Chemokines and their receptors in the metastatic behaviour of human pancreatic ductal adenocarcinoma.

Thesis

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CHEMOKINES and their RECEPTORS in the METASTATIC BEHAVIOUR of HUMAN PANCREATIC DUCTAL ADENOCARCINOMA

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Abstract

The aim of this thesis was to investigate the role of chemokines and their receptors in human pancreatic adenocarcinoma and to explore whether chemokine receptors and their ligands are involved in tumor dissemination. The repertoire of chemokine receptors expressed in 11 pancreatic adenocarcinoma cell lines tested included CXCR4 and CX3CR1. Their significance was therefore further investigated.

Expression of CXCR4 was higher in lines derived from metastases. The chemokine CXCL12 induced chemotaxis in CXCR4-positive cell lines, which was inhibited by an anti-CXCR4 specific antibody and by the antagonist AMD3100. Trans-endothelial migration, Matrigel invasion and activation of matrix metalloproteases were also enhanced by CXCL12. Proliferation was stimulated by CXCL12 in CXCR4-positive cell lines and partially inhibited by the inhibitor AMD3100, indicating an autocrine loop. The addition of exogenous CXCL12 inhibited apoptosis induced by serum starvation. These data demonstrate that autocrine or paracrine loops centred on the CXCR4/CXCL12 axis promote pancreatic cancer cell migration, matrix degradation and invasion, proliferation and survival.

The function of the chemokine receptor CX3CR1 was investigated in the context of the peculiar propensity of pancreatic cancer to disseminate and grow along nerve fibers, as its chemokine ligand Fractalkine/Neurotactin/CX3CL1 is expressed by neuronal structures. CX3CR1-positive tumour cells migrated in a dose-dependent manner to CX3CL1 and this effect was blocked by specific anti-CX3CR1 antibodies. CX3CR1-positive tumour cells adhered to endothelial and neuronal cells stimulated with TNFα/IFNγ, known to induce Fractalkine expression. Neuronal derived Fractalkine elicited migration of CX3CR1-positive pancreatic tumour cells. The CX3CL1 chemokine was detected in vivo in surgical sections of pancreatic cancer nerve

metastasis. These results suggest that the CX3CR1/Fractalkine axis could be involved in the dissemination of pancreatic tumour cells via nerve structures.

In conclusion, the data presented here support the hypothesis that a selected set of chemokine receptors are expressed in carcinoma of the pancreas and are involved in tumour cell migration and invasion. For CXCR4, promotion of cell survival and proliferation was observed. For CX3CR1, a role in perineural tropism is suggested.

This Thesis is dedicated to my sister

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INTRODUCTION

Chapter 1

Pancreatic Adenocarcinoma

1.1 Pancreatic cancer

Generalities

Human pancreatic cancer is a neoplasia primarily of ductal origin, which represents a major oncological challenge in the 21st century. It ranks as the fourth most frequent type of solid tumor in men (exceeded by lung, colorectal and prostate cancer) and the fifth cause of cancer death in women (exceeded by breast, colorectal, lung and ovarian-uterine cancer). The reasons for this high mortality rate are strictly correlated to the limited knowledge about the biology of this tumor. Despite advances in surgical as well as non-surgical treatment efforts, it remains a tumor with poor prognosis and a 5-year survival rate of 3-8% (1, 2).

Pancreatic cancer often presents clinically, with non-specific signs and symptoms, therefore difficult to diagnose. Known **risk factors** for pancreatic cancer are cigarette smoking, age (over 80% of the cases develop between 60 and 80), chronic inflammation (see **Paragraph 1.2**), diabetes and diet; not surprisingly, it is not associated to consistent environmental or occupational risk factors, as the pancreas is protected from direct contact with the environment and does not play a significant role in detoxification of xenobiotics (as in the liver) nor does it filter and concentrate toxins (as in the urinary system) (3).

According to the TNM system, the **staging** of pancreatic cancer (Stage 0 to Stage IVB) is determined by 3 factors: **T** (location and size of the tumor, ranging from TX to T4), **N** (evidence of metastases in lymph nodes close to the cancer, NX to N1) and **M** (evidence of distant metastases, MX to M1).

Specifically, there are 5 stages of **tumor size** in the current TNM classification:

TX: primary tumor cannot be assessed;

T0: no evidence of primary tumor;

Tis: (carcinoma *in situ*) is very early stage pancreatic cancer;

T1: tumor size in the pancreas is 2cm or less in any direction;

T2: tumor size is more than 2cm across in any direction;

T3: the cancer has started to grow in surrounding tissues around the pancreas, in the duodenum or the bile duct

T4: the cancer has grown further in the stomach, spleen, large bowel or nearby large blood vessels.

While the classification based on lymph node involvement is:

NX: regional lymph nodes cannot be assessed;

N0: no lymph nodes containing cancer

N1: there are cancer cells in a single lymph node (N1a) or more (N1b)

And finally, according to metastasis:

MX: distant metastasis cannot be assessed;

M0: the cancer has not spread into distant organs, such as the liver or lungs

M1: cancer has spread to other organs

Table 1.1 summarizes the characteristics of pancreatic cancer stages in this system.

Stage	Tumor	Lymph nodes	Metastasis
Stage 0	Tis	N0	M0
Stage I	T1	N0	M0
	T2	N0	M0
Stage II	T3	N0	M0
	T3	N0	M0
Stage III	T1	N1	M0
	T2	N1	M0
	T3	N1	M0
Stage IV	T4	Any N	M0
Stage V	Any T	Any N	M1

Table 1.1 Stage grouping for primary tumors of the exocrine pancreas.

Therapeutic treatments

Depending upon the type and stage, pancreatic cancer may be treated with different therapeutic approaches. As reported by the American Cancer Society, **surgery** may be necessary to remove the tumor (a section or entire pancreas and/or the small intestine). The type of surgery depends on the stage of the cancer, the location and size of the tumor, and the person's health. Types of surgery for pancreatic cancer include the **Whipple procedure** (if the tumor is located at the head of the pancreas (the widest part); the head of the pancreas, part of the small intestine, bile duct, and stomach, and other tissues will be removed), the **distal pancreatectomy** (if the tumor is located in the body and tail of the pancreas, both of these sections of the pancreas will be removed, along with the spleen), the **total pancreatectomy** (the entire pancreas, part of the small intestine and stomach, the common bile duct, the spleen, the gallbladder, and some lymph nodes will be removed). Generally, surgery can be pursued only if the cancer is localized; when cancer is found in distant lymph nodes and has spread too far to be cured, **palliative surgery** might be done to prevent or relieve symptoms if the cancer. For example, palliative surgery can be used to relieve blockage of the bile duct (which is cause of jaundice, pain and problems with digestion) (4). For those patients with adenocarcinoma of the pancreas who are not candidates for surgery or who have a recurrence of the cancer after surgical resection, **chemiotherapy** is often prescribed as a standard therapy. 5-Fluorouracil and Gemcitabine have been for used many years as chemotherapy; recently, it has been reported that Raltitrexed-Oxaliplatin regimen may constitute a treatment opportunity in gemcitabine-resistant metastatic pancreatic cancer (5).

Pancreatic cancer biology

The pancreas consists of two separate functional units –exocrine and endocrine pancreas- that regulate the major physiological processes of digestion and glucose metabolism. The **exocrine pancreas** is formed by acinar and duct cells (**Fig 1.1**). The acinar cells represent the bulk of the pancreatic tissue and produce digestive enzymes; they are organized into grape-like clusters, forming the termini of the branching duct system. The ducts add mucus and bicarbonate to the mixture and form a network of increasing size that empty in the duodenum. The **endocrine pancreas** consists of four specialized cell types that are organized into islets, secreting hormones into the bloodstream. The α and β cells regulate the usage of glucose through the production of glucagon and insulin, respectively. Pancreatic polypeptide and somatostatin that are produced in the PP and δ-cells modulate the secretory properties of the other pancreatic cell types.

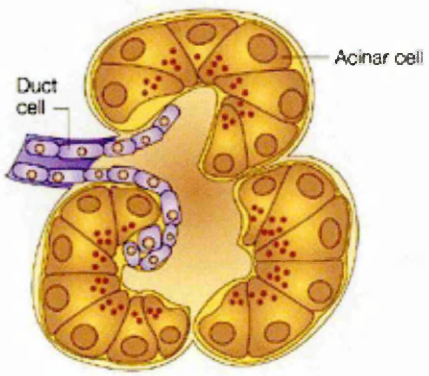


Figure 1.1 Anatomy of the exocrine pancreas. Duct cells are organized into a branching network ending with clusters of acinar cells.

Given the number of cell lineages, the pancreas can sustain several different tumor types, defined by their histological resemblance to the normal counterpart; all of these types show distinct clinical behaviour and genetic profiles (**Table 1.2**). Pancreatic adenocarcinoma -a tumor type with ductal-cell histology- is the most common type of cancer of the pancreas, accounting for greater than 85% of pancreatic neoplasms (6).

Tumor type	Frequency	Histological features
Adenocarcinoma	85%	Ductal morphology
Acinar-cell carcinoma	2%	Zymogen granules
Endocrine tumors	2%	Hormone production
Serous cystadenoma	2%	Ductal morphology, cystic growth

Table 1.2. Types of pancreatic neoplasms. Different tumor types are defined by their histological resemblance to the normal counterpart

A careful molecular and pathological analysis of evolving pancreatic adenocarcinoma has revealed a distinctive pattern of genetic lesions, i.e. a signature molecular profile of this malignancy; it consists of mutations in KRAS, CDKN2A, TP53 and SMAD4/DPC4 (7, 8). Ongoing studies are trying to define the contribution of such lesions to the biological features and evolution of the disease. Pancreatic adenocarcinoma is rarely observed spontaneously or following carcinogen administration in the laboratory mouse but genetic engineering has allowed the generation of strains that harbour germ line oncogenic lesions that are found in human pancreatic adenocarcinoma (6). For instance, transgenic mice expressing activated KRAS (9) or c-Myc (10) in the acini develop acinar carcinomas.

Although molecular pathology and genetic studies have provided an outline of the cellular perturbations associated with pancreatic adenocarcinoma, some key questions need to be answered; in particular what is the **cell of origin** of pancreatic cancer. As mentioned before, pancreatic adenocarcinoma cells strongly resemble pancreatic-duct cells, displaying cuboidal shape, ductal antigen expression and growth in tubular structures. Indeed, there is general agreement that the pancreatic ductal epithelial cell gives rise to this malignancy; recent studies of cell renewal and

differentiation and various rodent models of pancreatic damage have lead to the hypothesis that pancreatic-ductal cells are potential facultative stem cells with the capacity to differentiate into both endocrine and exocrine lineages. Such a proliferating cell with unlimited replicative potential would be a prime candidate for oncogenic mutation and tumorigenesis (6).

Analysis on resected pancreatic tissue from cancer patients have allowed for the compilation of a temporal map of the genetic lesions within the ductal epithelium occurring during the course of tumor progression. It has been shown that pancreatic adenocarcinoma progresses through a series of advancing morphological stages (now designated pancreatic intraepithelial neoplasia, **PanIN**): first, low cuboidal ductal cells become tall columnar due to extensive mucin production. Next, these epithelial cells start to show a certain degree of nuclear atypia and an enhanced proliferation rate which progressively increases and eventually leads to ductal cell shedding into the lumen of the ducts which facilitates tumor metastasis (6, 11). These morphological alterations correlate with increasing genetic abnormalities, among which activating KRas mutations are the earliest and more conserved (**Fig 1.2**).

Recently, the concept of pancreatic adenocarcinoma originating from the ductal epithelial cells has been questioned; there is evidence (the expression of non-ductal lineage marker, including endocrine factors and pancreatic enzymes in pancreatic tumor cells) that transdifferentiation of other pancreatic cell types, such as acinar cells, might serve as an alternative route to pancreatic adenocarcinoma (6, 11).

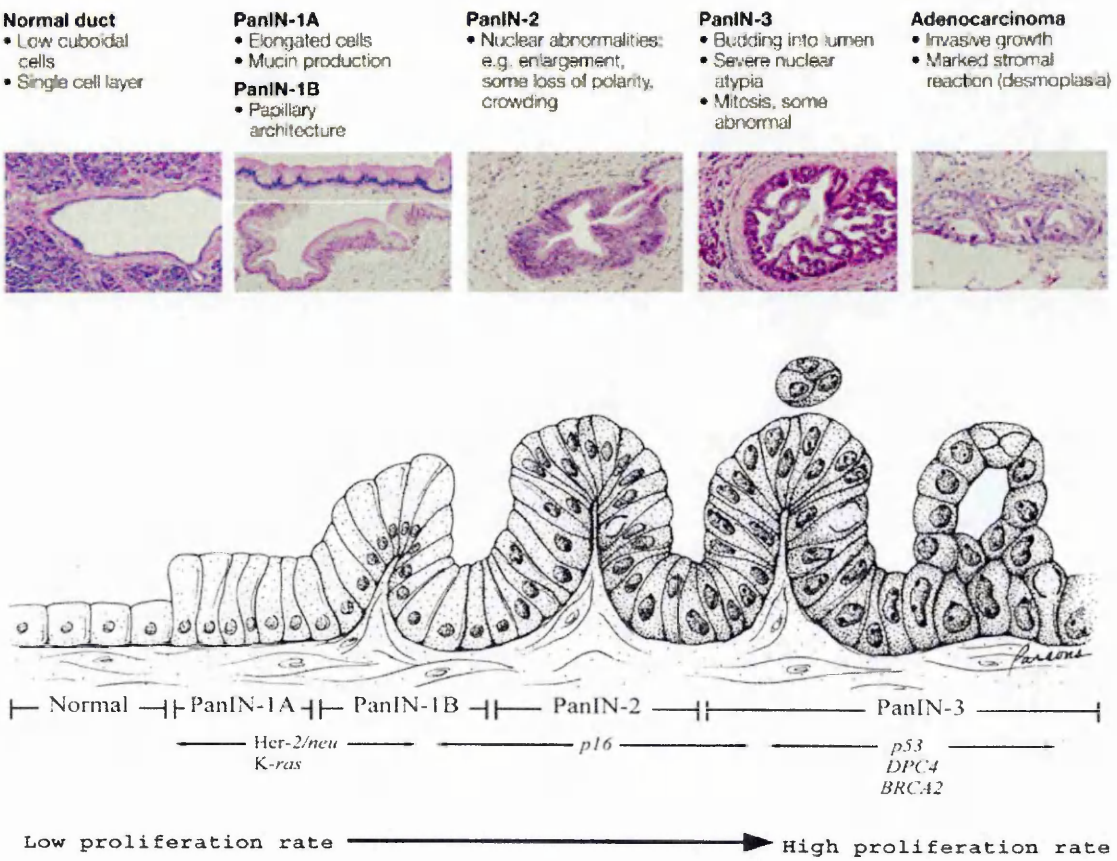


Fig 1.2. Genetic progression model of pancreatic adenocarcinoma. Pancreatic adenocarcinoma progresses through a series of morphological stages, each corresponding to distinct genetic lesions. (*Adapted from Bardeesy et al* (6))

1.2 Pancreatic Cancer and inflammation

Microenvironmental cellular interactions seem to be important in the pathogenesis of pancreatic adenocarcinoma; notably, these tumors show an extensive proliferation of stromal fibroblasts and deposition of extracellular-matrix components (**desmoplasia**) that seem to promote growth and invasiveness (**Fig 1.3**). The molecular basis of this phenotype is not resolved, nor it is clear whether the response is part of the tumorigenic programme or whether it represents a form of host defence against the tumor. This is in line with the general concept that interactions between tumor cells and surrounding stroma play a critical role in tumor growth (12-15). Under normal circumstances, the interaction between normal epithelium and normal stroma helps to

maintain tissue integrity. However, in cancerous tissue, the interaction between cancer cells and surrounding stroma combined with cellular signals such as transforming growth factor β (TGFβ), results in the formation of abnormal stroma, disruption of tissue integrity, and hence invasion and ultimately metastasis. In particular, tumor cells are thought to programme an oncogenic stroma that, in turn, contributes to their sustaining, through paracrine signalling, angiogenesis and protection from immune attack. The importance of tumor-stromal interactions in the aggressive behaviour of pancreatic cancer is supported by experimental evidence that the invasive potential of pancreatic cancer cells can be greatly enhanced by coculture with stromal fibroblasts (16). Moreover, this concept is supported by the observation that the less aggressive mucinous type of pancreatic carcinoma is associated with very little stromal reaction around the tumor (17).

Inflammatory cell mediators

Two cell components of the stroma can have a major role in the carcinogenic process: fibroblasts, and leukocytes. **Fibroblasts**, responsible for the synthesis, deposition and remodelling of much of the extracellular matrix (ECM), are recognized as a source of growth factors that influence the growth of carcinoma cells; most of these factors are predominantly stimulators of proliferation and can play a role in promoting the carcinogenic process (e.g. FGF (fibroblast growth factor), IGF (insulin-like growth factor), EGF (epithelial growth factor), HGF (hepatocyte growth factor)). Indeed, several studies have shown that fibroblasts, upon modulation by cancer-derived factors and given the appropriate environment, can differentiate in myofibroblasts, which can in turn produce pro-invasive signals (13).

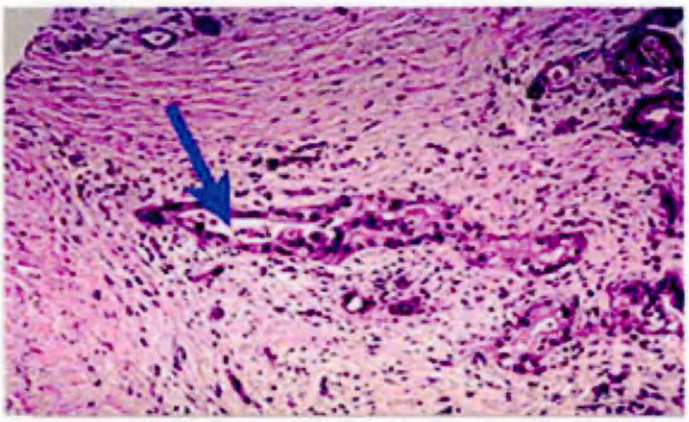


Fig 1.3. A cancerous duct. The arrow indicates the carcinoma (Haematoxylin and Eosin × 100).

The second important player in the stroma-cancer cell crosstalk is represented by the inflammatory component, i.e. **leukocytes**. In fact, although it has long been known that cancer frequently arises in areas of chronic inflammation, only in the last few years has a functional relationship between inflammation and cancer been established (18); examples include colon carcinoma, associated with inflammatory bowel disease (19) and stomach cancer, following *Helicobacter pylori* infection (20). Inflammatory cells influence cancer initiation and promotion by secreting cytokines, growth factors and chemokines, which stimulate proliferation of epithelia and generate reactive oxygen species that can cause DNA damage (18). Among different leukocyte cell types, macrophages are the most abundant and an important component of the stroma of neoplastic tissues. Several lines of evidence demonstrate that tumor-associated macrophages (TAM) can have dual functions in their interactions with neoplastic cells and are a key component of inflammatory circuits which promote tumor growth and progression (18, 21).

Chronic pancreatitis and pancreatic cancer

Given the link between inflammation and cancer (18, 21), it seems reasonable to investigate whether a connection between chronic pancreatitis and the development of pancreatic adenocarcinoma does exist. **Chronic pancreatitis**, the most prevalent disorder of the exocrine pancreas, is an inflammatory disease associated with a gradual damage of the organ (11). In patients with hereditary pancreatitis (a hereditary form of the disease, accounting for <1% of all forms of pancreatitis and caused by a mutation of the trypsinogen gene on chromosome 7), the risk of developing pancreatic cancer is 53 times the risk in unaffected individuals; moreover, epidemiological data suggest an increased risk of pancreatic cancer in patients with sporadic chronic pancreatitis that correlates with the duration of inflammation (22). However, despite the deleterious effect of chronic inflammation on tissue integrity, only a small percentage of pancreatic cancers are due to chronic pancreatitis.

The pathophysiology of pancreatitis involves the aberrant release of proteolytic enzymes that cause acinar cell injury and a subsequent inflammatory response which in most cases is associated with gradual resolution. In chronic pancreatitis, the acinar injury is believed to recur, causing persistent infiltration of inflammatory cells, eventually leading to atrophy and fibrosis. Chronic inflammation occurring simultaneously with cell proliferation would be an ideal landscape for malignancy to develop.

Cytokines: new molecular mediators in pancreatic adenocarcinoma

Also for pancreatic adenocarcinoma, the inflammatory mechanisms responsible for the development of the neoplasia rely on the release of several cytokines, transcription factors and inflammatory enzymes. Indeed, a number of cytokines are found increased in pancreatic cancer (e.g. TNFα, IL-1β, IL-6, IL-8, CCL2) (22-27).

Each cytokine can evoke a cascade of events in inflammatory cells, including the synthesis and release of other cytokines and growth factors and affect the host response to tumor cells. Moreover, some cytokines can provide positive signals for pancreatic cancer cell growth, including EGF, IGF, IL-1α, which originate in peri-tumoral inflammatory cells, but may also be produced by the pancreatic cancer itself, thus exerting an autocrine action (28, 29). TNFα has been proposed to increase survival of pancreatic cancer cells (30). In addition, a peculiar aspect of pancreatic cancer cells is their loss of responsiveness to growth inhibitory cytokines, including TGFβ (28). This growth factor deserves attention, as it plays a dual role in cancer microenvironment; in fact, while promoting tumor growth through stromal and angiogenesis support and impairing immune surveillance, TGFβ can also act as a growth inhibitor of most epithelial cells. However, mutations in the TGFβ signalling pathway downstream of the receptor may impair TGFβ mediated growth inhibition, while retaining other pathway components, including the ones favouring tumor invasion and metastasis (14, 31). Thus, loss of responsiveness to TGFβ as observed in pancreatic cancer, is associated to acquisition of a tumoral phenotype (28).

As for other carcinomas, also for pancreatic cancer a connection between inflammation and cancer progression is the transcription factor NFkB, which regulates the transcription of several inflammatory genes as well as cell cycle. Not surprisingly, NFkB is found constitutively activated in a high percentage of pancreatic adenocarcinomas, inhibiting apoptosis in these cells and cytokines, such as TNFα, may favour development and growth of malignant cells through NFkB activation (30). Also IL-8 expression is induced in vitro through NFkB activation; this chemokine, constitutively expressed at high levels in pancreatic cancer cells, produces an autocrine growth stimulatory effect in certain cell lines (32) and inhibition of its activity in

transplanted pancreatic tumor cells leads to suppression of tumor growth and metastasis in nude mice (33).

Although leukocytes are the main source of inflammatory mediators, still also pancreatic tumour cells can produce a number of cytokines and growth factors. In vitro established pancreatic cancer cell lines represent a valuable tool for the characterization of inflammatory mediators. The first part of results of my thesis will be focused on the characterization of pancreatic cancer cell lines, with attention to the release of inflammatory mediators (See Chapter 4).

1.3 Invasion and metastasis in pancreatic cancer

The devastating evolution of pancreatic cancer is not only due to the high proliferating potential of pancreatic cancer cells, but also to the ability of these cells to metastasize even when the primary tumor spread is limited. Metastasis is the main cause of treatment failure and death for pancreatic cancer patients. This is due to the difficulty of removing metastasis through conventional surgery; indeed, often in pancreatic adenocarcinoma metastases involve vital organs, (including liver, celiac plexus, some lymph nodes, depending on their location and large blood vessels, such as the portal vein), therefore excluding surgery as a treatment option. From these considerations it is evident that controlling metastasis is a major goal to improve the life expectancy of pancreatic cancer patients.

Recently, microarray expression profiling of metastatic cells and in vivo video microscopy are providing new advancement in the characterization of metastasis (34-36). However, despite its importance in the aggressiveness of pancreatic cancer, how metastatic progression develops is still unknown and very little knowledge is available about the molecular mechanisms regulating this process.

A multistep process

The metastatic process consists of a series of steps all of which must be successfully completed to give rise to a metastatic tumor. As a primary tumor grows, it needs to develop a blood supply –the **angiogenesis** process- that can support its metabolic needs and can also provide tumor cells an escape route to leave the primary mass and enter the circulation (process known as **intravasation**). The cells need to survive in the circulation until they can arrest in a new organ; here they might **extravasate** from the circulation into the surrounding tissue. Once in the new site, cells must initiate and maintain growth to form pre-angiogenic micrometastases; this growth must be sustained by the development of new blood vessels in order for a macroscopic tumor to form (37). The same steps are supposed to take place in pancreatic cancer metastasis formation.

The capability of sustained **angiogenesis** is a feature of cancer which dictates malignancy. The formation of new blood vessels is permissive for local and systemic expansion of the tumor mass and can be induced by multiple molecules, released by both cancer and stromal cells. Numerous factors controlling the formation of tumor vascularisation have been found overexpressed in pancreatic cancer, including vascular endothelial growth factor (VEGF), IL-8 and members of the TGF and FGF family, as already said. Tumor cells produce and secrete these molecules, which bind receptors expressed on endothelial cells, thus guiding the process of new vessel formation. In particular, as far as VEGF is concerned, pancreatic cancer cells express also the cognate receptors VEGFR-I and VEGFR-II and the ligand/receptor system consisting of VEGF and VEGF-RII has been proposed to be of biologic significance in the pathogenesis of pancreatic cancer growth (38, 39).

Sooner or later during the development of pancreatic cancer, some pioneer cells detach from the primary mass and move out, invade adjacent tissues and circulate to

distant organs, where they may form new colonies. Migration and invasion of cancer cells into surrounding stroma are likely to be prevented by cell-cell and cell-matrix interactions, thus disruption of these adhesive connections leads to increase motility of tumor cells, which detach from the primary lesion. Therefore, adhesion molecules on the cell surface play an important role in tumor cell migration and regulate the potential for epithelial cells to metastasize, being adhesion molecule interactions also able to convey regulatory signals to the cell (40). Several classes of proteins are participating when cells exhibit an invasive or metastatic phenotype, including cell-cell adhesion molecules (**CAMs**), **cadherins** and **integrins**. E-cadherin function is lost in a majority of pancreatic tumors (41); generally, the presence of E-cadherin is considered as an important suppressor of invasion and metastasis and its functional elimination may represent a key step in the acquisition of this phenotype. ICAM-1 and VCAM-1 expression has been found increased in pancreatic cancer samples in comparison with normal tissues; they likely contribute to cancer cell migration and spread to distant organs (42). Finally, changes in integrin expression are also evident in invasive and metastatic pancreatic cancer cells (43).

The role of Lymphangiogenesis in cance metastasis

Like in normal tissues, both vascular and lymphatic vessels are present in malignant tissues. Lymphoangiogenesis, namely the proliferation of new lymphatic vessels, is an important initial step in tumour metastasis. Tumour cells spread via the lymphatic system to regional lymph nodes and finally into larger lymphatic vessels, wich re-enter into the blood vascular system; therefore, similarly to angiogenesis, lymphoangiogenesis provides new vessels that malignant cells can use to escape the confines of the primary tumour (44). For several years, the study of lymphatic vessels has been difficult, most likely due to a lack of appropriate molecular markers that could

be used to discriminate between lymphatics and blood vessels. The discovery of lymphatic vessel cell-surface markers, such as VEGFR3, podoplanin, LYVE-1 and CD34 has allowed the purification and study of these vessels (45). Lymphoangiogenesis is controlled in part by members of the vascular endothelial growth factor (VEGF) family, mostly VEGF-C and VEGF-D and their cognate receptors on lymphatic endothelium, VEGFR-3 (44). In addition to the two VEGF family members, fibroblast growth factor 2 (FGF2), platelet derived growth factor B and hepatocyte growth factor (HGF) stimulate lymphatic vessel growth (45). Growth factor stimulation of lymphatic vessels enhances lymphatic metastasis. Several studies have found positive correlations between VEGF-C and VEGF-D expression and vascular invasion, lymphatic vessel and lymph node involvement and distant metastasis; VEGF-C expression in tumour cells may be induced by growth factors or proinflammatory cytokines, and some may be derived by inflammatory cells in tumours. Lymphatic vessels in pancreatic cancer have not been studied well, however, lymphoangiogenesis appears important in metastasis of pancreatic cancer (40). Morphological studies clearly indicate alterations of lymphatic vessels in the periphery of the tumours, which can be due to overexpression of VEGF-C, as excess of VEGF-C causes lymphatics to enarge. VEGF-C expression has been associated with increased lymphatic vessel invasion and lymph node metastasis, but not with decreased patient survival in pancreatic cancer (46).

The role of extracellular proteases

The second class of players in transforming pancreatic cancer into an invasive and metastatic phenotype are **extracellular proteases**; in the 1970s it was first recognized that tumor-derived proteases, e.g. plasminogen activators and metalloproteinases, play an important role in the cleavage of basement membranes and extracellular matrix molecules and thus assist in the processes of invasion and metastasis of cancer cells (47). Later, it became accepted that also non-tumoral stromal cells may contribute to this process (48). Proteases can also play a role in the early phases of tumor development through their ability to proteolitically activate several molecules, releasing them from the matrix; examples include VEGF and TGFβ (49-53).

The MMP family can be broadly subdivided into five groups: collagenases, stromelysins, elastases, membrane-type (MT) MMPs and the gelatinases. Although the classification system was developed on the basis of substrate specificity, it is now recognized that there is some overlap between some members of the family (e.g. MMP-2 can cleave fibrillar collagen similar to the collagenases) (**Table 1.3**). The multigene family of metal containing proteases shares several common characteristics: (a) each degrade at least one component of the basement membrane; (b) their catalytic activity depends on the presence of zinc ions at the catalytic active site; (c) they are inhibited by metal chelators and tissue inhibitors known as tissue inhibitors of metalloproteinases (TIMPs); and (d) they are secreted as zymogens and require activation extracellularly, usually accompanied by loss of a 10-kDa amino-terminal domain (54-56). The **collagenases** catalyze degradation of fibrillar forms of collagen (i.e. type I, II, III). The **stromelysins** have relatively broad substrate specificity, catalyzing degradation of many different substrates in the ECM, including proteoglycans (core protein), laminin, fibronectin. The group of **membrane-type MMPs** possess a transmembrane domain and catalyze activation of progelatinase A and degrade a variety of ECM substrates. The

cytokine-induced **Gelatinase B** (MMP-9) and the constitutively expressed **Gelatinase A** (MMP-2) are also known as type IV collagenases and degrade gelatin (denatured collagen) and type IV, V, VII, IX, X collagen. Type IV collagen is particularly abundant in basement membranes, which are the membranes separating organ parenchyma from the underlying stroma. Four endogenous specific inhibitors of MMPs (tissue inhibitor of metalloproteases, **TIMP**) have also been described: TIMP-1 to TIMP-4; they inhibit protease activity by forming high-affinity complexes with the active MMPs.

Given the important role played by MMPs in cancer progression, there has been great interest in the development of MMP inhibitors (MMPIs) (57-59); as metalloproteases are also involved in the migration of endothelial cells during angiogenesis, MMPIs can affect metastasis both by acting directly on tumor cell invasion, but also by inhibiting angiogenesis.

Preclinical studies testing the efficacy of MMP suppression in tumor models were very exciting and synthetic metalloproteinase inhibitors (MPIs) rapidly developed; however, the results of some of these trials have been disappointing and the clinical development of MPIs had to overcome unforeseen problems (60). Batimastat (BB-94), a broad spectrum inhibitor, became the first MMPI to be tested in humans (61); it was then replaced by Marimastat (BB-2516), another peptido-mimetic MMPI orally available (62-64). Despite some limitations, Marimastat has proved to be as effective as conventional therapy (gemcitabine) in treatment of pancreatic carcinoma patients (65).

MMP	ECM substrate
MMP-1 (Interstitial Collagenase) MMP-8 (Neutrophil Collagenase) MMP-13 (Collagenase-3) MMP-18	Fibrillar collagens
MMP-3 (Stromelysin-1) MMP-10 (Stromelysin) MMP-11 (Stromelysin-3) MMP-7 (Matrilysin)	Laminin, fibronectin, non-fibrillar collagens
Metalloelastase	Elastin
MMP-14 (MT1-MMP) MMP-15 (MT2-MMP) MMP-16 (MT3-MMP) MMP-17 (MT4-MMP)	Gelatinase A, fibrillar collagens, proteoglycans, ECM glycoproteins Gelatinase A Gelatinase A
MMP-2 (Gelatinase A) MMP-9 (Gelatinase B)	Type I, IV, V and fibrillar collagens, gelatin Type IV, V collagen, gelatin
MMP-19	Gelatin

Table 1.3 The Matrix Metalloprotease Family. The original classification system was developed on the basis of substrate specificity.

Regulation of the MMPs occurs at three levels: alteration of **gene expression** (one of the most potent family of inducers are phorbol esters, such as PMA, as well as inflammatory cytokines, IL1, IL-8, TNFα), **activation of zymogens** (by agents such as trypsin 2, cathepsins, elastase) and inhibition of **tissue inhibitors** of metalloproteases. Alteration of all three levels of control have been associated with tumor cell progression. There is often a balancing effect within the corresponding physiological inhibitors of MMPs, to create the environment necessary for either physiological or pathological processes.

The main function of MMPs is degradation of the extracellular matrix, a mechanism required for many physiologic functions, including angiogenesis, wound healing, bone resorption and mammary involution, as well for pathological conditions, including cancer. Indeed, in either case the rate-limiting step is the breakdown of

connective tissue barriers. The difference between them is that physiological invasion is regulated, whereas tumorigenic invasion appears to escape any limiting control. Correlating with this observation, MMPs are invariably upregulated in the stromal compartment of invasive epithelia; for instance, MMP-2 expression is increased in several tumors and strongly correlates with nodal status and tumor stage (66, 67). MMP9 derived from hematopoietic cells of host origin, has been shown to contribute to skin carcinogenesis (68). In addition, MMP9 has complex effects beyond matrix degradation, including promotion of angiogenesis and release of growth factors (69, 70). Several studies in pancreatic cancer indicate that proteases are upregulated (in particular MMP-2 and MMP-9), protease inhibitors are downregulated (TIMP1 is reduced in tumors with lymph node metastasis) and inactive zymogen forms of proteases are converted into active enzymes (71). Moreover, the plasminogen activator/plasmin system has been implicated in tumor invasion and metastasis; tissue type plasminogen activator (**t-PA**) and urokinase type plasminigen activator (**u-PA**) and their respective receptors, annexin II and u-PAR, have been demonstrated to contribute to the invasive behaviour of pancreatic cancer (72)). There is also evidence that certain pancreatic cancer cells induce uPA expression in stromal cells, which then bind to the urokinase receptor (uPAR) expressed on the cancer cells (73). This likely enables cancer cells to migrate through tissue barriers.

Organ selective spreading

In common with many other cancers, pancreatic adenocarcinoma also displays preferential organ metastatization; **locoregional lymph nodes**, **liver**, especially with tumors in the tail and the body of the pancreas, the **celiac plexus** (a network of many nerves grouped around the aorta, causing back pain when pressed upon by a growing tumor), **superior mesenteric vessels**, closely associated to the pancreas and **portal**

vein. The location of the metastases determines whether the cancer can be surgically removed or not, therefore having an important impact on the outcome of the disease.

One of the principal causes of metastasis in pancreatic adenocarcinoma is the high frequency of local recurrence; indeed, although surgery has been shown to be an effective therapeutic approach, the tendency to recurrence usually leads to the death of the patient within 1 or 2 years after surgery. It has been proposed that pancreatic cancer may have a multifocal origin and that tumors left in the remaining pancreas after a Whipple operation may be the source of local recurrence, but this hypothesis has not found support, because total pancreatectomy does not change the outcome (74). Microscopic tumor metastases already existing in the liver at the time of surgery are a possible source of recurrent disease, but it is unlikely that they are the explanation for tumor recurrence in the retroperitoneum (75).

A possible source of recurrence may be represented by neural invasion. Detailed pathohistologic studies of a large series of resected pancreatic ductal adenocarcinoma have shown that one of the most persistent characteristics is **perineural invasion** (76-78). Although perineural invasion also occurs in other types of cancer, this phenomenon is most consistently observed in pancreatic cancer. This seems to be due to anatomical reasons; indeed, the pancreas hosts a large amount of neural tissue, including ganglia, and it is in close proximity to abundant neural plexi and ganglia in the retroperitoneum. This observation is highlighted in the hamster pancreatic cancer model, where perineural invasion can be found in virtually all cases (79, 80).

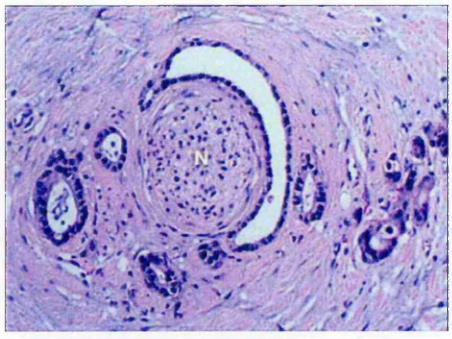


Fig 1.4 Pancreatic cancer perineural invasion. Hematoxylin Eosin staining of a surgical section of pancreatic cancer adenocarcinoma with tumour cells infiltrating an intrapancreatic nerve (magnification x100). N indicates the nerve.

