also express chemokines and their receptors (Balkwill 2004, Romagnani et al. 2004, Kiefer & Siekmann 2011, Mukaida & Baba 2012). Importantly, chemokines are secreted by both the stromal and the malignant part of the cancer tissue, and functionally, the chemokine...
Table 1: General overview of CXC chemokines involved in the regulation of angiogenesis and lymphocyte recruitment.

<table>
<thead>
<tr>
<th>CXC L ELR</th>
<th>Previous nomenclature</th>
<th>CXC R</th>
<th>Receptor expression</th>
<th>Function</th>
<th>General origin</th>
<th>Common disease</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>GROα/MGSA-α</td>
<td>CXCL1</td>
<td>CXCR2</td>
<td>Epi/endo</td>
<td>Chemotaxis of neutrophils, angiogenesis, promotes tumour growth and metastasis</td>
<td>Neutrophils, platelets, monocytes</td>
<td>Thymus, natural killer cell, monocytes</td>
</tr>
<tr>
<td>+</td>
<td>GROβ/MGSA-β</td>
<td>CXCL2</td>
<td>CXCR2</td>
<td>Epi/endo</td>
<td>Chemotaxis of neutrophils</td>
<td>Neutrophils</td>
<td>Lung, pancreatic, ovarian cancers</td>
</tr>
<tr>
<td>+</td>
<td>GROγ/MGSA-γ</td>
<td>CXCL3</td>
<td>CXCR2</td>
<td>Epi/endo</td>
<td>Chemotaxis of neutrophils</td>
<td>Neutrophils</td>
<td>Neutrophils</td>
</tr>
<tr>
<td>+</td>
<td>PF4</td>
<td>CXCL4</td>
<td>CXCR3B</td>
<td>Endo</td>
<td>Anti-angiogenic, inhibits tumour vascularisation</td>
<td>Thymus, natural killer cell, monocytes</td>
<td>Melanoma, ovarian cancer</td>
</tr>
<tr>
<td>+</td>
<td>ENA-78</td>
<td>CXCL5</td>
<td>CXCR3</td>
<td>Endo</td>
<td>Angiogenesis</td>
<td>Neutrophils</td>
<td>Gastrointestinal tumour</td>
</tr>
<tr>
<td>+</td>
<td>Granulocyte chemotactic protein 2</td>
<td>CXCL6</td>
<td>CXCR1, CXCR2</td>
<td>Epi/endo</td>
<td>Angiogenesis</td>
<td>Neutrophils</td>
<td>Pancreatic cancers</td>
</tr>
<tr>
<td>+</td>
<td>NAP-2, PBP</td>
<td>CXCL7</td>
<td>CXCR2</td>
<td>Epi/endo</td>
<td>Mediates angiogenesis via MAPK and mTOR pathway</td>
<td>Neutrophils, platelets, monocytes</td>
<td>Thymus, natural killer cell, widespread</td>
</tr>
<tr>
<td>+</td>
<td>IL8</td>
<td>CXCL8</td>
<td>CXCR1, CXCR2</td>
<td>Epi/endo</td>
<td>Neutrophil chemoattractant and activating factor</td>
<td>Neutrophils</td>
<td>Neutrophils</td>
</tr>
<tr>
<td>+</td>
<td>Monokine induced by IFN-γ (MIG)</td>
<td>CXCL9</td>
<td>CXCR2</td>
<td>Endo</td>
<td>Attract tumour-infiltrating lymphocytes, inhibit endothelial cell migration and proliferation</td>
<td>Thymus, natural killer cell, widespread</td>
<td>Thymus, natural killer cell, widespread</td>
</tr>
<tr>
<td>+</td>
<td>IFN-γ-inducible protein 10 (IP10)</td>
<td>CXCL10</td>
<td>CXCR3</td>
<td>Endo</td>
<td>Promote anti-tumour effects by attracting T-lymphocytes</td>
<td>Thymus, natural killer cell, widespread</td>
<td>Thymus, natural killer cell, widespread</td>
</tr>
<tr>
<td>+</td>
<td>IFN-inducible T-cell α chemotactant (LTAC)</td>
<td>CXCL11</td>
<td>CXCR3, CXCR7</td>
<td>Epi/endo</td>
<td>Inhibit endothelial cell migration and proliferation</td>
<td>Thymus, natural killer cell</td>
<td>Clear cell ovarian cancers</td>
</tr>
<tr>
<td>+</td>
<td>SDF-1α/β</td>
<td>CXCL12</td>
<td>CXCR4, CXCR7</td>
<td>Endo/epi</td>
<td>Neovascularisation during foetal organogenesis, Tumour cell migration and proliferation</td>
<td>Widespread</td>
<td>Widespread</td>
</tr>
<tr>
<td>+</td>
<td>BLC/BCA-1</td>
<td>CXCL14</td>
<td>CXCR5</td>
<td>Unknown</td>
<td>Migration of T-cells</td>
<td>B cells</td>
<td>Human lung cancer</td>
</tr>
<tr>
<td>+</td>
<td>BRAK/bolekine</td>
<td>CXCL16</td>
<td>CXCR6</td>
<td>Epi</td>
<td>Soluble CXCL16 enhances proliferation and migration. Transmembrane CXCL16 inhibits proliferation</td>
<td>Activated T-cells</td>
<td>Activated T-cells</td>
</tr>
</tbody>
</table>

