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in molecular medicine

Chemokines in cancer

Mitchell J. Frederick and Gary L. Clayman

Chemokines are small, chemotactic cytokines that direct migration of leukocytes, activate inflammatory responses and participate in many other pleiotropic functions, including regulation of tumour growth. Chemokines modulate tumour behaviour by three important mechanisms: regulation of tumour-associated angiogenesis, activation of a host tumour-specific immunological response, and direct stimulation of tumour cell proliferation in an autocrine fashion. All of these mechanisms are promising points of cancer intervention, and preclinical mouse models suggest that chemokine antagonists and agonists could become important in the development of new anticancer therapies.

Advances in bioinformatics and the rapid expansion of expressed sequence tag databases have led to a virtual explosion in the identification of new chemotactic cytokines (chemokines) within the past five years (Ref. 1). Chemokines constitute a large superfamily of secreted proteins (with relatively low molecular weight) that cause directed migration of leukocytes (Ref. 2). In humans, there are approximately 50 known chemokines, and it is now clear that they are involved in a plethora of homeostatic and disease processes including haematopoiesis, lymphoid organ development, inflammation, leukocyte trafficking, allergies, wound healing, growth of new blood vessels (angiogenesis), cancer development, and tumour metastasis (Refs 1, 2, 3). Several excellent published reviews examine the general function of chemokines and their receptors in these diverse biological processes (Refs 1, 2, 3, 4, 5, 6), including a review by Wang et al. published in 1998 (Ref. 7) that discusses chemokines in the context of tumour growth and metastasis. This article also examines the role chemokines play in cancer but incorporates more-recent findings, including data suggesting that dogma regarding the immunogenicity of tumours is beginning to change.

The chemokine superfamily

Nearly all members of the chemokine superfamily contain four conserved cysteine amino acid residues, with the separation of the first two cysteine residues determining the subfamily (Fig. 1). The CXC subfamily contains a single nonconserved amino acid separating the first

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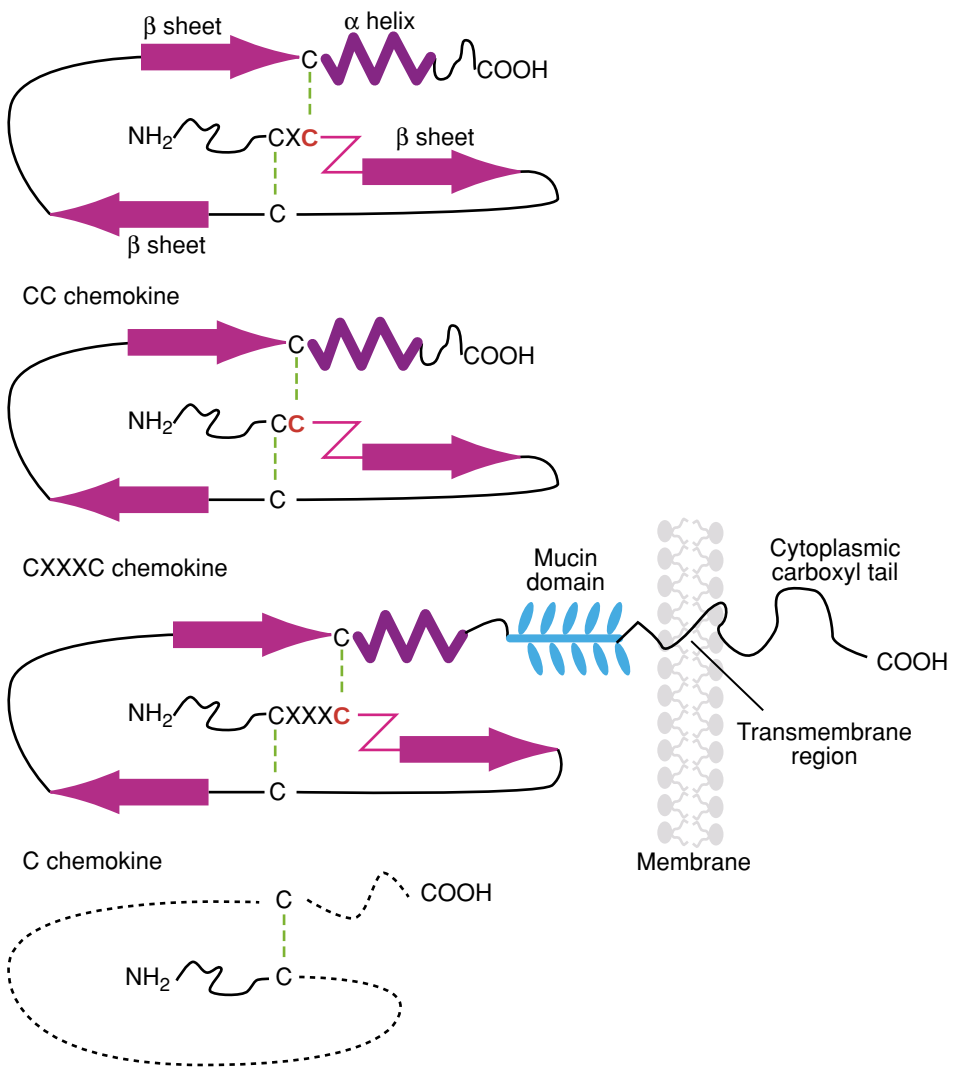
Institution URL: http://www.mdanderson.org/

Accession information: (01)00330-1a.pdf (short code: txt001mfh); 18 July 2001
ISSN 1462-3994



Schematic illustrations of the major secondary structural motifs of the four chemokine subfamilies

Figure 1. Schematic illustrations of the major secondary structural motifs of the four chemokine subfamilies. In each of the four subfamilies (a–d), the N-terminus (NH_2) is followed by a small stretch of disordered structure (thin black wavy line). After the second conserved cysteine residue (C in red) in the first three subfamilies (a–c), there is a small loop ending in a single turn of an alpha helix (pink Z shape), which is then followed by three anti-parallel beta sheets (thick magenta arrows) separated by turns and loops (thin black curved lines). In the first three subfamilies, an alpha helix (purple zigzag) occurs near the C-terminus (COOH) and is followed by another disordered region (black). Disulphide bonds between the conserved cysteine residues are represented by a dashed green line. The CXXXC chemokine is exceptional compared with the other subfamilies in that it can occur as a transmembrane protein as shown, or as a soluble molecule following cleavage (not shown). A mucin domain, which extends the CXXXC chemokine away from the cell membrane, is depicted as a blue branched structure, and the transmembrane region and cytoplasmic carboxyl tail are also shown. The structure of the C chemokine (d) is mostly drawn with dotted lines, because it is largely unknown **(fig001mfh)**.

Accession information: (01)00330-1a.pdf (short code: txt001mfh); 18 July 2001
ISSN 1462-3994 ©2001 Cambridge University Press

two cysteine residues, while the CC and CXXXC subfamilies have either none or exactly three nonconserved amino acids between the cysteines. The fourth subfamily, known as the C chemokines, is unique in that only two of the four cysteine residues (i.e. the first and third) are present. A list of all chemokines reported in published papers, grouped by subfamily, along with their respective receptors, chemotactic targets, and other biological functions, is presented in Tables 1 and 2. The CXC chemokines are further subdivided according to the presence or absence of another conserved three amino acid motif (Ref. 3), Glu-Leu-Arg (the ELR motif), the implications of which are discussed later.