Pour et al support the idea that all pancreatic cancers show perineural invasion if several sections have been scrutinized histologically. This observation and other existing data strongly suggest that tumor cells residing in the celiac and superior mesenteric ganglia indeed represent the main source of pancreatic cancer recurrence (74).

Novel concepts on metastasis: proposed role for chemokines and their receptors

For many years, researchers have tried to give an explanation for the phenomenon of organ-specific metastasis. In particular, the observation that many types of cancer display organ-specific pattern of metastasis but these organs sometimes do not correspond to those found in the drainage site has prompted to hypothesize the existence of organ-specific attractant molecules. These mediators would be responsible for tumor cell chemoattraction, stimulating the migrating tumor cells to invade the walls of blood vessels and finally enter the organs.

Chemokines, small cytokines endowed with chemotactic activity, through the interaction with specific receptors, have been found to perfectly fit with this theory; indeed, recent studies have shown that tumor cells express patterns of chemokine receptors that match chemokines specifically expressed in organs to which these cancers commonly metastasize (81-83).

In this thesis I have investigated the expression of chemokine receptors in human pancreatic adenocarcinoma to test the hypothesis that chemokine receptors could play a role in pancreatic cancer dissemination.

Chapter 2

Chemokines and their Receptors

2.1 The chemokine family

Structure and nomenclature

Chemokines are chemotactic cytokines that activate specific receptors expressed on cellular membranes. Chemokine reeptors sense a chemical gradient and mobilize cells in the gradient direction. The principal targets of chemokines are bone marrow-derived cells and, as motility is an essential part of their function, chemokines play pivotal roles in coordinating leukocyte navigation. However, chemokines are not only simple chemotactic factors. These proteins can regulate several cellular activities, including maintainance of homeostasis, angiogenesis/angiostasis, cellular differentiation and activation, wound healing, tumor growth and metastasis, lymphocyte homing and development of lymphoid tissue, and regulation of the immune response. All cell types, including structure cells, have the potential to actively participate in chemokine production.

The chemokine family (**Table 2.1**) comprises about 50 members; most are low molecular weight molecules (8-10 kDa), all consisting of roughly 70-130 amino acids, with four conserved cysteines (84-88). Two main subfamilies, CXC (α) and CC (β) chemokines are distinguished, according to the position of the first two cysteines, which are separated by one amino acid (CXC) or adjacent (CC). The cysteines form two disulfide bonds which confer to the chemokines their characteristic three-dimensional folding, with a flexible N-terminal loop connected to the more structured core of the molecule (3 β-sheets) and a terminal α-helix. The CXC chemokine family can be further subdivided according to the presence or absence of a conserved tripeptide motif glutamic acid-leucine-arginine (ELR) at the N-terminus of the protein, before the CXC domain; this motif is not simply structural but seems to be linked to function, giving specificity for neutrophil chemotaxis and angiogenesis. Two variants of the chemokine

structure paradigm have been described: Lymphotactin-α (XCL1) and lymphotactin-β (XCL2), the two members of the C (γ) family, have two instead of the ususal four conserved cysteines and Fractalkine (CX3CL1), also called Neurotactin, has three amino acids between the first two cysteines, giving rise to the one-member CX3C (δ) subfamily. A systematic nomenclature for chemokines became necessary as more and more new molecules were found. This classification relies on the principle established for their receptors, defined as CXC, CC, XC and CX3C followed by R and a number; thus chemokines are defined by the same structure-related acronyms followed by L (for ligand) and the number of their gene (designated long ago as SCY -small secreted cytokines- and numbered chronologically) (87, 89).

There are also differences in the genomic organisation of the chemokine families. Many of the genes for the CXC chemokines, which act on neutrophils are located on chromosome 4, while a majority of the genes for the CC chemokines, which act on monocytes are clustered on chromosome 17 (89). These genes may have arisen by duplication and divergence from primordial chemokine genes but remained in clusters, supporting the idea that their functions are to some extent related. The remaining chemokines are in new chromosomal locations away from these two main clusters. These genes are more conserved between species and have highly specific functions, suggesting that they are older in evolutionary terms (89).

Like cytokines, chemokines are secreted proteins; only two of them, CXCL16 and CX3CL1 are synthetized with a typical transmembrane sequence, which anchores them to the cell membrane, and the chemokine domain suspended by a mucin-like stalk.

Chemokine Ligands	Other Names	Chemokine Receptors
CXC Subfamily		
CXCL1	GROα/MGSA-α	CXCR2>CXCR1
CXCL2	GROβ/MGSA-β	CXCR2
CXCL3	GROγ/MGSA-γ	CXCR2
CXCL4	PF4	CXCR3B
CXCL5	ENA-78	CXCR2
CXCL6	GCP-2	CXCR1, CXCR2
CXCL7	NAP-2	CXCR2
CXCL8	IL-8	CXCR1, CXCR2
CXCL9	Mig	CXCR3A
CXCL10	IP-10	CXCR3A
CXCL11	I-TAC	CXCR3A
CXCL12	SDF1 α/β	CXCR4/ CXCR7
CXCL13	BCA-1	CXCR5
CXCL14	BRAK/bolekine	Unknown
CXCL15	Murine lungkine	Unknown
CXCL16	Bonzo ligand	CXCR6
C Subfamily		
XCL1	Lymphotactin/SCM-1α/ATAC	XCR1
XCL2	SCM-1β	XCR1
CX3C Subfamily		
CX3CL1	Fractalkine	CX3CR1
CC Subfamily		
CCL1	I-309	CCR8
CCL2	MCP-1/MCAF/TDCF	CCR2
CCL3	MIP-1α/LD78α	CCR1, CCR5
CCL4	MIP-1β	CCR5, CCR8
CCL5	RANTES	CCR1, CCR3, CCR5
CCL6	C10	CCR1
CCL7	MCP-3	CCR1, CCR2, CCR3, CCR5
CCL8	MCP-2	CCR1, CCR2, CCR3, CCR5
CCL9/10	MIP-1γ	CCR1
CCL11	Eotaxin	CCR3
CCL12	Murine MCP-5	CCR5
CCL13	MCP-4	CCR1, CCR2, CCR3
CCL14	HCC-1	CCR1, CCR5
CCL15	HCC-2/Lkn-1/MIP-1δ	CCR1, CCR3
CCL16	HCC-4/LEC/LCC-1	CCR1
CCL17	TARC	CCR4, CCR8
CCL18	DC-CK1/PARC/AMAC-1	Unknown
CCL19	MIP-3β/ELC/exodus-3	CCR7
CCL20	MIP-3α/LARC/exodus-1	CCR6
CCL21	6Ckine/SLC/exodus-2	CCR7
CCL22	MDC/STCP-1	CCR4
CCL23	MPIF-1/CKβ8-1	CCR1
CCL24	Eotaxin-2/MPIF-2	CCR3
CCL25	TECK	CCR9
CCL26	Eotaxin-3	CCR3
CCL27	CTACK/ILC	CCR10
CCL28	MEC	CCR10

Table 2.1. The chemokine family (*adapted from Allavena et al Crit Cancer Treatment Review 2005*)

Functional classification

Beside structure, chemokines can also be grouped according to their function; in fact, they can be produced by leukocytes and tissue cells either constitutively or after induction, thus a former classification grouped chemokines into the functional subfamilies termed "inflammatory" and "homeostatic". Thus, **homeostatic chemokines** usually guide the trafficking of leukocytes under steady state conditions, during immune surveillance of healthy peripheral tissues and control the architecture of secondary lymphoid organs; by contrast, **inflammatory chemokines** are produced and control the recruitment of effector leukocytes under conditions of inflammation, immune reactions, tissue injury and tumors. In particular, tumors are characterized by the constitutive expression of inducible chemokines (90); examples include CCL2 production in breast and pancreatic cancer, melanoma, sarcomas and lung tumors (91)(**Fig. 2.1**).

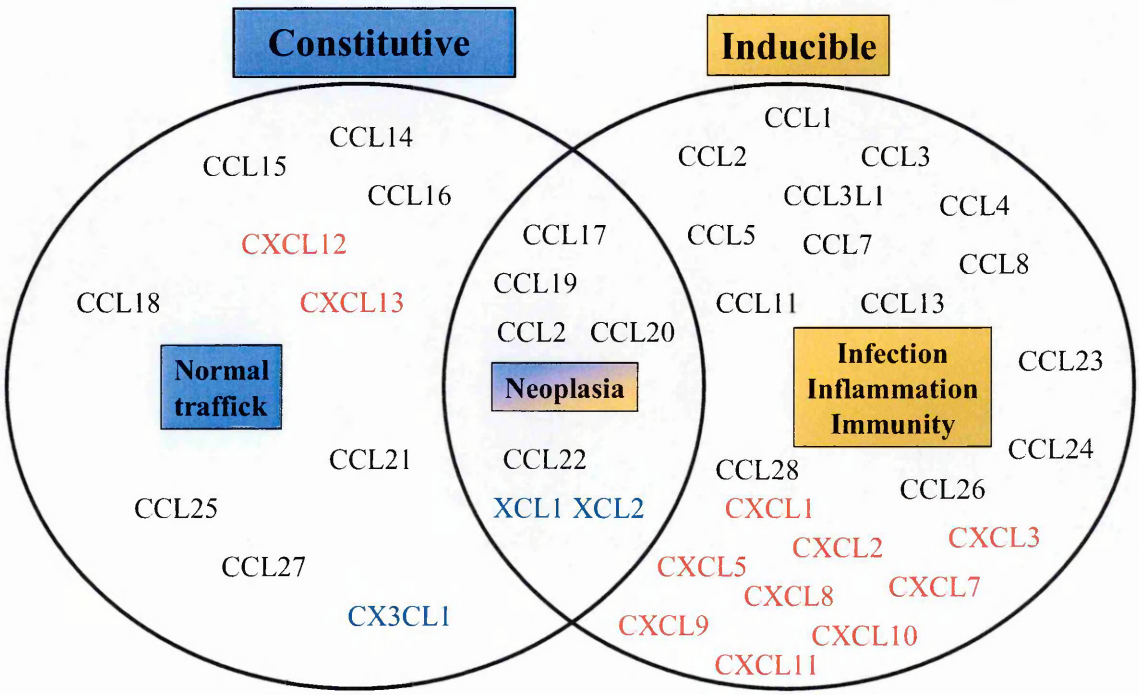


Fig. 2.1 Chemokine functional classification. Tumors are characterized by the constitutive expression of inducible chemokines (adapted from (90)).

Many chemokines have broad target cell selectivity. In particular, expression of inducible chemokines can be elicited by almost any stimulus that alters cellular homeostasis; they can be thought of as vertebrate cellular "SOS response" that recruits leukocytes to areas of tissue injury. Recent findings, however, indicate that several chemokines cannot be assigned unambiguously to either one of the two functional categories and therefore are referred to as "**dual function**" chemokines (92). Dual function chemokines participate in immune defense functions (i.e. are upregulated under inflammatory conditions, examples include the interferon-inducible chemokines CXCL9, CXCL10, CXCL11) and also target non-effector leukocytes, including precursors and resting mature leukocytes, at sites of leukocyte development and immune surveillance (for instance CCL1, CCL25, CXCL16) (92). Many dual function chemokines are highly selective for lymphocytes and have a role in T-cell development in the thymus, as well as in T-cell recruitment to inflammatory sites. Remarkably, dual-function and homeostatic chemokines usually bind to a single receptor, expressed mainly on lymphoid cells, in contrast to inflammatory chemokines, which bind to multiple receptors (see below).

Chemokine and receptor specificity

Chemokines exert their biological activity on leukocytes by binding to seven transmembrane domain, G-protein coupled receptors (see below). The principal target of chemokines are leukocytes and these small proteins tightly regulate their traffick, such as during their recruitment into sites of inflammation or positioning in secondary lymphoid organs (87, 89). In general, different chemokine classes tend to exhibit different ranges of leukocyte specificity; schematically, CXC-ELR+ chemokines (ELR is a highly conserved amino acid motif: Glu-Leu-Arg) are the major chemoattractants for neutrophils, whereas CXC-ELR- chemokines attract lymphocytes and monocytes

but have little or no action on neutrophils. Chemokines belonging to the CC family act primarily on monocytes, but they can also attract lymphocytes, NK cells, basophils and eosinophils. CX3C and C chemokines act on lymphoid cells (T cells and NK cells) and Fractalkine is also active on monocytes. Despite this specificity, **redundancy** in the action on target cells is an intriguing feature of chemokines: no chemokine is uniquely active on one leukocyte population and usually a given leukocyte population has receptors for and responds to different chemokines. This confers robustness to the system, as variations in the amount or quality of any chemokine or receptor would have bearable consequences for basal trafficking of leukocytes (85, 93, 94). Moreover, the interaction of chemokines with their receptors is characterized by considerable **promiscuity**. Most ligands interact with more than one receptor and most known receptors have been reported to interact with multiple ligands; only CXCR4, CXCR5, CXCR6, CCR6, CCR9 and CX3CR1 bind to only one chemokine. Probably all cell types can produce chemokines under appropriate conditions and usually, a cell produces many chemokines concomitantly in response to the same stimulus (**polyspeirism**) Once again, this receptor promiscuity and polyspeirism contribute to the robustness of the chemokine network, essential features which act to retain the chemokine system function, even if genetic or epigenetic alterations affecting individual components occur.

Chemokine Receptors

Structure and nomenclature

Chemokines induce cell migration and activation by binding to specific G-protein-coupled cell surface receptors on target cells. Ten CC (CCR1-10), seven CXC (CXCR1-7, the last is a very recent acquisition), one CX3C (CX3CR1) and one XCR (CXR1) receptors have been identified (86, 89). Receptor expression is a crucial determinant of the spectrum of action of chemokines. The profile of chemokine-receptor expression on an individual cell is determined by its lineage, stage of differentiation and microenvironmental factors, such as chemokine concentration, the presence of inflammatory cytokines, low oxygen tension (hypoxia). Indeed, some receptors are restricted to certain cells (e.g. CXCR1 is predominantly restricted to neutrophils), whereas others are more widely expressed (e.g. CCR2 is expressed on monocytes, T cells, natural killer cells, dendritic cells, and basophils). Moreover, chemokine receptors can be constitutively expressed on some cells, whereas inducible in others (e.g CCR1 and CCR2 are constitutively expressed on monocytes, but are expressed on lymphocytes only after IL-2 stimulation); again, the expression of some chemokine receptors can be restricted to a cell state of activation and differentiation (e.g. CXCR3 is expressed on activated Th1 T lymphocytes, whereas CCR3 is preferentially expressed on Th2 lymphocytes) (95). Although initially studied on leukocytes, some chemokine receptors are also expressed in nonhematopoietic cells, including neurons, astrocytes, epithelial and endothelial cells. These observations suggest that the chemokine system has other roles in addition to leukocyte chemotaxis.

As already pointed out for chemokines, the remarkable feature of the chemokine receptor family is their promiscuity as far as ligand binding is concerned; however, although relatively few receptors bind only one ligand, CC receptors bind only CC

chemokines and CXC receptors bind only CXC chemokines. This ligand-receptor restriction may be related to structural differences between CC and CXC chemokines, which have similar primary, secondary and tertiary structures but different quaternary structures (96).

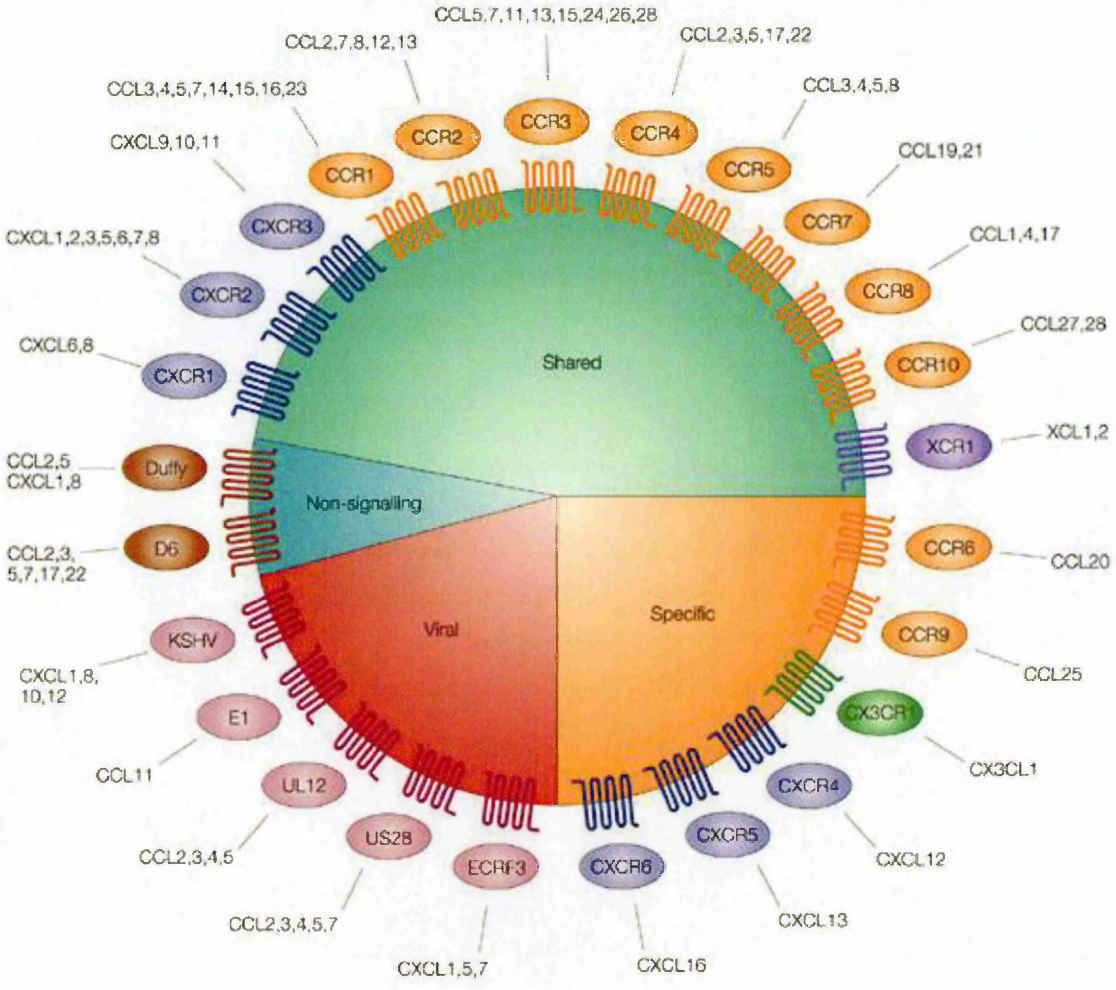


Fig. 2.2. Chemokine Receptor family. Ligand specificity is represented, with many receptors binding more than one single ligand.

Chemokine receptor signaling

The first studies on **chemokine signaling** showed that functional responses were prevented by pre-treatment of the cells with *Bordetella pertussis* toxin, indicating that the receptor was coupled to GTP-binding proteins of the G_i type (97), which eventually turned out to be the rule for all the chemokine receptors. Although the details of the chemokine signalling pathways appear to vary slightly depending on the cellular context, the general rule is that, on chemokine binding, the G protein activated by GTP dissociates in α and Bγ subunits, the latter activating two major signal transduction enzymes **PLC** (both the isoenzymes β2 and β3 (98) and **PI3Kγ**. PLC cleaves PIP2 yielding to two second-messengers: IP3, (which induces the release of calcium ions from intracellular stores, leading to a transient rise of the free calcium concentrations) and DAG (which activates several isoforms of PKC). While the rise in the concentration of intracellular calcium has been widely used to test the responsiveness of chemokine receptors to different chemokines, activation of PKC isoenzymes is stimulated by almost any surface receptor and therefore is not special for chemokine-induced signal transduction. However, PKC activation by chemokines contributes to receptor phosphorylation which leads to desensitization and inhibition of functional responses (99) (see later). In myeloid cells, migration is not dependent on PLCβ activity, but requires activation of PI3Kγ (100, 101); PI3Kγ rapidly generates PIP3 and initiates the activation of another kinase, PKB (102). In a chemotaxing cell, signaling components responsible for the formation of cell polarity, directional sensing and F-actin polymerization, such as PI3K, PIP3, the small GTPase Rac, PKB, translocate to the leading edge of the cell, where they contribute to local actin polymerization, whereas the mediators of actomyosin contraction are recruited to the trailing edge (103)(**Fig. 2.3**). Another important player is Cdc42, a small GTPase, which is recruited to the leading edge, where it has the essential role to exclude from the leading edge a

phosphatase, PTEN, negative regulator of PI3Kγ. Without Cdc42, cells exhibit a random walk, rather than directed migration (104-106). Thus, upon chemokine stimulation, PI3K localizes anteriorly, whereas PTEN localizes posteriorly and this spatial and temporal regulation determines the polarity of the migrating cell (107).

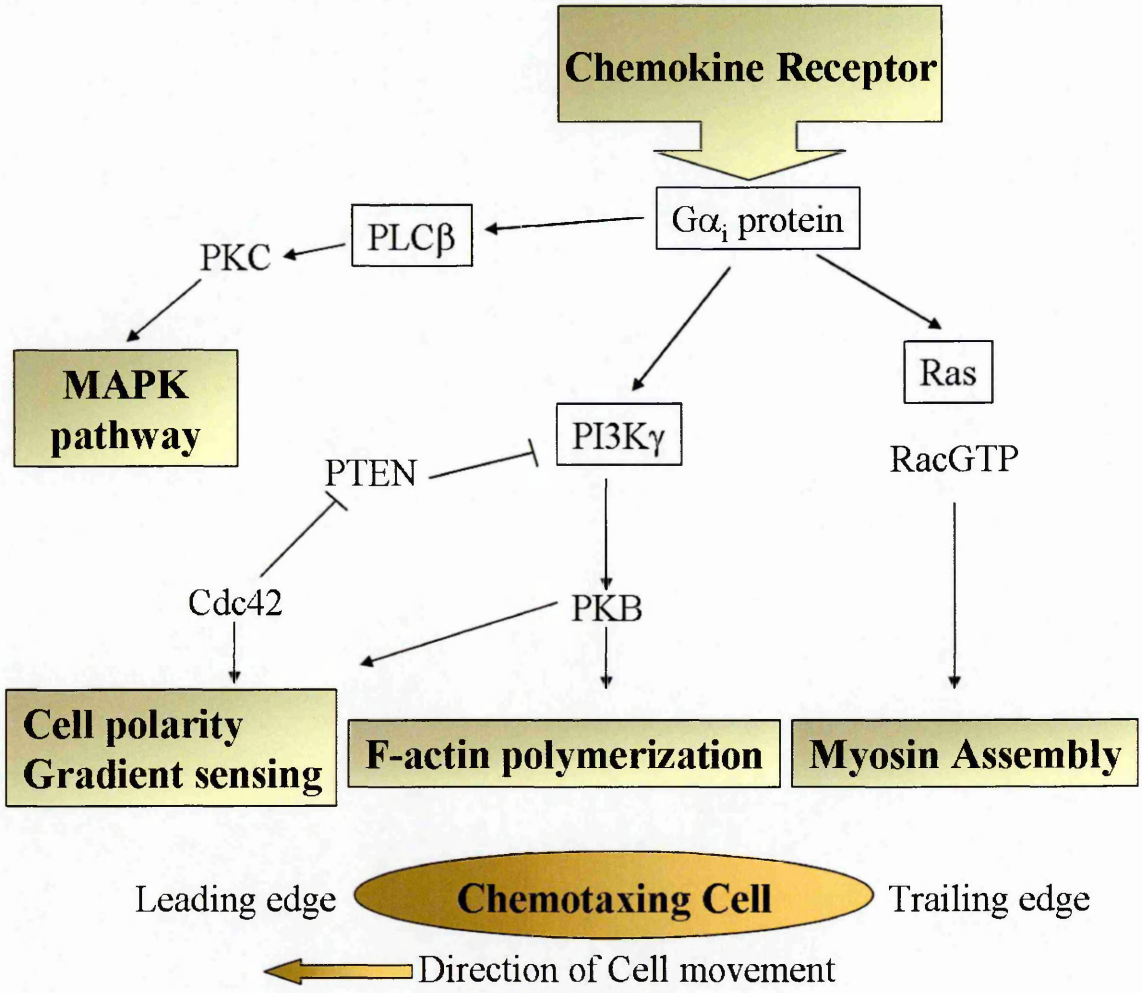


Fig 2.3. Signaling through chemokine receptors. Different signalling components localize either in the leading edge or in the trailing edge, determining cell movement (*adapted from Tanaka et al,* (108)).

There is increasing evidence that chemokine receptors can also activate several intracellular effectors downstream of G coupling, including the low molecular weight protein Ras and Rho and the mitogen-activated protein (MAP) kinase pathway (109,

110). Recently it has been suggested that, like cytokine receptors, chemokine receptors are also able to signal through the JAK-STAT pathway (111, 112). Upon ligand binding, the receptor dimerizes and catalyses the phosphorilation of the JAK kinases which in turn phosphorylate the receptor itself; this event brings to STAT molecule recruitment, dimerization and translocation to the nucleus where it triggers the expression of cytokine dependent genes.

Chemokine receptor signalling is transient, and the rapid termination of receptor activity is achieved by three mechanisms: receptor inactivation, desensitization and internalization. **Receptor inactivation** is mediated by the intrinsic GTPase activity of the Gα subunit, which hydrolyzes GTP and reunite with Gβγ to return to the initial conformation of inactive heterotrimers. **Desensitization** is caused by receptor phosphorilation, through G-protein-coupled receptor kinases, among which PKC and finally **internalization** is caused by β-arrestin or adaptin-2 mediated receptor sequestration and internalization, through clathrin-coated pits or caveolae. The speed of response recovery is determined by the fate of internalised receptors, either lysosomal degradation or dephosphorylation and cell surface recycling; the pathway leading to either degradation or recycling following receptor internalization are determined by the guardians of all vesicular machinery, Rab GTPases (113) and by the rate of *de novo* chemokine receptor synthesis.

CXCR4 is a unique receptor in this regard, as it does not share with the other chemokine receptors the common feature of rapid inactivation; indeed, it possesses the remarkable property of being able to induce prolonged signalling (114). Stimulation of prolonged signalling of Akt/PKB and ERK2 is likely to contribute to this phenomenon (115). Recently, Tilton et al have raised various possibilities to explain this fact, the most likely being that CXCR4 remaining at the surface is not desensitised (as is usual with chemokine receptors) but can continue to transduce a signal in response to

CXCL12, despite receptor internalisation. Receptor recycling could also contribute to this effect (115).

Signal transduction downstream chemokine receptors is much more complicated than the one above described; indeed, a given receptor will activate signalling pathways influenced by those from other receptor systems.

Beside the conventional signalling receptors, other chemokine binding molecules with high structural similarity to chemokine receptors have been described, namely the Duffy antigen receptor for chemokines (DARC) (116), D6 (117, 118) and CCX-CKR (119). These receptors do not elicit migration or conventional cellular responses, but still retain the capability to bind chemokines with high affinity. They are also referred to as "silent receptors" and have been suggested to favour transfer of chemokines across endothelial barriers and/or to act as decoy receptors which dampen inflammatory reactions (120).

2.3 *In vivo* biology of Chemokines and Receptors

The eponymous function of chemokines and their receptors is to mobilize cells in a gradient direction. This biological function is particularly important in leukocyte recruitment at sites of inflammation, although it is now well known that many different cells express chemokine receptors and are therefore able to respond to chemotactic stimuli. The process of recruitment of circulating leukocytes at sites of inflammation begins with the chemokine and chemokine receptor interaction, which eventually culminates in the final cell movement.

Multistep model of leukocyte extravasation

Binding to endothelium occurs through complex interactions of adhesion molecules and chemokines, exposed on endothelial cells and their receptors, expressed by the moving cell. Leukocytes tether to vessel walls using **selectins**, molecules that are specialized for adhesion under shear stress; these molecules have been demonstrated to be expressed also by other cell types, therefore presumably involved in the binding to the endothelium of different types of cells. This process, also referred to as **rolling**, is the first weak stop signal for circulating cells. Then a second class of adhesion molecules, **integrins**, is responsible for firm adhesion; they are structurally designed for rapid conformational changes regulating their affinity and leading to firm arrest of cells on the vessel wall and then transendothelial migration. Chemokines, bound to **glycosoaminoglycans** on the surface of endothelium, are thought to provide the signals that convert the low-affinity, selectin-mediated interaction into the high-affinity, integrin –mediated interaction that leads to extravasation of leukocytes.

Blocking of leukocyte adhesion to endothelium by pertussis toxin, a G-protein–signalling inhibitor, confirms this hypothesis (121).

Role of chemokines in physiologic processes

Chemokine biological function is particularly important in **leukocyte recruitment** at sites of inflammation and therefore, the primary role of chemokines in guiding leukocyte migration bears the important consequence that blocking chemokines or their receptors profoundly affects all those inflammatory responses requiring leukocyte recruitment.

The trafficking of Dendritic cells (DC) and lymphocytes through secondary lymphoid organs is finely regulated by chemokine receptors. DC can take up antigens in

peripheral tissues and migrate to draining lymph nodes where they present antigen to naïve T cells, triggering the T cells to proliferate and differentiate. These activated T cells can then migrate to the inflamed tissues to perform their effector functions. The movement of T cells and DC between lymph nodes and the periphery may be controlled by differential chemokine receptor expression (122). Circulating monocytes and immature DC can express receptors for chemoattractants such as fMLP (representative of bacterial proteins) and C5a (generated in the complement cascade) as well as chemokine receptors including CXCR4, CCR1, 2, 3, 4, 5 and 6 (123, 124). Using these receptors, immature DC can migrate towards sites where there is a high concentration of inflammatory chemokines such as CCL2, CCL3 and CCL5. Immature DC can also respond to constitutively expressed chemokines such as CXCL12, and this may be important for localising DC in tissues under normal conditions. At sites of inflammation, immature DC are activated by inflammatory cytokines such as TNF-α and IL-1 (125), which results in DC maturation. This causes a switch in their chemokine receptor expression. Maturing DC lose their responsiveness to CCL3, 4, 5, 7 and 20 (126, 127), but concomitantly upregulate CCR7 and gain responsiveness to CCL19 and CCL21 which are expressed in T cell rich areas of secondary lymphoid organs including lymph nodes, spleen and tonsils.

Naïve T cells can express CXCR4 and CCR7, which may account for their localisation in secondary lymphoid organs. Upon interaction with antigen-presenting DC, naïve T cells are activated and alter their chemokine receptor profile. Activated T cells can express CXCR3, CXCR4, CCR1, 2, 3, 4, 5, 6, 7 and 8 (128, 129). Activated T cells can polarise to T helper 1 (Th1, expressing IFN-γ and IL- 12) and T helper 2 (Th2, expressing IL-4) subsets, which differ in their cytokine production and function during an immune response and also express different chemokine receptors. CCR5 and

CXCR3 are preferentially expressed on Th1 cells, while Th2 cells preferentially express CCR3, CCR4 and CCR8 (128, 129), although this differential chemokine receptor expression is not clear-cut. Finally, naïve B cells can express CXCR5 and CCR7 (130) which direct them to the follicles of secondary lymphoid organs, where the ligands CXCL13, CCL19 and CCL21 are expressed. Upon maturation to plasma cells, B cells downregulate CXCR5 (131) and CCR7 (132), but have increased sensitivity to CXCL12 (through CXCR4) which regulates plasma cell positioning in the spleen and lodgement in the bone marrow.

Various indications in the literature suggest that chemokines have a role in the development of different biological responses that goes beyond cell recruitment. Chemokines have been shown to play a direct role also in definition of the cytokine milieu during both inflammatory and immune responses as well as in important mechanisms such as hematopoiesis. Thus, chemokines not only support differential leukocyte recruitment, but also directly affect target cell functions. Chip-based gene expression profile analysis in chemokine-activated monocytes revealed that CC chemokines induce specific transcriptional programs in target cells, demonstrating that chemokine effects on target cells include induction of transcriptional events (133).

Chemokines not only attract **T cells**, but they may also have roles in regulating T cell biology, influencing Th1/Th2 **polarisation** (134). CCL2 can suppress Th1 responses and cause an increase in IL-4 (Th2 cytokine) production by activated and memory T cells in vitro (134, 135). CCL2 addition to macrophages in vitro can also decrease IL-12 (Th1 cytokine) expression (136). CCL2 may therefore promote Th2 polarisation both directly and indirectly by increasing IL-4 and decreasing IL-12 production, respectively. In contrast, addition of CCL3 to in vitro cultures of activated T

cells promoted the development of IFNγ-producing cells (135) and hence Th1 differentiation. Similarly, CCL3, 4 and 5 production by monocyte-derived DC can promote the development of IFNγ-producing cells (137). However, experiments in mice deficient in the chemokines CCL2 and CCL3 and the chemokine receptors CCR1 and CCR2 have been less conclusive, and in some instances given opposite results, depending on the experimental protocols used (138-141).

More work is required to further elucidate the role of chemokines in the differentiation of T cells, and also the contribution of chemokines produced by T cells themselves. This may have implications for the use of chemokine receptor antagonists in the treatment of inflammatory disease.

Chemokines play also an important role in **hematopoiesis**. Stem cells and progenitor cells (HPC) in the bone marrow are subjected to the influence of a variety of different cytokines, resulting in either stimulation or inhibition of proliferation (142). Chemokine receptor expression by HPC may regulate the homing of these cells within the bone marrow during differentiation and maturation, and their mobilisation into the circulation. In particular, haematopoietic progenitor cells express CXCR4 and can migrate in response to CXCL12 (143); therefore, this chemokine/receptor pair plays an important role in the balance between retention and mobilization of progenitor cells in the bone marrow and this function is accomplished through a mechanism of receptor desensitization and downregulation, with CXCL12 being constitutively produced in high amounts in bone marrow. Moreover, they can also be important for the retention of HPC in the bone marrow, during B lymphopoiesis; retaining B cell precursors in the bone marrow would enable their regulated differentiation into mature B cells (144). Beside hematopoiesis, CXCR4 and CXCL12 are important in development and embryogenesis, as clearly demonstrated by knockout mice (145, 146). Both CXCR4 and CXCL12 deficient mice die in utero, pointing out their role in development and

embryogenesis; this may also explain why CXCL12 is such a highly conserved chemokine. These mice have severely reduced numbers of B cell progenitors and myeloid progenitor cells, suggesting that the CXCL12/CXCR4 pair is responsible for B cell lymphopoiesis and bone marrow myelopoiesis. Surprisingly, T lymphopoiesis is unaffected in these mice. They also have severe heart defects, including defective cardiac ventricular septum formation and a disorganised cerebellum. Evidence have been collected that mice deficient in either CXCR4 or CXCL12 have defective formation of the large blood vessels supplying the GI tract, possibly due to defective regulation of vascular branching and/or remodelling processes in endothelial cells (145).

Chemokine in vivo functions are not limited to immunity and inflammation; given their broad spectrum activities, it not surprising that they play a role in several types of human pathologies, including asthma, cardiovascular diseases, transplantation, neuroinflammation, HIV-associated diseases and neoplasia (147). Here I give a general overview of the role of chemokines in some important pathologies.

Chemokines in Pathology

Asthma is a chronic disease of the small airways where chronic inflammation leads to reversible airway obstruction. The leukocyte infiltrate characterizing allergen response consists of eosinophils, mononuclear cells (particularly Th2 cells), basophils and mast cells. Among different chemokine receptors expressed on the leukocytes associated with asthma, CCR3 seems to play a prominent role (148), since it is the receptor for CCL11 (eotaxin); this chemokine was first described due to its ability to attract eosinophils which are closely correlated with lung dysfunction clinically (149). Indeed, protein and mRNA expression of CCR3 are elevated in the bronchial mucosa and skin of patients with asthma (150), where it is expressed on eosinophils, basophils,

mast cells and airway epithelial cells (151).

Multiple sclerosis (MS) is a demyelinating autoimmune disease mediated by CD4+ T cells specific for one or more autoantigens in the central nervous system, which produce a variety of destructive inflammatory mediators. Beside auto-reactive T lymphocytes, MS lesions contain a variety of cell types, including monocytes/macrophages and T cells which are subsequently recruited, activated microglia and activated cerebrovascular endothelium (152). During active MS attacks there are significantly increased levels of CXCL9, CXCL10, and CCL5 in cerebrospinal fluid (153) and CCL2, CCL7 and CCL8 have been found immunohistochemically in MS lesions (154). Various chemokine receptors are also expressed in MS lesions: CCR2 and CCR5 are found on macrophages, microglia and T cells, and CCR3 is also found on reactive astrocytes (155). MS patients heterozygous or homozygous for the CCR5Δ32 allele (which encodes a non-functional form of CCR5) have delayed disease onset of approximately 3 years compared with affected siblings (156), suggesting that CCR5 may be a target for therapy. CXCR3 is expressed by more than 90 % of CD3+ T cells in cerebrospinal fluid, and >99 % of T cells in perivascular accumulations in active lesions (153). The presence of CXCL9 and CXCL10 in MS lesions may account for the recruitment of CXCR3 positive T cells; CXCR3 may also be a target for therapeutic intervention in MS (157).