Epi, epithelial cell expression; Endo, endothelial cell expression.
ligand and receptor pairs can exert a direct effect on tumour proliferation and survival (Erreni et al. 2009, Ha et al. 2011, Boimel et al. 2012). The chemokines from stromal cells may influence the survival of malignant cells by binding to the functional receptors acquired on the cancerous cells, enhancing metastasis in a chemokine-rich environment (Mantovani et al. 2010, Balkwill 2012).

This review will discuss the emerging roles of the CXC chemokines in the pathogenesis of EOC, in the context of their inflammatory, angiogenic and metastatic properties.

The role of CXC chemokines in normal ovarian physiology

The surface epithelium of the ovary is a natural extension of the peritoneal lining and is directly exposed to any metabolic and environmental stress present in the peritoneal cavity (Maccio & Madeddu 2012). Regular cycling and release of ova from the ovary causes the ovarian epithelium to undergo regular cycles of wounding and repair throughout a female’s reproductive life. This process is generally considered to induce an inflammatory response and triggers (Murdoch et al. 2010) the secretion of a large number of chemokines, cytokines, enzymes and growth factors (Murdoch & McDonnel 2002).

A model of CXC chemokine-coordinated regulation of angiogenesis and inflammation after wounding of a normal epithelial cell layer is summarised in Fig. 2 (Griffioen & Molema 2000, Spinetti et al. 2001, Romagnani et al. 2004), and a more detailed review of wound repair involving chemokines can be found in Romagnani et al. (2004). At the wound site, platelets release growth factors including vascular endothelial growth factor (VEGF), platelet-derived growth factor and various cytokines including CXCL7, CXCL1, CXCL5 (which initiate neutrophil recruitment) and CXCL4 (to assist in blood-clot formation) (Griffioen & Molema 2000, Spinetti et al. 2001, Romagnani et al. 2004). Wounded epithelial cells then express CXCL8, inducing an inflammatory response and resulting in angiogenesis and the sprouting of new blood vessels with high CXCR2 expression (Romagnani et al. 2004). The epithelial layer also produces angiostatic CXCL9, -10 and -11, arresting the migration of proliferating CXCR3+ endothelial cells and leading to lymphocyte recruitment during the period of healing (Romagnani et al. 2004). CXCL8 is also involved in re-epithelialisation of the wound site (not shown in Fig. 2; Romagnani et al. 2004). Other non-CXC chemokines are also involved in wound healing; for example, CCL2 expression during wound healing also recruits CCR2+ macrophages and monocytes to the inflamed ovarian epithelium (Fader et al. 2010). The role of non-CXC chemokines in this process is reviewed elsewhere (Charo & Ransohoff 2006).

Chemokines and cytokines also modulate ovarian function via their involvement in follicular development and ovulation and the formation, function and death of the corpus luteum (Auersperg et al. 1998). CXCL8, for example, is significantly elevated in normal folliculogenesis, suggesting a role in mediating neutrophil attraction (Ness & Modugno 2006). Moreover, anti-CXCL8 antiserum can suppress hCG-induced ovulation and neutrophil activity in women undergoing IVF (Ness & Modugno 2006). CXCL8 levels also correlate with collagenases, which are crucial to the remodelling of the cervical matrix (Auersperg et al. 1998). CXCL1, another neutrophil attractant, is present in periovulatory follicular fluid and is expressed by ovarian stromal cells. On completion of ovulation, the ruptured epithelium undergoes repair to form the corpus luteum, characterised by significantly increased infiltration of leukocytes and modulated by pro-angiogenic factors in the follicular fluid such as CXCL8 and CCL2 (Merritt & Cramer 2011).

Recent studies have shown that CXCL12 is expressed in the cells of the normal ovarian surface epithelium and the epithelium of the fallopian tube (Machelon et al. 2011). Interestingly, CXCL12 is absent in the follicles and the oocytes (Machelon et al. 2011, Merritt & Cramer 2011). Constitutive expression of CXCL10 has also been reported in normal ovary, with cyclic increases observed during ovulation (Wong et al. 2002, Zhou et al. 2004). While the precise roles played by these chemokines in the physiology of the ovary are still unclear, their presence firmly establishes their importance in normal ovarian function.