Chemokines are secretory proteins, with the precursor molecules containing identifiable hydrophobic signal peptides. The CXXXC chemokine fractalkine/neurotactin (CX3CL1) is exceptional in that it is membrane bound (Ref. 7). In addition to chemokines produced by cells, several chemokines are encoded by

Table 1. Characteristics of human CXC chemokines (tab001mfh)

Common name(s)[a]	Receptor(s)	Chemotactic function[b]	Cancer-related functions
Glu-Leu-Arg (ELR)-positive			
GRO-α	CXCR2	Np, T, B, Ba, EC	Angiogenic, growth factor for melanoma tumours
GRO-β	CXCR2	Np, Ba, EC	Angiogenic, growth factor for melanoma tumours
GRO-γ	CXCR2	Np, Ba, EC	Angiogenic, growth factor for melanoma tumours
ENA-78	CXCR2	Np, EC	Angiogenic
GCP-2	CXCR1, CXCR2		
NAP-2	CXCR2	Np, EC	Angiogenic
IL-8	CXR1, CXCR2	Np, T, B, Ba, EC	Angiogenic, tumour growth factor
Glu-Leu-Arg (ELR)-negative			
PF4	Unknown	Np	Causes tumour necrosis, angiostatic, inhibits tumour growth
IP-10	CXCR3	Tac, M, NK, Eo	Angiostatic, inhibits tumour growth
MIG	CXCR3	Tac, Eo	Angiostatic, inhibits tumour growth
I-TAC	CXCR3	Tac	Angiostatic
BRAK/BMAC/MIP-2γ	Unknown	Np, DC, M, B	Downregulated in certain cancers
BLC	CXCR5	B	
SDF-1	CXCR4	T, B, DC, M, Ba, EC	Angiogenic

[a] Common names are those most frequently cited in the literature over the past three years. For the new nomenclature see Ref. 8.
[b] The listing of cell types that undergo chemotaxis in response to each chemokine is based on reported results in the literature rather than extrapolation from chemokine receptor expression. Abbreviations: B, B cell; Ba, basophil; DC, dendritic cell; EC, endothelial cell; Eo, eosinophil; M, monocyte; NK, natural killer cell; Np, neutrophil; T, T cell; Tac, activated T cell.

Accession information: (01)00330-1a.pdf (short code: txt001mfh); 18 July 2001
ISSN 1462-3994

https://doi.org/10.1017/S1462399401003301 Published online by Cambridge University Press

Table 2. Characteristics of human CC, C and CXXXC chemokines (tab002mfh)

Common name(s)[a]	Receptors	Chemotactic function[b]	Cancer-related functions
CC chemokines			
I-309 (TCA3)	CCR8	T, M, NKac, Np	Induces anti-tumour immunity
MCP-1	CCR2, CCR9, CCR10, CCR11	T, M, B, NK, DC, Ba, EC	Angiogenic
MIP-1α	CCR1, CCR5	T, M, DC, Eo	Induces anti-tumour immunity
MIP-1β	CCR5, CCR8, CCR9	T, M, NK, DC	
RANTES	CCR1, CCR3, CCR5, CCR9	T, M, NK, DC, Eo, Ba	
MCP-3	CCR1, CCR2, CCR3,	T, M, NK, DC, Np, Eo	
MCP-2	CCR2, CCR3, CCR9, CCR11	Tac, M, NKac, DC, Eo, Ba	
Eotaxin	CCR3, CCR9	T, Eo, Ba	
MCP-4	CCR2, CCR3, CCR9, CCR11	M, DC, Eo, Ba	
HCC-1	CCR9	M	
HCC-2/MIP-5	CCR1	T, M, DC, Eo	
HCC-4/NCC-4/LEC	CCR1, CCR8	M, DC	Induces anti-tumour immunity
TARC	CCR4, CCR8	T, NK	
PARC	Unknown	T	
ELC	CCR7	T, B, NK, DC	Induces anti-tumour immunity
LARC	CCR6	T, B, DC, Eo	Induces anti-tumour immunity
MDC	CCR4	T, NK	
TECK	CCR9	T, B, DC	
Eotaxin-3	CCR3	Eo, Ba	
ALP/Eskine	CCR10	T	
CCL28	CCR10	T	
Eotaxin 2	CCR3	Eo, Ba	
MPIF-1	CCR1	M, DC	
SLC	CCR7	T, B, NK, DC	Induces anti-tumour immunity
C chemokines			
Lymphotactin	XCR1	T, NK	
CXXXC chemokines			
Fractalkine/ neurotactin	CX3CR1	T, NKac, M	

[a] Those most frequently cited in the literature within the past three years (see Ref. 8 for new nomenclature).
[b] The listing of cell types that undergo chemotaxis in response to each chemokine is based on reported results in the literature rather than extrapolation from chemokine receptor expression. Abbreviations: B, B cell; Ba, basophil; DC, dendritic cell; EC, endothelial cell; Eo, eosinophil; M, monocyte; NKac, activated natural killer cell; NK, natural killer cell; Np, neutrophil; T, T cell; Tac, activated T cell.

Accession information: (01)00330-1a.pdf (short code: txt001mfh); 18 July 2001
ISSN 1462-3994 ©2001 Cambridge University Press

viral genes and are grouped into a fifth family (for details see the Chemokine Family website at http://cytokine.medic.kumamoto-u.ac.jp/CFC/CK/Chemokine.html).

Chemokines have often been co-discovered by multiple investigators and therefore have numerous names in the literature, which can be a source of confusion. A new standardised systemic nomenclature has been adopted (Ref. 8) and is used here in parentheses when ligands are first mentioned. The common names listed in Tables 1 and 2 represent those most frequently cited in the literature over the past three years. A complete list of alternative common names along with the new nomenclature can be found at http://cytokine.medic.kumamoto-u.ac.jp/CFC/CK/Chemokine.html.

Chemokines can be made by virtually every nucleated cell type under appropriate conditions (Ref. 4). Most chemokines are expressed in response to a stimulus, but some are constitutively expressed in a tissue-specific manner. Thus, the distinctions between inducible and constitutive expression might depend simply upon the origin of cells under study. For example, the chemokine BRAK (CXCL14; originally named BRAK because of its expression in breast and kidney) is constitutively expressed at high levels in normal squamous epithelium, but might require a stimulus to be expressed by inflammatory cells and certain cancers (Ref. 9).

Chemokine receptors

Chemokines exert their migration-inducing properties on leukocytes through binding to chemokine receptors that belong to the seven-transmembrane domain G-protein-coupled rhodopsin superfamily (Ref. 7). Chemokine receptors have received considerable attention because of the key findings that some act as co-receptors for human immunodeficiency virus (HIV), which causes acquired immune deficiency syndrome (AIDS) (Ref. 1). Although important, this discovery is not discussed here as it is not directly related to common mechanisms operating in cancer.

In addition to their seven hydrophobic transmembrane domains, chemokine receptors have an N-terminus outside the cell, three extracellular and three intracellular loops, and a C-terminus containing serine and threonine phosphorylation sites in the cytoplasm. Several chemokine receptors have been cloned and many have the capacity to bind more than one chemokine within a subfamily (Refs 5, 6). The mouse CC chemokine secondary lymphoid-tissue chemokine (SLC/CCL21) is exceptional because it can bind not only the CCR7 receptor but also CXCR3 used by members of the CXC chemokine subfamily (Ref. 10). Binding of ligand to chemokine receptors initiates a cascade of G-protein-coupled intracellular signal transduction events (Ref. 7), leading to activation of phospholipases, hydrolysis of phosphatidylinositol (4,5)-bisphosphate, formation of inositol trisphosphate and diacylglycerol, intracellular calcium (Ca^{2+}) flux, and activation of protein kinase C and the mitogen-activated protein kinases. Five CXC chemokine receptors, 11 CC receptors, and one receptor each for the C and CXXXC chemokines have thus far been identified (Tables 1 and 2).