Rheumatoid arthritis is characterized by the presence of a mixed inflammatory cell infiltrate into synovium-lined joints, in response to autoantigens. The success of anti-TNF-based therapy has indicated the critical role played by this cytokine in arthritis, presumably mediated by the induction of chemokines. Synovial fluid from pathological joints contains high levels of CCL2, CCL3, CCL5 and CXCL10 and both

synovial-lining cells and leukocytes are the source. Leukocytes are also the target, expressing CCR2, CCR5, CCR2 and CXCR3 (158, 159).

Accumulating evidence indicates that chemokines play a central role in cardiovascular disease and in particular in **atherosclerosis** (160), a chronic inflammatory disease of the blood vessel wall, characterized by the accumulation of mononuclear cells. It is a multifactorial disease, with risk factors such as smoking, hypertension, hypercholesterolemia, family history and diabetes; however, there is consensus on the origin of atherosclerotic plaques from an inflammatory response to the arterial damage, occurring either because of hypercholesterolemia or shear stress. One of the chemokines found involved in this pathology is CCL2: indeed, CCL2 and CCR2 deficient mice have 60-85% less arterial lipid deposition than wild type mice, in hypercholesterolemia models and this is consistent with the role of CCL2 in leukocyte recruitment. Recent evidence demonstrate that a broad spectrum CC-chemokine blockade reduced atherosclerosis in an Apo-E knockout mice (161, 162).

Another important chemokine in atherosclerosis is CX3CL1, namely Fractalkine: this chemokine is produced by endothelial cells after inflammatory cytokine stimulation and can mediate leukocyte adhesion and infiltration into the vascular wall, as well as NK-cell-mediated endothelium injury. High levels of mRNA for CX3CL1 and other 16q13-chromosome-linked chemokines have been observed in human arteries with advanced atherosclerotic lesions (163). The ability to recruit leukocytes and its expression in vascular cells strongly suggest a pivotal role for Fractalkine in the pathophysiology of atherosclerosis (164). Moreover, in humans, gene polymorphisms at amino acids 249 and 280 of the Fractalkine receptor have been reported (165-167), causing amino acid substitution: valine (V) instead of isoleucine (I) in position 249 and methionine (M) instead of threonine (T) in 280. Homozygosity for

V249-CX3CR1 has been found to be associated with increased risk for acute coronary events in comparison to I249 heterozigosity (167). The molecular explanation is that the V249 variant displays higher binding affinity to the chemokine, corresponding to an enhanced ability of monocytes to adhere to the vascular endothelium (168).

As the chemokine system plays an essential role in host defense, it is not surprising that chemokines and their receptors may be involved in **rejection of allogeneic transplants**. In particular, after an early non specific release of inflammatory chemokines attracting neutrophils and monocytes, CXCR3 and CCR5 ligands appear, several days after the transplants, consisting with their orchestrating the movement of cells involved in acute rejection (169). Moreover, it has been demonstrated that CX3CL1 expression is enhanced in rejecting cardiac allografts (170); in addition, the treatment of organ recipients with polyclonal anti CX3CR1 blocking antibodies markedly prolonged survival of MHC-mismatched heart allografts (170).

As far as bone marrow transplant is concerned, CXCR4 and its ligand chemokine CXCL12 are very important. As comprehensible given their role in the balance between stem cell precursor retention and mobilization in the bone marrow, CXCR4/CXCL12 play a role in bone marrow engrafment of CD34+ cells and this may have clinical implications with regard to therapeutic stem cell transplantation.

CD4 is the primary cell surface receptor used by human immunodeficiency virus (**HIV**) to penetrate T lymphocytes and macrophages. The chemokine receptors CXCR4 and CCR5 function as co-receptors which, along with CD4, allow viral envelope fusion and entry (171, 172). A range of chemokine receptors have now been shown to have co-receptor activity in vitro, including CCR1, 2, 3, 4, 5, 8, 9, CXCR2, 4, 5, 6 and CX3CR1

(173), but so far only CXCR4 and CCR5 have been shown to act as co-receptors in vivo, hence X4 tropic and R5 tropic viruses are described. Since chemokine receptors act as co-receptors for HIV entry, endogenous chemokine production can regulate HIV replication. In 1995, Cocchi et al published that CCL3, CCL4 and CCL5 have CD8+ T-cell-derived HIV inhibitory activity (174). Also, individuals homozygous for the Δ32 allele of CCR5, who are deficient in cell surface CCR5 expression, can remain uninfected despite exposure to HIV (175, 176). These observations suggest that chemokines and their receptors which are involved in HIV infection are potential targets for the development of new drugs to treat HIV. For example, small molecule antagonists of CCR5 and CXCR4, which can block HIV entry, are entering clinical trials (177, 178).

2.4 Chemokines in Cancer

Since their discovery, the field of chemokines was strongly connected to cancer biology. Indeed, in the early 1980s it had been noted that tumor supernatants contained chemo-attractants active on monocytes (179); the Tumor-derived Chemotactic Factor isolated in the culture supernatants of tumor cell lines was lately identified as CCL2 (180). The role of chemokines in tumor biology has dramatically developed in the last decade and has expanded from the regulation of leukocyte attraction within the tumor mass to the promotion of tumor cell survival, proliferation and dissemination (82, 83, 181).

As already said, primary tumor growth, invasion and metastasis to distant organs are dependent on a highly orchestrated series of events, including cellular transformation, a pro-angiogenic environment, local tumor cell growth, invasion

through the extracellular matrix (ECM) and vascular basement membrane, entry into the circulation and eventually non-random tumor-cell metastasis to distant organs. In addition to their role in regulating leukocyte trafficking, chemokines have been shown to be involved in each of these events.

Chemokines in the tumor microenvironment

In the tumor microenvironment chemokines are produced both by stromal cells (fibroblasts, endothelial cells and infiltrating leukocytes) and by the tumor itself. CCL2 is probably the CC chemokine most frequently secreted by cancer cells. Human tumors shown to express CCL2 *in vivo* include sarcomas, gliomas, lung tumors, carcinomas of the breast, cervix and ovary, melanoma and pancreas (91) Several lines of evidence, including correlation between production and infiltration in murine and human tumors, passive immunization and gene modification, indicate that CCL2 plays a pivotal role in the recruitment of monocytes in neoplastic tissues, as discussed below (91, 133).

As mentioned above, tumors are generally characterized by the constitutive expression of chemokines belonging to the inducible realm. The molecular mechanisms accounting for constitutive expression have been defined only for CXCL1 and involve NFκB activation. Melanoma cells display high expression of NF-κB-inducing kinase (NIK) and this phenotype is responsible for constitutive activation of IκB kinase and MAPK signaling cascades, as well as for constitutive activation of NF-κB (182, 183) This may represent a general mechanism underlying constitutive expression of inflammatory chemokines in tumors.

Role of chemokines in tumor progression.

Beside CCL2, a variety of other chemokines have been detected in neoplastic tissues as products of tumor cells or stromal elements. These include CCL5, CXCL12,

CXCL8, CXCL1, CXCL13, CCL17 and CCL22. CCL5 is produced by breast carcinoma and melanoma (184, 185). In breast cancer CCL5 expression by tumor cells correlates with a more advanced stage of disease, suggesting that CCL5 may be involved in breast cancer progression (184, 186). Melanoma is probably the most studied cancer type in which CXC chemokines and in particular CXCL1 and related molecules (CXCL2, CXCL3, CXCL8 or IL-8) have been demonstrated to play a role in tumor progression (187). They do so by direct stimulation of neoplastic growth, promotion of inflammation and induction of angiogenesis. CXCL1 was initially identified and purified from supernatants of melanoma cell lines and characterized as an autocrine growth factor (188, 189). Blocking of CXCL1, or its receptor CXCR2, with specific antibodies inhibited the growth of melanoma cells in vitro (189). Conversely, the over-expression of CXCL1 (190), CXCL2 or CXCL3 (191) in various melanoma cell lines increased their ability to form colonies in soft agar and their tumorigenicity in nude mice. A few other studies have proposed a similar role for CXCL8 related chemokines in head and neck (192) pancreas (193) and Non-Small-Cell Lung Cancer (NSCLC) (194). Autocrine and paracrine expression of CCL20 has been reported in pancreatic cancer (195). Finally, CXCL13 is a B cell chemokine and is highly expressed in Helicobacter pylori-induced lymphoma (196) and a role for CXCL13 in the localization of tumor cells has been suggested.

Chemokines regulate angiogenesis.

Angiogenesis is a key event in tumor growth and progression and chemokines have a major impact on the regulation of neovascularization in tumor tissues. As already said, the N-terminus of several CXC chemokines contains a highly conserved amino acid motif (Glu-Leu-Arg: ELR motif), which immediately preceeds the first cystein (197). ELR+ chemokines have potent angiogenic activity. The angiogenic members

include CXCL1 through CXCL8, with the exception of CXCL4. These chemokines act through a common receptor, CXCR2. Although some ELR+ chemokines bind both CXCR1 and CXCR2, it is widely accepted that only CXCR2 mediates the angiogenic activity and, accordingly, endothelial cells express only CXCR2 (198) Another important ligand-receptor pair is CXCL12 and CXCR4. Even if CXCL12 is a non-ELR chemokine, its activity has been implicated in neo-angiogenesis (199, 200).

The importance of ELR+ chemokines in supporting angiogenesis during the neoplastic progression has been established in a variety of tumor cell types, including prostate and ovarian carcinoma and NSCLC (201-203). Both in mouse tumor models and in surgical specimens obtained from tumor patients, expression of CXCL5 and CXCL8 was associated with increased neovascularization and inversely correlated with survival. Conversely, depletion of CXCL5 resulted in attenuation of tumor growth and angiogenesis. The finding of the unique use of CXCR2 receptor, despite the redundancy of ELR+ chemokines, provides a good opportunity to target this receptor for therapeutic interventions.

On the other hand, another series of CXC chemokines lacking the ELR motif (non-ELR) are characterized by the ability to block or inhibit angiogenesis. The angiostatic members are CXCL4, CXCL9, CXCL10 and CXCL11. The three latter chemokines are interferon-inducible and bind the CXCR3 receptor (86, 204). Recent observation has demonstrated that CXCR3 exists in two different isoforms: CXCR3A and CXCR3B, which differ in their NH2 terminus (205) CXCR3B (205), which is more expressed than CXCR3A in endothelial cells, appears to mediate the angiostatic activity of IFN-inducible chemokines. In addition, CXCL4 was shown to bind to CXCR3B (205). Non-ELR CXC chemokines have been shown to inhibit angiogenesis in several tumor models. Over-expression of CXCL9 and CXL10 in tumor cells leads to

spontaneous tumor regression in lymphoma cells (206) NSCLC (207) and melanoma (208).

Therefore, the balance of ELR+ versus non-ELR chemokines produced in the tumor microenvironment may determine the degree of angiogenesis surrounding and inside the tumor tissue and the consequent tumor progression.

Chemokines mediate leukocyte recruitment in tumors

The local production of inflammatory chemokines by tumor and stromal cells would be expected to cause the recruitment of various types of leukocytes to the tumor tissue. Indeed, tumors are composed of cancer and stromal cells, which sometimes are very developed and can even outnumber neoplastic cells. Besides fibroblasts and endothelial cells, leukocytes (especially macrophages and T lymphocytes) are the most represented cell types. Because these inflammatory cells secrete a variety of biologically active molecules, they are likely to regulate neoplastic processes that affect the growth and spread of tumor cells.

CXCL8 and related chemokines act primarily on **neutrophils**. In spite of constitutive production of these ligands by tumor cells, neutrophils are not a major and obvious constituent of the leukocyte infiltrate. However, these cells, though present in minute numbers, may play a key role in triggering and sustaining the inflammatory cascade, for instance by releasing angiogenic molecules (209).

Tumor-Associated Macrophages (TAM) derive from monocytic precursors circulating in the blood (210) Experimental evidencesuggests that tumor infiltrating macrophages may facilitate tumor growth and progression, as, although they can potentially display tumor cytotoxicity, they are believed to have primarily pro-tumor functions (**Fig 2.4**) (21, 210, 211). In breast cancer, tumor cells produce CCL5, and the

level of expression correlates with the extent of macrophage infiltration and lymph node metastasis (184). In a mouse model, the long-term administration of a CCL5 antagonist, Met-CCL5, significantly reduces the subcutaneous growth of CCL5-producing syngeneic mouse breast cancer cells without affecting their proliferative ability, but concurrently inhibits leukocyte infiltration in the tumor (212). In addition to being a target for chemokines, TAM are a source of a selected set of these mediators (CCL2, CCL17, CCL18, CCL22). CCL18 was recently identified as the most abundant chemokine in human ovarian ascites fluid (213). When the source of CCL18 was investigated, it was tracked to TAM, with no production by ovarian carcinoma cells. CCL18 is a CC chemokine produced constitutively by immature DC and attractant for naive T cells, by interacting with an unidentified receptor (87). Attraction of naive T cells in a peripheral microenvironment dominated by M2 macrophages and immature DC is likely to induce T cell anergy.

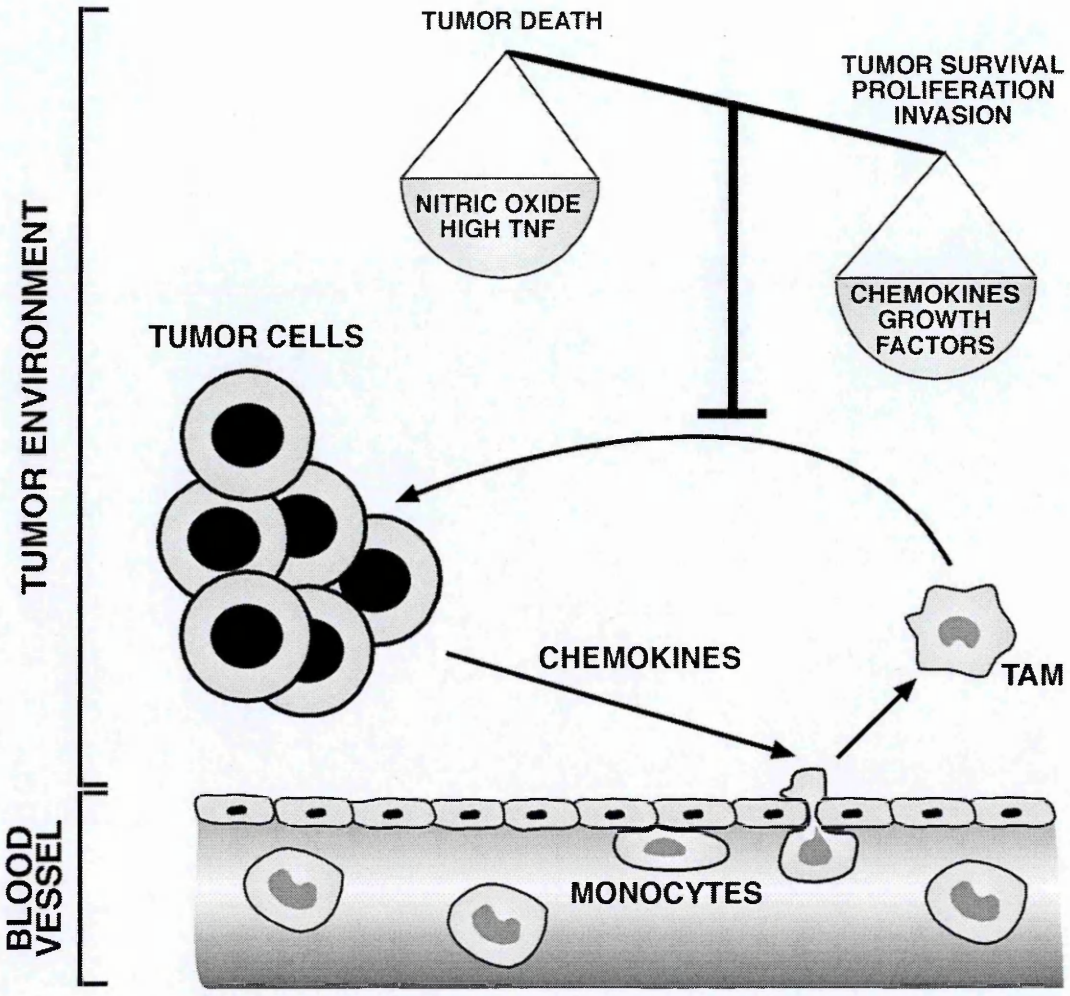


Fig 2.4. Tumor-derived chemokines actively recruit circulating blood monocytes at the tumor site. In the tumor micro-environment monocytes differentiate into Tumor-Associated Macrophages (TAM), which have an ambiguous role in their relationship with cancer cells, but most frequently have pro-tumoral functions.

Also **T lymphocyte** recruitment at the tumor site has an important role and chemokines are part of amplification and regulation systems of polarized T cell responses. Some chemokines may enhance innate and specific host immunity against tumors but, on the other hand, other chemokines may contribute to escape from the immune system, by recruiting Th2 effectors and regulatory T cells (214, 215).

Nasopharingeal and ovarian carcinoma express CCL5 and CCL3, ligands of CCR5; they have been proposed to regulate T cell infiltration (216, 217); transduction of CCL5

in tumor cells resulted in loss of tumorigenicity due to activation of anti-tumor immunity (216). Despite this evidence, in vivo expression of CCL5 is associated with advanced disease in breast and cervical cancer (184, 218). Also expression of CXCL9 has been associated with heavy infiltration of T lymphocytes in human melanoma (219) and in mouse tumor models (220, 221). CXCL10 was reported to be an important factor for IL-12-mediated anti-tumor response through the recruitment and activation of CD8 lymphocytes (222) and NK cells.(223) The chemokine CCL21 recruits dendritic cells and lymphocytes (naive T, NK cells and a subset of memory T cells), and displays anti-neoplastic effects when transduced in tumor cells(224-226) or injected locally (227). Similar results were reported for CCL19, which shares with CCL21 the same receptor CCR7 (228).

A variety of **dendritic cells** (DC) subsets are also found in tumor tissues and chemokines are involved in their recruitment (229, 230). Although usually rare cells, DC have been detected in several tumor types, including lung, prostate, nasopharynx, kidney, thyroid, breast, ovary carcinoma and melanoma (231, 232)

Finally, another interesting example of tumor leukocyte interaction is Hodgkin's lymphoma. Reed-Sternberg cells in Hodgkin's lymphoma have been shown to express CCL22 and CCL17 (233, 234). These chemokines recognize CCR4 which is preferentially expressed on Th2 lymphocytes and on T regulatory cells (85, 235). Interestingly, in the same tumor, stromal cells produce CCL11, which attracts eosinophils and Th2 cells. Therefore, in this human tumor, neoplastic elements and stroma use complementary tools to recruit immunocompetent cells associated with polarized type II responses, unable to mediate anti-tumor immunity. In the same vein of driving into tumors polarized Th2 cells, the oncogenic virus human herpesvirus 8 (HHV8), involved in the pathogenesis of Kaposi's sarcoma and hematological malignancies, encodes three CC chemokines (vMIPI, II and III) which interact with

CCR3, CCR4 and CCR8 expressed on Th2 cells and T regulatory cells (235). Consistently with these *in vitro* observations, Kaposi's sarcoma is infiltrated by CD8+ and, to a lesser extent, CD4+ cells with a predominant Th2 phenotype. Therefore, HHV8 virus-encoded chemokines represent a strategy to subvert antiviral/antitumor immunity by favouring the recruitment of inefficient cells and cells with suppressive activity. In addition to viral chemokines, HHV8 encodes for a chemokine receptor homologue, ORF74, also known as KSHV vGPCR, showing similarity with CXCR2 (236). This receptor triggers a constitutive signal which is further increased by CXCL8 and CXCL1, providing a good example of a direct role of chemokines and receptors in neoplastic transformation. Indeed, over-expression of KSHV vGPCR alone resulted in the development of lesions resembling Kaposi's sarcoma (237).

Chemokines and extracellular proteases

The field of chemokines in tumor biology has dramatically developed in the last decade and has expanded from the regulation of leukocyte attraction within the tumor mass to the promotion of tumor cell survival, proliferation and mobilization (91).

It has long been known that tumor-derived proteases can cleave the extra-cellular matrix molecules and lead to the dissolution of the basement membrane, thus facilitating the process of tumor cell invasion. What it has remained unknown until very recently is that chemokines are potent inducers of enzymes and receptors which degrade the extracellular matrix and favour tumor invasion (49, 238, 239). As I have already discussed before, a variety of proteolytic enzymes, in particular the tissue type plasminogen activator (t-PA), the urokinase-type plasminogen activator (u-PA) and the large family of matrix-metalloproteinases (MMPs) have been implicated in this degradation (68, 240); indeed, the activity of these enzymes has been associated with more aggressive neoplastic behaviour. Confirming the role of chemokines in the

activation of these enzymes, in a gene expression analysis, the chemokine CCL5 specifically induced gene expression of various MMPs, especially MMP9, along with the uPA receptor (133). CXCL8 expression by human melanoma cells induces transcriptional activation of expression of the gene encoding MMP2 and augmented collagenase activity in these tumor cells, which leads to increase invasiveness (241). In prostate cancer, CXCL8 over-expression induces the expression of MMP-9, leading to increased tumor cell invasiveness and metastatic potential in nude mice (242).

Not only tumor cells produce proteases. Leukocytes, in particular macrophages, are potent producers of proteases and strong evidence demonstrates that chemokines activate TAM to release MMPs in the tumor micro-environment. A number of studies have highlighted the role of TNF in the regulation of MMP activity in monocytes and chemokines have been demonstrated to activate MMP activity in monocytes through TNFα pathway (238).

The role of CCL2 in pancreatic cancer

Several studies have indicated that CCL2 is primarily responsible for the recruitment of monocytes at the tumor site. Indeed, CCL2 levels correlated with the abundance of TAM in several types of adenocarcinoma, including ovarian, breast and pancreas (25, 243-245). Interestingly, CCL2 production has been detected also in TAM, indicating the existence of an amplification loop for their recruitment (244). In accordance with the potential dual role of TAM, the gene transfer of CCL2 into tumors had contrasting effects. At least three reports indicated reduced tumorigenicity (246-248). The results of another study pointed to an opposite effect: the number of spontaneous lung metastases was augmented in animals injected with CCL2-transfectants compared to those injected with parental cells (249, 250). The impact of

CCL2 on tumor growth in a nontumorigenic melanoma system revealed a biphasic effect. Low-level CCL2 secretion, with "physiological" accumulation of TAM promoted tumor formation, while high CCL2 secretion resulted in massive macrophage infiltration into the tumor mass and in its destruction (250). Similarly, a high inoculum of CCL-2-transfected melanoma cells showed retarded tumor growth, while a small inoculum was more tumorigenic (251). These results are consistent with the "macrophage balance" hypothesis (21, 211). Moreover work in gene-modified mice has shown that CCL2 can orient specific immunity in a Th2 direction; although the exact mechanism for this action has not been defined, it may include stimulation of IL-10 production in macrophages (141).

In the last couple of years, we have been interested in the role of CCL2 in pancreatic adenocarcinoma. As I said above, CCL2 is found in many epithelial cancers; we have found that CCL2 is secreted by some pancreatic carcinoma cell lines, while these tumor cells never express CCR2, the functional receptor for CCL2 (**Fig 2.5**). Moreover, inflammatory cytokines such as IFNγ, IL1β, TNFα synergistically up-regulate its expression (data not shown).

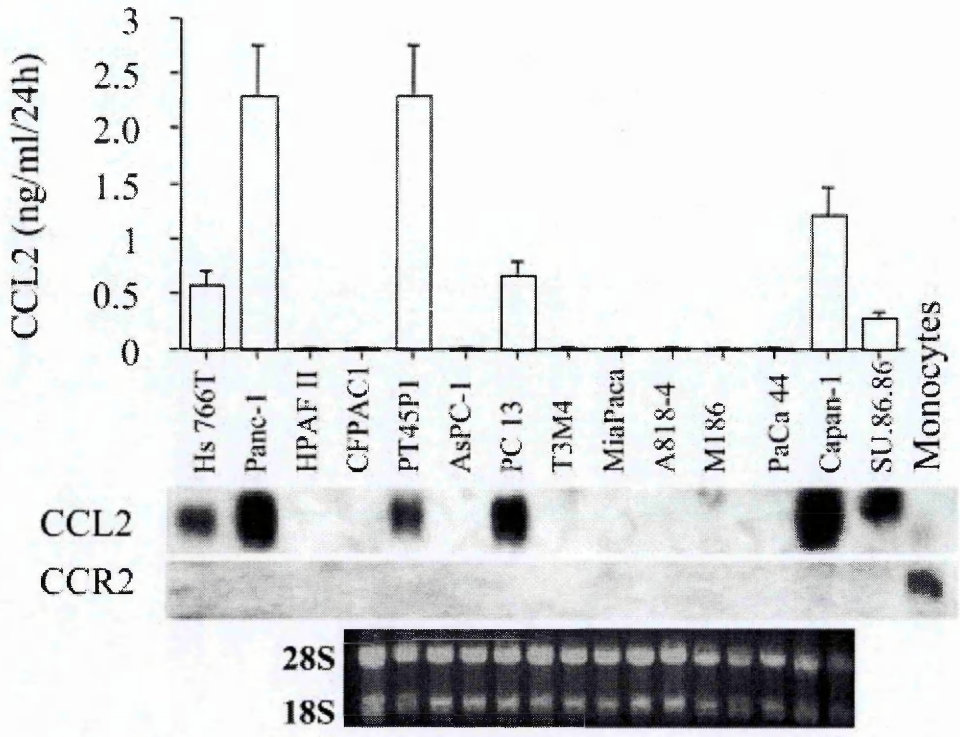


Fig 2.5. CCL2 in pancreatic adenocarcinoma cell lines. mRNA analysis revealed that 6 out of 14 pancreatic cancer cell lines analyzed express the chemokine CCL2. ELISA assay confirmed the result at the protein level. None of the cell lines express the corresponding receptor (*from Monti et al* (25)).

The expression of CCL2 was detected also in supernatant of primary tumors (**Fig 2.6**) as well as in surgical sections of pancreatic cancers (**Fig.2.7** (25)).

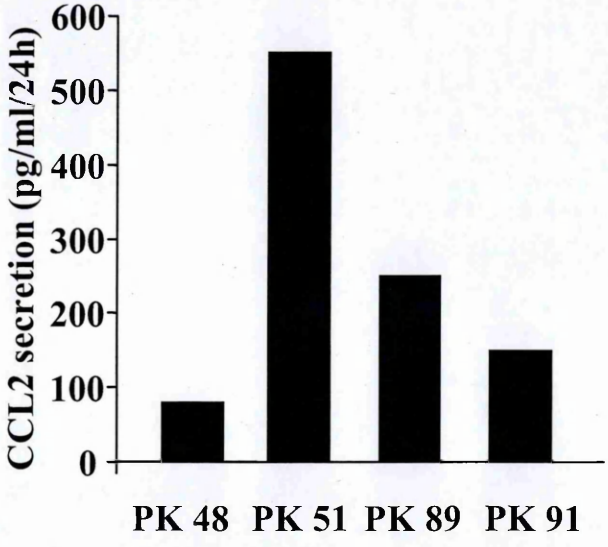


Fig. 2.6 Expression of CCL2 in human pancreatic cancer *in vivo*. CCL2 expression was determined at the protein level in supernatants of 4 human pancreatic tumor cell primary culture by ELISA.

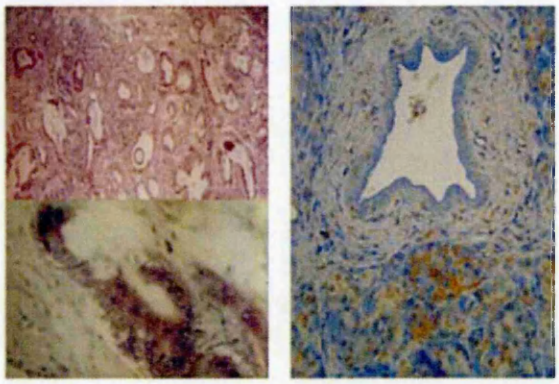


Fig. 2.7 Expression of CCL2 in human pancreatic cancer *in vivo*. Immunoistochemical localization of CCL2 in human pancreatic cancer cells(left) and normal tissues (right). Representative examples (x200; x400) of paraffin sections stained with antibodies to CCL2 (5D3-F7).

The tumor associated CCL2 is released into the circulation of tumor- bearing patients (**Fig. 2.8**) (25).

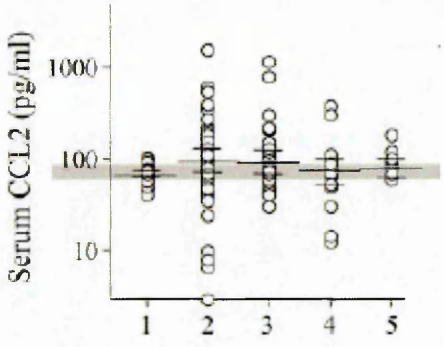


Fig. 2.8 Median levels of serum CCL2 in pancreatic diseases. *Lane 1*, healthy subjects (median value=67 pg/ml). *Lane 2*, patients with pancreatic ductal adenocarcinoma (median value=105 pg/ml). *Lane 3*, patients with other pancreatic adenocarcinoma (median value=90 pg/ml). *Lane 4*, pancreatic benign tumors (median value=70 pg/ml). *Lane 5*, pancreatic inflammatory diseases (median value=80 pg/ml).

Serum CCL2 levels are positively correlated with intratumoral macrophage infiltration (**Table 2.2**) (25).

CCL2 serum levels	Mϕ (CD68+)
Low (57<pg/ml)	**24 ± 4%**
Normal	**46 ± 8%**
High (91>pg/ml)	**57 ± 12%**

Table 2.2. Correlation between CCL2 serum levels and macrophage infiltration. (Low group, n=27; Normal group, n= 47; High group, n=76). The amount of leukocytes infiltrating the tumor was evaluated as immunostained cells at x200 magnification (average of 10 random fields) by using a Leitz Diaplan microscope.

Taken together, these data strongly indicate that CCL2 is expressed and secreted in vitro and in vivo by pancreatic tumor cells, thus confirming what has been observed also for other carcinomas, in particular in ovarian cancer. Tumor derived CCL2 could promote the recruitment of leukocytes, in particular monocytes/macrophages, as indicated by the correlation between CCL2 levels and CD68-positive cells; therefore this chemokine could play a central role in the crosstalk between tumor cells and their microenvironment.

2.5 Chemokines and receptors in tumor spread and progression

Novel concepts in organ selective metastasis

For many years, the prevailing explanation of the metastatic process was that some tumor cells somehow escape from the primary mass, enter lymph or blood circulation and stop in small blood vessels, where they would give rise to secondary tumors. This mechanical model predicts that the formation of a metastatic lesion depends on the number of tumor cells delivered to an organ. This does not fit with the observation that many types of cancer display an organ-specific pattern of metastasis but these organs sometimes do not correspond to those found in the drainage site. In other words, the selective metastasis pattern a particular tumor displays cannot be explained simply by blood and lymph flow. Examples are breast cancer preferential metastatization to liver, brain and lung, prostate cancer spreading to bone. Pancreatic cancer displays a peculiar recurrence along nerve structures.

Some theories have been put forward to explain the phenomenon of organ selective metastasis. In 1889, an English surgeon, Stephen Paget, on the basis of his numerous observations of cancer patients, proposed the **seed and soil theory**, according to which

the metastasis pattern is due to the dependence of the seed (the cancer cell) on the soil (the secondary organ). This idea was challenged in the 1920s by James Ewing, who suggested that circulatory patterns between a primary tumor and specific secondary organs were sufficient to account for organ-specific metastasis. These theories are not mutually exclusive, and current evidence supports a role for both of them: both mechanical factors (how many cells are delivered to an organs) and seed-soil compatibility factors (does the organ preferentially support or suppress the growth of the specific cancer-cell type) contribute to the ability of specific types of cancer to spread to various target organs, by acting at different stages of the metastatic process. The initial steps are likely to depend on blood-flow patterns, as most circulating cancer cells arrest by size restriction; in fact, capillaries are small (typically 3-8 μm in diameter) and designed to allow the passage of red blood cells (7 μm in diameter and highly deformable), whereas many cancer cells are quite large (20 μm or more in diameter). However, once cells have been seeded to an organ, their subsequent growth will depend on the compatibility of the seed with the soil that they encounter in the organ, therefore on the molecular interactions between cancer cells and the environment of the new organ.

Numerous candidates have been proposed as mediator molecules of the interaction between cancer cells and the organ, in particular organ specific growth factors and adhesion molecules. Recently, a **chemo-attraction theory** (also referred to as **homing theory**) has been proposed, according to which organ-specific attractant molecules enter the circulation, stimulating the migrating tumor cells to invade the walls of blood vessels and enter the organs. Chemokines and their receptors have been found to perfectly fit with this theory; in fact, recent studies have shown that tumor cells express patterns of chemokine receptors that match chemokines specifically expressed in organs to which these cancers commonly metastasize. In particular, the concept that a

particular chemokine-receptor pair may promote organ-specific tumor metastasis was first experimentally addressed by Muller et al (81). Therefore, the identification of molecular addresses (i.e. chemokines) or adhesion receptors (selectins) on endothelial cells in vascular beds of distal organs that specifically trap circulating malignant cells supports the active arrest view of the homing theory (252). As chemokines are involved in the homing of leukocytes, it seems reasonable to suppose that they contribute to the homing of cancer cells to specific secondary sites, thereby promoting organ specific metastasis. This theory is strengthened by the observation that chemokine signalling results in the transcription of target genes involved not only in cell motility, but also in cell invasion, interaction with the extracellular matrix and survival (91, 181).

Tumor cells express chemokine receptors

While the expression of chemokines in human and experimental tumors has been the object of intense investigation, the expression of chemokine receptors has been pursued to a much lesser extent, until very recently.

Tumor cell motility is a pivotal step in the intricate process leading to the formation of metastases, and tumor cells that have increased metastatic potential are more motile than non-metastatic tumor cells. Morphological studies of rat sarcoma cells have shown that the structure of the actin network relates to the degree of the malignancy, and determines the cell motility (253). Moreover, in MCF-7 breast cancer carcinoma cell line, the level of F-actin showed a significant increase after treatment with the CC chemokine MIP-1α and MIP-1β (254).. In the same work, confocal microscopy further indicated a redistribution of the cytoskeletal F-actin within 45 min after chemokine stimulation with movement of F-actin towards the periphery of the cells in a polarized manner. These data suggest that chemokines can attract tumor cells.

Medium conditioned with mouse lung microvessel endothelial cells possesses chemotactic activity for a highly lung metastasizing variant of the RAW117 murine large cell lymphoma cell line, but not for the poorly metastatic parental cell or a liver metastasizing variant. The chemotactic activity was purified and identified as JE, the murine counterpart of CCL2 (255). Another interesting murine tumor model suggested the involvement of chemokines in organ selective metastatization: ESb-MP cell line, a variant of the highly metastatic cell line ESb, (derived from a murine T cell lymphoma) displayed frequent in vivo metastasization to the kidney, while the parental cell line rarely infiltrate this organ; moreover, it migrated in response to kidney organ conditioned media, to which the parental cells did not respond. This raised the possibility that a kidney derived chemotactic factor may be involved in the attraction of ESb-MP cells in vitro and may account for the kidney specific localization of ESb-MP metastases in vivo. JE/CCL2 and CCL5 chemokines were purified from murine kidney derived mesangial cell supernatant as inducers of in vitro migration of ESb-MP variant cells but not of the parental cells (256). Further studies with radiolabeled chemokines revealed that cell surface expression of chemokine receptors is necessary but may not be sufficient for functional responsiveness. In fact, cells must possess also the proper molecular array to transduce a receptor triggered signalling cascade.

Earlier studies already pointed out that tumor cells express functional chemokine receptors. Some tumor cell lines migrated in response to CXCL8 and related chemokines, and antibodies against CXCR2 were able to inhibit the growth of melanoma cells in vitro (189). Other inflammatory chemokines have been tested and induced motility of malignant cells of hematopoietic and epithelial origin (257). CCR4 is often expressed in adult T-cell leukemias that preferentially invade the skin, where one of the CCR4 ligands, CCL17, can be expressed (258). CCR3 is expressed in CD30+ cutaneous lymphomas, and its ligand CCL11 is often expressed in the tumor cells and

tumor-associated skin lesions (259). As far as solid tumors are concerned, the most potent chemoattractants for human breast adenocarcinoma cell lines were CCL3, CCL4, CCL5 and CCL2. Breast carcinoma cells also express CXCR4, the receptor for CXCL12, and this receptor has been recently implicated in the process of metastasis (81). Since then, many other tumors of different lineages have been evaluated and shown to express chemotactic receptors (**Table 2.2**) (83, 91) .