Epithelial ovarian cancer

EOCs account for between 80 and 95% of all ovarian tumours diagnosed and exhibit the highest mortality rate of any gynaecological tumour type (Roett & Evans 2009). EOCs are commonly classified according to histopathological appearance as clear cell, endometrioid, mucinous or serous; amongst these, serous epithelial tumours are the most commonly diagnosed tumour type (Kobel et al. 2008). However, wide varieties of less common ovarian neoplasms exist and can include mixed, undifferentiated or Brenner-type tumours (Kaku et al. 2003, Arnoiannaki et al. 2011). While traditionally subgrouped according to FIGO stage and grade, a growing body of evidence now suggests that low- and high-grade tumours have different aetiologies arising from unrelated, underlying molecular pathologies (Giks et al. 2008, Kobel et al. 2008). Low-grade tumours typically exhibit micropapillary structures and originate from borderline tumours. These tumours are believed to progress through benign and borderline stages to malignancy and are generally associated with good prognosis. They also have well-defined mutational pathways and often express mutated KRAS or BRAF (Cho 2009). By contrast, a
significantly large number of TP53 gene mutations have been found in high-grade serous tumours (Milner et al. 1993), and they also have a poorly defined mutational pathway with mutated TP53 expression found in up to 80% of cases (Vang et al. 2009). They appear to arise spontaneously, metastasise early in their progression and are associated with poor prognosis (Cho 2009, Cho & Shih Ie 2009, Roett & Evans 2009).

Figure 2 An overview of CXC chemokine involvement in normal wound repair (expanded details found in Romagnani et al. (2004)). (A) Generation of blood clot and neutrophil recruitment: (A1) growth factors, cytokines and chemokines are released by clotted platelets, while CXCL4, -5 and -7 initiate neutrophil recruitment to the wound site; (A2) epithelial cells at the wound site express CXCL8, while endothelial cells express CXCL1; (A3) neutrophils expressing CXCR2 migrate into the wound site along the CXCL1/CXCL8 chemotactic gradient. (B) Recruitment of lymphocytes and monocytes: (B1) epithelial cells continue to express CXCL8, promoting the growth of new blood cells. Epithelial cells also express CXCL9, -10 and -11 to limit and regulate endothelial cell proliferation and new blood vessel growth; (B2) CXCL9 and -10 recruit lymphocytes during wound healing; (B3) ongoing CXCL1 expression by endothelial cells promotes continued neutrophil recruitment, while CXCL9, -10 and -11 regulate blood vessel growth and the migration of proliferating endothelial cells; (B4) CXCL4 helps promote and maintain blood clotting.

Balancing inflammation, T-cell recruitment, angiogenesis and angiotaxis: the role of chemokines in ovarian cancer development and progression

Inflammation and the ovarian surface epithelium

Similar to other tumour types, chronic inflammation is an important condition underlying the growth and progression of ovarian tumours (Ness & Cottreau 1999,}.
were associated with favourable prognosis via recruit-
ment of fimbriae of the tube (Medeiros et al. 2006, Crum et al. 2007, Coffelt & Scandurro 2008, Maccio & Madeddu 2012). As key mediators of inflammation, chemokines play two major roles: i) they are responsible for the recruitment and activation of immune cells into sites of malignancy (and possibly areas of pre-neoplastic growth) and ii) they mediate pro- and anti-angiogenic effects, processes required for the vascularisation and progression of tumours. In addition, the multiple biological effects exerted by CXC chemokines mean that each of these processes is inextricably linked. Therefore, it is of paramount importance to identify molecules that are involved and their contribution towards anti-tumour activity and in preventing recurrence – or, conversely, how their activity may promote tumour growth and metastasis.

Although an accepted view is that a majority of ovarian cancer cases originate from the ovarian surface epithelium, emerging evidence suggests the involvement of the fimbriae of fallopian tubes. Crum et al. showed that fallopian tubes and ovaries from women with BRCA mutations often contained precursor lesions in the fimbriae of the tube (Medeiros et al. 2006, Crum et al. 2007). By contrast, Clarke et al. have demonstrated an association between BRCA and inflammation in serous ovarian carcinomas. BRCA mutations (or epigenetic loss) were associated with favourable prognosis via recruitment of intraepithelial CD8+ (and to a lesser extent CD3+ T-) tumour-infiltrating lymphocytes (TILs). This novel association between intraepithelial T-cell infiltration and BRCA mutation in serous EOC has also been observed with certain breast cancer types and can lead to a favourable patient prognosis (Clarke et al. 2009). Chronic inflammation from diseases such as hepatitis, human papilloma virus in the cervix and ulcerative colitis are well known to cause cancer of the tube (Demopoulos et al. 2001). Similarly, it has been suggested that proliferation and hyperplasia in the fallopian tube are associated with lesions that may be carcinogenic in nature (Moore & Enterline 1975). Compelling evidence for this hypothesis was recently presented by Kim et al. (2012) who demonstrated, using a Dicer–Pten knockout mouse, that serous epithelial tumours can arise in the fallopian tube and rapidly metastasise to the ovary. Mice in this study exhibited a 100% mortality rate from tumours that bore a striking resemblance to clinical disease (Kim et al. 2012).