Chemokines modulate angiogenesis

Angiogenesis is the growth of new blood vessels from already existing vessels and microcapillaries (Ref. 11). It is a process that normally takes place during embryonic development and wound healing, but is also required for many solid tumours to grow beyond 2 mm in diameter (Refs 11, 12). Vascularisation of a tumour is also required for rapid growth (Ref. 12). Several published reviews address the role of chemokines in regulating angiogenesis (Refs 3, 13, 14, 15).

Early studies demonstrating that platelet factor 4 (PF4/CXCL4) could inhibit endothelial cell proliferation, angiogenesis in the chick chorioallantoic membrane assay, and tumour growth in immunodeficient mice were among the first to show that a chemokine could inhibit angiogenesis (Ref. 16). Subsequently, interleukin 8 (IL-8/CXCL8) was the first chemokine discovered to stimulate endothelial cell chemotaxis, proliferation and in vivo angiogenesis (Ref. 17). Both PF4 and IL-8 are members of the CXC chemokine subfamily, but they differ in that IL-8 contains the ELR motif thought to be necessary for causing chemotaxis of neutrophils. The observation that other ELR^+ CXC chemokines could stimulate angiogenesis, whereas ELR^- CXC chemokines [particularly those induced by interferon (IFN)] inhibited angiogenesis, led to the hypothesis that the ELR motif is critical for determining the effect a CXC chemokine has on angiogenesis (Ref. 18). The functional role of the ELR motif has been demonstrated by mutational

Accession information: (01)00330-1a.pdf (short code: txt001mfh); 18 July 2001
ISSN 1462-3994

analysis (Ref. 18). Substitution of the ELR motif in IL-8 with the amino acids TVR derived from IFN-γ-inducible protein (IP-10/CXCL10) or DLQ derived from PF4 resulted in an ELR⁻ IL-8 mutant that was unable to stimulate endothelial chemotaxis or in vivo angiogenesis and that actually behaved as an inhibitor of angiogenesis. In contrast, addition of the amino acids ELR to the CXC chemokine MIG/CXCL9 (monokine induced by IFN) converted the protein from an angiostatic to an angiogenic molecule. The presence of both stimulators and inhibitors of angiogenesis among the CXC chemokine subfamily led to the postulate that CXC chemokines form a balanced network of angiogenic and angiostatic regulators that is disrupted in cancer (Ref. 15). Figure 2 illustrates how the balance of angiogenic and angiostatic factors can affect tumour growth.

Chemokines that stimulate angiogenesis

All ELR⁺ CXC chemokines, including growth-regulated oncogene (GRO-α, -β and -γ/CXCL1–CXCL3), ENA-78 (CXCL5), granulocyte chemotactic protein (GCP-2/CXCL6), neutrophil-activating protein 2 (NAP-2/CXCL7) and IL-8, stimulate endothelial cell chemotaxis in vitro and angiogenesis in vivo (Ref. 3). The ability of ELR⁺

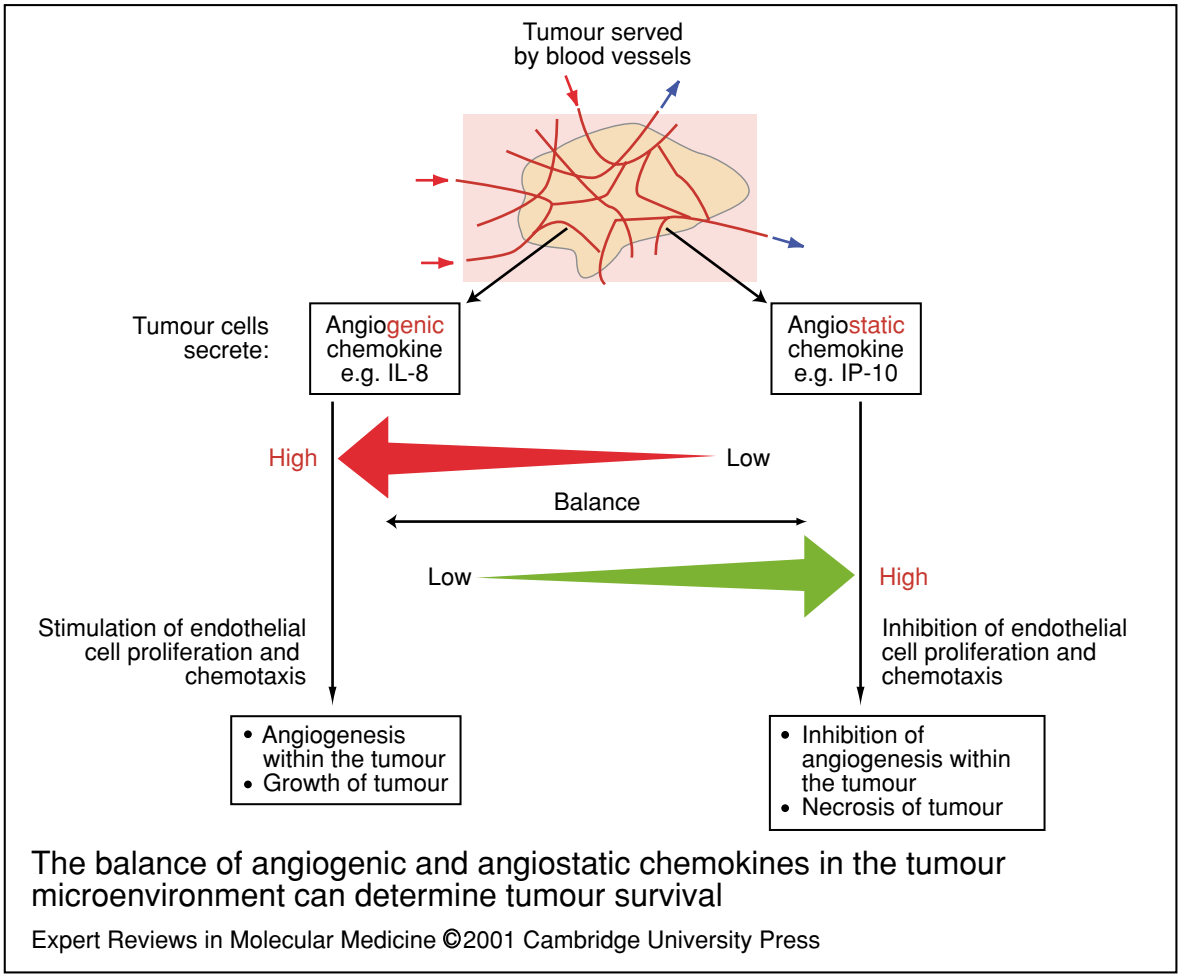


Figure 2. The balance of angiogenic and angiostatic chemokines in the tumour microenvironment can determine tumour survival. Chemokines can stimulate or inhibit proliferation and chemotaxis of the endothelial cells of the blood vessels that serve a tumour. Where a tumour secretes greater amounts of an angiogenic chemokine [e.g. interleukin 8 (IL-8)] than an angiostatic chemokine, angiogenesis is stimulated, leading to new blood vessel formation and continued tumour growth. An excess of angiostatic chemokines [e.g. interferon-γ-inducible protein 10 (IP-10)] in the tumour microenvironment inhibits neovascularisation, leading to tumour necrosis **(fig002mfh)**.