Tumor type	Chemokine receptor expressed	
	Most frequent	Other receptors
Breast	**CXCR4**	**CCR7**
Ovary	**CXCR4**	**CCR9**
Prostate	**CXCR4**	**CCR9**
Melanoma	**CXCR4**	**CXCR3, CCR7, CCR10**
NSCLC	**CXCR4**	**CCR7**

Table 2.3.Chemokine receptors expressed by human tumor cells. CXCR4 is the most frequently found in different histological types of malignancies (*adapted from Balkwill F* (91))

Overall these results support the concept that chemokines could direct tumor cell migration in vivo: malignant cells bearing chemokine receptors on their cell surface would be endowed with the capability to respond to chemokine gradients and selectively migrate to specific organs where the chemokine is present.

In this thesis I have investigated if chemokines and their receptors are involved in human pancreatic adenocarcinoma metastasis and progression.

Chapter 3

Materials and Methods

3.1 Cells and Tissues

Cell lines

All cell lines were grown in pyrogen-free conditions in a humidified atmosphere of 5% CO_2 and 95% air at 37°C. The cells were grown in tissue culture plastic flasks or plates (Falcon, Bedford, MA), passaged when confluent by trypsinization (trypsin/EDTA, 0.05/0.05 w/v)and seeded at an appropriate density (usually, less than $5x10^4$ cells/ml).

Human pancreatic carcinoma cell lines AsPC-1, Capan-1, MiaPaCa-2, Panc-1, Hs766T, were purchased from the American Type Culture Collection, (Rockville, MD), A8184, PT45, HPAF, CFPAC, PaCa44, T3M4 were kindly provided by Prof. Scarpa (Department of Pathology, University of Verona, Verona, Italy). Four cell lines were obtained from primary tumors (PT45, PaCa44, MiaPaCa2, Panc1), three from ascites (AsPC 1, A8184, HPAF) and four from metastasis (T3M4 and Hs766T from lymph node metastasis, CFPAC and Capan 1 from liver metastasis,) (26). The cell lines were maintained in DMEM (Gibco, Scotland, UK) supplemented with 10% FBS (Hyclone, Logan, UT). The immortalized epithelial cell line derived from normal human pancreatic ducts HPDE6, kindly obtained from Dr. Ming-Sound Tsao (University of Toronto, Toronto, Ontario, Canada), has been previously shown to maintain the phenotypic and genotypic characteristics of normal human pancreatic ducts (260). The ovarian cell line OVCAR-3 (derived from primary tumour) was cultured in RPMI medium (Gibco, Scotland, UK); the ovarian cancer cell lines OVCAR-4 (derived from a primary tumour), SK23, SKN (obtained from SKOV3 p53 transfection (261)), A2780 (derived from a primary tumour), IA9, SKOW3 (derived from ascites), the breast cancer cell lines MCF-7, MDA-MD435 and MDA-MD231 (derived from lung metastasis), and the colon cell lines HCT116 (derived from a primary tumour) and SW620 (derived from

lymph node metastasis) were cultured in DMEM 10% FBS. These cell lines were already available in the lab.

Prior to their use in functional assays (migration, invasion) or flow cytometry, cells were detached without trypsin, to avoid surface receptor cleavage; medium and serum were removed and cells washed thoroughly with physiological saline solution and left 10 minutes at 37°C in medium 1% FBS.

Neuroblastoma (SKN-BE and SY5Y), and astrocytoma cell lines, already available in the lab, were cultured in DMEM 10% FBS; the glioma cell line H4 in OptiMEM medium (Gibco), supplemented with 10% FBS. To analyze Fractalkine mRNA expression, cell lines were stimulated with TNFα (10 ng/ml), interferon-γ (1000 U/ml) and RNA extracted after 24 hours. For adhesion assays, cell monolayers were stimulated with TNFα (10 ng/ml), interferon-γ (1000 U/ml) overnight and medium replaced before starting with the assay. Finally, to collect supernatants for CX3CL1 measurement, cells were seeded at 10^6 cells/ml in six-well plates in DMEM 10% FBS and cultured for 18 hours before replacing medium and stimulating them with TNFα/IFNγ. After overnight incubation, medium was replaced with serum-free medium and conditioned supernatants collected after 24 hours.

Primary tumors

Table 3.1 reports the origin of the surgical specimens analyzed and the clinico-pathological features of the corresponding patients. Primary tumors from surgical specimens were cut into little pieces with a scalpel and enzymatically digested with trypsin (0.125%) for 2 hours at 37°C, as previously described (25); additional purification on a density gradient (Ficoll) and by adherence on tissue plastic were

performed. Cells were plated on Primaria plates (Falcon, NJ) at a density of $1x10^6$ cell/ml and maintained in DMEM supplemented with 10% FBS. Tumor cells grew as adherent cells with epithelial morphology and were >95% positive for expression of cytokeratine 7, as assessed by intracellular staining with a FITC-labeled mouse anti-human anti-cytokeratin 7 monoclonal antibody (clone CK3-6H5, Miltenyi Biotec, USA). After 24-48 h, cells were prepared for RNA extraction, performed with Trizol (Life Technologies, Inc.), following the manufacturer's instructions.

Patient N°	Age	Sex	Tumor site	TNM Stage	Vessel invasion	Perineural invasion	Lymph node invasion	Size (cm)	Hystology
PK 93	70	M	Head	II	Yes	Yes	No	3.2	Adenocarcinoma
PK 96	76	F	Head	I	Yes	Yes	No	3.6	Adenocarcinoma
PK 97	56	M	Tail	III	Yes	Yes	No	4.3	Adenocarcinoma
PK126	63	M	Head	I	No	No	Yes	2.9	Adenocarcinoma
PK 132	55	F	Head	II	No	Yes	No	3.5	Adenocarcinoma
PK 136	59	F	Head	II	No	No	No	2.7	Adenocarcinoma
PK 135	65	M	Tail	III	Yes	Yes	Yes	4.1	Adenocarcinoma

Table 3.1. Origin of surgical specimens processed to obtain primary tumors. Clinico-pathological features of patients are listed.

PBMC

PBMC were used as internal control for RNA analysis.

To this aim, they were purified by Ficoll-Hypaque density centrifugation. Briefly, buffy coats from healthy donors from the Blood Transfusion Service (Desio Hospital) was diluted 1:1 with sterile saline and centrifuged for 10 minutes at 1000 rpm. After centrifugation, supernatant containing platelets was thrown away and 0.9% Sodium Chloride added till 35 ml. Then, 15 ml of Ficoll-Hypaque (GIBCO, Scotland UK) were laid underneath cell suspension using a syringe with a long needle and centrifuged for 20 minutes at 1750 rpm. The mononuclear cell layer was removed using a sterile pasteur, and washed thoroughly by resuspension in sterile saline. PBMC were then used for RNA extraction.

HUVEC

Human endothelial cells were obtained from umbilical vein (HUVEC) and cultured as described previously (262, 263). Briefly, cells were collected from umbilical cords, rinsed inside with physiological saline solution. A solution of collagenase 1A (SIGMA) was injected into the cord. After a 20 minute incubation at 37°C, the cord was rinsed with medium containing 20% FCS and the collected solution centrifuged. Cells were routinely used confluent at 2^{nd}-6^{th} passage. Cells were maintained in E199 medium with 20% bovine serum, supplemented with endothelial cell growth supplement (100 µg/ml; Collaborative Research Inc, Lexington, MA) and heparin (100 µg/ml; Sigma Chemical Co, St Louis, MO). The purity of EC cultures was checked by expression of von Willebrand factor and found to be greater than 99% positive. HUVEC were used both in transmigration and adhesion assays and stimulated with TNFα/IFNγ for mRNA analysis by Northern Blot.

3.2 Reagents

Where indicated, cell lines were treated with IL-1β (10 ng/ml), TNFα (10 ng/ml), interferon-γ (500 U/ml), all purchased by Peprotech, for 8 hours. Hypoxia was generated by culturing cells for 4 hours in an atmosphere-controlled culture chamber (Bellco Glass) containing a gas mixture composed of 94% N_2, 5% CO_2 and 1% O_2. Cells were cultured in 6-well plates in DMEM 10% FBS. Desferrioxamine (0.4 mmol/L, SIGMA) was used in the same experiment as chemical compound to mimic hypoxia.

AMD3100 (Sigma, **Fig 3.1**) is a byciclam compound (1,4,8,11-tetraazacyclotetradecane octahydrochloride)(264); it is currently used as a CXCR4 chemokine receptor antagonist in HIV patients and for stem cell mobilization from the

bone marrow in transplanted patients. AMD3100 is extremely specific in its affinity for the CXCR4 receptor: this depends at least in part on an electrostatic interaction between the basic (positively charged) nitrogens of the cyclam moieties and the acid (negatively charged) carboxylates of the aspartic acid residues located at positions 171, 182, 193 and 262 of the CXCR4 receptor. AMD3100 does not interact with a variety of chemokine receptors other than CXCR4 (265).

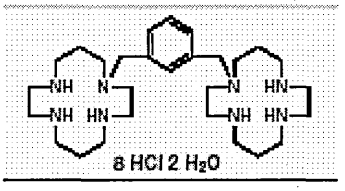


Fig 3.1. Structure of the byciclam AMD3100.

3.3 Methods for analysing mRNA expression

Total RNA extraction from fresh or cultured cells was performed with Trizol (Gibco), following manufacturer's instructions. Three different methods were used to evaluate mRNA expression, depending on the sensitivity required by the analysis. Fractalkine expression in endothelial cells and neuronal cell lines was analyzed by Northern Blot. Chemokine receptors on pancreatic cancer cell lines were previously analyzed by RT-PCR with specific primers, followed by semi-quantitative Real-Time PCR, used to compare cell lines and get eventual difference between their expression levels. Specifically:

Northern Blot

cDNA for CX3CL1 was prepared as previously described (266), and subsequently used for probe labelling. 10 μg of total RNA were subjected to

electrophoresis through a 1 % agarose-formaldehyde gel (1 % agarose [Gibco], 6 % formaldehyde, 20 mM MOPS, 0.5 μg/ml ethidium bromide) then blotted by capillary transfer onto nylon membrane (Hybond N+, Amersham, UK). After transfer, the membrane was heated for 2 hours to 80°C to allow crosslinking. The membrane was placed in a suitable hybridisation tube and pre-hybridised for 1-2 hours at 42 °C with 20 ml of hybridisation buffer (0.2 M sodium phosphate buffer pH 7.2, 100 μg/ml salmon sperm, 7 % SDS 45 % formamide). cDNA probes were labelled by random priming using the Megaprime DNA labelingsystem (Amersham, Buckinghamshire, UK) according to the manufacturer's instructions. Prior to use, the radiolabelled probe was heated to 100 °C for 5 minutes, then quenched on ice for up to 30 min. Probe was then added to 20 ml of hybridisation buffer (approximately 1 x 10^6 cpm/ml of buffer), which was poured on to the membrane in place of the pre-hybridisation buffer. Hybridisation was performed overnight at 42 °C. Following hybridisation, membranes were washed twice with 2 x SSC, 0.1 % SDS for 5 min at room temperature, twice with 0.1 x SSC, 0.1 % SDS for 15 min at 68 °C and finally once with 2 x SSC for 10 min at room temperature. The membrane was then wrapped in Saran wrap and exposed overnight to Kodak Biomax MS film with an intensifying screen, at –70 °C.

RT-PCR

cDNA was synthesized by random priming from 1μg of total RNA with GeneAmp RNA PCR kit (Applied Biosystems), according to the manufacturer's instructions. The following primers were used for the subsequent PCR: human CXCR4 (sense: 5' AGC TGT TGG CTG AAA AGG TGG TCT ATG 3'; antisense: 5' GCG CTT CTG GTG GCC CTT GGA GTG TG 3'); human β-actin (sense: 5' AAG ATG ACC CAG ATC ATG TTT GAG 3'; antisense: 5' GGA GCA ATG ATC TTG ATC TTC 3'). PCR was performed with AmpliTaq DNA Polymerase (Applied Biosystems) following the

manufacturer's instructions. Cycling conditions: 26 cycles (20 cycles for β-actin) of 1 min at 94°C, 1 min at 56°C, 1 min at 72°C. PCR products were resolved by electrophoresis on 2% agarose gels and visualized by ethidium bromide staining.

Real-Time PCR

Real-Time PCR was performed using SYBR Green dye and GeneAmp® 5700 Sequence Detection System (PE Biosystems, Foster City CA). The sequences of primer pairs specific for each gene (Invitrogen) were designed with Primer Express® Software (Applied Biosystems). 2μl of cDNA (obtained from the amplification of 1 μg RNA in a total volume of 50 μl)was used as the template; 12.5μl of 2X SYBR Green PCR Master Mix (Applied Biosystem) was mixed with template and primers. The total reaction volume was 25μl. Cycling conditions were 10 min at 95° C, 40 cycles of 15 s at 95° C and 1 min at 60°. Experiments were performed in triplicate for each sample. mRNA was normalized to β-actin mRNA by subtracting the cycle threshold (Ct) value of β-actin mRNA from the Ct value of the gene (ΔCt). Fold difference (2^-ΔΔCt) was calculated by comparing the ΔCt with either the ΔCt of the cell line HPDE6 or with that of unstimulated cells.

The sequences of primer pairs were as follows: **human CCR2** (sense: 5' CAT CGG TTA TTT TGG CGG AA 3'; antisense: 5' GGT GAC CGT CCT GGC TTT TAA 3'); **human CCR6** (sense: 5' TGC CAC GTG CAA GTT GCT TAA 3'; antisense: 5' AGC AGC ATC CCG CAG TTA AAG 3'); **human CCR7** (sense: 5' TGC ATC AGC ATT GAC CGC TA 3'; antisense: 5' TAT CCA GAT GCC CAC ACA GGA 3'); **human CXCR2** (sense: 5' CAG TCC TTT GGC TTC ATC GTG 3'; antisense: 5' GGT GAA TCC GTA GCA GAA CAGC 3'); **human CXCR4** (sense: 5' CAA GGC CCT CAA GAC CAC AAT 3'; antisense: 5' CCC AAT GTA GTA AGG CAG CCA A 3');

human CX_3CR1 (sense: 5' TGA TTT GGC TGA GGC CTG TTA T 3'; antisense: 5' GGA CAG GAA CAC AGT CCC AAA G 3'); **human β-actin** (sense: 5' TCA CCC ACA CTG TGC CCA TCT ACG A 3'; antisense: CAG CGG AAC CGC TCA TTG CCA ATG G 3').

3.4 Flow Cytometry

Pancreatic cancer cells were removed from flasks non-enzymatically, after three washes and a 10 minute incubation at 37°C with physiological saline. Nearly 300000 cells were then resuspended in ice cold washing buffer (phosphate-buffered saline (PBS) containing 1% human serum) and incubated with 10 μg/ml fluorescin-labelled mouse anti-human CXCR4 antibody (12G5; R&D Systems, Minneapolis, MN) or 3 μg/ml PE-labeled mouse anti-human CX_3CR1 antibody (MBL, Watertown, MA) or antibody anti CX_3CL1 (clone 81506, R&D, Minneapolis, MN, USA) for 30 minutes at 4°C. Cells were analysed with a FACScalibur flow cytometer.

3.5 ELISA assay

CCL2 was measured with antibodies developed in our laboratory; sensitivity of the test was 4 pg/ml. Commercial kit by R&D were used for all the other cytokines; the sensitivity of the assays was as follows: CCL5: 2 pg/ml, CCL22: 1 pg/ml, CXCL8: 4 pg/ml, CXCL12: 4 pg/ml, VEGF: 4 pg/ml, HGF: 16 pg/ml, TGFb: 4 pg/ml, IL1b: 2 pg/ml, IL6: 1 pg/ml, IL10: 4 pg/ml, TNFa: 2 pg/ml. Cells were plated at the same density and medium replaced when 80% confluence was reached; supernatants were collected after 24 hours. At least three different experiments were performed and the median of the values calculated.

To measure CX3CL1 in supernatants, neuroblastoma cells were seeded at 10^6 cells/ml in six-well Costar plates (Corning, Inc.) and cultured for 18 hours before replacing medium and stimulating them with TNFα/IFNγ in DMEM without FBS. After overnight incubation, medium was replaced with serum-free medium and conditioned supernatants collected after 24 hours. The amount of CX3CL1 released in the supernatant after 24 hours was quantified by ELISA with commercial kit (R&D, Minneapolis, MN, USA). At least three experiments were performed and the media ± SE calculated; p value was calculated with Student t Test analysis.

3.6 Immunohistochemical analysis

Paraffin sections of three pancreatic cancer patients (PKwere deparaffinised in xylene (2 x 10 min) and taken down through graded alcohols (100%, 90%, 70%, 5 min each).

Endogenous peroxidase was blocked by adding 100 µl of 3% H_2O_2 (in H_2O) to each section for 30 minutes, in the dark.

Sections were then microwaved in boiling 0.01M sodium citrate pH 6.0 for 10 min to retrieve antigen, and rinsed in 400 ml PBS. The slides were laid out in a humidified box and excess PBS was blotted from around each section using a tissue.

To each section, 100-200 µl primary antibody at appropriate dilution was added to cover the section, specifically, the rabbit polyclonal anti human CX3CR1 antibody was diluted 1:350 in PBS, while the goat anti-human anti CX3CL1 Ig 1:50 in PBS. The slides were incubated for 1 hour in a humidified box at room temperature.

Sections were washed twice in PBS for 3 min, prior to incubate them with 100-200 µl of secondary antibody (EnVision HRP rabbit/mouse, DakoCytomation).

After washing, the peroxidase substrate 3,5-diaminobenzidine (DAB; Liquid DAB + Substrate Chromogen System, DakoCytomation) was prepared, by diluting one drop in 500 µl of buffer and incubated for 5 minutes with the slides.

Sections were washed in distilled water and counterstained for 2 seconds in Hematoxilin (Mayer, DIAPATH) diluted 1:5 in H2O.

The slides were dipped in distilled water and dehydrated through graded alcohols (70%, 90%, 100%, 2 min each).

3.7 Methods for analysing cell migration

Chemotaxis

Many different assays are available to measure and quantify the process of *in vitro* cell migration; they basically depend on the principle that chemokine signals transmitted through their GPCR cognate receptors provide directional cues and stimulate an enhanced rate of cell locomotion. These in vitro assays have been designed to reflect the guiding principle and the driving force of leukocyte migration from the blood to the tissues and in the extravascular tissues. Mechanistically, this entails the ability of chemokines to activate leukocyte integrins and to convert the initial loose, rolling interaction of leukocytes with the endothelial cells into firm adhesion and spreading.

The method of chemotaxis is based upon active migration of test cells through a filter with pores of a precise size. The filter is placed in a chamber to create two compartments, as originally introduced by Boyden (267). Cells are added to the upper compartment, whereas the lower compartment is filled with the chemotactic substance (**Fig 3.2**).

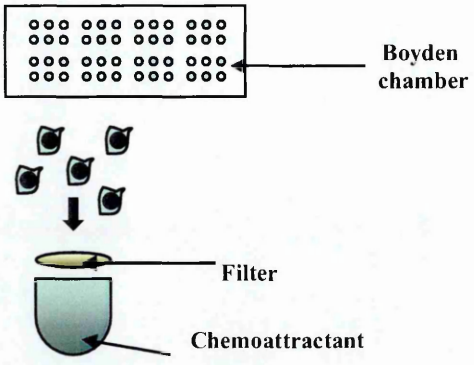


Fig 3.2 Chemotaxis assay with a modified Boyden chamber.

As a consequence, a chemotactic gradient is created and cells penetrate through the pores of the filter to the lower compartment. The number of migrated cells indicates the potency of the chemotactic substance. A commercially available commonly used device is the 48-well chemotaxis chamber (the so called modified Boyden Chamber, Neuroprobe), which allows to test different cells and chemoattractant in the same assay. The microchamber consists of a top and bottom acrylic plate, sealed by a silicon gasket. The upper wells, containing cells are separated from the lower wells (containing chemoattractant) by a micropore membrane. The filter separating the two chamber compartments can vary; depending on the cell type, different filter materials and pore sizes should be used. Cellulose ester filters allow to measure the migration distance into the filter, whereas polycarbonate membranes are used to determine the number of cells migrated through the pores. Usually, PVP (polyvinyl pyrrolidone)-pretreated membranes are used, while for cells displaying reduced adhesion, PVP-free membranes can be used whose lower surface has been coated with matrix proteins (collagen, fibronectin, gelatin). Different size pores are available, ranging from 3 to 13 μm. At the end of the assay, whose time can vary depending on cells (1h to overnight), filters are fixed and stained. Usually the final result is calculated from the average counts of at

least three wells. The chemotactic activity can be expressed either as **chemotactic index** (percentage of the maximal number of cells migrated to the control chemoattractant (e.g. assay medium)) or as **net migrated cells** (the number of cells migrated to the control medium is subtracted from the number of cells migrated to the chemoacctractant). As expected, all the assays used to measure chemotaxis have been set up for different leukocyte populations, therefore established protocols are available. As cancer cell chemotaxis is a relatively recent acquisition, we have adapted a classical protocol to our purposes (**Protocol 1**). Basically, larger pores and longer migration time have been required for cancer cells to get an appreciable migration.

Protocol 1: Chemotaxis

1. resupend cells in appropriate medium (DMEM, 1% FCS) and dilute to 700000 cells/ml;
2. prepare serial dilutions of chemoattractant in the same medium;
3. add 28-30μl of the chemoattractant to the lower compartment of the microchamber;
4. put the filter (13μm pore for Hs766T, 8μm pore for the other cell lines) on the bottom plate and reassemble the microchamber;
5. add 50 μl of the cell suspension to each well (corresponding to 20000-40000 cells);
6. incubate chamber at 37°C in a 5% CO_2 incubator for the time required (Overnight for Hs766T, 8h for the other cell lines)
7. dismount the microchamber unit, wet the non-migrated cell side of the membrane with PBS and wipe the cells off this filter side;
8. fix the cells which adhere to the lower surface of the membrane with 70% methanol, dry and stain with Diff-Quick;
9. place the membrane on a microscope slide to dry and count cells at 400X magnification, in 10 oil immersion fields for each well.

For each cell line, I standardized migration conditions and applied minor modifications. In particular, filters were always coated with fibronectin (5 μg/ml, Sigma); pore size ranged from 13μm to 8 μm ,depending on the cell line properties. Chemokines used as chemoattractants in the lower compartment were purchased from Peprotech, (Rocky Hill, NJ). Net migrated cells over control cells were counted in ten microscope high power fields (magnification:x1000). At least eight spots were counted for each

experimental group; values are the mean ± SE of three different experiments and p value calculated by Student T Test. Where indicated, cells were incubated with a blocking anti-CXCR4 mAb (12G5, R&D; 10μg/ml), or with AMD3100 (Sigma, 1μg/ml) or anti-CX3CR1/CX3CL1 mAb (polyclonal, Torrey Pines, Houston, TX and clone 81506, R&D, respectively).

Transmigration assay

Human endothelial cells were grown to confluence on polyvinylpyrrolidone (PVP)-free polycarbonate filters (12 μm pore) and mounted on Boyden chambers over a second filter (**Fig. 3.3**).

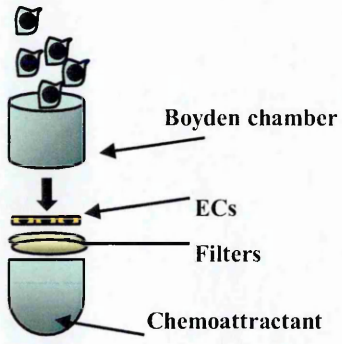


Fig 3.3 Transmigration assay with a Boyden chamber. Endothelial cells are grown on the filter.

^{51}Cr-labeled tumour cells were seeded in the upper compartment and coincubated with endothelial cells monolayers for 16 hours at 37°C. Nonadherent cells were gently washed away and adherent cells were removed with a cotton swab. The radioactivity in the double filter and in the lower compartment referred to transmigrated cells. The adherent cells were considered to comprise both cells bound to endothelial cells as well as those that had transmigrated. Values are the mean ± SE of three different experiments and p value calculated by Student T Test.

Invasion Assay

Cell invasion protocol was a kind gift of Dr. Maura Poli (Mario Negri Institute Bergamo, Italy); briefly, it was examined using a 48 well modified Boyden chamber and a reconstituted extracellular matrix membrane (Matrigel, Becton Dickinson) (**Fig. 3.4**).

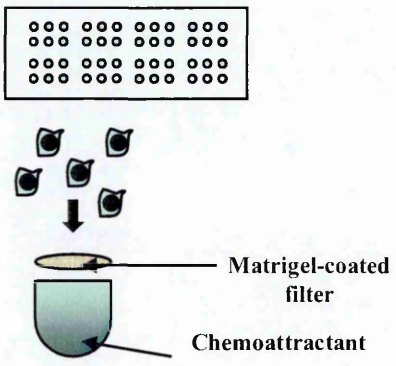


Fig 3.4 Invasion assay with a modified Boyden chamber. A Matrigel layer is deposed on top of the filter.

Cell invasion chambers were prepared by carefully placing onto a polycarbonate PVP-free filter 10 μl of Matrigel (0.5 μg/ml) and incubating at 37°C for 30 minutes to allow Matrigel polymerization; then 45 μl of cell suspension was added to each well and incubated at 37°C overnight. Migrated cells were evaluated as for the chemotaxis experiment.

3.8 Gelatinase activity assay

The activity of selected metalloproteases in pancreatic cancer cell and monocyte supernatants was assessed by using a MMP Gelatinase Activity Assay Kit (Chemicon, International, Inc.), according to manufacturer's instructions. **Fig 3.5** illustrates the test principle. Briefly, the kit utilizes a biotinylated gelatin substrate, which is cleaved by active MMP-2 and MMP-9 (gelatinase) enzymes. Remaining biotinylated fragments are

then added to a biotin-binding 96-well plate and detected with streptavidin-enzyme complex. Addition of enzyme substrate results in a colored product, detectable by its OD (450 nm). The activity detected in the supernatants can be easily quantified by comparison with a MMP-2 positive control, (APMA-activated human MMP2 enzyme). This kit provides a quick and sensitive system for evaluating the gelatinase activity in cell supernatants and other biological fluids. Moreover, unlike traditional zymography, this assay measures MMP activity in solution, which is often different than the apparent activity observed on zymographs, where the MMP enzymes are physically separated from their natural inhibitors. Pancreatic cancer cell lines were cultured and stimulated with CXCL12 100 ng/ml for 24 hours and supernatants collected for the MMP assay. Values are the mean ± SE of three different experiments performed. P value was calculated by Student T Test.

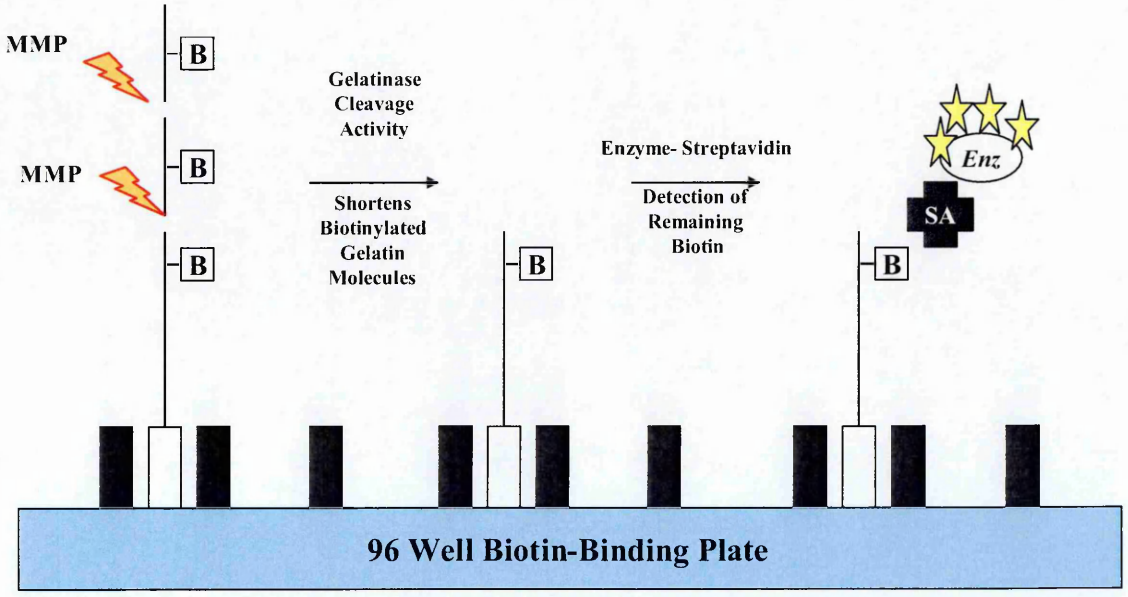


Fig 3.5. MMP activity assay. Gelatinase activity in supernatants is measured by assessing their capability to cleave a biotin-labeled gelatine substrate.

3.9 Adhesion assay

Adhesion of tumour cells to neuroblastoma cell monolayers was studied as described previously, with minor modifications (263, 268). Neuroblastoma cells were grown to confluence in flat-bottomed 6-well plates. ^{51}Cr-labeled tumour cells (Amersham, UK) were coincubated with neuroblastoma monolayers at 37°C for 1 hour in DMEM 1% FBS, under slow agitation, to prevent aspecific attachment. At the end, nonadherent cells were washed away and adherent cells were solubilized with 1 mL of 0.1% sodium dodecyl sulfate and radioactivity was counted in a gamma counter. Results represent the percent of adherent cells ± SE of three replicates/group. P value was calculated by Student T test.

3.10 Methods for analysing cell proliferation and apoptosis

CFSE dilution assay

Proliferation was measured in vitro using the vital dye 5-(and-6)-carboxyfluorescein diacetate succinimidyl ester, mixed isomer (5-(6)-CFDA, SE [CFSE], Molecular Probes, Inc., Eugene, OR); this method is currently used to investigate lymphocyte proliferation and based on the dilution of the dye incorporated, upon serial cell division (269, 270). Briefly, cells were labelled with CFSE (0.5 μM) before seeding in 12-wells plates. Stimuli were given after 12 hours of resting in medium without serum. After 3 days, cell division was indicated by decreased CFSE fluorescence intensity, as assessed by flow cytometry. At least three different experiments were performed and one representative reported.

Cell Cycle Analysis

For cell cycle analysis, cells were seeded in 12 well plates and let adhere for 12 hours, after which medium was replaced with DMEM 1% FBS. After 72 hours, they were collected, washed once with PBS and fixed with 70% ethanol at -20°C for 24 hours. Fixed cells were washed three times with PBS and incubated for 1 hour with a Propidium Iodide (PI) solution (Sigma Chemical Co.; 20μg/ml) containing RNAase A (Sigma Chemical Co.; 0.1 μg/ml). Cells were then subjected to cell cycle analysis for determining DNA contents by flow cytometry. Cell debris were excluded on the basis of forward versus side scatter. Doublets and clumps were excluded by gating on a bivariate distribution of AUX (PI peak pulse) versus the PI integrated signal. Data from 10,000 events were collected in the final gated histograms. The cell histogram was divided into 3 regions according to cell cycle phase: G0/G1, G2/M and sub-G1 peak (PI fluorescence from fractional DNA content of apoptotic cells), which defined the proportion of apoptotic cells. At least three different experiments were reported and one representative was reported.

Apoptosis Assay

Staining with Annexin V is the method to evaluate earlier apoptotic events, such as loss of plasma membrane asymmetry.

The percentage of cells undergoing apoptosis was determined using fluorescein-conjugated human annexin-V (Pharmingen, BD Biosciences). Cells were plated in 12-well plates and cultured for 18 hours. Then they were incubated for 24 hours under apoptosis-inducing conditions (serum deprivation) with or without CXCL12 (100 ng/ml). Cells were collected and stained at room temperature in the dark for 15 minutes in 200 μl buffer containing FITC-annexin-V (5 μl); after incubation they were subjected

to FACS analysis. Annexin-V^+ cells correspond to apoptotic cells. Values are the mean ± SE of three different experiments performed.

3.11 Stable infection with a CX_3CR1-GFP viral vector

A viral vector carrying the sequence coding for human CX_3CR1 fused to the sequence for the reporter gene GFP was prepared. A former plasmid vector was generated and tested and subsequently inserted into the viral vector.

Plasmid encoding the CX3CR1-GFP fusion protein preparation

Human CX_3CR1 cDNA (encoding the Ile^{249}-Met^{280} variant) subcloned in the mammalian expression vector pCDNA3 was a kind gift of Philippe Deterre (Pasteur Institute, Paris, France). To generate the fusion protein CX_3CR1-GFP, the pEGFP-N1 vector and CX_3CR1 cDNA were prepared by digestion with restriction enzymes to generate complementary ends. Briefly, a forward primer was appositely designed to be specific to the 5' end of CX_3CR1 cDNA and HindIII tailed; by the same way, a reverse primer specific for the 3' end was designed and BamH1 tailed. The vector was amplified by PCR with the primers, digested with HindIII/BamH1 and subcloned in the mammalian vector pEGFP-N1 (BD Biosciences, Clontech, Milan, Italy) therefore codifying for a chimeric protein CX_3CR1-GFP.

After ligation of the foreign DNA and pEGFP-N1 with the enzyme DNA ligase, the resulting vector was introduced into bacterial cells (DH5α) by transformation and cells containing foreign DNA selected by screening for kanamycin.

A receptor-negative pancreatic tumor cell line, MiaPaCa2, known to grow in nude mice, was then transiently transfected with the construct and checked for transgene

expression by FACS analysis; both GFP and CX_3CR1 expression were analysed, the latter by PE-labeled anti human CX_3CR1 antibody.

Viral vector construction

The generated CX_3CR1-pEGF-N1 vector was expanded by transformation of bacterial cells and purified with Marligen Columns (Marlingen, Heidelberg, Germany), yielding endotoxin free DNA. Final concentration was approximately 1 μg/μl. The viral vector (pRRLsinPPT.CMV.GFPpre) was then kindly prepared by Maria Luisa Malosio in collaboration with Naldini's group (San Raffaele Hospital, Milan). Briefly, 293T cells, selected as good recipients of DNA, are cotransfected with four plasmids (each carrying a sequence for a viral particle component: two 3rd generation core packaging plasmid, a Self Inactivating transfer vector plasmid, an Envelope plasmid and the plasmid CX_3CR1-pEGF-N1). This passage allows the rescue of the recombinant HIV genome with the gene of interest, CX3CR1-GFP and packaging into viral particles. The calcium-phosphate precipitation method is used to transfect cells. After 16 hours, medium is replaced with a fresh one to begin virus collection; after 48 hours, supernatant, containing viral particles, is collected and concentrated by ultracentrifugation; the final pellet is resuspended in a very small volume (1/500 of the starting volume of medium), splitted into small aliquots and stored at -80°C. The subsequent passage is titration of the lentiviral vector: Hela cells are plated and ten-fold dilutions of the viral stock are added. After 72 hours, cells are harvested and analyzed by flow cytometry to calculate the titer. The infectivity of the vector preparation is calculated by the ratio of transduction units (TU)/ml and should be $>10^4$. TU/ml value is calculated by (N° of cells analyzed) x (% of cells GFP+) x (10^N), N=(-) viral dilution factor (see (271-274) for reference). After titration, the appropriate amount of vector ($3x10^6$ TU in 2 ml medium) was used to infect MiaPaCa2 cells. Due to the high efficiency of

infection procedure, no selection was required and CX_3CR1-GFP MiaPaCa2 almost 99% pure were obtained and used for experiments.

Aim of the Study

In the last two decades there has been increasing evidence for a role of chemokines in tumour biology. Tumours constitutively produce chemokines, which have been found to exert a variety of biological activities, including leukocyte recruitment, promotion or inhibition of angiogenesis, activation of matrix metalloproteases, growth promoting activity and inhibition of apoptosis. While the significance of chemokine expression in human and experimental tumours has been the object of intense research activity, the expression of chemokine receptors on tumour cells has been investigated to a lesser extent. The hypothesis that tumour cells may use chemokines to determine metastatic destinations has recently begun to be investigated. If this hypothesis is correct, a corollary is that the expression of chemokine receptors in tumour cells could not be random, but selective chemokine receptors might be expressed, which recognize ligand chemokines present in high amounts at the site of metastasis

Human pancreatic adenocarcinoma is a highly aggressive, early metastatic disease; at the time of diagnosis, more than 80% of the patients show tumours locally extended beyond the pancreas and metastases in regional lymph nodes. Peculiar of this tumour is its dissemination to peripheral nerves. Surprisingly, in spite of the clinical importance of this process, the molecular events driving the tumour cell spreading are only in part understood.