Retrograde menstruation via the fallopian tube is a common condition, where the flow of endometrial fluid bathes the tubes with inflammatory molecules such as CXCL8 (interleukin-8 (IL8)), tumour necrosis factor alpha (TNF-α) and granulocyte–macrophage colony-stimulating factor (GM-CSF). Each of these is elevated in ovarian carcinomas (Strandell et al. 2004). It is well established that a combination of tubal ligation and hysterectomy is protective against ovarian cancer (Green et al. 1997, Seidman et al. 2002). Together, these findings strongly support the notion that inflammation in the fallopian tube, the ovarian epithelium or both are contributing factors in the development of ovarian cancer.

The inflammation-mediated release of chemokines, cytokines and growth factors can also promote migration and proliferation of leukocytes, epithelial and endothelial cells (Fig. 2; Mukaida & Baba 2012). At a molecular level, there is evidence that pro-inflammatory cytokines such as IL1, IL6 and TNF-α are linked to a poor prognosis in EOC patients (Clendenen et al. 2011, Maccio & Madeddu 2012). Oxidative stress, cell necrosis and rapid cell division are hallmarks of chronic inflammation (Ness & Modugno 2006). Rapid cell division gives rise to a possibility of replication error and the need for DNA repair, such as at the key regulatory site TP53, increasing the risk of mutagenesis. This is further supported by the high rate of TP53 mutation commonly observed in high-grade serous EOC (Merritt & Cramer 2011).

Recently, inflammatory cytokine production in the human ovarian cancer microenvironment has been described in terms of an autocrine cytokine network. The TNF network includes TNF-α, IL6 and CXCL12 (Kulbe et al. 2012); in particular, the CXCL12/CXCR4 axis plays a major role in the proliferation and metastasis of ovarian tumour cells (Barbieri et al. 2010). Enhanced TNF network activity has been associated with genes involving angiogenesis, inflammation and leukocyte infiltration. Targeting members of the TNF network via siRNA or neutralising antibodies reduced experimental peritoneal ovarian tumour growth, as well as angiogenesis in human ovarian tumour biopsies and mouse xenograft models (Kulbe et al. 2012).

The nuclear factor-κB (NF-κB) pathway is also important in modulating autocrine and paracrine signalling between inflammatory markers in the cancer microenvironment (Son et al. 2007). Notably, NF-κB regulates the CXCL12/CXCR4 autocrine signalling in ovarian cancer cell lines (Miyanishi et al. 2010). Over-expression of prostaglandins, leading to altered expression of cytokines and chemokines, increases the invasiveness of ovarian tumour cells (and other tumour types) (Aiilakkio-Tallberg et al. 1988, Subbaramaiah et al. 1997, Ness & Modugno 2006). The expression of cytokines and chemokines in inflamed malignancies of the female genital tract may, therefore, lead to dysfunctional autocrine loops, resulting in increased tumour progression and invasion.

**Recruitment of T-cells to epithelial ovarian tumours**

In the normal anti-tumour immune response, interactions between CXCL9 (MiG), CXCL10 (IP10) and their receptor CXCR3 influences the chemotraction of natural killer (NK) cells, activated Th1 cells, gamma-delta T-cells, macrophages and dendritic cells towards tumours (Strieter et al. 2006, Whitworth & Alvarez 2011). The importance of CXCL9 and CXCL10 to
generate an anti-tumour response is supported by their roles as mediators of IL12, a potent immunoregulatory cytokine (also being trialled as a therapeutic agent in ovarian cancer patients, although with modest results) (Whitworth & Alvarez 2011). Additionally, in an investigation into links between coagulation and inflammation, it was shown that culture of cells derived from ovarian cancer ascites and peripheral blood mononuclear cells (PBMCs) from healthy donors also induced significant up-regulation of inflammatory cytokines (IL1β and IL6), along with CCL2 and CXCL8 (Naldini et al. 2011). This suggests that the ovarian cancer microenvironment is inflammatory in nature and can direct PBMCs to increase the release of inflammatory chemokines and cytokines.