Accession information: (01)00330-1a.pdf (short code: txt001mfh); 18 July 2001
ISSN 1462-3994 ©2001 Cambridge University Press

chemokines to induce angiogenesis appears to be independent from their ability to recruit inflammatory cells, since angiogenesis takes place in the rat corneal micropocket assay in the absence of infiltrating leukocytes (Ref. 3). Both GRO-α and IL-8 have been shown to stimulate endothelial cell proliferation in vitro (Refs 17, 19, 20).

One ELR⁻ CXC chemokine that actually stimulates, rather than inhibits, angiogenesis is stromal-derived factor 1 (SDF-1/CXCL12). The SDF-1 receptor CXCR4 is expressed on endothelial cells, which undergo chemotaxis in response to SDF-1 (Ref. 21). Levels of mRNA for the SDF-1 receptor on human endothelial cells are upregulated in response to vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF), which are non-chemokine angiogenic factors (Ref. 22). Moreover, SDF-1 induces angiogenesis from cross-sections of leukocyte-free rat aorta in vitro (Ref. 23) and the formation of capillary-like structures by endothelial cells in culture (Ref. 24). Another difference between ELR⁻ CXC chemokines that inhibit angiogenesis and SDF-1 is that the latter is not induced by IFN.

Recently, a member of the CC chemokine subfamily has also been reported to stimulate angiogenesis directly. Monocyte chemotactic protein 1 (MCP-1/CCL2) attracts monocytes, as well as activated natural killer (NK) and T cells. MCP-1 causes chemotaxis of human endothelial cells, and induces blood vessel formation in the chick chorioallantoic membrane model and in the mouse matrigel plug assay (Ref. 23). At least part of the angiogenic mechanism of MCP-1 appears to be a direct effect on endothelial cells, as MCP-1 causes rat aortic sprouting in the absence of leukocytes. Consistent with this hypothesis is the observation that human endothelial cells express the MCP-1 receptor CCR2 (Ref. 23).

Chemokines that inhibit angiogenesis

Among the CXC chemokines that do not contain the ELR motif, those that are induced by IFN are highly angiostatic. For example, MIG, IP-10, and IFN-inducible T cell alpha chemoattractant (I-TAC/CXCL11) inhibit growth of new blood vessels stimulated by either VEGF or angiogenic CXC chemokines in the rat corneal micropocket assay (Ref. 3). IP-10 and MIG also inhibit the in vitro chemotaxis of endothelial cells (Ref. 14). IFNs are known to inhibit angiogenesis, and therefore induction of ELR⁻ CXC chemokines might be one of the primary mechanisms underlying this (Refs 25, 26). Not all angiostatic CXC chemokines are induced by IFN. For example, PF4, which is not induced by IFN, is another ELR⁻ CXC chemokine that inhibits angiogenesis in the chick chorioallantoic membrane assay (Ref. 16), attenuates endothelial cell chemotaxis (Ref. 18) and blocks in vitro proliferation of endothelial cells (Ref. 27).

Evidence that chemokines regulate tumour growth

Experimental evidence suggests that chemokines regulate tumour growth in humans and other animals by various mechanisms, including modulation of angiogenesis, activation of a tumour-specific immune response, and autocrine growth stimulation. Evidence for these individual mechanisms is presented separately here, but it should be pointed out that all three processes can operate simultaneously in vivo.

Imbalance of chemokines modulating angiogenesis regulates tumour growth

The Eastern concept of 'Yin and Yang' (i.e. that everything is controlled by two equal but opposing forces) is a recurring motif throughout biology, as processes such as angiogenesis must be temporally turned on and off during the life of an organism. Moore et al. (Ref. 15) have proposed that 'angiogenesis is regulated by a complex balancing act between opposing angiogenic and angiostatic factors'. Considerable observations and experimental data suggest that this hypothesis is true. Belperio et al. (Ref. 3) have reviewed much of the data supporting a role for CXC chemokines in modulating the angiogenesis of tumours in vivo, and some key examples of this effect will be cited here.

IL-8

Elevated levels of the angiogenic CXC chemokine IL-8 have been detected in a variety of tumours, including non-small-cell lung carcinoma (NSCLC) (Ref. 28), metastatic melanoma (Ref. 29), ovarian carcinoma (Ref. 30) and colon carcinoma (Ref. 31). Tissue homogenates derived from NSCLC contain chemotactic activity for endothelial cells and induce angiogenesis in a corneal assay, which can be partially neutralised by antibodies to IL-8 (Ref. 28). Growth and tumour vascularity of the IL-8-producing NSCLC adenocarcinoma cell line A549 in severe combined immunodeficient

Accession information: (01)00330-1a.pdf (short code: txt001mfh); 18 July 2001
ISSN 1462-3994

(SCID) mice can be significantly blocked by systemic delivery of neutralising antibodies to IL-8 (Ref. 32). As neutralising antibodies to IL-8 have no effect on A549 cell growth in vitro, IL-8 does not behave as an autocrine growth factor for this tumour line and probably contributes to tumour growth in vivo by stimulating angiogenesis. In addition to its angiogenic function, IL-8 can also activate expression of the degradative enzyme metallomatrix protein 2 and enhance metastatic potential of melanoma cells (Ref. 33).

Ascites fluid collected from ovarian cancer patients contains IL-8 capable of stimulating angiogenesis (Ref. 34), and correlations have been found between levels of IL-8 production by ovarian carcinoma cell lines and ability of these lines to grow and vascularise in mice (Ref. 35). Similarly, serum from patients with prostate cancer contains elevated IL-8 levels (Ref. 36), and antibody-mediated neutralisation of the IL-8 produced by the prostate carcinoma cell line PC-3 has been shown to reduce tumour growth and tumour-related angiogenesis in a SCID mouse model (Ref. 37). Thus, evidence from a variety of human cancers implicates IL-8 as an angiogenic CXC chemokine that promotes the growth of tumours in vivo.

ENA-78

The ELR$^+$ CXC chemokine ENA-78 also contributes to the in vivo growth and angiogenic potential of NSCLC (Ref. 38). Freshly isolated human NSCLC biopsy specimens have elevated levels of ENA-78 protein compared with normal lung controls, as detected by ELISA (enzyme-linked immunosorbent assay), and immunohistochemistry has confirmed NSCLC tumours as the source of expression. Homogenates of human NSCLC specimens are angiogenic in the rat corneal micropocket assay, and the development of vasculature can be blocked by antibodies that neutralise ENA-78. Systemic delivery of antibodies that neutralise ENA-78 significantly inhibits in vivo tumour growth, lung metastasis, and tumour-associated endothelial cell density of an ENA-78-expressing NSCLC line in SCID mice. As neutralising antibodies to ENA-78 fail to attenuate in vitro tumour proliferation, it appears that ENA-78 behaves in a manner similar to IL-8 in NSCLC by promoting tumour growth via increased angiogenesis (Ref. 38).

GRO-α and GRO-γ

Human melanoma tumours express the CXC chemokines GRO-α and GRO-γ, as measured by ELISA and immunohistochemistry (Ref. 39). The mechanism and significance of GRO protein expression in melanoma tumours has been reviewed by Luan et al. (Ref. 39). The expression of all three GRO proteins can be detected at the mRNA level. Overexpression of any of the GRO proteins following transfection of non-tumourigenic immortalised mouse melanocytes results in the ability to form highly vascular tumours in nude mice. Conditioned medium from tumourigenic transfectants is angiogenic in the rat corneal micropocket assay, and neutralising antibodies to the appropriate GRO protein block the angiogenic response. Repeated administration of neutralising antibodies to GRO proteins also blocks in vivo tumour growth of GRO-expressing transfectants, which is accompanied by a reduction in the number of viable endothelial cells in tumours. These studies suggest that melanoma expression of GRO proteins contributes to tumour growth in part by stimulating angiogenesis. GRO proteins are also autocrine growth factors in vitro, as discussed later.