The principal aim of this thesis was to investigate the role of chemokines and their receptors in pancreatic cancer and to understand if receptors and their ligand chemokines are involved in tumor dissemination.

Specifically, I have extensively analyzed the expression of a number of chemokine receptors on tumor cell lines of pancreatic adenocarcinoma and on freshly

isolated tumour cells obtained from surgical specimens. Later on, I have concentrated my efforts on the significance and biological role of receptors/ligands in this tumor. The process of metastasis involves the escape of tumor cells from the primary mass via lymphatic and blood vessels, transport to and arrest in a target organ and growth of metastasis in the target organ; all of these steps are strictly regulated by chemokines. Therefore, I have tried to investigate whether chemokines and their receptors could affect each of these processes. I have performed in vitro studies of adhesion of tumor cells to endothelial cell monolayers, transendothelial migration assays and invasion assays through Matrigel-coated filters. Finally, I have investigated whether selected chemokines can increase resistance to apoptosis in injured tumor cells or regulate their cell cycle progression.

After a preliminary biological characterization of eleven pancreatic adenocarcinoma cell lines, focused on molecular properties and capability of releasing cytokines and chemokines, I screened a panel of chemokine receptors on these tumor cell lines and also on freshly isolated tumor cells from pancreatic adenocarcinoma surgical specimens.

CXCR4 emerged as the most expressed receptor in human pancreatic adenocarcinoma, I have focused on the role of this chemokine receptor in pancreatic tumor cells. mRNA expression, regulation and functional activity have been analyzed with available in vitro assays.

The initial screening has revealed that some pancreatic cancer cell lines and freshly isolated tumor cells express also the chemokine receptor CX3CR1. Given the peculiarity to disseminate along nerve fibers, I have tested the hypothesis that the chemokine Fractalkine/Neurotactin, highly expressed in neuronal tissues, and its

receptor CX3CR1 expressed by tumor cells, are involved in the peculiar tropism of pancreatic adenocarcinoma cells for neural tissues. After mRNA analysis on cell lines and primary tissues, the functional role of the receptor has been investigated. Moreover, the CX3CR1 receptor has been successfully infected into a CX3CR1-negative cell line and functional assays are being performed with CX3CR1- and parental cell line. Specifically, I'm going to use CX3CR1-transfected tumout cells in *in vivo* experiments aimed at investigating the role of CX3CR1 in the growth and metastatic potential of pancreatic cancer.

The obtained results reveal for a role of chemokines and their receptors in pancreatic cancer cells migration and growth; these finding contributes to a better understanding of the biology of this carcinoma and raises the possibility that the system of chemokines/receptors may be a valuable therapeutic target.

RESULTS

Chapter 4

Preliminary characterization of pancreatic adenocarcinoma cell lines and isolation of pancreatic tumour cells from surgical specimens

4.1 Collection and characterization of pancreatic cancer cell lines

During my project, I have based the study of cell biology of ductal adenocarcinoma of the pancreas on *in vitro* investigations, employing various stable pancreatic ductal carcinoma cell lines (PDCL), commonly available through cell culture collections. The use of cell lines is necessary to obtain information on a homogeneous population of pancreatic tumour cells that are common to researchers worldwide. However, the various cell lines can reveal a great deal of diversity, so that generalized interpretation of results needs to be viewed cautiously. Therefore, the characterization of PDCL used in *in vitro* experiments has been a necessary preliminary step of the project.

The molecular and biological analysis of 11 established cell lines is shown in **Table 4.1**. Most cell lines (9/11) were grade G2/G3 and only 3 G1.

Cell Line	Source	Grade	In vitro grade	Tumorigenic (nude mouse)	Transplanted tumor grade
Panc-1	**Primary tumor**	**G3**	**G3**	**Yes**	**G3**
MiaPaCa2		**G3**	**G3**	**Yes**	**G3**
Paca44		**G2**	**Nt**	**Yes**	**G2/3**
PT45		**G3**	**G3**	**Yes**	**G3**
Capan-1	**Metastasis**	**G1**	**G1**	**Yes**	**G1**
CFPAC		**G1**	**Nt**	**Yes**	**?**
Hs 766T		**G2**	**Nt**	**Yes**	**?**
T3M4		**G2**	**G3**	**Yes**	**G2/G3**
AsPC-1	**Ascites**	**G2**	**G2**	**Yes**	**G1/G2/G3**
HPAF		**G1**	**G2**	**Yes**	**G1**
A8184		**G2**	**G2**	**Yes**	**G2**

Table 4.1. Biological analysis of 11 human pancreatic adenocarcinoma cell lines. For each cell line, source, grade, in vivo tumorigenicity and grade of trasnplanted tumour are reported.(A*dapted from* (26))

All the cell lines expressed many of the typical genetic lesions which represent the molecular profile of pancreatic adenocarcinoma (7, 8), including mutation in KRAS, p53, p16 SMAD4/DPC4 (**Table 4.2**).

Cell Line	K-ras	p53	p16	DPC4/smad
Panc-1	+	+	+	-
MiaPaCa2	+	+	+	-
Paca44	+	+	+	-
PT45	+	+	+	-
Capan-1	+	+	+	+
CFPAC	+	+	+	+
Hs 766T	?	+	?	+
T3M4	+	+	+	-
AsPC-1	+	+	+	+
HPAF	+	+	+	-
A8184	+	+	+	-

Table 4.2. Molecular profile of pancreatic adenocarcinoma cell lines. All the cell lines express many of the typical genetic lesions representing the molecular profile of pancreatic adenocarcinoma (275, 276).

I next focused my attention on the release of immunoregulatory molecules, i.e. cytokines, angiogenic and growth factors, chemokines. In fact, self-sufficiency of growth-promoting factors and release of immuno-modulatory and pro-angiogenetic factors are hallmarks of the pathogenesis of pancreatic cancer. Tumour cell lines cultured for 24h under standardized conditions have been tested for the ability to secrete soluble factors using ELISA. I have measured the following molecules:

- chemokines: CCL5, CCL2, CXCL8, CXCL12, CCL22;

- cytokines: IL-6, IL-10, TNFα, IL-1β;

- pro-angiogenetic and growth-promoting factors: VEGF, TGFβ, HGF

A summary of the results is reported in **Table 4.3** and **Table 4.4**.

Cell Line	CCL2	CCL5	CCL22	CXCL8	CXCL12
Panc-1	2290	107	0	0	28
MiaPaca2	0	847	20	20	24
Paca44	0	91	70	0	24
PT45	2290	27	0	480	19
Capan1	970	107	60	10	17
CFPAC	0	14	20	40	27
Hs766T	580	n.t.	20	0	1326
T3M4	0	n.t.	1200	130	28
AsPC1	0	n.t.	20	0	59
HPAF	0	0	20	0	27
A8184	n.t.	42	n.t.	n.t.	28

Table 4.3. Chemokine production by human pancreatic adenocarcinoma cell lines. CCL2, CCL5, CCL22, CXCL8, CXCL12 were measured by ELISA. Results are expressed as pg/ml for tumour cell monolayers (24h culture). *n.t.* not tested (A*dapted from* (26)). Data are the median of three different experiments.

VEGF, CCL2, CCL5 and TGFβ are the more frequent factors released by pancreatic cancer cell lines; less frequent is the secretion of CXCL8, CCL22, IL-6 and the secretion of CXCL12, IL-10 and HGF. TNFα and IL1β are always undetectable. Some of these tumor-derived factors, including VEGF, TGFβ, IL-10 and IL-6 have immunomodulatory effects and have been well described as relevant in pancreatic cancer progression, inducing angiogenesis, metastasization and stroma reaction (40). Moreover, chemokines may influence the extent and phenotype of the leukocyte

infiltrate within tumour mass but also may have multiple effects on tumor growth, angiogenesis and metastasis.

Cell Line	VEGF	HGF	TGFβ	IL-1β	IL-6	IL-10	TNFα
Panc-1	140	0	40	0	0	0	0
MiaPaca2	60	0	40	0	0	0	0
Paca44	110	310	180	0	0	0	0
PT45	1070	0	90	0	530	0	0
Capan1	190	0	30	0	40	0	0
CFPAC	90	0	260	0	600	40	0
Hs766T	0	0	0	0	0	80	0
T3M4	90	0	0	n.t.	90	0	0
AsPC1	70	0	0	0	0	0	0
HPAF	40	0	40	0	0	0	0
A8184	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.

Table 4.4. Secretion of soluble factors by human pancreatic adenocarcinoma cell lines. Inflammatory cytokines (IL-1β, IL-6), IL-10, pro-angiogenetic factor (vascular endothelial growth factor (VEGF)), growth promoting factor (transforming growth factor β (TGFβ)), hepatocyte growth factor (HGF) were measured by ELISA. Results are expressed as pg/ml for tumour cell monolayers (24h culture). *n.t.* not tested . (A*dapted from* (26)). Data are the median of three different experiments.

4.2 Primary tumour isolation

It is known that cell lines may display different cell behaviour with respect to the original tumor and in order to overcome this problem, the use of freshly isolated pancreatic cancer cells in short-term culture (primary cultures) may represent a valid

alternative. It may be a more representative model of *in vivo* conditions compared to long standing cell lines. Therefore, I have tried to isolate pancreatic tumor cells from surgical specimens of resected patients. Due to the high stromal reaction characterizing pancreatic adenocarcinoma tumor, surgical samples are generally enriched in fibroblasts, leukocytes and non-tumoral cells, which make tumor cell isolation very hard. Moreover, leukocyte contamination may be very confusing in the type of analysis I was udertaking, as they express chemokines and their receptors in very high amounts.

To this aim, I have set up a method to isolate the minority of tumor cells from the bulk of stromal cells. Tumour is cut in little pieces and subjected to mechanical agitation with trypsin (0.125%); this way neoplastic ducts detach from the rest of the tissue in groups of cells which can be separated on a density gradient (Ficoll). The obtained cells (usually 90% tumour cells and 10% fibroblasts) are further purified by adherence on tissue plastic, by taking advantage of the strong adherence of fibroblasts. They can be either immediately lysed for RNA extraction or stained with antibodies.

CK7 is a specific marker of epithelial cells which is not present on fibroblasts (277, 278). To assess the purity of pancreatic tumor ducts preparations, I stained cells with an anti-cytokeratin 7 antibody and performed a FACS analysis. Most all the cells were Cytokeratin-7 positive. Two representative profiles are shown in **Fig 4.1**.

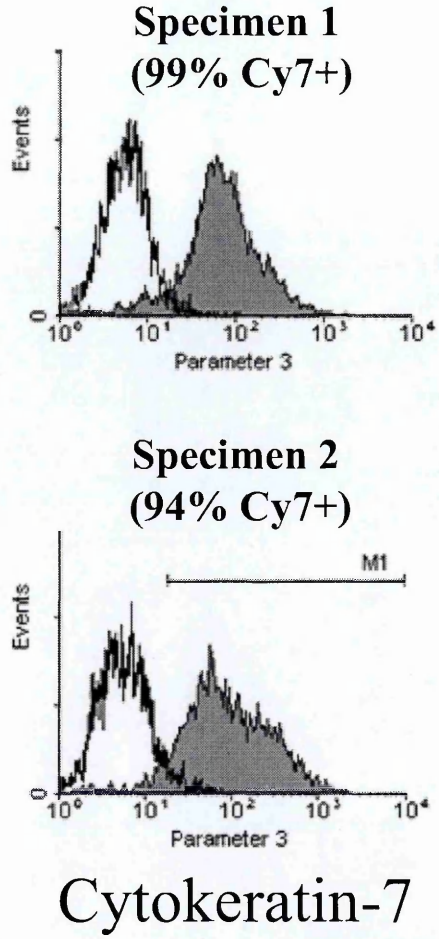


Fig. 4.1. Cytokeratin expression in pancreatic cancer tumor cells from surgical samples. Two surgical specimens (PK93 and PK96, see **Table 3.1** for clinico-pathological features) are represented of at least 8 analyzed; 94-99% of cells are Cytokeratin-7-positive.

4.3 Chemokine receptor overview

Recent evidence demonstrate that tumour cells themselves express chemokine receptors, which possibly supports tumour cell survival and invasion. Therefore, it seemed interesting to investigate the expression of a number of chemokine receptors in human pancreatic adenocarcinoma cell lines

RNA was extracted by tumour cell lines grown in monolayers and subjected to analysis by Real-Time PCR for the expression of different chemokines receptors. A first

experiment was designed to obtain an overview of the chemokines receptors expressed by tumour cell lines. To this aim, we evaluated CCR2, CCR6, CCR7, CXCR2, CXCR4 and CX_3CR1. The chemokine receptor CXCR4 was expressed in more than half of cell lines (6/11), in some of which it was present in high amounts. Also CX3CR1, the chemokine receptor binding Fractalkine was expressed in 6/11 cell lines, although in lower quantity. The chemokine receptor CCR7 was expressed in 4 cell lines and CCR6 was significantly expressed only in two cell lines. CCR2 and CXCR2 were not significantly expressed. **Table 4.4** summarizes these results.

	CCR2	CCR6	CCR7	CXCR2	CXCR4	CX3CR1
PanC1	-	-	-	-	-	+
MiaPaCa2	-	-	-	-	-	-
PaCa44	-	-	-	-	-	-
PT45	-	-	-	-	-	-
Capan1	+	+	+	+	+	+
CFPAC	-	-	-	-	+	-
Hs766T	-	-	+	-	++	+
T3M4	-	-	+	-	+	-
AsPC1	-	+	+	-	+	+
HPAF	-	-	-	-	+	+
A8184	-	-	-	-	+	+

Table 4.4 Chemokine receptor analysis in eleven pancreatic tumor cell lines. mRNA extracted by cell lines was analysed by Real-Time PCR with specific primers. Chemokine receptor amount, normalized to the housekeeping gene β-actin, was expressed as a fold increase over the cell line with the lowest expression; fold below 10 was considered negative (-), fold above 10 was considered positive (+); (++) indicates fold above 1000.

After this preliminary analysis, we decided to focus our attention on CXCR4 and CX_3CR1 and their role in pancreatic cancer progression.

I will discuss these results more in detail in the following Chapters.

Chapter 5

CXCR4 in Pancreatic Adenocarcinoma

5.1 Introduction and goal

This chapter will focus on the expression and function of the chemokine receptor CXCR4 and its chemokine ligand CXCL12 in human pancreatic adenocarcinoma. My preliminary experiments clearly indicated that CXCR4 was the most frequently expressed among the chemokine receptors tested. Therefore, the aim of the experiments I am going to present was to gain some insight into the possible role of this chemokine receptor in tumor spreading. Metastasis is a complex multi step process in which migration to a distant site is only one of the passages; indeed metastatic tumor cells may also have a variety of properties endowing them with tissue invasion and growth ability; I have therefore extensively analyzed possible functions exerted by CXCL12 in pancreatic cancer cell lines, trying to reproduce in vitro the main steps involved in metastasis.

5.2 *CXCR4* mRNA analysis

CXCR4 expression in pancreatic tumor cell lines

I first evaluated the expression of *CXCR4* by RT-PCR in eleven established PDCL. *CXCR4* mRNA expression was clearly detected in six out of eleven lines, with different amounts of *CXCR4* transcripts; in particular, Hs766T showed a very high expression, quite comparable to the expression in normal PBMCs, used as positive control (**Fig 5.1**).

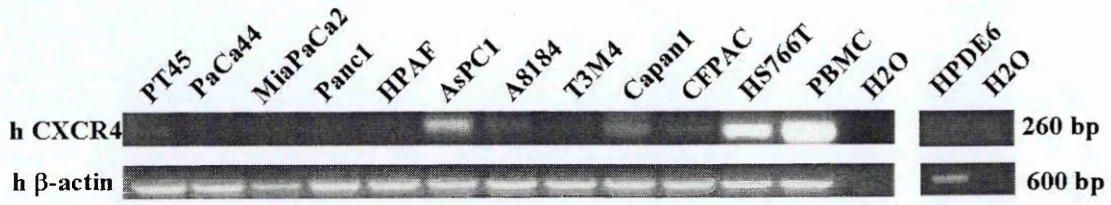


Fig 5.1 CXCR4 in pancreatic cancer cell lines. RT-PCR expression of *CXCR4* and *β-actin* mRNA in human pancreatic adenocarcinoma cell lines and in the immortalized epithelial cell line HPDE6, derived from normal human pancreatic ducts. Human PBMCs were used as positive control. Reaction was stopped at 26 cycles for CXCR4 and at 20 cycles for β-actin. One representative analysis of three performed is reported.

I tested the expression of *CXCR4* mRNA also in an immortalized epithelial cell line derived from normal human pancreatic ducts (HPDE6). This cell line has been previously shown to maintain the phenotypic and genotypic characteristics of normal human pancreatic ducts (260). HPDE6 showed no detectable expression of *CXCR4* transcripts.

To better appreciate the differences between cell lines, the expression of *CXCR4* mRNA was evaluated by semi quantitative Real Time PCR; the cell line HPDE6 was used as a reference. This second analysis confirmed the expression of *CXCR4* in the six positive tumor cell lines (at least 20-fold compared with HPDE6 cells). The cell lines Hs766T, AsPC1 and Capan1 showed the highest expression (1165-, 122- and 86-fold, respectively (**Fig 5.2**).

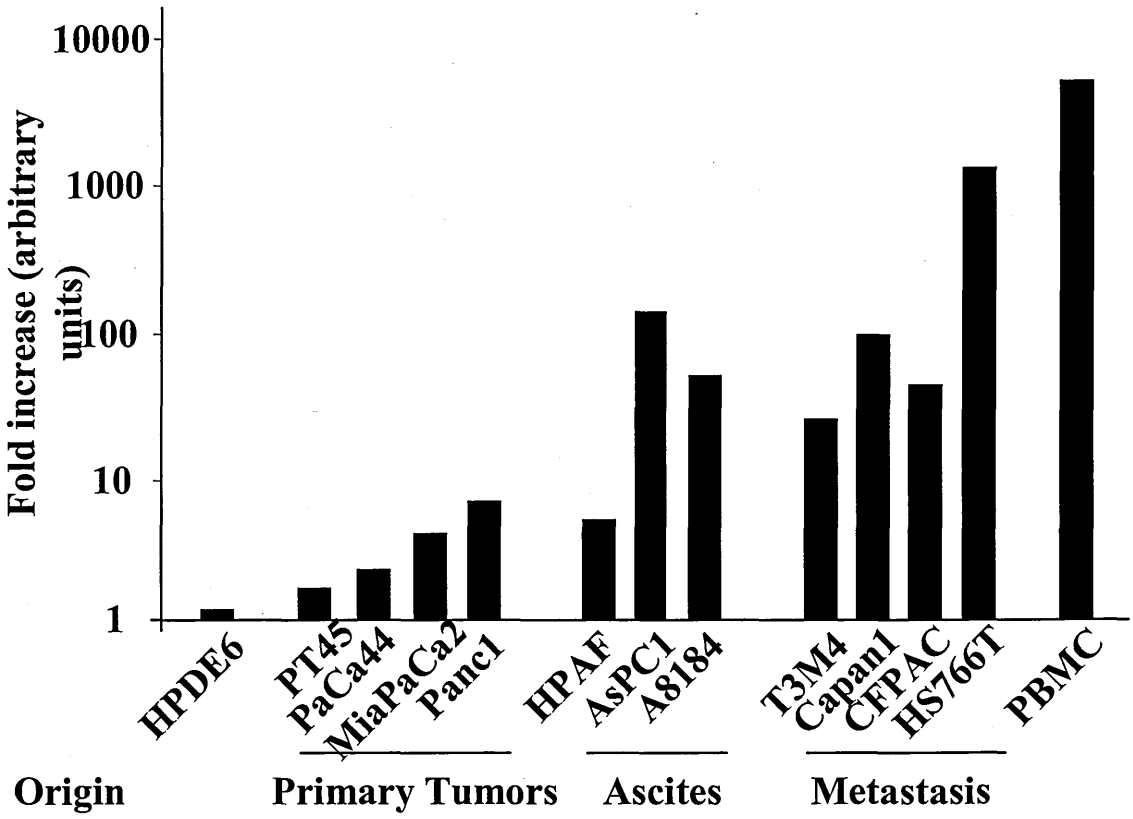


Fig 5.2 CXCR4 in pancreatic cancer cell lines. Semi quantitative Real-Time PCR was used to better appreciate differences between cell lines. For each cell line, the amount of *CXCR4* mRNA, normalized to *β-actin*, is expressed as relative to the cell line HPDE6. One representative analysis of three performed is reported.

This second analysis proved to be very important and revealed that, interestingly, *CXCR4* is expressed at higher levels in cell lines originating from metastatic or ascitic lesions, compared to cell lines derived from primary tumors.

Regulation of *CXCR4* expression in pancreatic tumor cell lines

Cytokines are potent modulators of chemokine receptor expression and are frequently present in the tumor microenvironment. Also some tumor cells, as I discussed in Chapter 4, are able to secrete cytokines and chemokines. I was therefore

interested in evaluating the effect of some inflammatory or immunomodulatory cytokines on *CXCR4* expression in pancreatic tumor cells. RNA was analyzed after cell stimulation with IL-10, IL-6, HGF, IFNγ, IL-1β and TNFα . Modulation by HGF is of interest as it has been demonstrated that HGF increases the expression of chemokines by papillary carcinoma of the thyroid (279). In two cell lines tested (AsPC1 and H766T), IL-10, IL-6, HGF, and the combination of IL-1β and TNFα did not modify *CXCR4* expression (not shown), while IFNγ consistently down regulated *CXCR4* transcript (**Fig 5.3**).

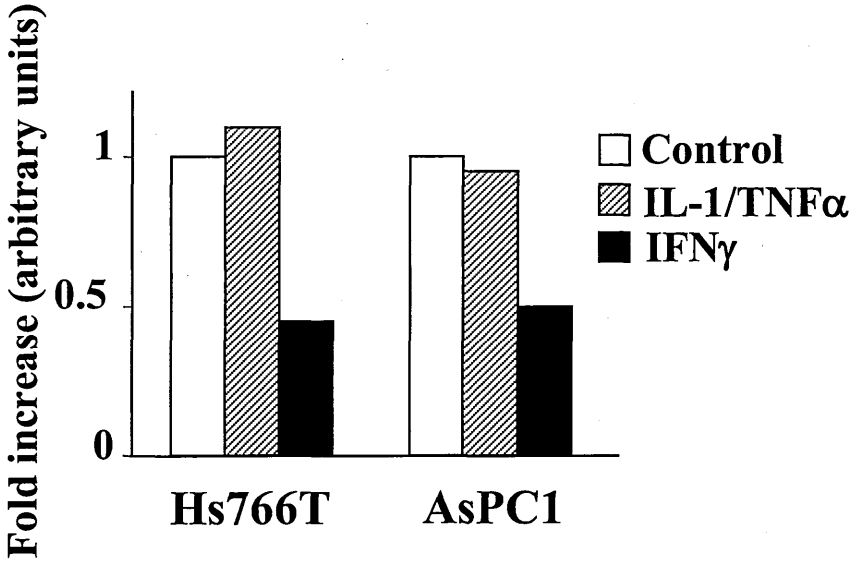


Fig 5.3 *CXCR4* modulation. Regulation of *CXCR4* mRNA by inflammatory cytokines in cell lines was assessed by Real-Time PCR. Cells were treated for 8 hours with a combination of TNFα (10 ng/ml) and IL-1β (10 ng/ml) or IFNγ (500U/ml). One representative analysis of two performed is reported.

Regulation of *CXCR4* expression by Hypoxia

Recently, our group (280) demonstrated that hypoxia, a low oxygen tension condition frequently present in tumoral necrotic areas, regulates the expression of

CXCR4 in monocytes as well as in ovarian cancer cells. Hypoxic conditions can be reproduced in vitro by colturing cells in an atmosphere-controlled culture chamber containing a gas mixture composed of 94% N_2, 5% CO_2 and 1% O_2. I tested the effect of hypoxia in pancreatic cancer cell lines and found that, in line with the previous results, hypoxia up regulated *CXCR4* mRNA in pancreatic cancer cell lines (**Fig 5.4**). As the use of the culture chamber implies some manipulation, an internal control is usually recommended; we used Desferrioxamine, an iron chelator, which mimics hypoxia conditions. In 4 out of 6 cell lines, the effect was similar to the one observed in low oxygen colture, as CXCR4 was upregulated compared to control conditions. In Hs766T and A8184, the upregulation was much higher than in hypoxic culture, possibly meaning that the effect due to the culture chamber was underestimated and further confirming that hypoxia upregulates CXCR4.

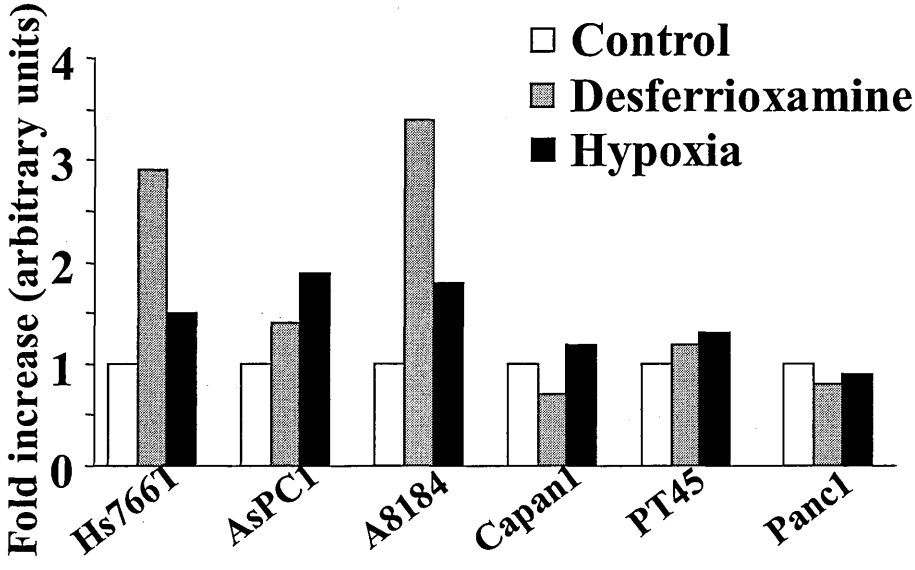


Fig 5.4 *CXCR4* modulation by hypoxia in cell lines, as assessed by Real-Time PCR. Cells were cultured in low-oxygen tension (Hypoxia) or treated with Desferrioxamine as a control (0.4 mmol/L) for 4 hours. Hypoxia consistently up regulated *CXCR4* mRNA in pancreatic cancer cell lines. One representative analysis of two performed is reported.

Expression of *CXCR4* in freshly isolated pancreatic tumor cells

Pancreatic tumour cells from surgical specimens (see **Table 3.1** for clinico-pathological features) of resected patients were isolated and routinely checked for purity from stromal components (see **Fig.4.1**). In 7 different tumor samples, the amount of *CXCR4* mRNA, normalized to β-actin, was evaluated by Real Time PCR, and expressed as relative to the cell line HPDE6, used as reference, as for the above experiments. Moreover, I also analyzed the RNA derived from a preparation of freshly isolated normal pancreatic ducts.

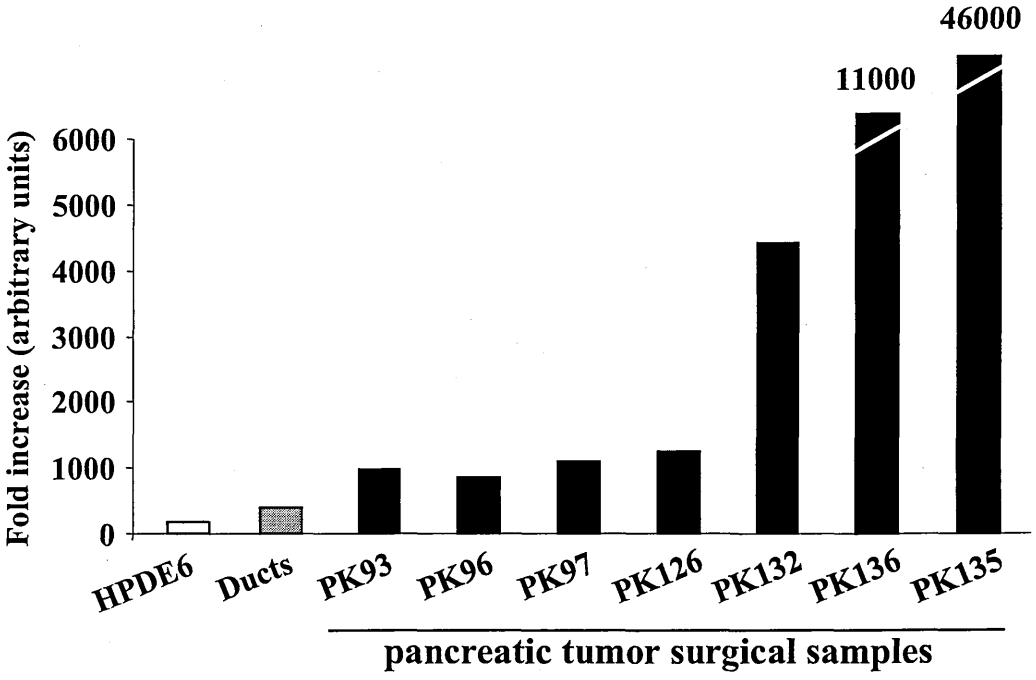


Fig 5.5 *CXCR4* mRNA expression in tumor cells from surgical samples of resected pancreatic cancer patients. The amount of *CXCR4* mRNA, normalized to *β-actin*, is expressed as relative to the cell line HPDE6 (white bar). Human epithelial pancreatic ducts were isolated from the pancreatic tissue of a multiorgan donor (grey bar). For some tumor samples, numbers on top of bars indicate the fold increase relative to expression of *CXCR4* in HPDE6. One representative analysis of two performed is reported.

As shown in **Fig. 5.5**, freshly isolated tumor cells showed much higher levels of *CXCR4* compared to HPDE6 cells. Freshly isolated normal pancreatic ducts expressed substantial amounts of *CXCR4*, although always at lower levels compared with tumor samples.

CXCR4 surface expression

To confirm that mRNA transcripts correspond to receptor expression on the surface of tumor cells, I evaluated CXCR4 cell-surface expression by FACS analysis. Five cell lines that were scored positive by RT-PCR (Hs766T, AsPC1, Capan1, A8184, and CFPAC) had high surface expression of CXCR4. Representative FACS profiles are shown in **Fig 5.6**.

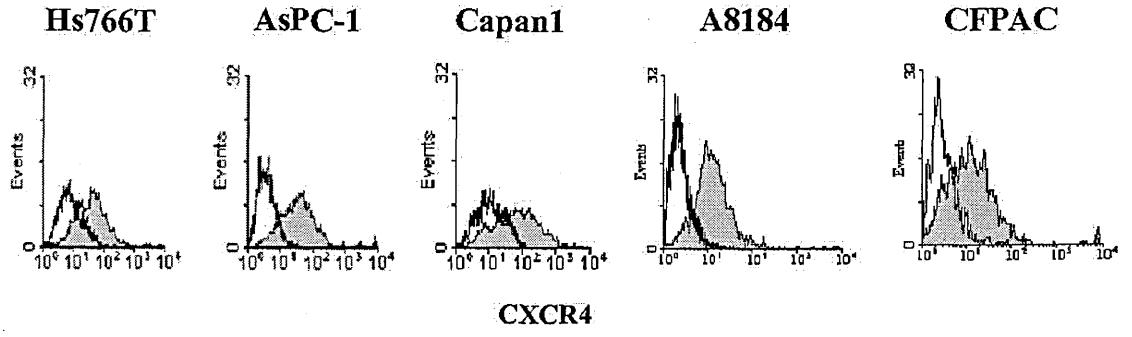


Fig.5.6 Surface expression of CXCR4 on pancreatic cancer cell lines detected by flow cytometry. Cells were removed from flasks non-enzimatically and incubated with 10 mg/ml FITC-CXCR4 anti-human antibody (clone 12G5). One representative analysis of four performed is reported.

5.3 CXCR4 functional activity

Setting up migration assays

Chemotaxis (migration towards a chemical gradient) is the eponymous function of chemokines. Therefore, the first functional assay I performed was the migration of tumour cells in response to chemokines, in chemotaxis assays.

Here, I report chemotaxis experiments performed for the cell line Hs766T. Different pore filters, (8 and 13 μm), were used and three times of migration (4, 8 and 16 hours). As shown in **Fig 5.7**, an appreciable migration was achieved with 13 μm pore filters in 16 hours, as the CXCL12-elicited migration was significantly higher than the basal one, while no appreciable migration was observed with 8 μm pores and a shorter time was not optimal. For other cell lines (e.g. MiaPaCa2, PT45, CFPAC), in contrast, migration in this condition was not optimal and an aspecific migration was observed also in response to the control medium. For these cell lines, 8 hour/8 μm pore was the best combination (data not shown). Finally, A8184 cell line migration was performed in 4 hour/8 μm pore conditions, as basal migration was too high, if longer times were used.

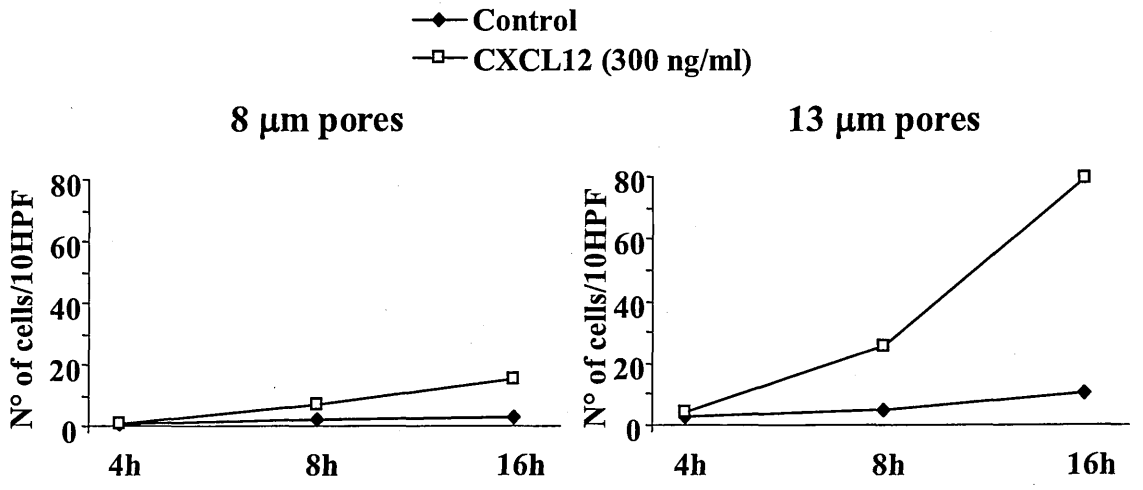


Fig. 5.7. Setting up of migration assay for Hs766T cell line. Two different pore sizes (8 and 13 μm) and two times (8 and 16 hours) were combined and the best condition selected for future assays.

CXCL12 stimulates pancreatic cancer cell chemotaxis

To verify that CXCR4 is functional in pancreatic cancer, selected cell lines were tested in chemotaxis as well as other migration assays. The cell line Capan1 was excluded for its characteristic to disaggregate in large clusters. **Fig 5.8** shows that the CXCR4-positive cell lines Hs766T, AsPC1, A8184 and CFPAC did migrate in response to CXCL12 in a classical chemotaxis assay, with an optimal response at 300 ng/ml. In contrast, the CXCR4-negative cell lines PT45 and MiaPaCa2 did not migrate to CXCL12 (**Fig. 5.8**). We recently reported that none of the 11 pancreatic tumour cell lines express CCR2 (25). In line with this finding, different concentrations of CCL2 did not induce a chemotactic response neither in Hs766T nor in AsPC1 cells.