Recent gene expression studies identified CXCL10, CXCL11 and their receptor CXCR3 as amplified in a subset of ovarian cancer patients from a sample of 489 high-grade, serous epithelial tumours. These studies defined this group of tumours as ‘immunoreactive’ (CGARN 2011), suggesting an important role for these chemokines in the tumour microenvironment. A primary function for these chemokine ligands is the trafficking of leukocytes and the recruitment of activated CD4+ Th1 cells, CD8+ T-cells and NK cells towards sites of inflammation (Clark-Lewis et al. 2003). High concentrations of CXCL9 and CXCL10 are also associated with the presence of TILs, and this is a positive predictor of patient outcome with patients exhibiting lower recurrence, less dissemination and longer survival (Zhang et al. 2003, Sato et al. 2005, Tomsova et al. 2008, Kryczek et al. 2009). By contrast, negative patient outcomes are associated with tumour infiltration by regulatory T-cells (Treg) that mediate immune tolerance (Curiel et al. 2004, Sato et al. 2005, Kryczek et al. 2009). Sato et al. (2005) demonstrated that high intraepithelial CD8+/CD4+ T-cell and CD8+ T-cell/Treg ratios resulted in improved survival in EOC. Moreover, neither intraepithelial CD4+ or other CD3+ TILs alone were associated with survival in this study (Sato et al. 2005). Others have more recently shown that longer survival is positively correlated with both CD8+ and CD3+ TILs (Hwang et al. 2012). However, CD8+ positive TILs showed a more consistent association with patient survival in this meta-analysis (Hwang et al. 2012).

EOC tissue and ascites fluid contain high levels of CD4+ CD25+ FOXP3+ Treg cells, which are not present in normal ovarian tissues (Curiel et al. 2004). FOXP3 is a marker of Treg cells, crucial for maintaining immunologic homoeostasis by preventing autoimmunity in a normal immune environment (Kelley & Parker 2010). In a study of immune cell infiltration into tumours, a high level of the chemokine CCL22 (secreted by tumour-associated macrophages) was detected in tumour ascites, possibly inducing Treg trafficking into tumours and suppressing the response of TILs (Curiel et al. 2004). In an apparent contradiction, however, under certain conditions in the tumour microenvironment, CXCL9, CXCL10 and the cytokine IL17 can also be produced by tumour-associated macrophages and contribute to positive outcomes in EOC (Kryczek et al. 2009).

An explanation as to why TILs are effective at improving EOC patient outcomes can be partially attributed to the recent identification of T helper 17 (Th17) cells. These cells can contribute to pathogenesis of autoimmune disease and initiate tumour growth in certain models but drive anti-tumour immunity in some epithelial malignancies (Zou & Restifo 2010, Wilke et al. 2011). Th17 cells are postulated not to be immunosuppressive, as they do not express FOXP3 (Wilke et al. 2011). An important study by Kryczek et al. (2009) showed that in addition to NK cells and Th1 effector

**Figure 3** Promotion of a favourable outcome in EOC involving CXC chemokines. (1) Th17 cells (without Treg suppression in this example) secrete IL17 and anti-tumour promoting IFN-γ, after induction by tumour-associated macrophages secreting IL1β (Kryczek et al. 2009); (2) IL17 secretion and IFN-γ induce tumour cells to secrete CXCL9 and CXCL10; (3) angiostatic chemokines establish a gradient whereby adaptive and innate immune cells migrate to the tumour site. A high CD8+ T-cell to low CD4+ T-cell/Treg ratio can lead to positive outcomes in EOC (Sato et al. 2005).
T-cells, Th17 cells secreting IFN-γ and IL17 were correlated with the elevation of Th1-type chemokines CXCL9 and CXCL10 in the ovarian cancer microenvironment. CXCL9 and CXCL10 were produced by primary ovarian cancer cells and macrophages, leading to the recruitment of CXCR3-expressing effector CD8+ T-cells and NK cells to the tumour. A significant association was found with the presence of IL17 in patient ascites, with high IL17 levels (median patient survival 78 months) significantly improving survival time compared with low IL17 levels (median patient survival 27 months) (Kryczek et al. 2009). The levels of CXCL9 and CXCL10 were directly correlated with tumour and tumour-infiltrating NK cells and CD8+ T-cells, highlighting the importance of these ELR-chemokines in limiting the spread of EOC. Figure 3 attempts to summarise the effect of increased CXCL9 and CXCL10 concentrations in the EOC microenvironment, which are reported to lead to a positive survival outcome.