PF4 and IP-10

The CXC chemokine PF4 was the first chemokine shown to modulate angiogenesis (Ref. 16). Intralesional injection of this angiostatic chemokine suppresses the tumour growth of murine melanoma cell lines in immunocompetent mice and of human tumour cell lines in nude mice (Ref. 40), which is consistent with its role as a downregulator of angiogenesis. However, perhaps the best-studied angiostatic chemokine is IP-10. Luster and Leder (Ref. 41) were among the first investigators to examine the potential anti-tumour effects of IP-10. They reasoned that IP-10 could be one of the secondary cytokines that mediate the in vivo anti-tumour effects of lipopolysaccharide (LPS). They demonstrated that transfection of a murine mammary adenocarcinoma or a plasmacytoma line with cDNA encoding IP-10 led to rejection of transfected tumours in syngeneic mice, but not in immunodeficient nude mice. Because rejection occurred in immunocompetent mice only and was accompanied by infiltration of lymphocytes, recruitment of inflammatory cells appeared to be the main mechanism involved. However, other evidence surfaced suggesting

Accession information: (01)00330-1a.pdf (short code: txt001mfh); 18 July 2001
ISSN 1462-3994

that IP-10 could also mediate tumour rejection by virtue of its angiostatic properties. Using a mouse matrigel neovascularisation model, IP-10 was shown to inhibit angiogenesis stimulated by bFGF in vivo (Ref. 42), and repeated intratumoural injection of Burkitt lymphoma tumours with IP-10 was found to cause significant tumour necrosis in nude mice (Ref. 43). Despite the extensive necrosis, there was no significant difference in size between IP-10-treated and control-treated Burkitt lymphoma tumours. Thus, initial experiments suggested that the anti-tumour effect of IP-10 was more robust in immunocompetent mice, but seemed to involve at least two mechanisms depending upon the mouse model employed.

An important determinant of the mechanism behind the IP-10-mediated anti-tumour response seems to be the tumour cells themselves. This is illustrated by the role of IP-10 in the biology of NSCLC, which was examined by Arenberg et al. (Ref. 44). When they divided human NSCLC biopsy specimens into histological type, they found that squamous carcinomas expressed elevated levels of IP-10 compared with normal lung, whereas adenocarcinomas had normal levels. The established NSCLC line A549 (adenocarcinoma origin) expressed little IP-10, grew well and metastasised to the lungs in immunodeficient SCID mice. However, the NSCLC line Calu-1 (squamous origin) secreted relatively high levels of IP-10 and grew poorly in SCID mice. Therefore, tumour growth in immunodeficient mice was inversely correlated with levels of IP-10 production. Homogenates from IP-10-producing Calu-1 cells were poorly angiogenic in the rat corneal micropocket assay, unless neutralised with antiserum to IP-10. Neutralising antibodies to IP-10 also significantly enhanced the growth of Calu-1 cells in SCID mice, demonstrating that endogenous production of IP-10 impaired the growth of certain tumours even in immunodeficient mice. Consistent with this hypothesis, repeated intratumoural injection of the fast-growing A549 adenocarcinoma with IP-10 slowed the growth of tumours, reduced the level of tumour-associated endothelial cells and inhibited spontaneous metastasis to the lungs in SCID mice.

MIG

The angiostatic chemokine MIG has also been shown to be capable of inhibiting tumour growth, tumour-associated angiogenesis and metastasis (Ref. 45).

Anti-tumour properties of IL-12 are related to CXC chemokine induction

IL-12 has potent anti-tumour properties and is a known stimulator of IFN-γ production (Ref. 46), which itself induces both IP-10 and MIG (Ref. 2). Early studies established that the anti-tumour properties of IL-12 were dependent upon induction of IFN-γ (Ref. 47). This finding led investigators to explore whether the anti-tumour effects of IL-12 were mediated through secondary cytokine induction of the CXC chemokines IP-10 and MIG. Antibodies to either IP-10 or MIG are able to attenuate the IL-12-mediated growth inhibition of tumours in both immunocompetent (Ref. 26) and immunodeficient mice (Ref. 25), supporting the hypothesis that IL-12 works through secondary induction of IP-10 and MIG. In the nude mouse model, significant tumour necrosis was observed in response to IL-12, which is similar to what has been observed following direct intratumoural injection of either IP-10 (Ref. 43) or MIG (Ref. 48). However, significant infiltration by $CD8^+$ T cells was apparent in the immunocompetent model. Because IL-12-treated tumours were rapidly rejected in the immunocompetent model it was not possible to determine whether angiostatic mechanisms were also operative. Markers of NK cell activity were detectable in IL-12-treated tumours in nude mice, raising the possibility that recruitment and activation of NK cells could also contribute to slowed tumour growth. Thus, it appears that both angiostatic and immunological mechanisms could be operating following induction of IP-10 and MIG by IL-12.

Requirements for the immune system in the IL-12-mediated rejection of tumours was examined using a SCID mouse model depleted of NK cell activity with anti-asialo GM1 serum. Pancreatic adenocarcinoma tumours transfected with IL-12 failed to grow or initiate angiogenesis, as observed in a skinfold chamber model, when SCID mice were depleted of NK cell activity (Ref. 49). These experiments emphasise that an intact immune system is not absolutely required for the angiostatic and anti-tumour properties of IL-12. One explanation for this result is that cells outside of the immune system could produce IP-10 or MIG in response to IL-12. This is supported by the observations of other

Accession information: (01)00330-1a.pdf (short code: txt001mfh); 18 July 2001
ISSN 1462-3994

investigators that cultured renal cell carcinoma cells can produce IP-10 and MIG in response to IFN-γ, and that explanted renal carcinoma cells produce mRNA for IFN-γ and IP-10 following incubation with IL-12 (Ref. 50).

Chemokines can activate tumour-specific immunity

One of the greatest challenges in developing tumour vaccines is getting the host to recognise tumours that might be poorly immunogenic for a number of reasons. There is now considerable experimental evidence that certain chemokines, particularly those from the CC family, are able to activate a tumour-specific immune response capable of mediating rejection of ordinarily 'non-immunogenic' cancers. This is particularly significant, as one of the major obstacles in using immunotherapy to treat cancer has been the low immunogenicity of spontaneously arising human tumours (Ref. 51). Certain members of the CC chemokine family are chemotactic for either monocytes and dendritic cells (antigen-presenting cells) or T cells (including cytotoxic T cells, and T helper 1 cells). These cell types are almost certainly involved in generating specific immunity. Hallmarks of immunity include protection from subsequent tumour challenge following initial tumour rejection, adoptive transfer of memory effector cells to protect naive animals, or induction of certain functions of immunised T cells in vitro by subsequent re-exposure to tumours.

One of the earliest studies demonstrating that a chemokine could activate tumour-specific immunity employed the mouse CC chemokine TCA3 (CCL1) (Ref. 52), which is chemotactic for monocytes and T cells. Two different mouse myeloma cell lines were transfected with TCA3 and were rejected by immunocompetent syngeneic mice. Up to 10 weeks following rejection, mice were immune to tumour formation by the appropriate untransfected parental cell line, but not to challenge with the other myeloma cell line. Moreover, immunity could not be achieved using irradiated untransfected parental cells in place of TCA3-secreting cells. Thus, the acquired immunity necessitated TCA3 expression during vaccination, was highly tumour specific and was relatively long lived. Similarly, syngeneic mice were able to reject a colon adenocarcinoma cell line engineered to express the CC chemokine macrophage inflammatory protein 1α (MIP-1α/CCL3), which is chemotactic for monocytes, T cells and dendritic cells (Ref. 53). Again, mice that had rejected the transfected tumours were immune to subsequent inoculation with the parental adenocarcinoma cell line that did not express MIP-1α. The rejected tumours were found to be infiltrated by macrophages and neutrophils.