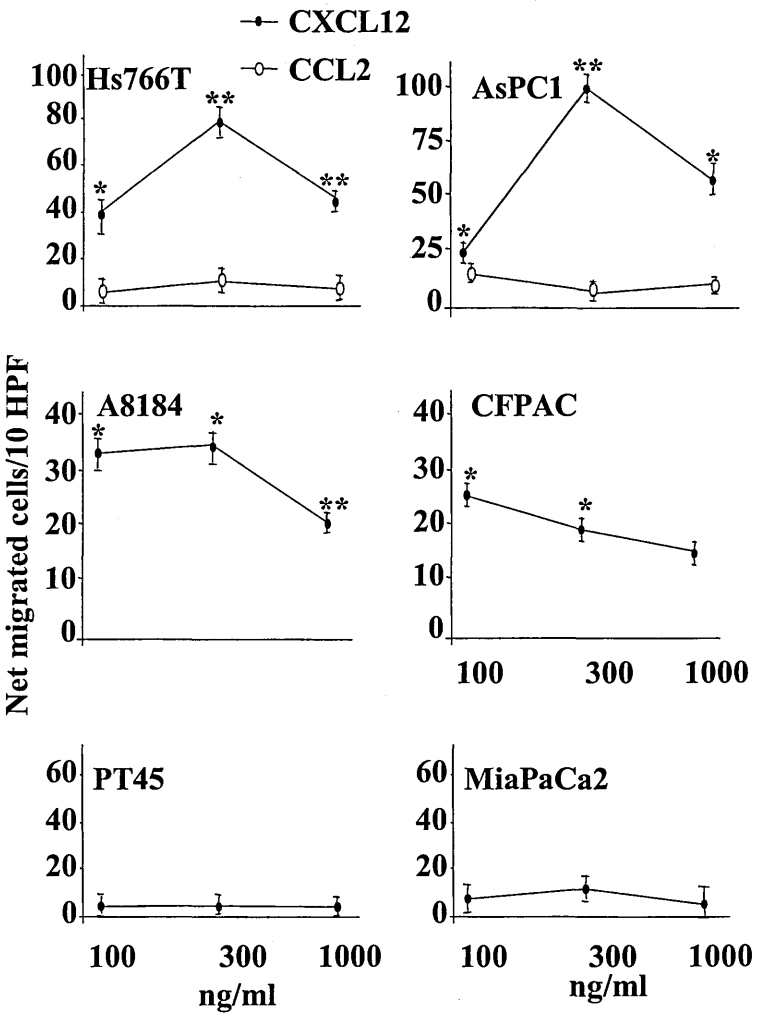


Fig 5.8 CXCL12 stimulates pancreatic cancer cell chemotaxis. CXCR4-positive cell lines (Hs766T, AsPC1, CFPAC, and A8184) migrated in classical chemotaxis assays to different concentrations of CXCL12 in a dose-response manner, whereas CCL2 did not induce a chemotactic response in the cell lines Hs766T and AsPC1. The CXCR4-negative cell lines, PT45 and MiaPaCa2 did not migrate to CXCL12. Shown are net numbers of migrated cells counted in 10 high power fields over basal migration (in the absence of chemokine). Basal migration was 120 cells/10 HPF for Hs766T, 23 cells/10 HPF for AsPC1, 27 cells/10 HPF for CFPAC, 25 cells/10 HPF for A8184, 47 cells/10 HPF for MiaPaCa2 and 27 cells/10HPF for PT45 (*$p<0.02$ *versus* control; **$p<0.001$ *versus* control, calculated by Student t Test). Migration conditions were as follows: 16 hours and 13 μm pore filters for Hs766T; 16 hours and 8 μm pore filters for AsPC1; 8 hours and 8 μm pore filters for CFPAC, PT45 and MiaPaCa2; 4 hours and 8 μm pore filtrs for A8184. Values are the mean ±SE of eight replicates. One representative experiment of three performed is shown.

To confirm that the migratory activity observed in chemotaxis assays was specifically mediated by CXCR4 engagement, I tried to inhibit it by incubating cells

with an anti-CXCR4 monoclonal antibody. Pretreatment of Hs766T cells with the blocking antibody completely blocked cell migration in response to CXCL12 (**Fig. 5.9**). A similar effect was observed by pretreating cells with a CXCR4-selective inhibitor, AMD3100.

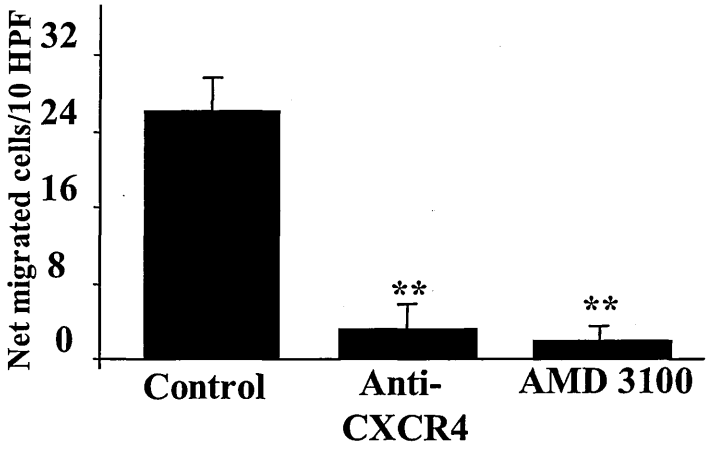


Fig. 5.9 CXCL12 induced migration is mediated by CXCR4. Pre-treatment of Hs766T cells with a blocking anti-CXCR4 mAb (10 μg/ml) or with AMD3100 (1 μg/ml) significantly reduced cell migration in response to 300 ng/ml of CXCL12 (**p<0.01 *versus* control, Student t Test). Values are the mean ± SE of eight replicates. One representative analysis of two performed is reported.

CXCL12 stimulates pancreatic cancer cell adhesion and transmigration

Tumor cell adhesion to endothelial cells and transendothelial migration are key steps in the process of tumor invasion and metastasis. CXCL12 significantly enhanced adhesion to human umbilical vascular endothelial cells and transendothelial migration of the CXCR4-positive cell line Hs766T (**Fig 5.10**).

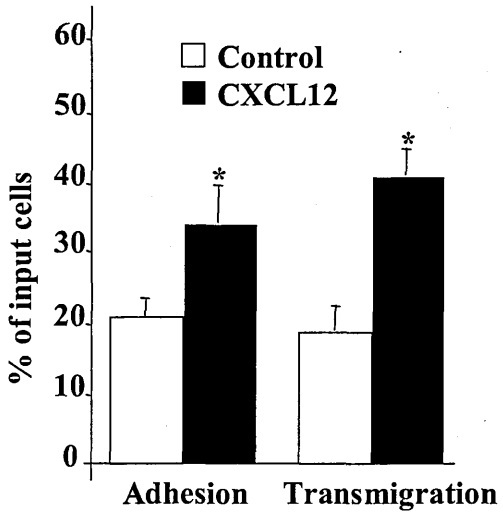


Fig 5.10 CXCL12 stimulates adhesion and transmigration through endothelial cells in the CXCR4-positive cell line Hs766T. HUVEC were grown on polycarbonate filters. 51Cr–labeled tumour cells were seeded in the upper compartment and trans-endothelial migration assessed after 16 hour incubation in the presence of CXCL12 (300 ng/ml) in the lower compartment. Nonhaderent cells were washed away and the radioactivity in the filter referred to adhered cells, while the radioactivity in the filter and in the lower compartment referred to transmigrated cells. Values are the mean ± SE of three different experiments performed (**p<0.02 *versus* control, Student t Test).

CXCL12 stimulates gelatinase activity

Chemokines are potent activators of matrix metalloproteases (MMPs) (49, 133, 238, 239). Hence, it was of interest to measure the activity of MMP2 and MMP9 in the supernatants of CXCL12-treated tumor cells. Freshly isolated human monocytes were used as positive control. The gelatinase activity of MMPs from untreated tumor cells was very low (**Fig. 5.11**). CXCL12 significantly increased MMP activity in Hs766T and AsPC1, with levels of activity similar or higher than human monocytes, while CXCL12 did not affect the MMP activity in the CXCR4-negative cell line MiaPaCa2. The activity of metalloproteases is of major importance in the digestion of the extracellular matrix and has been implicated in the metastasizing ability of tumor cells

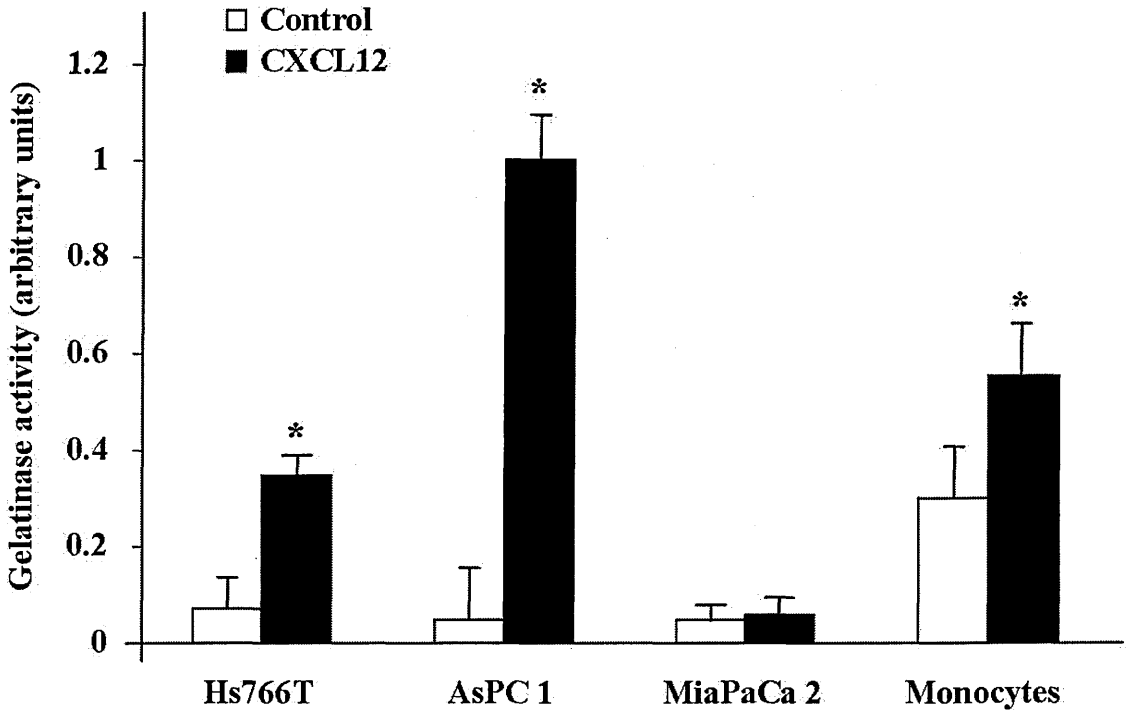


Fig 5.11 CXCL12 stimulates the activity of selected metalloproteases. Induction of MMP2 and MMP9 gelatinase activity; the assay used does not discriminate between MMP2 and MMP9 and measures the overall gelatinase activity of supernatantts tested. Three pancreatic cancer cell lines were stimulated with 100 ng/ml CXCL12 for 24h, in synthetic medium (X-Vivo) and supernatants were tested for metalloprotease activity in ELISA. CXCL12 induces metalloprotease activity in two CXCR4-positive cell lines (AsPC1 and Hs766T) but not in the CXCR4-negative cell line (MiaPaCa2). Human monocytes were used as positive control. Values are the mean ±SE of three different experiments performed (*$p<0.01$ *versus* control, Student t Test).

In line with the finding that CXCL12 induces MMP activity, tumor cells stimulated with CXCL12 showed enhanced ability to invade Matrigel-coated filters **(Fig. 5.12)**.

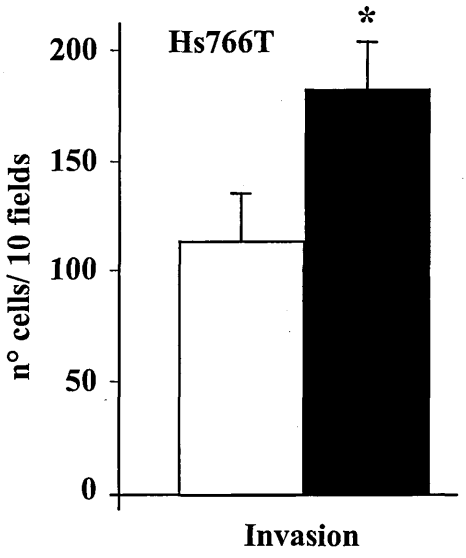


Fig 5.12 CXCL12 stimulates invasion. Invasion of Matrigel coated filters was assessed after 16 hours (*p<0.01 *versus* control, Student t Test). Shown is one representative experiment of two performed.

5.4 The axis CXCR4/CXCL12 role in pancreatic cancer cell proliferation and survival

CXCL12 stimulates pancreatic cancer cell line proliferation

I investigated whether the expression of CXCR4 on tumor cells had an effect on their proliferation. Tumors frequently produce chemokines, thus I previously evaluated whether pancreatic cancer cells produced CXCL12 (Chapter 4). Of eleven cell lines tested, only Hs766T produced significant amounts of the chemokine (1326 pg/ml/10^6 cells, Table 4).

The effect of CXCL12 on tumor cell proliferation was assessed on CFSE-labelled cells. Under optimal culture conditions (in the presence of 10% serum), addition of CXCL12 increased proliferation in the cell line AsPC1 (CXCR4-positive, CXCL12-non producing), but not in Hs766T cells, which produces the chemokine.

Treatment with AMD3100 greatly inhibited spontaneous proliferation only in Hs766T cells, which is in line with the presence of the endogenous chemokine (**Fig. 5.13**).

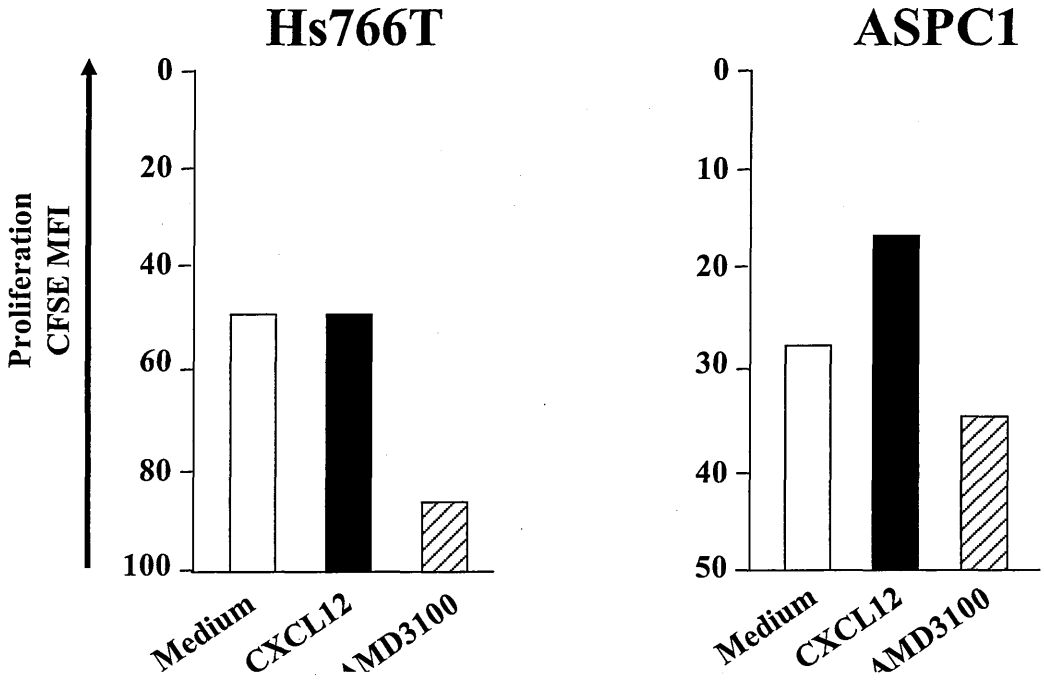


Fig 5.13. CXCL12 enhances proliferation in Hs766T and AsPC1 cell lines, under optimal conditions. Cells were labeled with CFSE and fluorescence measured after 3 days. Proliferation is indicated by decrease in mean fluorescence (MFI). In the presence of 10% serum, CXCL12 enhances the proliferation of AsPC1 cells (compare black bar with white bar) and addition of the CXCR4-antagonist AMD3100 reverts this effect (grey bar). In the CXCL12-producing cell line Hs766T, AMD3100 inhibits proliferation (compare dashed bar with white bar). Each sample was obtained by mixing a triplicate, to get a representative number of cells. One representative of three experiments is shown.

Under suboptimal culture conditions (absence of serum), the presence of CXCL12 greatly enhanced cell proliferation in both Hs766T and AsPC1 cell lines (**Fig. 5.14**). This is in agreement with our finding that CXCL12 release was reduced by 90% in serum-free conditions (not shown). The enhancing effect of CXCL12 on cell

proliferation was strongly inhibited by treatment with AMD3100 in AAsPC1 cells and to a lesser extent in Hs766T cells.

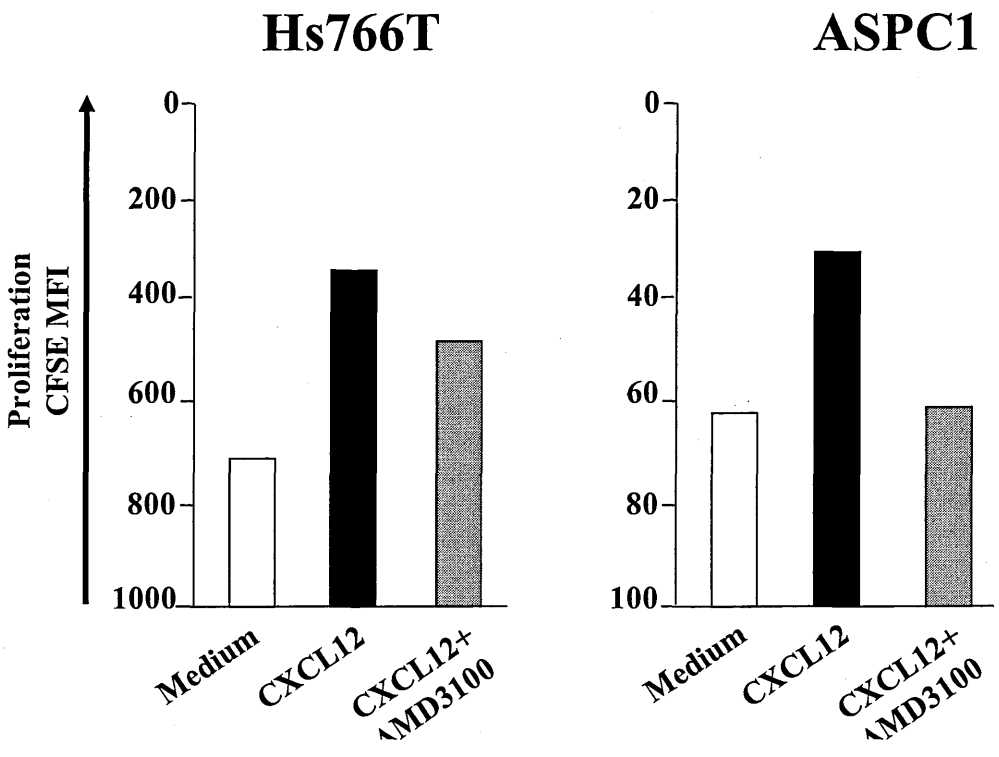


Fig 5.14. CXCL12 enhances proliferation in Hs766T and AsPC1 cell lines, under sub-optimal conditions. Under conditions of serum deprivation (culture in medium 1% FBS), CXCL12 restores the proliferation of Hs766T and AsPC1 cells (black and white bar); this effect is partially inhibited by the AMD3100 inhibitor (grey bar). Each sample was obtained by mixing a triplicate, to get a representative number of cells. One representative of three experiments is shown.

These results indicate that CXCL12 stimulates pancreatic cancer cell line proliferation, and, in at least one representative CXCR4-positive cell line, the chemokine acts as an autocrine growth factor.

CXCL12 promotes survival of pancreatic cancer cells

An important feature of metastatic cells is the ability to regulate their survival. I therefore tested whether CXCL12 could rescue Hs766T and AsPC1 tumor cells from serum deprivation-induced death. Treatment of cells cultured in serum-free medium with CXCL12 reduced the percentage of propidium iodide (PI) positive cells by 46% in Hs766T and by 50% in AsPC1 (n=4, data not shown). We next performed cell cycle analysis. As shown in **Fig.5.15**, serum deprivation enhanced the proportion of apoptotic cells, as indicated by the sub-G1 peak detection (from 13% to 27% in Hs766T and from 2% to 11% in AsPC1). We used also IL-1β as an apoptotic stimulus, as we previously observed that this cytokine induces apoptosis in these cell lines (26). Addition of CXCL12 (100 ng/ml) significantly reduced spontaneous DNA degradation, as shown by decrease of sub-G1 peak (from 27% to 16% and from 11% to 4% for Hs766T and AsPC1 respectively; where IL-1β was used, from 21% to 16% and from 6% to 2%, in Hs766T and in AsPC1 respectively).

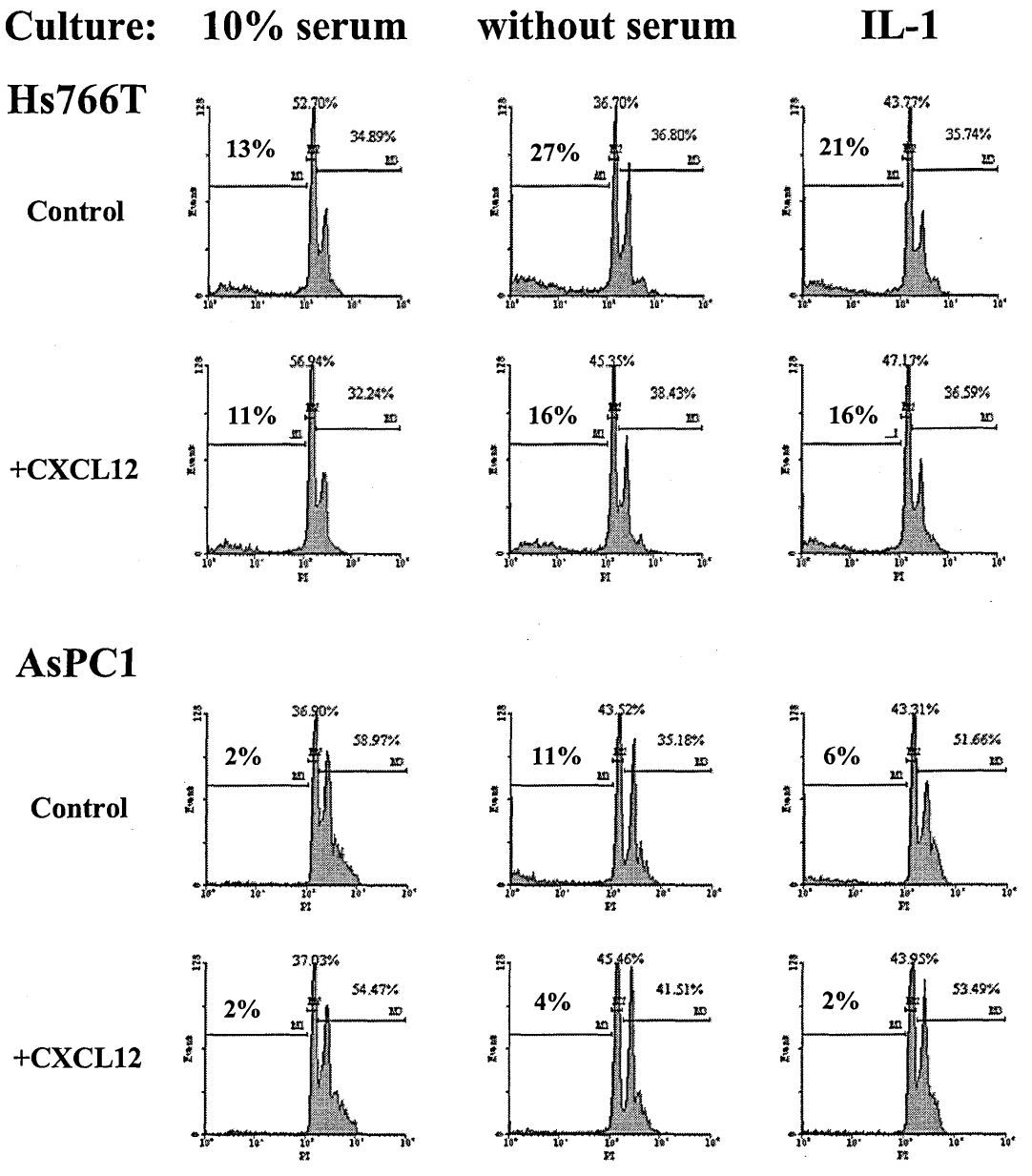


Fig 5.15 CXCL12 rescues Hs766T and AsPC1 cells from apoptosis induced by serum deprivation and IL-1β. Cell cycle analysis of cells cultured in normal or apoptosis-inducing conditions, with or without 100 ng/ml CXCL12. After 72 h cell cycle analysis was performed, by staining cells with PI. In all the apoptosis inducing conditions, addition of CXCL12 decreased sub-G1 peak. One representative of three experiments is shown.

To further address the question whether CXCL12 regulates survival of pancreatic cancer cell lines, we evaluated the percentage of annexin-V^+ cells under serum-deprived culture conditions. In both Hs766T and AsPC1 cell lines, treatment with CXCL12 decreased the percentage of apoptotic annexin-V^+ cells (from 19% to 15% in Hs766T

and from 17% to 12% in AsPC1), while CXCL12 had no significant effect in the CXCR4-negative cell line MiaPaCa2 (**Fig.5.16**).

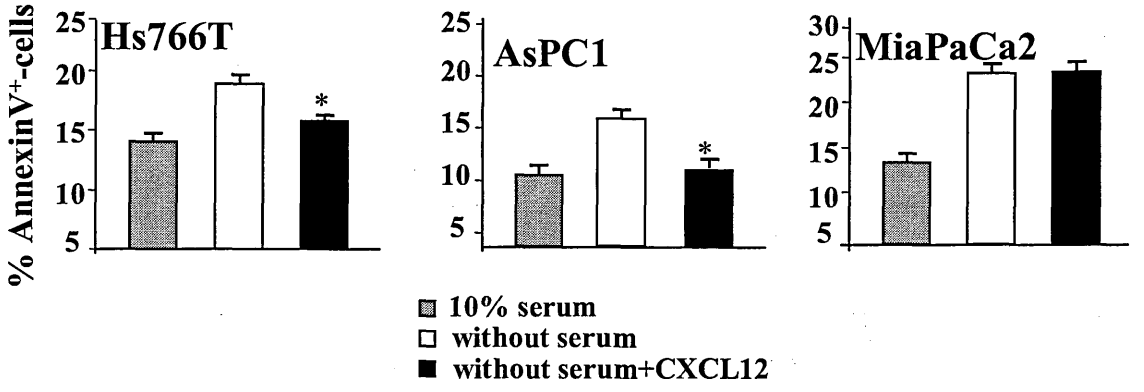


Fig.5.16. CXCL12 rescues Hs766T and AspC1 from apoptosis. Percentage of AnnexinV$^+$ positive cells in cell lines cultured in serum-free medium for 24 h with or without CXCL12. In Hs766T and AsPC1, CXCL12 (100 ng/ml) decreased number of apoptotic annexinV$^+$ cells, while CXCL12 had no significant effect in the CXCR4 negative cell line MiaPaCa2. Values are the mean ± SE of three different experiments performed (*p <0.01, Student t Test).

5.5 Discussion

In this Chapter, I presented data on the expression and functional role of the CXCR4/CXCL12 axis in human pancreatic adenocarcinoma.

From the first mRNA screening of eleven pancreatic tumor cell lines, I found that CXCR4 is the chemokine receptor mostly expressed. In particular, 6 of 11 cell lines tested express considerable levels of *CXCR4* transcripts, that was confirmed at the protein level in selected cell lines.

Interestingly, I found that *CXCR4* is differentially expressed in the pancreatic cancer cell lines depending on their origin. Only one out of three cell lines derived from primary tumors expresses the transcript, in very low amounts, while all but one cell lines derived either from ascites or metastasis express higher amounts of *CXCR4* mRNA. This result suggests an association between the expression of the receptor and the *in vivo* origin (either primary tumour or metastatic site) of tumor cells.

Also surgical specimens from pancreatic adenocarcinoma express *CXCR4* in higher amounts compared with an immortalized cell line derived form human pancreatic ducts (HPDE6) as well as compared to freshly isolated normal pancreatic ducts. The finding that tumor tissues have higher expression of *CXCR4* compared to the normal counterpart is in agreement with previous reports (281-283).

Surprisingly, the preparation of freshly isolated pancreatic ducts that was tested had relatively high levels of *CXCR4*. One possible explanation is that these pancreatic ducts were isolated from a multi-organ donor in irreversible coma. Although it is unknown whether this specific pathological condition affects the expression of chemokine receptors, it is well known, and I also confirm it in this report, that hypoxia

up-regulates *CXCR4*. It may be possible that the high *CXCR4* expression is the result of hypoxic conditions surely occurred before pancreas explants. High expression of *CXCR4* in pancreatic ducts has also been recently reported by Kajali et al in NOD-IFN-γ-transgenic mice, as well as in parental NOD mice (284). These authors also highlight the importance of the CXCR4-CXCL12 ligand-receptor axis in the survival proliferation and migration of ductal pancreatic ells.

CXCR4 is expressed on a variety of tissues and cell types, including leukocytes, haematopoietic progenitor cells, endothelial cells, epithelial cells and cells of the central nervous system (285). Its chemokine ligand, CXCL12, is a homeostatic chemokine; it is expressed constitutively in a range of tissues and does not appear to be regulated by pro-inflammatory cytokines (286, 287). Its ubiquitous expression may be due to the presence of a GC-rich sequence in the 5'-flanking region of the CXCL12 gene, a feature which is associated with 'housekeeping' genes (287).

Both CXCR4- and CXCL12-deficient mice have been generated and have contributed to the comprehension of the very broad spectrum of actions of this receptor/ligand pair (145, 146). Both the transgenic mice die *in utero* and present very similar phenotypes, a feature confirming that CXCL12 only acts through CXCR4; this has been the general consensus till some months ago, when another receptor for CXCL12 has been cloned, named CXCR7 (288).

Besides the usual functions attributed to a homeostatic chemokine/receptor pair, both CXCR4 and CXCL12 play a critical role in other physiological processes, including foetal development and organogenesis (severe heart defects, disorganised cerebellum are observed in knockout mice), vascularisation, and mobilization of haematopoietic stem cells (285). Moreover, they also have importance in pathological

conditions as HIVinfection, being CXCR4 a co-receptor for the virus entry, and in tumour metastasis, as here discussed.

Modulation of chemokine receptors has not been extensively investigated on tumor cells. It has been recently reported that hypoxia (low oxygen tension) up-regulates CXCR4 expression in endothelial and tumor cells (280). Indeed CXCR4 is an hypoxia inducible gene, being a target of Hypoxia-inducible factor 1 alpha (HIF) (289). In line with this finding we observed that CXCR4 expression was enhanced in two cell lines cultured under hypoxic conditions. As low oxygen tension is likely to occur in the neoplastic mass; hypoxia-induced up-regulation of CXCR4 may have direct *in vivo* relevance.

To assess whether cytokines present in the tumor microenvironment could modulate CXCR4, I tested IL-10, IL-6 and hepatic growth factor (HGF), (known to be produced by human pancreatic tumor and stromal cells) (290, 291). *CXCR4* mRNA transcripts were never affected by the above cytokines (data not shown). The inflammatory cytokines, TNFα and IL-1β were also ineffective; in contrast, treatment with IFNγ consistently reduced *CXCR4* expression. It is well established that IFNγ plays a crucial role in immune resistance against tumors (292). Therefore the IFNγ-mediated down modulation of *CXCR4* may contribute to inhibition of tumor growth and metastasis.

In line with other results that CXCR4 is implicated in promoting the migratory phenotype of a variety of tumors (81, 281-283, 293) in this study CXCL12 induced a chemotactic response in CXCR4-positive cell lines and this response was inhibited when CXCR4 receptors were blocked by a specific monoclonal antibody or by the

CXCR4 antagonist AMD3100. Tumor cell adhesion to endothelium and transendothelial migration are key events in the process of tumor invasion and metastasis and are chemokine-regulated steps. Adhesion to endothelial cells and transendothelial migration were enhanced by CXCL12 in CXCR4-positive pancreatic tumor cells.

Cancer dissemination can be viewed as a tissue remodeling process that involves proteolytic degradation of ECM in the surrounding normal tissue. Metalloproteases are a family of enzymes activated by chemokines, involved in the degradation of ECM and known to mediate cancer invasion and metastases (50-53, 55, 238). The initial interest in proteases was on their critical role in degrading the basement membrane, to permit the penetration by tumor cells of surrounding connective tissues and blood vessels; moreover, the ECM blocks tumor metastasis not only in the sense of being a physical barrier but also because it forms a self-protective, apoptosis resistant microenvironment (50). Later on it has been clear that proteases, and in particular matrix metalloproteinases, can target many non-ECM proteins, including growth factor receptors, cell-associated molecules, and cytokines VEGF and TGFβ represent two examples of such factors that are stored in a latent complex within the ECM and can be released by MMP proteolysis (53, 70), enhancing their bioavailability. As a result, the activity of proteases in cancer is far more complex than initially anticipated and includes tumor promoting as well as tumor-suppressive effects (52). Moreover, several lines of evidence suggest also a supportive role for ECM components in metastasis, possibly due to the capability of ECM components (e.g. fibronectin, laminin-5) of promoting migration and MMPs have been demonstrated to have a role in exposing cryptic domains within ECM molecules that can promote migration and metastasis (294).

Among the eleven types of metalloproteases, MMP-2 and MMP-9 participate in the degradation of type IV collagen, which is one of the major components of cellular basement membranes, whose elimination is essential to invade stroma and vessels; indeed, expression of MMP-2 and MMP-9 has been associated with venous invasion and hematogenous metastasis (72). Pancreatic cancer is strongly invasive, usually directed to large vessels, such as the portal vein, and the development of liver metastasis is generally dependent on venous invasion by primary tumor cells. Proteases are expressed in the extracellular milieu as inactive proforms that become activated through a variety of mechanisms that often involve a close collaboration among several families of proteases. Thus overexpression of proteases as detected by antibody staining does not necessarily mean an increase in proteolytic activity. For this reason, the availability of a functional assay measuring metalloprotease activity rather than expression, as the one I used in this work, acquires much importance.

In this study, I found that in the absence of chemokines, the gelatinase activity of MMPs from untreated tumor cells was very low. In contrast, in CXCL12-treated tumor cell lines, MMPs were significantly more active. In line with these findings, CXCL12 triggered tumor cell invasion through a Matrigel layer. Collectively, these results indicate that CXCR4 expression confers tumor cells with increased motility and invasion ability.

Although the functional significance of chemokine receptor expression by tumor cells has largely being investigated, in general, few biological assays have been performed, mainly aimed at demonstrating that chemokines enhance the migratory phenotype of tumor cells bearing their cognate receptor.

It is now well established that chemokines play other important roles besides chemotaxis; in particular, they may have direct or indirect growth stimulating effects on tumor cells. There is evidence that CXCL8 and CXCL1 are implicated as endogenous growth stimulating factors in melanoma as well as in tumors of different histologies(187, 188). A promoting effect of CXCL12 on tumor cell proliferation was reported also for ovarian carcinoma (295). Our group has extensively analyzed the expression and production of different chemokines in pancreatic cancer; some of the cell lines produce CCL2, CCL5 and CXCL8, with a heterogeneous pattern (25, 26). In this work, I found out that CXCL12 is produced only by Hs766T, the cell line expressing the highest levels of CXCR4. Interestingly, the CXCR4 antagonist AMD3100 inhibited proliferation in Hs766T, suggesting that the endogenous CXCL12 may function as an autocrine or paracrine factor. *In vivo,* CXCL12 can be produced by several cell types, including stromal and endothelial cells (283); moreover, this chemokine is produced in lymph nodes, where secondary localization of tumors, including pancreatic carcinoma, occurs. In *in vitro* experiments, CXCL12 enhanced the proliferation of the CXCR4-bearing cell lines, especially under sub optimal culture conditions. When the Hs766T cell line was cultured in serum-free medium, CXCL12 release was dramatically reduced and exogenous CXCL12 significantly stimulated cell growth, both in Hs766T and in AsPC1.

AMD3100 has been originally tested in HIV patients as CXCR4 is one of the major receptor for the virus, and showed to have a good safety profile (296, 297); moreover, it has been considered as a therapeutic tool for other pathologies in which CXCR4 may play a role. For instance, it has been shown to mobilize CD34+ stem cells from the bone marrow into the bloodstream; indeed, AMD3100 is actively pursued as a stem cell mobilizer for transplantation in patients with multiple myeloma and non-

Hodgkin's lymphoma (297). Some recent data have provided the scientific rationale for the clinical evaluation of AMD3100 also in cancer. In cells from adult glioblastoma and pediatric medulloblastoma, CXCR4-CXCL12 signalling induced chemotaxis and enhanced proliferation and survival (298). When tested in vitro in cell cultures, the selective CXCR4 antagonist (AMD3100) was shown to reverse these effects. When AMD3100 was used to treat mice bearing intracranial glioblastoma or medulloblastoma, tumor burden was significantly smaller in AMD3100-treated animals (298, 299).