**Angiostatic chemokine ligands: CXCL9 and CXCL10 expression can inhibit EOC progression**

The ELR⁻ chemokines CXCL9, CXCL10 and CXCL11 can influence tumour progression through their angiostatic effects (Strieter et al. 2006). Primary ovarian cancer cells stimulated with IL17 and IFN-γ can be induced to secrete CXCL9 and CXCL10 (Kryczek et al. 2009), while CXCL11 (and CXCL9) expression has been detected in serous ovarian carcinoma cell lines and tissue by immunohistochemistry and reverse transcriptase PCR (Furuya et al. 2007). These CXCR3-binding chemokines can inhibit endothelial cell migration and proliferation, attract cells involved in innate and adaptive immunity and increase MHC class I processing by tumour cells, thereby enhancing recognition by the immune system (Groom & Luster 2011, Wilke et al. 2011; Fig. 3).

CXC-mediated regulation of the EOC microenvironment appears to be primarily due to CXCL9 and CXCL10; the anti-tumour effect of CXCL11 in EOC is reportedly less efficient in mounting an anti-tumour response (Lasagni et al. 2003, Furuya et al. 2007). Similar to the expression of CXCL9 and CXCL10 ligands, increased expression of CXCR3 has been reported on the surface of EOC cells (Furuya et al. 2007) and clear cell ovarian cancer cells (Furuya et al. 2011). CXCR3 has been found to be expressed by endothelial cells while undergoing G2/M and late S phases of the cell cycle (Romagnani et al. 2001). Although the expression of CXCR3 in multiple cell types and specific cell cycle phases have been identified (Romagnani et al. 2001), their biological relevance has yet to be conclusively demonstrated (Lasagni et al. 2003, Giuliani et al. 2006, Gacci et al. 2009, Campanella et al. 2010).

**Angiogenic chemokine ligands can promote tumour development**

Pathological angiogenesis involves multiple cell types and growth factors and is observed in wound healing, arthritis and diabetic retinopathy. It is closely related to chronic inflammation and is an important event in the progression and spread of cancer (Keeley et al. 2008). ELR⁺ chemokines including CXCL8 (IL8), CXCL5 (ENA-78) and CXCL1 (GRO-α) are known to induce endothelial migration and proliferation through binding to CXCR2, leading to metastatic spread (Mukaida & Baba 2012). They can also act indirectly via the expression of CXCR2 on leukocytes, leading to the secretion of pro-angiogenic factors such as VEGF (Kiefer & Siekmann 2011). All three CXC chemokines...
have the ability to attract polymorphonuclear leukocytes including neutrophils (Mukaida & Baba 2012).

The significance of inflammation in the potential initiation and progression of EOC makes CXCL5 a potentially important (and largely unreported) factor in this disease. CXCL5 is expressed in both lung and pancreatic cancer and is up-regulated in endometriosis and ovarian carcinoma where it can attract neutrophils (Wislez et al. 2004, Furuya et al. 2007, Frick et al. 2008, Li et al. 2011). Once present, neutrophils secrete a broad spectrum of proteases including cathepsin G that can cleave CXCL5; N-terminal truncation of CXCL5 significantly enhances the chemotactic potency of CXCL5 both in vitro and in vivo (Wuyts et al. 1999, Mortier et al. 2010). Neutrophils isolated from the peripheral blood of ovarian cancer patients also produce increased reactive oxygen species and display increased adhesion to ovarian cancer cells through enhanced CD11b/CD18 expression on their cell surfaces (Klink et al. 2008). The adhesion of neutrophils to endothelial cells initiates remodelling of the endothelium, making it easier for tumour cells to metastasise (Wu et al. 2001, De Larco et al. 2004, Klink et al. 2008). Figure 4 attempts to summarise the influence of neutrophils and modified CXCL5 in the metastasis of ovarian tumour cells.

Both CXCL8 (Schutyser et al. 2002) and CXCL1 (Bolitho et al. 2010) are up-regulated in ascites fluid, while CXCL8 protein and anti-CXCL8 antibodies are also present in serum from advanced EOC patients (Lokshin et al. 2006). Stimulation by CXCL8 of various ovarian tumour cell lines leads to their proliferation in vitro (Wang et al. 2005) and is correlated with increased metastatic potential in SKOV3 cells (Yin et al. 2011). CXCL8 also has a direct role in angiogenesis, stimulating capillary tube formation via CXCR1 and CXCR2 (Li et al. 2003). In the context of advanced EOC, development of ascites and possible metastasis is promoted by angiogenesis through the key regulator VEGF (Masoumi Moghaddam et al. 2011). In an early study to determine the genes expressed in angiogenesis, five different human ovarian carcinomas were implanted into the peritoneal cavities of nude mice (Yoneda et al. 1998). The formation of ascites was associated with the expression of VEGF and CXCL8, and this was inversely associated with survival.