Generation of tumour-specific cytotoxicity has been reported using the poorly immunogenic adenocarcinoma cell line TSA transfected to secrete liver-expressed chemokine (LEC/CCL16) (Ref. 54), which is chemotactic for dendritic cells, monocytes, T cells, NK cells and neutrophils. Splenocytes removed from syngeneic mice that reject LEC-secreting tumours were able to kill untransfected parental TSA cells as assessed by in vitro cytotoxicity assays. Furthermore, splenocytes from immune mice secreted IFN-γ and IL-2 when challenged with parental TSA tumours in culture. The ability to induce IFN following re-exposure to tumour also raises the possibility that angiostatic chemokines might be produced and co-contribute to tumour rejection.

Development of tumour-specific $CD4^+$ memory T cells has been achieved in a mouse model using the CC chemokine EBI1-ligand chemokine (ELC/CCL19), which is chemotactic for T cells, B cells, dendritic cells, macrophage progenitors and NK cells. Vaccination of syngeneic mice using a murine breast carcinoma line stably transfected with ELC led to rejection of the tumour and immunity to re-challenge with untransfected parental tumours (Ref. 55). Initial anti-tumour activity towards ELC-expressing carcinoma cells required the contribution of both NK cells and $CD4^+$ T-cell subsets, but subsequent protection of naive mice following adoptive transfer of splenocytes required only the $CD4^+$ T-cell subsets.

By contrast, generation of a tumour-specific $CD8^+$ cytotoxic T-cell response has been elicited in syngeneic immunocompetent mice by intratumoural delivery of the CC chemokine MIP-3α (LARC/CCL20) via an adenovirus vector (Ref. 56). Initial anti-tumour activity occurred in wild-type and $CD4^+$-knockout mice, but not in $CD8^+$-knockout mice. MIP-3α is chemotactic for T cells and dendritic cells. Only splenocytes from mice treated with the MIP-3α-containing adenovirus could develop in vitro cytotoxicity after re-priming with uninfected parental cells. Furthermore, adoptive transfer of splenocytes derived from animals whose tumours were

Accession information: (01)00330-1a.pdf (short code: txt001mfh); 18 July 2001
ISSN 1462-3994

https://doi.org/10.1017/S1462399401003301 Published online by Cambridge University Press

treated with adenovirus expressing MIP-3α could protect naive animals from parental tumour growth.

Immune-modulating factors produced in the microenvironment within a tumour could potentially determine whether or not an immune response takes place. Chemokines can influence these factors by recruiting and activating leukocytes that elaborate secondary pro-inflammatory cytokine responses. Recently, intratumoural injection of SLC (chemotactic for T cells, monocytes and dendritic cells) into two weakly immunogenic lung cancer lines was shown to alter the tumour microenvironment towards a favourable immune response that was accompanied by a significant reduction of tumour size (Ref. 57). Reduction of tumour size following intratumoural administration failed to occur in either $CD4^+$- or $CD8^+$-knockout mice, indicating the requirement for T cells. Analysis of immune-modulating factors before and after SLC injections indicated that levels of the immunosuppressive molecules prostaglandin E_2, transforming growth factor β (TGF-β) and IL-10 all decreased in tumours. By contrast, an increase in the pro-inflammatory cytokines IFN-γ, IL-12, MIG, IP-10 and granulocyte–macrophage colony-stimulating factor was observed. Thus, delivery of an appropriate chemokine to the site of a tumour can tilt the balance of pro-inflammatory and immunosuppresive cytokines in the microenvironment to favour a positive immunological response by host effector cells against a tumour. A hypothetical model addressing how chemokines might influence the host response against a tumour is depicted in Figure 3.

Chemokines as autocrine growth factors

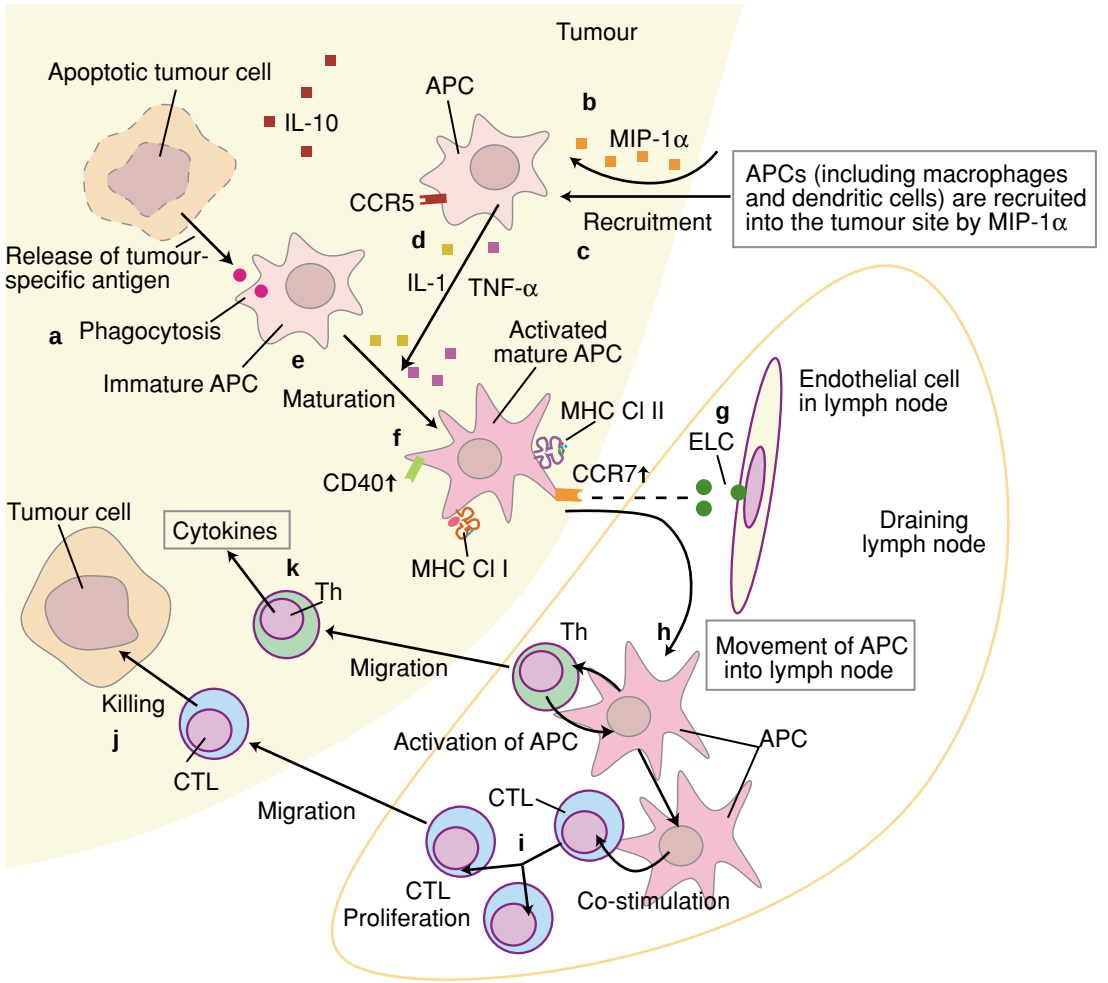
Autocrine growth factors are proteins that can bind receptors on the same cell that produced them, to stimulate proliferation. The GRO CXC chemokines were originally called 'melanoma growth stimulatory activity' polypeptides because, as the name suggests, these proteins are mitogenic for malignant melanoma cell lines in vitro. GRO-α was initially isolated from conditioned supernatant of the melanoma cell line Hs294T (Ref. 58), and is now known to be produced by many melanoma tumours. The significance of GRO chemokine expression in melanoma has been examined by Luan et al. (Ref. 39). Both GRO-α and its receptor CXCR2 are commonly expressed in malignant melanoma biopsies as assessed by immunohistochemistry. ELISA data suggest that GRO-α and GRO-γ are expressed by melanoma tumours in vivo, but expression of the alpha form tends to be higher. As antibodies to GRO-α were found to inhibit in vitro growth of melanoma cell lines, GRO-α fits all the criteria for an autocrine growth factor. GRO-α has also been shown to function as an autocrine growth factor for certain adenocarcinoma cell lines derived from the lung and stomach (Ref. 59).