An essential feature of tumour cells is the ability to regulate their survival and to resist to apoptosis. The chemokine CXCL12 has been shown to be involved in promoting survival of $CD34^+$ hematopoietic progenitors and T lymphocytes (132, 300) and of several types of cancer, including glioma, melanoma, NSCLC, renal and thyroid (91, 282, 299).

In this study, CXCL12 has been shown to protect $CXCR4^+$ pancreatic tumor cells from serum starvation-induced death or IL-1-induced damage, by decreasing the rate of apoptosis.

Collectively, these results show that expression of CXCR4 by pancreatic tumour cells mediates migration and invasion in the surrounding tissues and also promotes survival and proliferation of cancer cells.

5.6 Summary of results in Chapter 5

The results presented here demonstrate that metastatic pancreatic carcinoma cells express CXCR4 and that autocrine or paracrine loops centered on this chemokine receptor promote tumor cell migration, matrix degradation and invasion, proliferation and survival.

Specifically, the following results have been presented:

- pancreatic cancer cell lines express *CXCR4* mRNA; the expression is higher in cell lines derived form metastatic lesions compared with those derived from primary tumors;
- different inflammatory cytokines do not modify the expression, whereas IFNγ down regulates and hypoxia up regulates *CXCR4* transcripts;
- transcript expression is associated to surface expression in pancreatic carcinoma cell lines;
- *CXCR4* is expressed also in pancreatic cancer cells from primary tumors; all surgical (N=7) carcinoma samples tested express higher levels of *CXCR4* than normal pancreatic duct cells
- CXCR4 on pancreatic cancer cells is functional and mediates their migration to CXCL12; migration is selectively inhibited by anti-CXCR4 monoclonal antibody and by the antagonist AMD3100;
- CXCL12 also mediates transendothelial migration, Matrigel invasion and activation of metalloproteases;
- in CXCR4-positive cell lines CXCL12 stimulates cell proliferation and protects from apoptosis induced by serum starvation.

Chapter 6

CX3CR1 and Pancreatic Adenocarcinoma

6.1 Introduction and goal

This chapter will focus on the expression and function of the chemokine receptor CX3CR1 and its chemokine ligand CX3CL1/Fractalkine in human pancreatic adenocarcinoma. In the preliminary screening of chemokine receptors in pancreatic adenocarcinoma cell lines, I found that some cell lines express the receptor CX3CR1; its chemokine ligand, CX3CL1, also called Neurotactin, is found expressed in neurons and nerve fibers (301-304). Besides local and lymph node metastasis, hallmark of pancreatic cancer is a peculiar propensity to disseminate and grow along nerves. Therefore, the presence of CX3CR1 can assume a particular relevance in the context of pancreatic cancer tropism for neural structures. Hence, I wanted to test the hypothesis that CX3CR1 and its ligand CX3CL1/Neurotactin could have a role in pancreatic adenocarcinoma dissemination to nerves.

6.2 *CX3CR1* mRNA analysis

CX3CR1 expression in pancreatic tumor cell lines

In a first series of experiments, I evaluated the expression of CX3CR1 in eleven pancreatic adenocarcinoma cell lines by semi quantitative Real Time PCR. The immortalized epithelial cell line (HPDE6) derived from normal human pancreatic ducts was used as a reference of normal tissue. This analysis revealed that 6 pancreatic tumor cell lines express *CX3CR1*, with three cell lines (Capan-1, A8184, AsPC1) showing the highest expression (**Fig. 6.1**). Moreover, in each cell line, the expression of *CX3CR1* is higher than in the normal ductal epithelium, in which *CX3CR1* expression is not detected.

I reported in the previous chapter that human pancreatic tumor cells express functional CXCR4, it was of interest to verify whether CX3CR1 is co-expressed with CXCR4 on the same cell lines; two cell lines with the highest amount of *CX3CR1* (Capan-1 and AsPC1) are also high *CXCR4*-expressing, while A8184 expresses only *CX3CR1* at high levels. I also confirm in this analysis what was observed for *CXCR4*, i.d. the cell lines expressing the chemokine receptors are more frequently derived from metastatic lesions or ascites, while the negative cell lines derive from a primary tumor. This evidence, although based on a low casistic, seems to confirm our hypothesis that an association exists between chemokine receptor expression and the malignant potential of tumor cells (**Fig.6.1**).

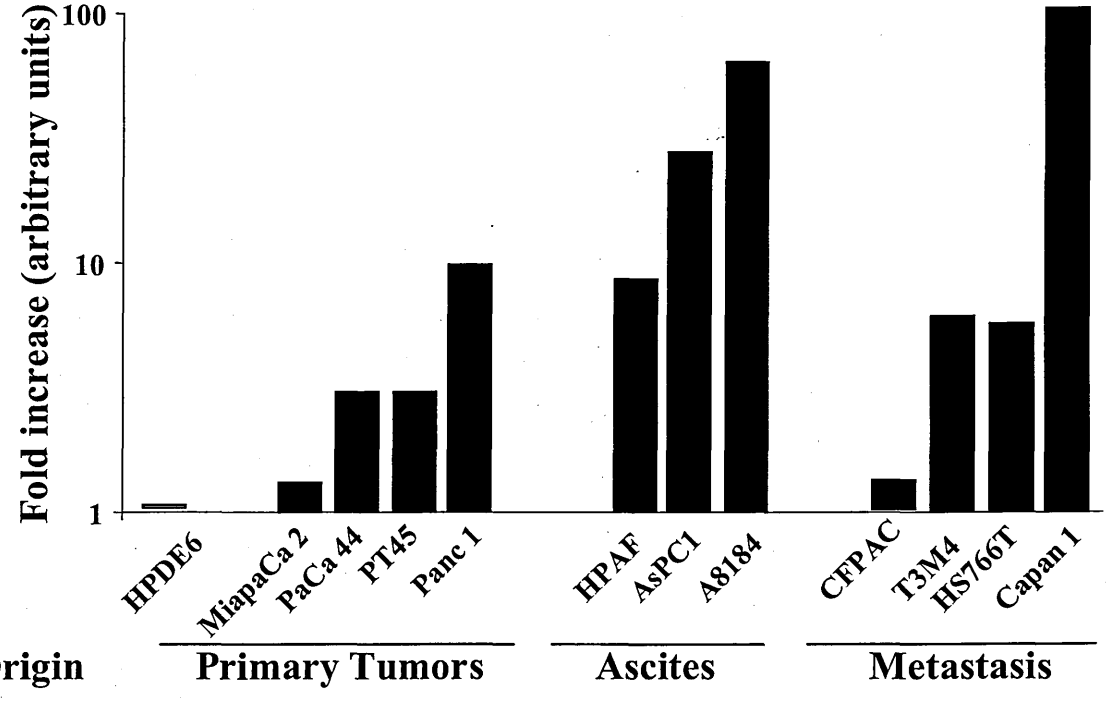


Fig 6.1 *CX3CR1* in pancreatic cancer cell lines. Semiquantitative Real-Time PCR of pancreatic cancer cell lines. For each cell line, the amount of *CX3CR1* mRNA, normalized to *β-actin*, is expressed as relative to the cell line HPDE6. One representative analysis of two performed is reported.

As no data are available in the literature regarding the expression of CX3CR1 by tumour cells, we asked whether the results described above were specific to pancreatic cancer. Therefore, I screened a panel of cell lines derived from other adenocarcinoma, including ovarian cancer cells, breast cancer and two colon carcinoma cell lines and found out that the expression is much lower compared to pancreatic cancer cells (**Fig 6.2**).

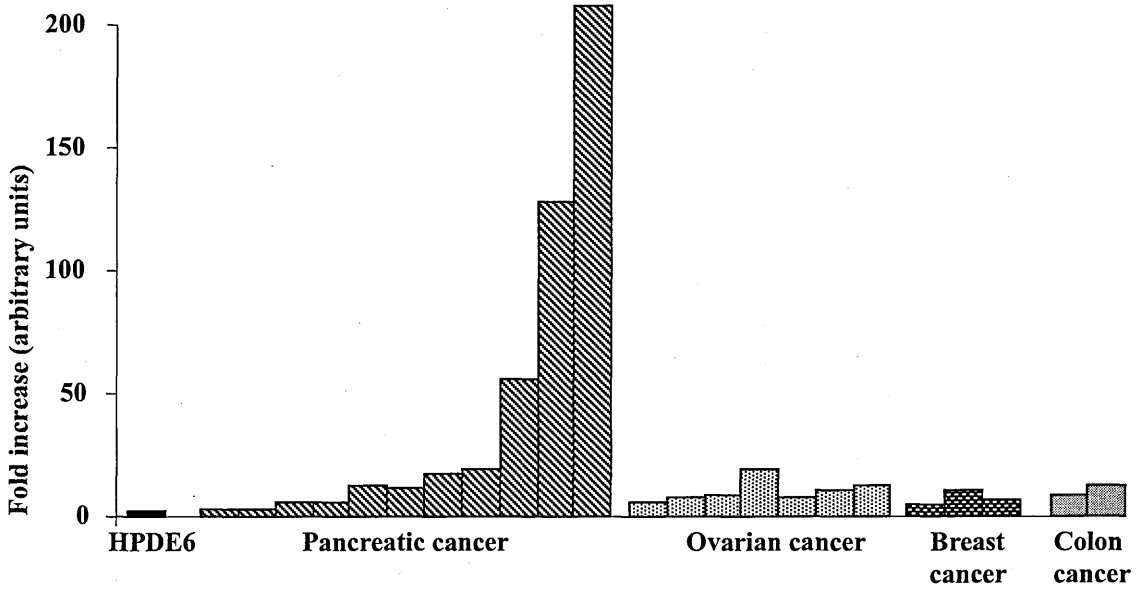


Fig 6.2 CX3CR1 in pancreatic and other tumour type derived cell linesc. Semiquantitative Real-Time PCR. For each cell line, the amount of *CX3CR1* mRNA, normalized to *β-actin*, is expressed as relative to the cell line HPDE6. One representative analysis of two performed is reported.

Expression of *CX3CR1* in freshly isolated pancreatic tumor cells

I next examined tumor cells from surgical samples of resected patients (see **Table 3.1** for clinico-pathological features). Tumor cells isolated by density gradients from the stromal component and checked for purity by staining with cytokeratin-7 were analyzed. In 7 different tumor samples, the amount of *CX3CR1* mRNA, normalized to

β-actin, was evaluated by Real Time PCR, and expressed as relative to the cell line HPDE6, used as reference, as for the above experiments. As shown in **Fig.6.3**, freshly isolated tumor cells showed much higher levels of *CX3CR1* compared to HPDE6. In addition, a cell preparation of freshly isolated pancreatic ducts was tested, in which *CX3CR1* expression is much lower than in primary tumors, although higher than the HPDE6 cell line.

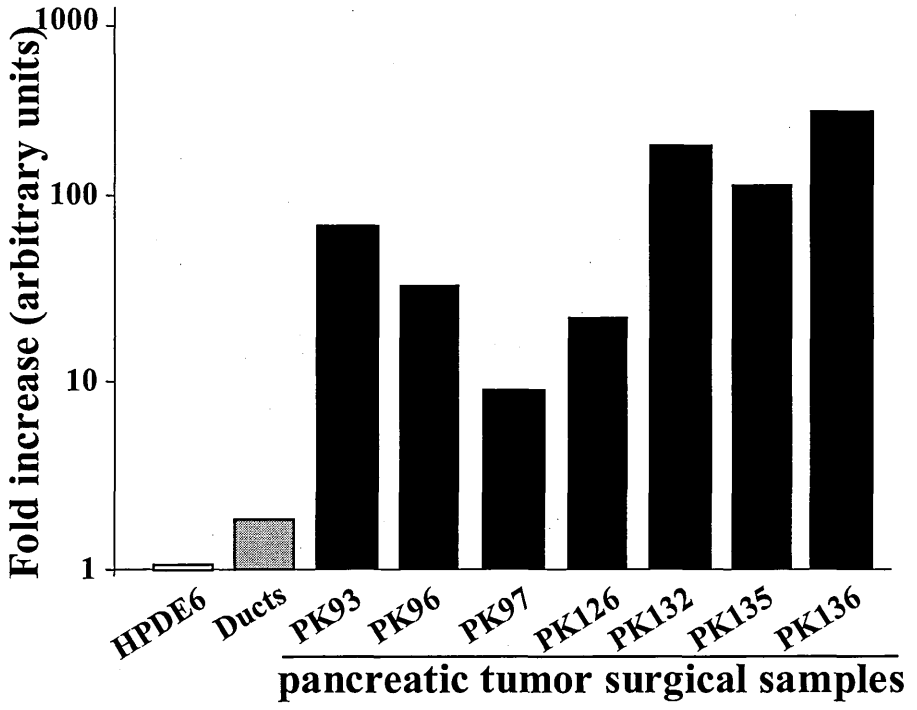


Fig 6.3 *CX3CR1* mRNA expression in tumor cells from surgical samples of resected pancreatic cancer patients. The amount of *CX3CR1* mRNA, normalized to *β-actin*, is expressed as relative to the cell line HPDE6 (white bar). Human epithelial pancreatic ducts were isolated from the pancreatic tissue of a multiorgan donor (grey bar). Shown is one representative analysis of two performed.

CX3CR1 surface expression

As for CXCR4, before starting to investigate the functional activity of CX3CR1 expressed by tumor cells, it was necessary to verify the expression of the receptor on the

surface of tumor cells. I evaluated CX3CR1 cell-surface expression by flow cytometry. Representative profiles of the cell lines A8184, AsPC1, Panc1, found to express the *CX3CR1* are presented in **Fig. 6.4**, showing that CX3CR1 is expressed on the membrane of pancreatic tumour cell lines.

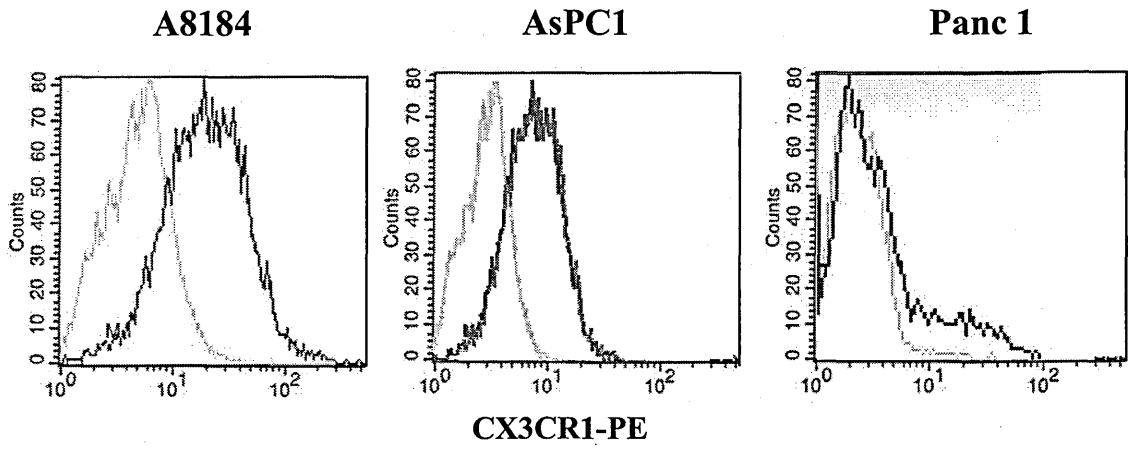
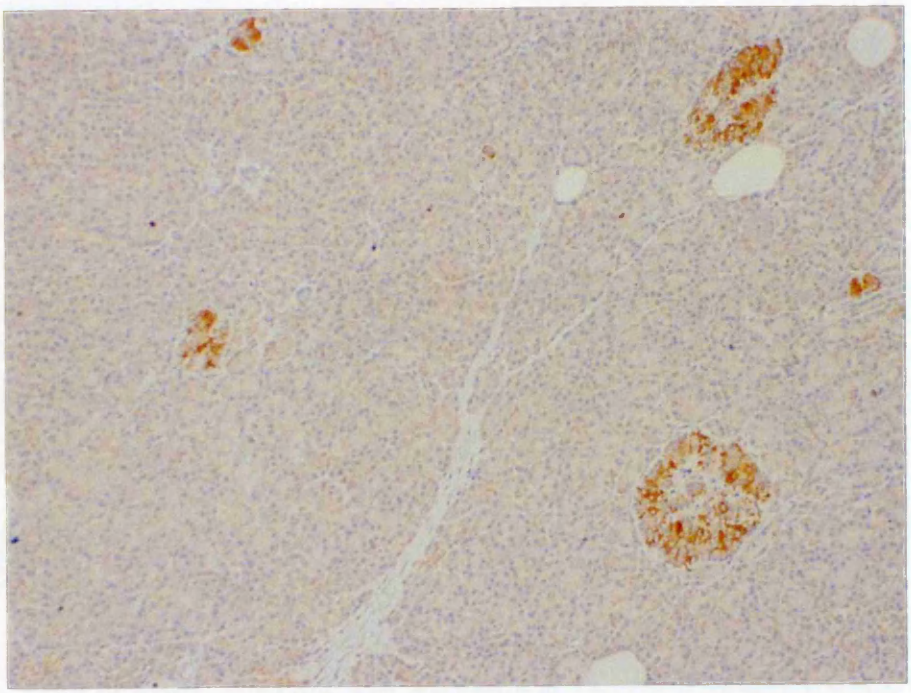


Fig 6.4 CX3CR1 is expressed at the surface of pancreatic cancer cell lines. Flow cytomety analysis of cell lines was performed with a PE-labeled anti-CX3CR1 antibody (3 μg/ml, clone 2A9-1). A PE-labeled mouse anti human isotype control (3 μg/ml) was used as control. Representative profiles are shown.

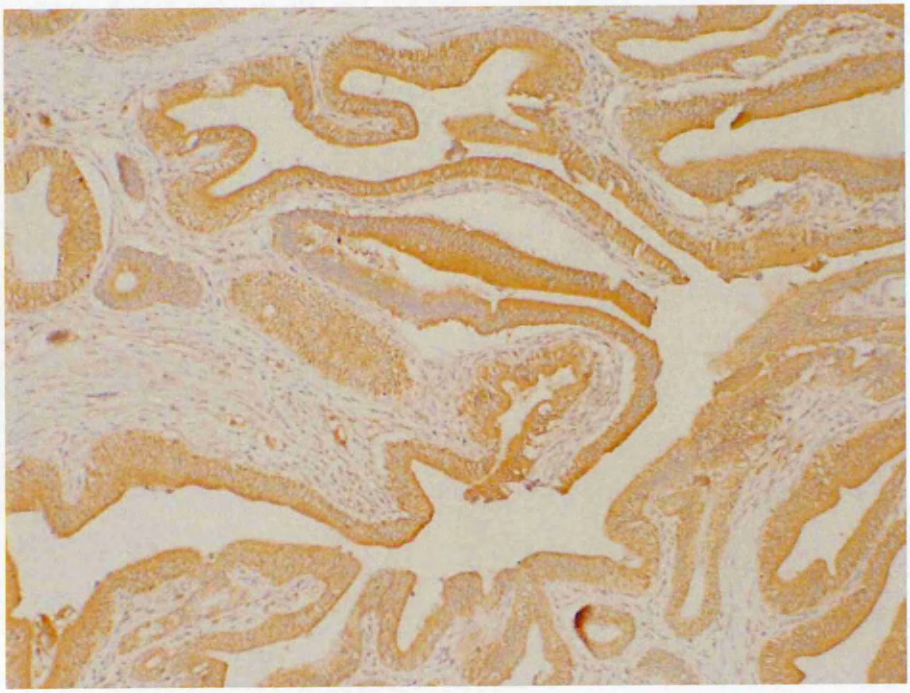
CX3CR1 *in vivo* expression

We also investigated, by immunohistochemistry, CX3CR1 protein expression in three surgical sections of pancreatic cancer patients (PK 93, PK96 and PK97, see **Table 3.1** for clinico-pathological features) and verified that CX3CR1 is expressed *in vivo* in pancreatic adenocarcinoma (**Fig 6.5, Panel B-D**). Most importantly, the expression of the receptor was detected in normal pancreatic ducts obtained from a multi-organ donor (**Fig 6.5, Panel A**).

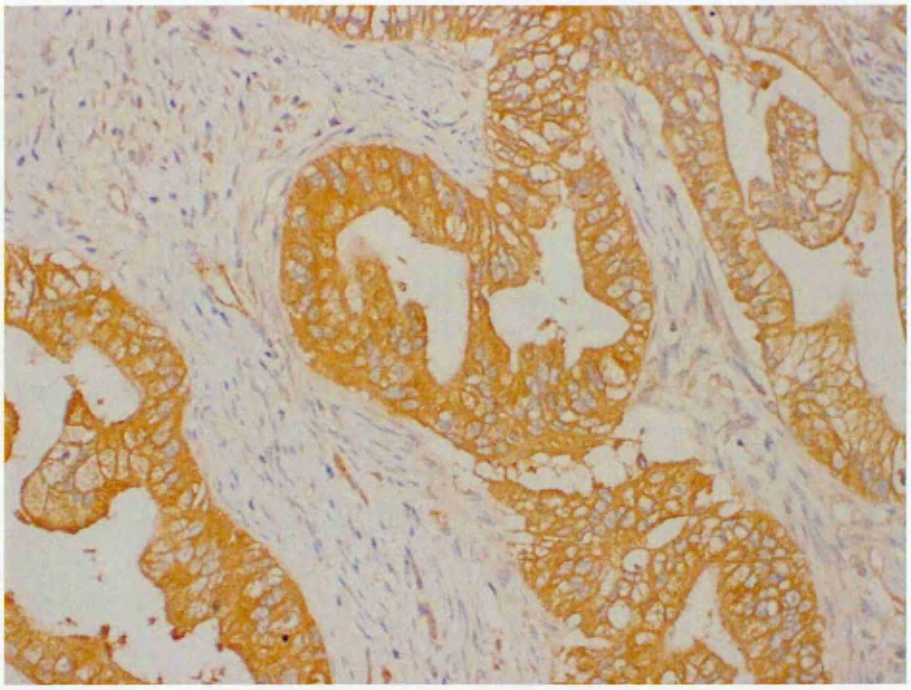
A



B



C



D

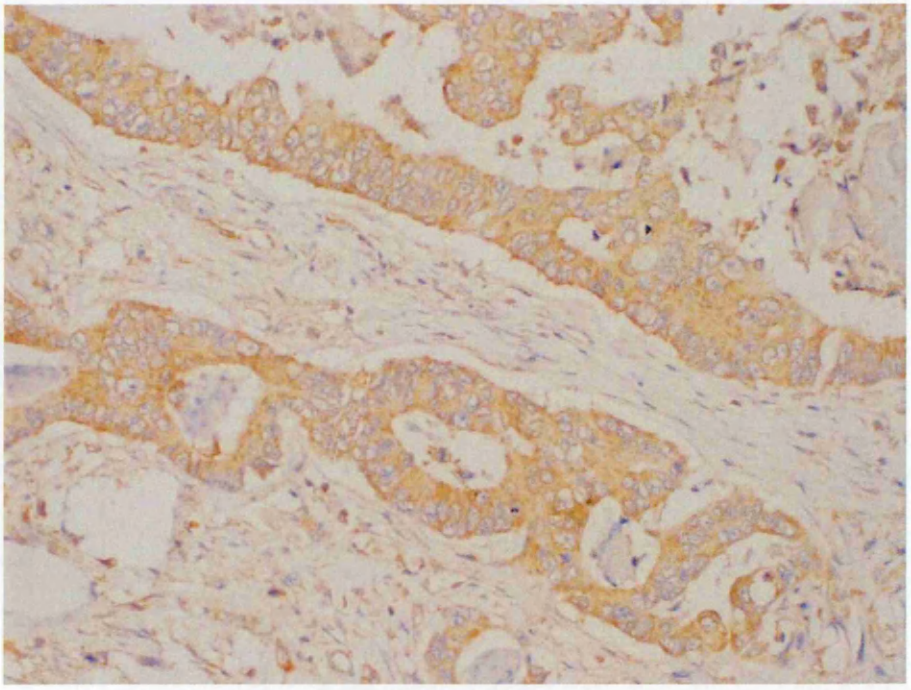


Fig 6.5 CX3CR1 expression by pancreatic cancer cells *in vivo*. Immunohistochemical analysis of three surgical sections of human pancreatic adenocarcinoma (PK 93, PK96, PK97, Panel B-D) and of a section of normal pancreatic ducts (Panel A) with an anti-CX3CR1 specific antibody.

6.3 CX3CR*1* functional activity

CX3CL1 stimulates pancreatic cancer cell chemotaxis

To confirm that CX3CR1 expressed in tumor cells is functional, I selected the cell lines with the highest receptor expression, A8184 and AsPC-1 (Capan 1, as said previously, was excluded for its characteristic to disaggregate in clusters) and tested in classical chemotaxis assays. **Fig. 6.6** shows that CX3CL1 elicited migration of tumor cells in a dose-response manner in both cell lines.

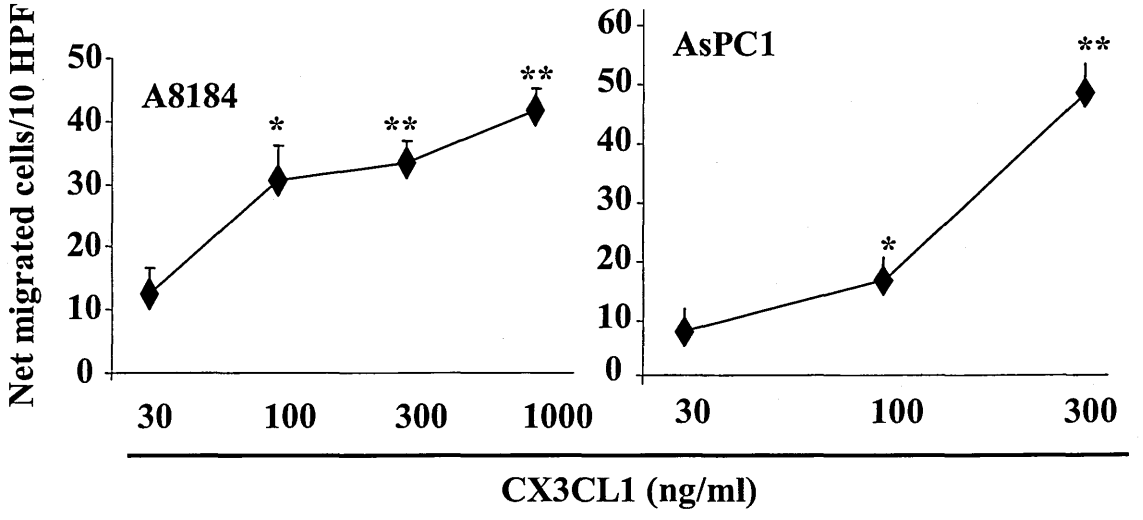


Fig 6.6 CX3CL1 stimulates pancreatic cancer cell chemotaxis. Selected CX3CR1-positive cell lines, A8184 and AsPC1 migrated in classical chemotaxis assays to different concentrations of Fractalkine in a dose-response manner. 8 μm pore filters were used and time of migration was 8 hours. Shown are net numbers of migrated cells over basal migration (in the absence of chemokine). Basal migration was 15 cells/10 HPF for A8184 and 7 cells/10 HPF for AsPC1, (*$p<0.02$ *versus* control; **$p<0.001$ *versus* control, Student t Test). Values are the mean ±SE of eight replicates. One representative experiment of three performed is shown

Moreover, cell migration was drastically inhibited by a blocking anti-CX3CR1 monoclonal antibody while was not affected by an irrelevant antibody (**Fig.6.7**).

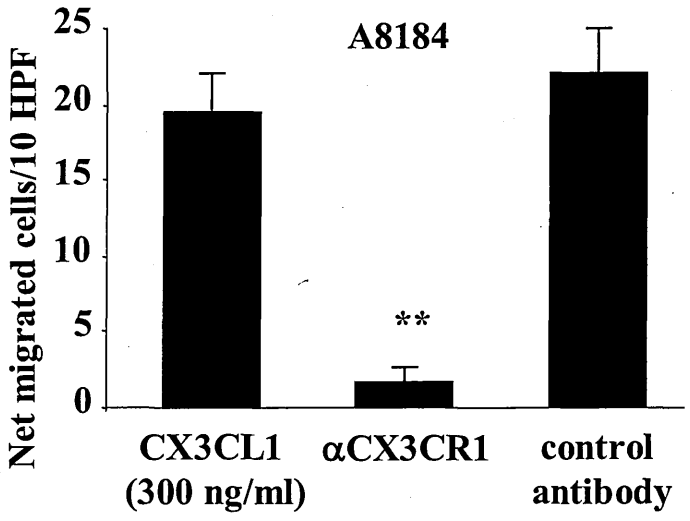


Fig. 6.7 CX3CL1 induced migration is mediated by CX_3CR1. Pre-treatment of A8184 cells with a blocking anti-CX3CR1 mAb (10 μg/ml) drastically reduced cell migration in response to 300 ng/ml of CX3CL1 (**p<0.01 *versus* control; Student t Test); an irrelevant antibody did not block CX3CL1-induced migration.

CXC3CL1 stimulates pancreatic cancer cell adhesion to endothelial cells

CX3CL1 is an unusual chemokine which can also function as an adhesion molecule. It was first described as produced by endothelial cells upon stimulation with inflammatory cytokines, the most potent stimulus being the combination of TNFα and IFNγ (266). CX3CL1 on endothelium mediates initial capture, firm adhesion and activation of circulating leukocytes. Therefore it was of interest to test a CX3CR1-expressing pancreatic cell line in adhesion assays to endothelial cell monolayers

Firstly, I verified by Northern Blot analysis that TNFα and IFNγ treatment of HUVECs (Human Umbilical Endothelial Cells), isolated following well established methods set up in our laboratory, (262) induced Fractalkine expression, and this was the case (**Fig. 6.8**).

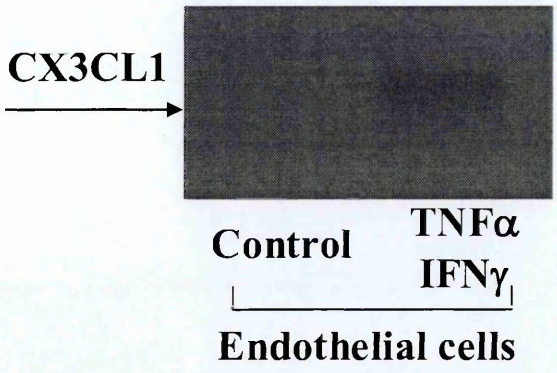


Fig. 6.8 TNFα/IFNγ induce CX3CL1 expression in endothelial cells. HUVEC cells were stimulated with TNFα (10 ng/ml) and IFNγ (1000 U/ml); RNA extracted after 24 hours was run in Northern Blot analysis. CX3CL1-specific mRNA was detected with cDNA ^{32}P-labeled probe.

Pancreatic tumor cell adhesion to the endothelial cell monolayer was enhanced upon stimulation of HUVECs with a combination of TNFα and IFNγ; this increase (nearly 70%) was partially reverted by pre-treatment of pancreatic cells with a CX3CR1 blocking antibody, thus demonstrating a specific role for CX3CL1 in adhesion to endothelial cells (**Fig 6.9**). In the same assay, adhesion of the cell line PaCa44, not expressing CX3CR1, to TNFα and IFNγ stimulated endothelial cells was not enhanced, thus supporting that the CX3CR1/CX3CL1 pair mediates pancreatic cancer cell adhesion to endothelial cells.

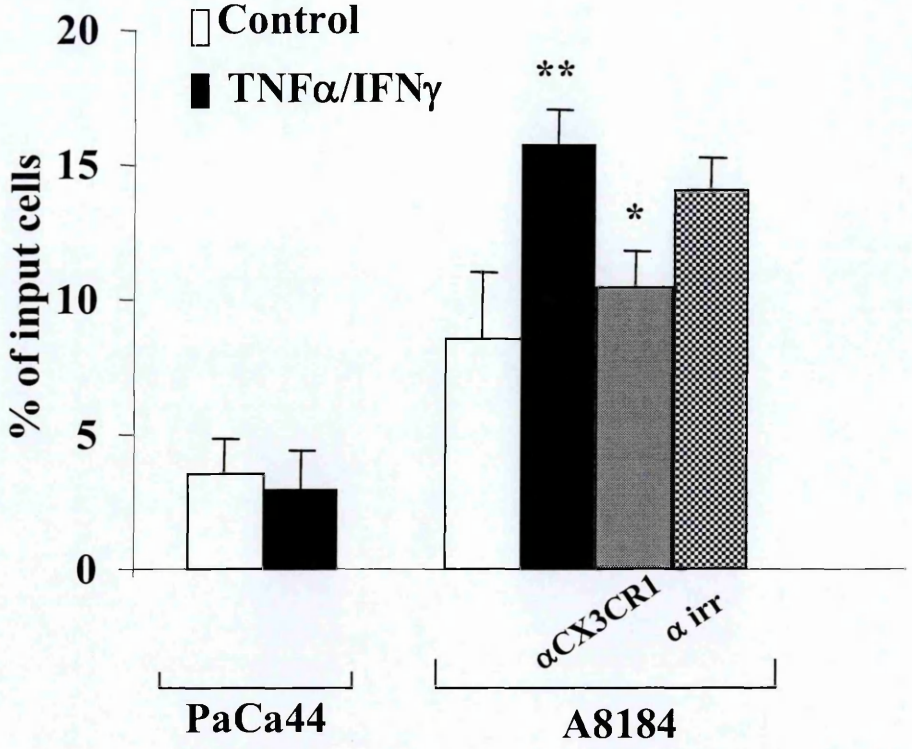


Fig 6.9 CX3CL1 mediates pancreatic cancer cell line adhesion to endothelium. Monolayers of HUVEC cells were stimulated with TNFα (10 ng/ml) and IFNγ (1000 U/ml) overnight and pancreatic cancer cell adhesion to the endothelial cell monolayer was assessed, at 37°C, for 1 hour. A8184 adhesion to the endothelial cell monolayer was enhanced upon stimulation of HUVECs with TNFα/IFNγ and this increase (nearly 70%) was partially reverted by pre-treatment of pancreatic cells with a CX3CR1 blocking antibody. Adhesion of the cell line PaCa44, not expressing CX3CR1, to TNFα and IFNγ stimulated endothelial cells was not enhanced. (*$p<0.02$ *versus* control **$p<0.001$ *versus* control, Student t Test). Mean values of two different experiments are reported.

6.4 *in vitro* pancreatic cancer cell nerve tropism

Human neuronal cells express and release CX3CL1

Our hypothesis of an involvement of CX3CL1/Neurotactin and its receptor in pancreatic cancer tropism for neuronal tissues arises from the observation that CX3CL1 is expressed in neurons (301-304). To better characterize the molecular mechanisms responsible for affinity of pancreatic tumor cells for neural structures, we tried to

generate an *in vitro* a model of nerve tropism. To do this, I screened a panel of 4 different cell lines of neuronal origin for the production of CX3CL1/Neurotactin, two neuroblastoma (SKN-BE, SY5Y), one glioma (H4) and one astrocytoma (TGSG). I measured CX3CL1 production both in basal conditions and after stimulation with inflammatory cytokines like TNFα, IFNγ, IL-1β.

A first Northern Blot analysis evidenced a neuroblastoma cell line (SKN-BE) producing CX3CL1 upon stimulation with TNFα/IFNγ (**Fig. 6.10**), while IL-1β did not exert this effect. Microglia is known not to produce CX3CL1, so it was not expected in the cell line H4.

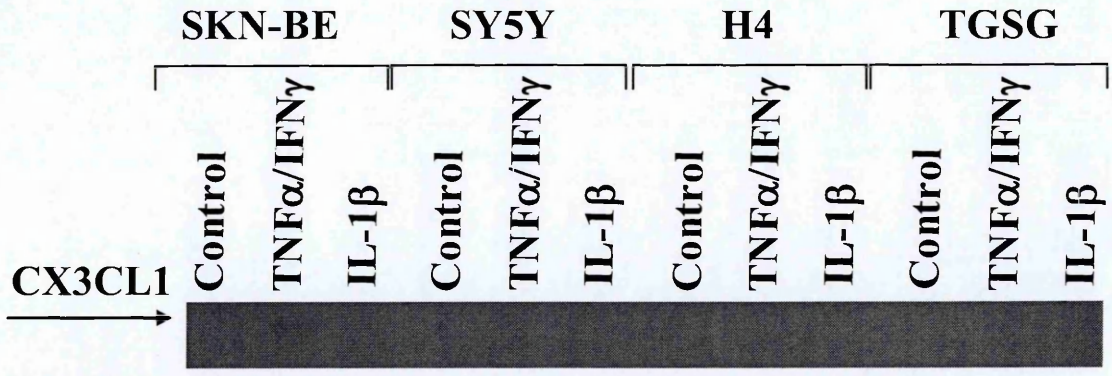


Fig. 6.10 TNFα/IFNγ induce CX3CL1 expression in SKN-BE cell line. Different neuron-derived cell lines were stimulated with TNFα (10 ng/ml) and IFNγ (1000 U/ml); RNA extracted after 24 hours was run in Northern Blot analysis. CX3CL1-specific mRNA was detected with cDNA ^{32}P-labeled probe.