Similar to CXCL5, CXCL1 has chemotactic activity for neutrophils and its N-terminal truncation leads to enhanced chemotaxis in vitro (Wuyts et al. 1999). Over-expression of CXCL1 in vitro also results in increased cell proliferation (Bolitho et al. 2010). CXCL1 has been shown to promote malignant transformation of epithelial cells through a complex RAS pathway-mediated transformation, which induces normal stroma to ‘re-program’ and become tumorigenic (Yang et al. 2006). Silencing of CXCL1 using siRNA eliminated RAS-induced transformation (Yang et al. 2006). In the same study, CXCL1 expression was elevated in both serum and tumour tissue from ovarian cancer patients (Yang et al. 2006). CXCL1 (in combination with CCL18) has also been suggested as a serum biomarker of ovarian cancer (Wang et al. 2011). Multivariate ELISA gave a sensitivity of 92% and specificity of 97% for detecting ovarian cancers, which was significantly higher than values obtained using CA 125 alone (Wang et al. 2011).

In normal ovarian function, both CXCL8 and CXCL1 are produced by the ovulating follicle to attract CXCR1+ and CXCR2+ leukocytes to assist with ovulation (Karstom-Encrantz et al. 1998). Using mouse models of peritoneal ovarian cancer, it has been shown that matrix metalloprotease-1 (MMP1) activation of the G protein-coupled receptor, protease-activated
receptor-1 (PAR1), is also an important stimulator of angiogenesis and metastasis in EOC (Agarwal et al. 2008). The paracrine control of chemokine production by CXCR1 and CXCR2 receptors on ovarian carcinoma endothelial cells is stimulated by the MMP1-PAR1 signalling system (Agarwal et al. 2010). After MMP1-PAR1 stimulation, it was shown that ovarian carcinoma cells both in vitro and in mice secreted CXCL8 and CXCL1, with the introduction of X1/2pal-i3 pepducin completely inhibiting angiogenesis (Agarwal et al. 2010). Inhibition of this pathway blocks tumour growth and metastasis in breast cancer models (Yang et al. 2008). The paracrine control of chemokine production CXCL1, with the introduction of X1/2pal-i3 pepducin cells both PAR1 stimulation, it was shown that ovarian carcinoma cells both in vitro and in mice secreted CXCL8 and CXCL1, with the introduction of X1/2pal-i3 pepducin completely inhibiting angiogenesis (Agarwal et al. 2010). Inhibition of this pathway blocks tumour growth and metastasis in breast cancer models (Yang et al. 2008); the MMP1-PAR1-CXCR1/2 pathway may therefore be a new target for preventing EOC-related angiogenesis.

Direct involvement of the ELR– chemokine CXCL12 in metastatic spread

In early studies to determine the influence of chemokine gradients on the metastatic spread of ovarian tumours, it was shown that the receptor CXCR4 induced calcium flux and could direct migration of tumour cells (Scotton et al. 2001, 2002). The expression of CXCR4 mRNA was detected in six ovarian cancer cell lines, ascites (19 of 20 samples), and primary ovarian tumours (eight of ten samples). The study showed that the cell lines displayed chemotaxis towards CXCL12, which was also present at high levels in ovarian cancer ascites fluid from 63 patients (Scotton et al. 2001). However, not all tumour cells in the primary ovarian tumours expressed CXCR4 mRNA, suggesting that other factors present in the tumour microenvironment contributed to receptor expression. After in vitro stimulation of the ovarian cancer cell line IGROV with CXCL12, cells could invade through Matrigel towards a CXCL12 gradient (Scotton et al. 2002). This also promoted the activation of Akt/protein kinase B, biphasic phosphorylation of p44/42 MAP kinase and induction of the cytokine TNF-α (Scotton et al. 2002).

The metastatic spread of EOC tumour cells to the peritoneal mesothelium also involves the CXCL12/CXCR4 axis (Kajiyama et al. 2008, Miyaniishi et al. 2010). Furthermore, a significant correlation has been shown between CXCR4 expression in both primary and metastatic ovarian tumours, as well as lymph node metastases (Jiang et al. 2006). In a mouse model of ovarian cancer, siRNA-mediated silencing of CXCL12 reduced tumour growth in vivo, and a CXCR4 antagonist (AMD3100) could increase T-cell-mediated anti-tumour responses (Righi et al. 2011). Human primary ovarian tumour cells also express CXCL12 and VEGF following exposure to the hypoxic tumour microenvironment (Kryczek et al. 2005). In this in vivo model, CXCL12 and VEGF promoted angiogenesis and the migration of human vascular endothelial cell expressing CXCR4 (Kryczek et al. 2005). Importantly, CXCL12 and CXCR4 are not detected in normal ovarian tissues (Jiang et al. 2006, Kajiyama et al. 2008), even in women with a family history of ovarian cancer (Scotton et al. 2002).