Many tumours have been shown to overexpress IL-8, compared with their normal, non-malignant tissue. IL-8 is an autocrine growth factor for certain melanomas (Ref. 60) as well as tumour cell lines derived from cancers of the colon, stomach, liver, pancreas and skin (Refs 59, 61, 62, 63). In biopsy tissue from ovarian carcinomas, neuroblastomas and squamous cell carcinomas of the head and neck, both IL-8 and its receptor are made by cancerous cells, suggesting that IL-8 could also function in an autocrine pathway for these tumours (Refs 30, 64, 65). The autocrine functions of GRO-α and IL-8 illustrate the pleiotropism of chemokines, as these particular molecules not only stimulate tumours to multiply, but also recruit endothelial cells in vivo to ensure that tumours develop an adequate vasculature as they grow. Thus, GRO-α and IL-8 are perhaps the most ideal tumour growth factors.

Downregulation of the novel CXC chemokine BRAK in cancer

Overexpression of chemokines that are advantageous to growth of tumours, such as IL-8 or GRO-α, has frequently been described. However, we recently cloned a gene that was downregulated in primary cultures of squamous carcinoma of the head and neck compared with non-malignant oral epithelial cells (Ref. 9). The differentially expressed gene was identical to the CXC chemokine BRAK, which was found to be ubiquitously expressed in normal tissue extracts and absent from a variety of established tumour cell lines (Ref. 66). In situ mRNA hybridisation studies (Ref. 9) demonstrated that in normal tissues BRAK is expressed at the highest levels by squamous epithelium derived from skin, cervix and the upper aerodigestive tract, whereas other normal tissues barely showed expression. Therefore, questions regarding whether BRAK is lost by tumours are best answered in cancers

Accession information: (01)00330-1a.pdf (short code: txt001mfh); 18 July 2001
ISSN 1462-3994 ©2001 Cambridge University Press



Host immune response to tumours can be altered by chemokine production

Figure 3. Host immune response to tumours can be altered by chemokine production. (a) An immature antigen-presenting cell (APC) phagocytoses tumour-specific antigen released by a neighbouring apoptotic tumour cell. In a non-immunogenic tumour (not shown), local secretion of the anti-inflammatory cytokine interleukin 10 (IL-10) by the tumour prevents the APC from maturing, exiting the local area, or presenting antigen to T helper cells. (b) However, increased local levels of the chemokine macrophage inflammatory protein 1α (MIP-1α), for example as a result of intratumoural therapeutic administration, might result in (c) increased recruitment of antigen-presenting cells (including dendritic cells and macrophages) to the tumour site. (d) Recruited macrophages release the pro-inflammatory cytokines tumour necrosis factor α (TNF-α) and IL-1, and in the presence of these pro-inflammatory cytokines (e) APC maturation now takes place. (f) As the APC matures, it begins to present the tumour-specific peptide in conjunction with both class I and class II major histocompatibility complex antigens (MHC Cl I and MHC Cl II). During APC maturation there is a change in chemokine receptor expression: functional CCR5 (the MIP-1α receptor) is lost, and CCR7 (which binds the CC chemokine ELC) is increased. Also, the CD40 activation antigen is upregulated on APCs. (g) Constitutive production of the chemokine ELC at the lymph node endothelium results in (h) movement of mature APCs into nearby draining lymph nodes. Once in the lymph node, the APC acts as a temporal bridge between T helper (Th) cells and cytotoxic T lymphocytes (CTLs), such that (i) the latter proliferate and (j) eventually leave the lymph node to kill the tumour. (k) Activated T helper cells also emigrate from the lymph node to the site of the tumour, leading to further production of pro-inflammatory cytokines **(fig003mfh)**.

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ISSN 1462-3994 ©2001 Cambridge University Press

derived from squamous epithelium. The majority of squamous cell carcinomas derived from the upper aerodigestive tract, as well as some cervical carcinomas, did indeed show loss of expression. A reversed expression pattern was noted in some colon adenocarcinoma tumours: certain colon adenocarcinoma specimens stained positive for BRAK mRNA, whereas the normal columnar epithelium from which they were derived was negative.

The fact that many stromal and inflammatory cell types surrounding and infiltrating tumours were often found to express BRAK (Ref. 9) suggested that this chemokine has a complex pattern of expression. Constitutive expression of BRAK is apparent in normal squamous epithelium, but is probably induced by local host factors in the case of other tissues. This is supported by our findings that freshly isolated monocytes and B cells do not make BRAK mRNA, but can be stimulated to do so by incubation with LPS. Interestingly, BRAK was recently found to be chemotactic for both B cells and monocytes (Ref. 67) – the inflammatory cells that were also found to produce BRAK (Ref. 9). At present, it is unclear what role BRAK plays in the biology of cancer. Further studies will need to address the potential of BRAK to modulate angiogenesis, proliferation and a possible anti-tumour response.

Clinical implications/applications

Cancer is a devastating disease for which standard therapy frequently fails. An estimated 5.2 million people die each year from cancer-related deaths around the world (Ref. 68). Two alternative and adjuvant therapies gaining popularity because of recent advances are anti-angiogenesis drugs and tumour vaccines. Chemokines are particularly promising molecules because they have proved useful as both anti-angiogenic agents and critical determinants for successful tumour vaccine development in preclinical animal models (Refs 3, 56, 57). Because many new chemokines have been discovered over the past five years, the timing is now ripe for these genes and their pathways to be assessed in human clinical trials for anticancer therapy.

To maximise the effectiveness of chemokine-based therapies, the problem of delivery mechanisms must be dealt with. Chemokines, as with other cytokines, are meant to function locally, and systemic delivery could result in unwanted side effects and toxicity. One promising avenue is delivery of chemokines by conventional gene therapy vectors, which has been successfully employed by several investigators in mouse models (Refs 45, 56, 69, 70). An advantage of using chemokines is that, unlike the case for tumour suppressor or 'death genes', it is theoretically not necessary for every tumour cell to be transduced. Thus, if a proportion of tumour cells secrete an angiostatic molecule, this might alter the balance of angiogenic factors in the tumour environment sufficiently to prevent neovascularisation and further cancer growth. This concept is even more apparent for the development of tumour vaccines, which initially requires interaction between only a subset of tumour cells, antigen-presenting cells and T cells.