Recently, sequential genetic changes were found in the TP53 allele as well as the CXCR4 gene locus (chromosome 2q21.3) of human transformed ovarian surface epithelial cell lines (Archibald et al. 2012). These were investigated to determine early events in the development of serous EOC. Following the screening of 50 serous EOC patient biopsies, the mutated TP53 expression gene signature (with decreased TP53 target gene expression) was associated with activation of epidermal growth factor receptor pathways, as well as high expression of CXCR4 genes (with corresponding mRNA and protein). This suggests that these events may occur early in the development of EOC (Archibald et al. 2012). Amongst other recently discovered genetic changes to potentially up-regulate CXCR4 expression in EOC is the abrogation of breast cancer metastasis suppressor 1 (BRMS1; Sheng et al. 2012). Suppression of BRMS1 by short-hairpin RNA transfection of OVCAR3 cell lines significantly enhanced CXCR4 expression at the mRNA and protein levels, with enhanced expression mediated by the NF-κB signalling pathway (Sheng et al. 2012).

Figure 5 offers a simplistic summary of the immune-suppressive tumour microenvironment in EOC. The lack of angiogenic ELR-chemokines results in unchecked angiogenesis due to the presence of Treg cells, while CXC chemokines and their receptors, in particular the CXCL12/CXCR4 axis, can promote tumour metastasis.

CXC chemokines as future therapeutic targets for EOC

Due to the importance of CXC chemokine signalling, there are active development strategies to exploit these pathways to reduce the morbidity and mortality associated with EOC. One strategy involves the stimulation of Th17 cells through the use of vaccines or immunotherapy to stimulate IFN-γ and IL17, resulting in elevated CXCL9 and CXCL10 levels (Munn 2009, Cannon et al. 2011, Goyne et al. 2011). However, animal or clinical trials are yet to be reported. The use of CXC chemokine inhibitors and antagonists for a variety of different cancers, including EOC (Zlotnik et al. 2011, Balkwill 2012), is emerging as a promising strategy for new treatments.

Of the CXC chemokine ligands and receptors involved in EOC, inhibition of CXCR4 has received a great deal of attention with several molecules found that are able to antagonise activity in response to CXCL12 (Barbieri et al. 2010). In two separate studies involving mouse models of EOC, AMD3100 (a clinically approved CXCR4 inhibitor) significantly reduced ovarian cancer cell growth (Ray et al. 2011, Righi et al. 2011). In mice, administration of AMD3100 significantly reduced

intrapерitoneal dissemination and angiogenesis (measured by vessel density with the tumour), increased T-cell-mediated anti-tumour immune responses and apoptosis and reduced FoxP3+ Treg cell numbers within the tumour (Righi et al. 2011). Subsequently, it was shown that AMD3100 blocked CXCL12/CXCR4 binding, resulting in prolonged survival and reduced tumour growth in mice with disseminated ovarian cancer (Ray et al. 2011). The use of this important chemokine inhibitor in human trials is yet to be reported.

Conclusion

A significant body of evidence indicates the involvement of chemokines in an EOC and suggests that initiation, progression and metastasis are strongly influenced by the CXC chemokines. In particular, their roles in inflammation, angiogenesis and immune cell recruitment have all been shown to exert profound influence in the tumour microenvironment; moreover, there is a complex interplay of these factors that influences clinical outcomes. Research to exploit these chemokine-signalling pathways, and translate the findings to a clinical environment, remains at a very early stage. Relevant studies in murine models using the inhibitor AMD3100 targeting the CXCR4/CXCL12 axis have been reported, but there are no human trials yet reported. Similarly, the enhancement of CXCL9 and CXCL10 expression through stimulation of TH17 cells, or CXCL5 inhibitor development to prevent neutrophil adhesion and potential metastasis, is also at an early stage.

The ground work for understanding the combined action and mechanisms of CXC chemokine activity in EOC is underway. Exploitation of CXC chemokine-signalling pathways to reduce EOC morbidity and mortality remains as a future avenue to potentially improve outcomes for patients with this disease.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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References


Bolitoh C, Hahn MA, Baxter RC & Marsh DJ 2010 The chemokine CXCL1 induces proliferation in epithelial ovarian cancer cells by transactivation of the epidermal growth factor receptor. Endocrine-Related Cancer 17 609–615. (doi:10.1677/ERC-10-0107)


Campanella GS, Colvin RA & Luster AD 2010 CXCL10 can inhibit endothelial cell proliferation independently of CXCR3. PLoS ONE 5 e12700. (doi:10.1371/journal.pone.0012700)


CXC chemokines in epithelial ovarian cancer