In the past, a major barrier in tumour immunology has been the lack of immunogenicity of cancer cells, especially of those arising spontaneously in humans (Ref. 51). The ability of tumours to escape the immune system involves both an active and passive process. Tumours actively secrete immunosuppressive molecules that thwart the immune system, and tumour-associated antigens are ordinarily of low immunogenicity (Ref. 51). The discovery and understanding of chemokine function is heralding a new era in cancer immunotherapy because of the ability to overcome both of these obstacles. Chemokines and cytokines (both pro-inflammatory and immunosuppressive) form a regulated network, such that expression of one molecule can dramatically shift the production of others from neighbouring cells. Therefore, it is possible to deliver a chemokine intratumourally and thereby downregulate local production of immunosuppressive molecules and upregulate secretion of pro-inflammatory ones. Particularly promising are the preclinical models that prove that chemokines can convert a poorly immunogenic tumour into an immunogenic one that elicits development of tumour-specific memory T helper cells and cytotoxic T cells.

Another approach being explored with chemokines is the development of drugs of low molecular weight that either mimic the behaviour of chemokines or antagonise their functions. The drugs can be either partial chemokines or small peptides that behave as receptor antagonists or agonists. These agents might have a significant advantage with respect to pharmacokinetics and minimisation of side effects. One such example is the hexapeptide antileukinate, which antagonises

Accession information: (01)00330-1a.pdf (short code: txt001mfh); 18 July 2001
ISSN 1462-3994

binding of ELR+ angiogenic chemokines to their CXCR2 receptor and has been shown to inhibit the growth of tumours effectively in vitro as well as in animal models (Ref. 59). Yet another approach is to combine chemokines with other inflammatory cytokines for a synergistic effect, such that smaller, less-toxic doses can be applied. This has been demonstrated in animals by the combined delivery of IL-12 and lymphotactin (CL1) (Ref. 70)

In summary, chemokines provide a new arsenal of anticancer weapons that can be implemented through a variety of strategies that make use of their anti-angiogenic and pro-inflammatory properties. A number of preclinical models have already been successful in utilising chemokines, and it should not be long before these start surfacing as Phase I trials in the treatment of human cancers.

Research in progress and outstanding research questions

Which receptors mediate chemokine modulation of angiogenesis? Although it is well established that CXC chemokines modulate angiogenesis, the receptors they use to do this and the mechanisms involved are currently being studied (Refs 3, 71). All ELR+ chemokines stimulate endothelial cell migration and are able to bind to the CXCR2 receptor, making this receptor a likely candidate for chemokine-mediated angiogenesis. Indeed, Addison et al. (Ref. 72) have demonstrated that CXCR2 is expressed on human microvascular endothelial cells, and antibodies to the receptor block both chemotaxis and in vivo angiogenesis stimulated by ELR+ chemokines. In addition, CXCR1 can function in IL-8-mediated chemotaxis of human microvascular dermal endothelial cells, as demonstrated by antibody blocking studies (Ref. 73). The receptor CXCR4 is probably responsible for SDF-1-stimulated angiogenesis, as binding by SDF-1 downregulates CXCR4, and results in measurable Ca^{2+} flux accompanied by endothelial cell chemotaxis (Ref. 24).

Little is known regarding the receptors responsible for inhibition of endothelial cell chemotaxis and angiogenesis mediated by ELR– CXC chemokines (Ref. 3). Expression of CXCR3, which binds IP-10, MIG and I-TAC, has been reported on human microvascular dermal endothelial cells and on human umbilical vein endothelial cells (Ref. 73). Nonetheless, it is unclear what role CXCR3 plays in inhibition of endothelial chemotaxis. In fact, human microvascular dermal endothelial cells exhibit a complex response to ELR– chemokines. Very high concentrations of IFN-inducible ELR– chemokines actually stimulate chemotaxis, yet similar concentrations of these chemokines significantly inhibit the chemotactic response to IL-8 (Ref. 73) and other angiogenic factors (Ref. 3). Consequently, the endothelial cell receptor mediating the angiostatic function of ELR– chemokines is an area of active study.

The receptor mediating PF4 inhibition of endothelial cell migration, proliferation and angiogenesis is also unknown (Ref. 3). In fact, the PF4 chemokine receptor mediating chemotaxis of neutrophils and monocytes is also not known (Ref. 71). PF4 can bind to angiogenic factors such as bFGF and the VEGF isoform VEGF165, thereby preventing them from interacting with their specific receptors (Ref. 74). However, PF4 is able to inhibit angiogenesis mediated by another VEGF isoform, VEGF121, without interfering with the ability of this molecule to bind to endothelial cells. There is evidence for a binding site on endothelial cells shared by IP-10 and PF4 that requires cell-surface heparin sulphate proteoglycans (Ref. 27). All CXC chemokines, including IP-10 and PF4, bind heparin sulphate via their basic C-termini. However, an earlier study demonstrated using the chick chorioallantoic membrane assay that an analogue of PF4 that lacks heparin-binding activity is a very effective inhibitor of endothelial cell proliferation in vitro and in vivo (Ref. 75). Therefore, the receptors and mechanisms of ELR– CXC chemokine inhibition of angiogenesis still require future study.

Acknowledgements and funding

We thank Robert Strieter (Department of Pulmonary and Critical Care Medicine, University of California, Los Angeles School of Medicine, USA) for expertly reviewing this paper and his many exceptional suggestions. This work was supported in part by grants from the National Institute of Dental Research 1-P50-DE11906 (93-9), National Institute of Health First Investigator Award R29 DE11689-01A1,Training of the Academic Head and Neck Surgical Oncologist Core Support Grant T32 CA60374-03 (GLC), the Betty Berry Cancer Research Fund, the Michael A. O'Bannon Foundation Cancer Research Fund and the Cancer Center Grant NIH-NCI-CA16672.

Accession information: (01)00330-1a.pdf (short code: txt001mfh); 18 July 2001
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Accession information: (01)00330-1a.pdf (short code: txt001mfh); 18 July 2001
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Further reading, resources and contacts

The Chemokine Family cDNA Database
http://cytokine.medic.kumamoto-u.ac.jp/CFC/CK/Chemokine.html

The Cytokines Online Pathfinder Encyclopaedia
http://www.copewithcytokines.de/cope.cgi

The following reviews provide further information on:

• Chemokine receptors and their ligands

Murphy, P.M. et al. (2000) International union of pharmacology. XXII. Nomenclature for chemokine receptors. Pharmacol Rev 52, 145-176, PubMed ID: 20164997

• Dendritic cell biology and trafficking, and potential use of dendritic cells in cancer therapy

Fong, L. and Engleman, E.G. (2000) Dendritic cells in cancer immunotherapy. Annu Rev Immunol 18, 245-273, PubMed ID: 20297046

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Features associated with this article

Figures

Figure 1. Schematic illustrations of the major secondary structural motifs of the four chemokine subfamilies (fig001mfh).
Figure 2. The balance of angiogenic and angiostatic chemokines in the tumour microenvironment can determine tumour survival (fig002mfh).
Figure 3. Host immune response to tumours can be altered by chemokine production (fig003mfh).

Tables

Table 1. Characteristics of human CXC chemokines (tab001mfh).
Table 2. Characteristics of human CC, C and CXXXC chemokines (tab002mfh).

Citation details for this article

Mitchell J. Frederick and Gary L. Clayman (2001) Chemokines in cancer. Exp. Rev. Mol. Med. 18 July, http://www-ermm.cbcu.cam.ac.uk/01003301h.htm

Accession information: (01)00330-1a.pdf (short code: txt001mfh); 18 July 2001
ISSN 1462-3994