The results were confirmed at the protein level, by analyzing chemokine production both in its membrane-bound and soluble form. CX3CL1 surface expression was upregulated by the concomitant treatment with TNFα and IFNγ in the neuroblastoma cell line SKN-BE (**Fig. 6.11**).

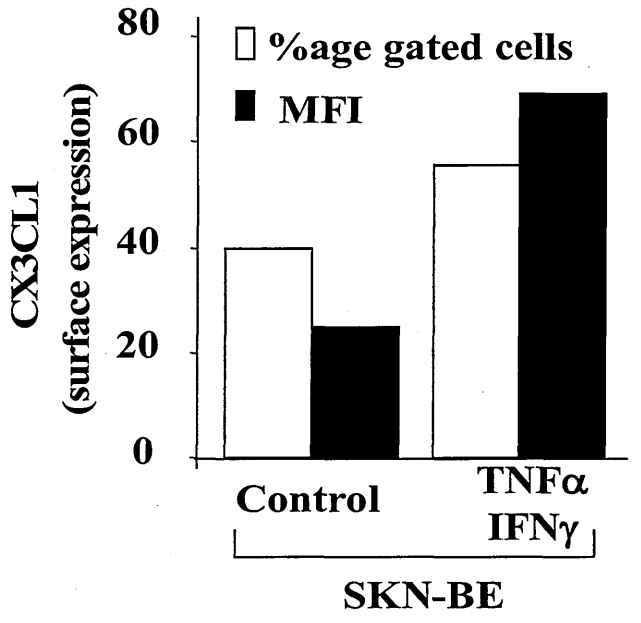


Fig 6.11 TNFα/IFNγ upregulate the surface expression of Fractalkine/Neurotactin in neuroblastoma cells. SKN-BE cells were stimulated for 24 hours and phenotype analysis performed with a CX3CL1 specific antibody, by FACS analysis. Percentage of positive cells (%age gated cells) and mean fluorescence intensity (MFI) are reported. One representative of three experiments is shown.

Moreover, CX3CL1 measurement in conditioned media of stimulated SKN-BE cells revealed that these cells are also able to release the chemokine in its soluble form. In line with the result obtained with Northern analysis, other neuronal cell lines did not produce the chemokine (**Fig.6.12**).

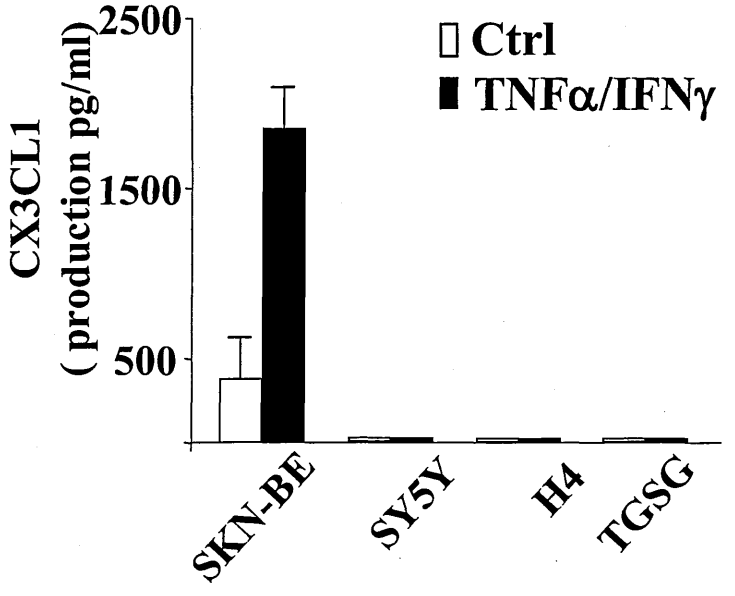


Fig 6.12 Fractalkine is released in the supernatant of neuroblastoma cells after TNFα/IFNγ stimulation. Only the cell line SKN-BE secreted CX3CL1/Neurotactin in the supernatant. The chemokine amount was measured by ELISA. Bars represent mean numbers of three different experiments .

CX3CL1 expression in human nerves and nerve metastasis

After I had verified in vitro that human neural cells express the chemokine Fractalkine, we thought it could be important to examine the expression of CX3CL1 *in vivo*. In particular, we focused our attention on surgical sections of pancreatic adenocarcinoma nerve metastasis and examined CX3CL1 protein expression by immunohistochemistry. **Figure 6.13** depicts CX3CL1 staining of sections corresponding to pancreatic cancer cells infiltrating nerve fibers.

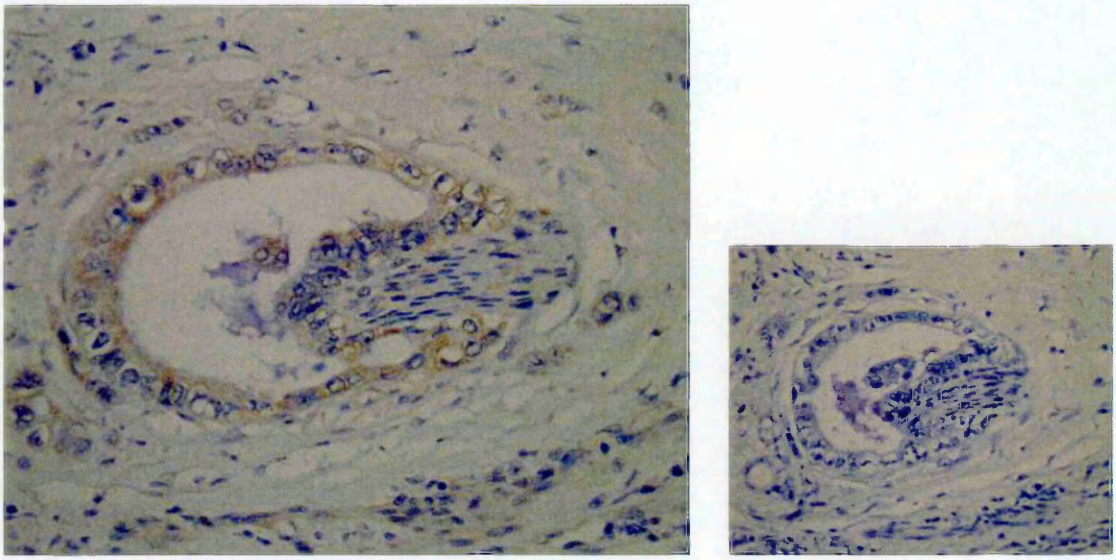


Fig 6.13. CX3CL1 is localized *in vivo* in pancreatic cancer nerve metastasis. Himmunoistochemical staining with a CX3CL1 specific antibody of a surgical section (PK93, see **Table.3.1** for clinico-pathological features) of pancreatic cancer tumour cells infiltrating nerve fibers. Picture on the right is the negative control (without primary antibody).

More specifically, Schwann cells inside the nerve are negative, while the staining is localized in intra axonal vesicles (**Fig 6.13, close-up).** Surprisingly, CX3CL1 seemed localized also in carcinoma cells (green arrow).

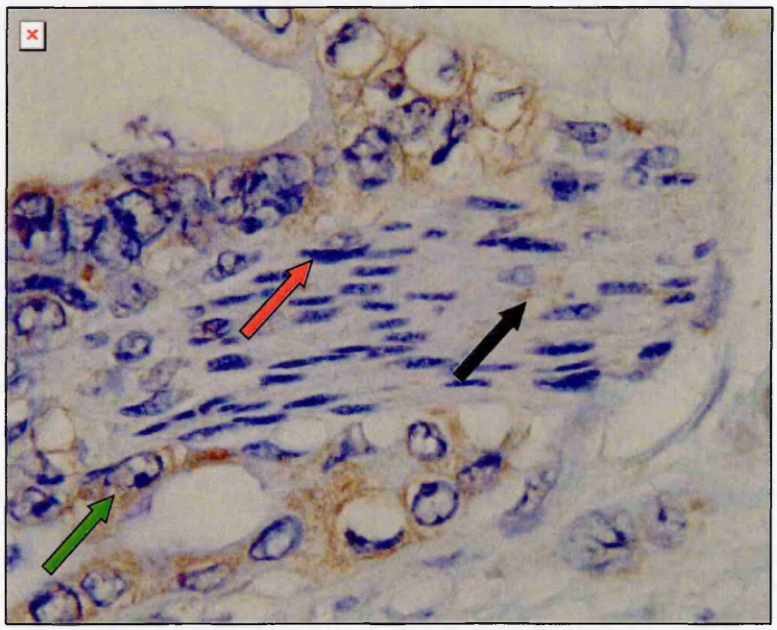


Fig 6.13, close-up. CX3CL1 staining is localized in intra axonal vesicles (black arrow); Schwann cells inside the nerve (red arrow) are negative, while staining is localized also in carcinoma cells (green arrow).

In the same analysis, we stained sections for the marker of proliferation Ki67; notably, cancer cells closed to nerves were highly stained, differently from other cells (**Fig. 6.14**).

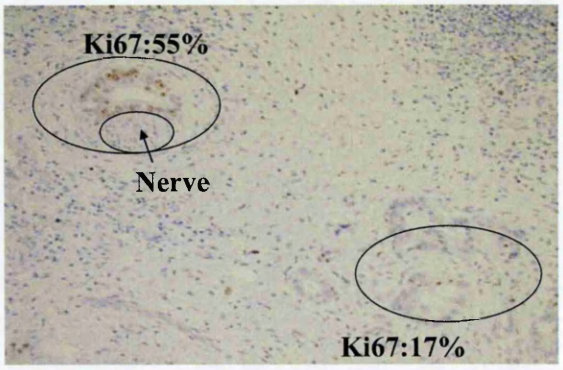


Fig 6.14 Ki67 staining on a surgical section of pancreatic cancer cells infiltrating nerves. Cancer cells close to nerves were highly stained, differently from other cells, indicating a higher proliferation rate.

This result suggests that the interaction between cancer cells and neural structures may positively affect pancreatic cancer cell proliferation.

Migration of Human Pancreatic tumor Cells to Neuronal Cell conditioned medium

As I had assessed that CX3CR1 positive pancreatic tumor cell lines migrate to recombinant CX3CL1, and that neuroblastoma cells are able to secrete CX3CL1/Neurotactin *in vitro*, it was of interest to verify if the natural, neuron-derived chemokine was functionally active and able to exert pancreatic cancer cell migration. Fractalkine concentration was measured in SKN-BE cell line supernatant, both in basal condition and after stimulation with TNFα/IFNγ (**Fig 6.12**); appropriately diluted (1:5) supernatants were tested as chemoattractants for A8184 cell line.

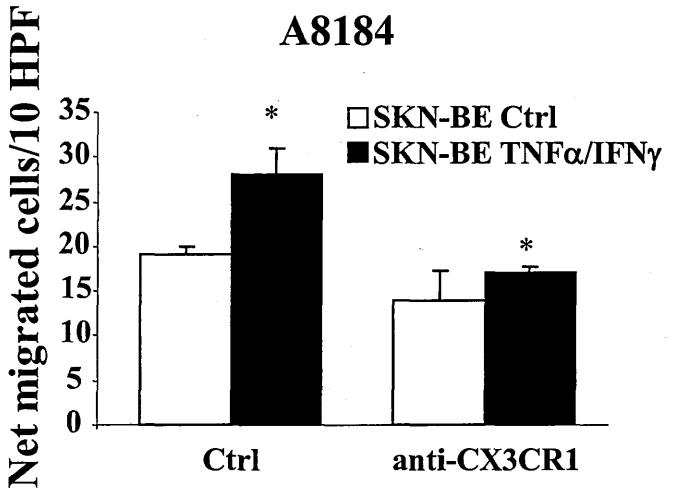


Fig 6.16 Neuroblastoma-derived supernatant containing CX3CL1/Neurotactin stimulates pancreatic cancer cell chemotaxis. A8184 cells migrated in classical chemotaxis assays to SKN-BE conditioned supernatant, diluted 1:5; migration was higher in the presence of TNFα/IFNγ stimulated supernatants. A8184 cell incubation with an anti CX3CR1-antibody partially blocked migration. Shown are net numbers of migrated cells over basal migration (in the absence of supernatant). Basal migration was 15 cells/10 HPF (*$p<0.02$ *versus* control, Student t Test). Values are the mean ±SE of eight replicates. One representative experiment of three performed is shown

TNFα/IFNγ stimulated SKN-BE conditioned supernatant elicited a chemotactic response in A8184 cells; migration was higher than the one observed in response to basal supernatant. Moreover, in the presence of a monoclonal anti CX3CL1 antibody migration in response to TNFα/IFNγ stimulated supernatant was decreased, demonstrating that A8184 migration was mostly Fractalkine mediated (**Fig.6.16**).

Human Pancreatic Tumor Cells adhere to Neuronal Cells expressing CX3CL1.

One peculiarity often clinically observed in pancreatic adenocarcinoma is their perineural invasion. Strikingly, cancer cells appear confined within the nerves, all along their route, even following their branching and without invading the surrounding tissue.

To investigate whether this interaction with nerves was CX3CL1, dependent I tested A8184 adhesion to neuroblastoma conditioned supernatant.

Pancreatic tumor cell adhesion to the neuroblastoma monolayer was enhanced upon stimulation of neuroblatoma with a combination of TNFα and IFNγ; this increase (nearly 100%) was partially reverted by pre-treatment of pancreatic cells with a CX3CR1 blocking antibody, thus demonstrating a specific role for CX3CL1/Neurotactin in the adhesion to neuroblastoma cells (**Fig.6.17**). In the same assay, SY5Y cells, the neuronal cell line not expressing the chemokine was tested and did not elicit pancreatic cancer adhesion after TNFα and IFNγ stimulation.

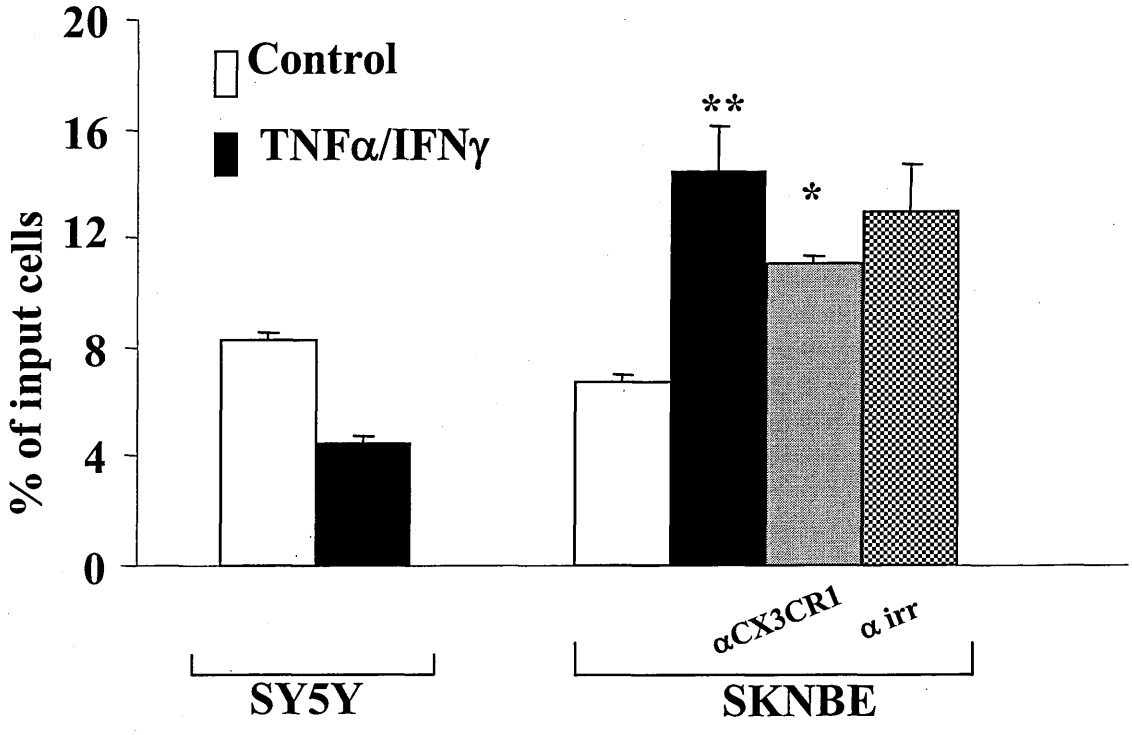


Fig 6.17 CX3CL1/Neurotactin mediates pancreatic cancer cell line adhesion to neuroblastoma cells. Pancreatic tumor cell line A8184 adhesion to a monolayer of the CX_3CL1 producing cell line SKN-BE was enhanced upon stimulation of SKN-BE with a combination of TNFα and IFNγ, known to upregulate its CX3CL1 expression (see **Fig 6.11**); this increase (nearly 100%) was partially reverted by pre-treatment of pancreatic cells with a CX_3CR1 blocking antibody. Adhesion was assessed after 1 hour of coincubation at 37°C. Adhesion of A8184 to the cell line SY5Y, not producing CX3CL1, was not enhanced by TNFα/IFNγ

stimulus. Values are the mean ± SE of two different experiments (*p<0.02 *versus* control **p<0.001 *versus* control, Student t Test).

6.5 Generation of a pancreatic cancer cell line stably expressing CX3CR1

To study the *in vivo* role of CX3CR1 in pancreatic tumor cells, we decided to generate a pancreatic cell line expressing exogenously the receptor CX3CR1. I prepared a viral construct carrying the sequence coding for human CX3CR1 fused to the sequence for the reporter gene GFP. This device would facilitate the recognition of tumor cells during the experiments, thanks to the fluorescence emitted by the green fluorescence protein. A similar GFP vector, lacking the CX3CR1 gene, was used as control (mock); the resulting parental MiaPaCa2 mock-infected cell line will be used in functional assays as control population.

For this experiment, I selected a receptor-negative pancreatic tumor cell line, MiaPaCa2, known to grow in nude mice and screened for the absence of chemokine receptors (e.g. CX3CR1, CXCR4, CCR2, CCR6) and stably infected it with the construct. After infection, cells were collected, expanded and tested for transgene expression.

To our purposes, the Fractalkine receptor should be expressed on the cell membrane, thus I selected FACS analysis to test the result of MiaPaCa2 infection; both GFP fluorescence and CX3CR1 presence on the surface membrane were checked (CX3CR1 by PE-conjugated anti-CX3CR1 antibody).

Figure 6.18 reports profiles of MiaPaCa2 not infected (left), infected with GFP (middle) and CX3CR1-GFP (right) vector respectively. Both the latter cell populations were 99% positive for the transgene.

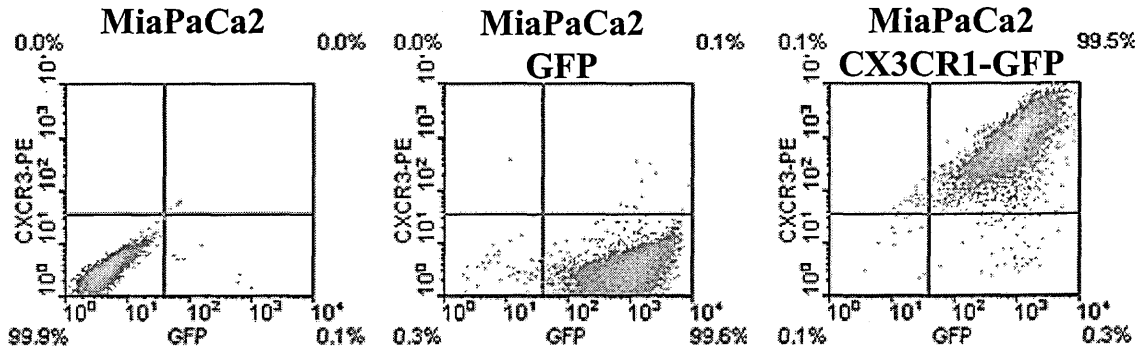


Fig 6.18 Transgene expression analysis of MiaPaCa2 stably infected. FACS profiles of MiaPaCa2 not infected (left), infected with GFP (middle) and CX3CR1-GFP (right) vector respectively. Both the latter cell populations were 99% positive for the transgene

MiaPaCa2 CX3CR1-GFP migration to CX3CL1

CX3CR1-GFP Miapaca2 and GFP-MiapPaCa2 were tested *in vitro* in chemotaxis assays for their capability to migrate to CX3CL1 gradients. CX3CL1 elicited migration only in the CX3CR1 positive cell line; notably, CX3CL1 was active at much lower doses (30 ng/ml and 100 ng/ml) than on A8184 and AsPC1, naturally expressing CX3CR1 (**Fig. 6.19**).

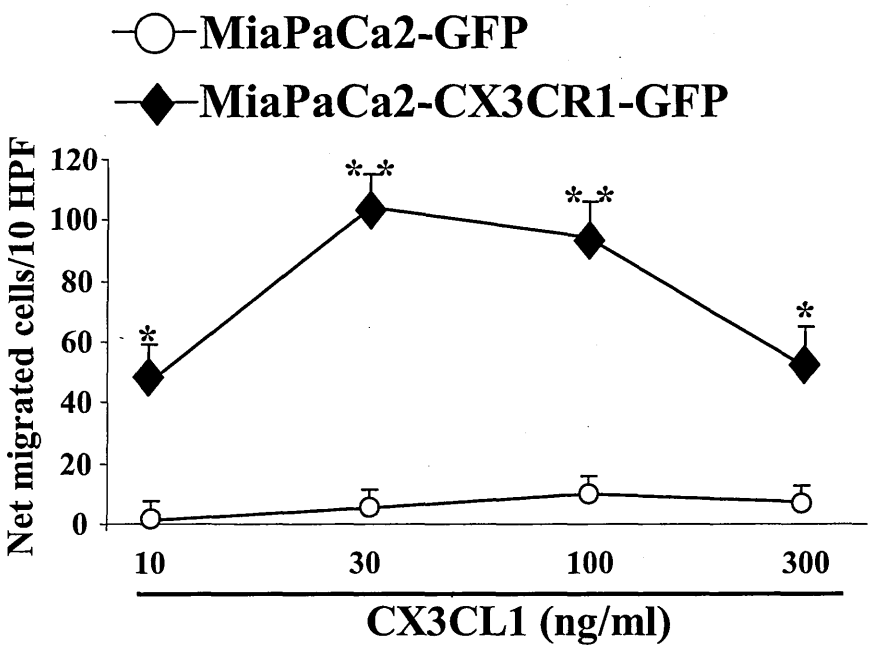


Fig 6.19 CX3CL1 elicits dose-response migration of MiaPaCa2 stably expressing CX3CR1. Shown are net numbers of migrated cells over basal migration (in the absence of chemokine). Basal migration was 27 cells/10 HPF for MiaPaCa2-CX3CR1-GFP and 40 cells/10 HPF for MiaPaCa2-GFP, Values are the mean ±SE of eight replicates (**p<0.001 *versus* control, Student t Test). One representative experiment of two performed is shown.

MiaPaCa2 CX3CR1-GFP sub-lines selection

We decided to select CX3CR1-GFP MiaPaCa2 growing sub-lines on the basis of surface expression of CX3CR1; this tool will help us in future experiments to better understand the relative importance of CX3CR1 expression in pancreatic cancer cells and the role in nerve tropism; in fact, at least two CX3CR1-transfected sub-lines and the parental cell line will be characterized both *in vitro* and in *in vivo* models of pancreatic cancer .

Instead of cloning the whole cell population to select sub-lines, we started from the subpopulation migrated to a CX3CL1 gradient in a Transwell migration assay. This strategy represents a cloning on functional basis. Cells passed through the filter during migration assay were collected and cloned to limiting dilution. **Fig. 6.20** reports FACS

profiles of two clones with similar in vitro growth kinetics (data not shown) and different levels of transgene expression.

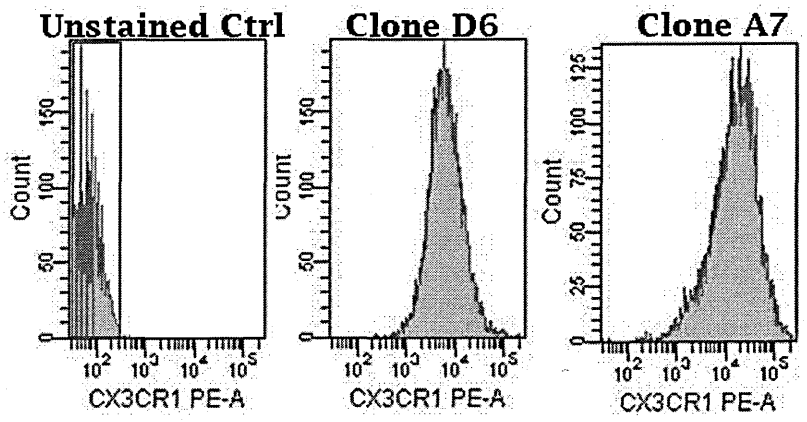


Fig. 6.20 Different CX3CR1 surface expression of two MiaPaCa2 CX_3CR1-GFP clones. MiaPaCa2 CX3CR1-GFP cloned by limiting dilution and two sub-clones selected for having different surface expression of the receptor. Mean fluorescence intensity was 10 x 10^3 for Clone D6 and 25 x 10^3 for Clone A7.

6.6 Discussion

In this Chapter I presented data on the expression and functional role of the chemokine receptor CX3CR1 in pancreatic adenocarcinoma and its possible involvement in the tropism of this cancer type for neuronal tissues.

The screening of pancreatic cell lines revealed that 6 of 11 cell lines tested express heterogeneous levels of *CX3CR1*, with three cell lines (Capan-1, A8184, AsPC1) showing the highest expression transcripts, that was confirmed at the protein level in selected cell lines. The expression of this particular receptor in pancreatic cancer had never been reported up to now; recently, CX3CR1 expression in prostate cancer has been documented and associated to prostate metastasis to bone marrow (305).

As observed for CXCR4, also CX3CR1 seemed most frequently expressed in those pancreatic cancer cell lines derived either from ascites or metastasis, supporting the hypothesis that CX3CR1 may be involved in tumour dissemination.

Also surgical specimens from pancreatic adenocarcinoma express *CX3CR1* in higher amounts compared with the immortalized cell line derived from human pancreatic ducts (HPDE6) as well as compared to freshly isolated normal pancreatic ducts.

In the world of chemokines, C and CX3C chemokines have been long regarded as two minor components, but the interest in these two classes has recently gained a new impetus, resulting from the identification of their receptors and the accumulation of data about their expression in several human diseases. Differently from the other

chemokines, XC and CX3C chemokines show less redundancy (303); CX3CL1/Fractalkine/Neurotactin binds only the receptor CX3CR1.

CX3CL1 is encoded as a transmembrane molecule, consisting of a chemokine domain linked to a mucin-rich stalk domain (299, 304); the cleavage of the full-length molecule results in the soluble form. As expected, the two different forms correspond two distinct biological functions: soluble CX3CL1acts as a chemoattractant, while the membrane-bound molecule, found expressed predominantly by epithelial cells (306), mediates adhesion of CX3CR1-bearing cells to cells expressing the ligand (304, 307, 308). We wanted to confirm that CX3CR1 expressed in tumor cells is functional, by testing both the biological activities mediated by the chemokine. Thus, we firstly verified that CX3CL1 elicits migration of pancreatic tumor cell lines in a dose-response manner (**Fig 6.6**) and this response was prevented by a CX3CR1 antibody (**Fig 6.7**).

Fractalkine was first described on endothelial cells upon stimulation with inflammatory cytokines (304, 309), the most potent stimulus being the combination of TNFα/IFNγ (266). CX3CL1 on endothelium mediates initial capture, firm adhesion and activation of circulating leukocytes. In our studies, CX3CL1 expressed on endothelial cells promoted CX3CR1 positive pancreatic tumor cell adhesion; the effect was CX3CL1-dependent, as blocked by a specific antibody and not observed in a CX3CR1-negative cell line (**Fig 6.9**). This result is particularly important as tumor cell adhesion to endothelium is a key event in the process of tumor invasion and metastasis; moreover, and most important, pancreatic cancer is particularly invasive through the hematogenous route, often giving metastasis to large vessels (72) and the involvement of CX3CL1 in tumor cell adhesion to endothelial cells might represent a possible mechanism of venous invasion.

Other cell types have been reported to produce the chemokine CX3CL1. In particular, the relatively high levels of Fractalkine in the brain and nerve terminations (310) has raised questions related to the function of this chemokine in the central nervous system; it has been proposed that CX3CL1 regulates cellular communication between neurons, producing the ligand and microglia, expressing the receptor (302, 311).

The expression of CX3CL1/Neurotactin in cells of the nervous system triggered our interest in the perspective of pancreatic cancer nerve tropism. Indeed, detailed pathohistologic studies of large series of resected pancreatic ductal adenocarcinoma have shown that one of the most persistent characteristics is perineural invasion (72). In studies conducted in a large series of patients with pancreatic cancer, the incidence of perineural invasion resulted to be 100%, most of which extended also to extrapancreatic nerves. Some other studies indicate that all pancreatic cancers show perineural invasion if several sections are histologically considered (74, 79), and this pattern of tumor spreading seems to be an early event, occurring in 75% of cases of stage I disease (77, 78). Collectively, many different observations support the idea that a tropism of pancreatic cancer cells to neural tissues really exists, observation confirmed also in a hamster model (74). The aggressive and unchecked spread through haematogenous and perineural routes accounts for the rapid and fatal progression of the disease; invasion of vessels and nerves is associated with poor prognosis in this disease and tumor growth along nerve fibers is the cause of severe pain in tumor patients. Moreover, growth of tumor foci around root ganglia in the spinal cord, is the first cause of tumor recurrence after surgery.

The peculiar tropism of pancreatic tumors for vessels and nerves prompted us to investigate whether Fractalkine and its receptor on tumor cells confer the ability to invade and disseminate along nerve fibers expressing the ligand CX3CL1.

To better characterize the molecular mechanisms responsible for affinity of pancreatic tumor cells for neural structures, we tried to reproduce *in vitro* a model of nerve tropism. We analyzed the biological interaction between a neuronal cell line expressing the chemokine and CX3CR1-positive pancreatic cancer cells and found out that neuronal-derived CX3CL1 is chemotactic for pancreatic cancer cells (**Fig 6.16**) and mediates their adhesion to neurons (**Fig 6.17**). Moreover, by immunohistochemistry we had evidence of the *in vivo* role of CX3CL1 in pancreatic cancer cell crosstalk with nerves; in surgical sections of intrapancreatic nerves infiltrated by pancreatic cancer metastasis, neural cells contain CX3CL1. Up to now, the chemokine has been reported in central neurons as well as in peripheral nerves (302, 310), but has never been analyzed in intra-pancreatic nerves.

Other molecules have been involved in the neural tropism of pancreatic cancer cells. In particular, the neurotrophin family of growth factors and their receptors, which have been found to be implicated in the paracrine growth and regulation of a number of neuronal as well as non-neuronal tumor types, including pancreatic adenocarcinoma. The expression of various neurotrophins (BDNF (brain-derived nerve growth factor), NT-3, NT-4, NT-5) and their receptors (Trk A, B, C) in pancreatic cancer cells have been demonstrated to play a role in their invasiveness (312). By the same way, Zhu and coll. have found that the expression of nerve growth factor and its receptor TrkA correlates with perineural invasion and pain in pancreatic cancer (313).

Part of the results in this Chapter is focused on the generation of a pancreatic tumour cell line stably expressing exogenous CX3CR1. This tool would be indispensable to define the involvement of the chemokine receptor CX3CR1 in the pattern of migration, adhesion and growth of tumor cells. The preliminary in vitro characterization of the CX3CR1 expressing cell line has confirmed what previously observed that CX3CR1 mediates pancreatic cancer cell chemotaxis to CX3CL1 gradient, as well as the parental mock cell line did not migrate to the chemokine (**Fig 6.19**). This cell line will be used in future *in vivo* models of nerve tropism.

To investigate whether receptor expression confers tumor cells with enhanced ability to spread and grow along nerve structures *in vivo*, we are planning to set up a model of nerve injury. Right sciatic nerves of immunodeficient mice will be crushed at the mid height and after 24 hours tumor cells (CX_3CR1-GFP MiaPaCa2 and mock GFP MiaPaCa2 tumor cells) will be injected in the tail vein. After 24-48 hours, sections of the injured (and control-lateral) nerves closed to the crush site will be taken and analyzed for the presence of GFP transgene by Real-Time PCR.

The crush model should maximize the expression of the chemokine ligand by nerves; as reported in the literature (310, 311, 314), in fact, a transient crush of sciatic nerve induces CX3CL1 expression. If the axis Neurotactin/CX3CR1 is indeed involved in the adhesion and dissemination of pancreatic tumor cells along nerve fibers, CX3CR1-transfected cells should localize preferentially to the site of nerve injury, where Neurotactin is upregulated.

We are also planning other *in vivo* experiments, in which CX3CR1-GFP MiaPaCa2 sub-clones expressing the surface receptor in different amounts will be transplanted

orthotopically in nude mice and the growth characteristics, pattern of tumor metastatization and overall survival will be evaluated.

The observation that perineural invasion occurs earlier than lymph node has recently lead to the suggestion that considering neural invasion as an independent factor would better correlate with survival (74). Targeting nerve invasion would therefore represent a valuable therapeutical tool; indeed, given the lack of success of conventional surgical intervention, less aggressive procedures targeting the tumor cells within the nerve could be a valid alternative strategy. Our results pointing out a role of CX3CR1 and its ligand CX3CL1 in pancreatic cancer nerve invasion suggest that this chemokine/ receptor pair could be considered as good candidates.

6.7 Summary of results in Chapter 6

In this Chapter, I have presented data suggesting a role of CX3CR1/ CX3CL1 axis in human pancreatic cancer neural tropism.

Specifically, the following results have been presented:

- pancreatic cancer cell lines express *CX3CR1* mRNA; the expression is higher in lines derived form metastatic lesions compared with those derived from primary tumors;

- transcript expression is associated to surface expression in pancreatic carcinoma cell lines;

- *CX3CR1* is expressed also in pancreatic cancer cells from primary tumors; all surgical carcinoma samples tested express higher levels of *CX3CR1* than normal pancreatic duct cells; moreover, CX3CR1 is expressed in vivo in pancreatic cancer cells, as assessed by immunohistochemical analysis;

- CX3CR1 on pancreatic cancer cells is functional and mediates their migration to CX3CL1/Fractalkine; migration is selectively inhibited by anti-CX3CR1 monoclonal antibody

- CX3CR1-positive tumor cells adhere to endothelial cells stimulated with TNFα/IFNγ, stimuli known to induce CX3CL1 expression; adhesion was prevented by pre incubation with an anti-CX3CR1 specific antibody

- in an in vitro model of nerve tropism, neuronal derived Fractalkine elicits CX3CR1-positive pancreatic tumor cell adhesion and migration

- in surgical sections of pancreatic metastasis to nerves, CX3CL1 is found localized in neuronal cells, suggesting a role in tumour cell and nerve crosstalk.

Chapter 7

Conclusions and future directions

7.1 Summary and overall conclusions

In the last decade, the role of chemokines in tumor biology has dramatically developed and has expanded from the regulation of leukocyte attraction within the tumor mass to the promotion of tumor cell survival, proliferation and dissemination (82, 83, 181). Experimental evidence have supported the concept that chemokines could direct tumor cell migration in vivo: malignant cells bearing chemokine receptors on the cell surface would be endowed with the capability to respond to chemokine gradient and selectively migrate to specific organs where the chemokine is present.

The data presented here demonstrate that a selected set of chemokine receptors are expressed in carcinoma of the pancreas and are involved in tumour cell migration and invasion. We found that the chemokine receptors CXCR4 and CX_3CR1 are functional in pancreatic adenocarcinoma. In particular, the CXCR4/CXCL12 axis promotes pancreatic tumor cell migration, matrix degradation and invasion, proliferation and survival. For CX3CR1, a role in perineural tropism is suggested as CX3CR1/Fractalkine axis seems to be involved in the dissemination of pancreatic tumour cells via nerve structures.

An increased understanding of the mode of action of chemokines on tumor cells and their microenvironment would be important to achieve significant therapeutic results in the management of cancer patients. If chemokine and receptor expression is an advantage for tumor cells, it is possible that these molecules will become target of therapeutic interventions. Indeed, chemokine and receptor antagonists are being developed and actively investigated. In the case of pancreatic adenocarcinoma, if CX3CR1 and CXCR4 receptors are really involved in invasion and metastasis, their

inhibition could potentially result in a controlling the metastatic behaviour of this aggressive cancer.

7.2 Targeting the chemokine system

Beside the results presented in this thesis, concerning the role of chemokine receptors in human pancreatic adenocarcinoma, an increasing number of studies have clearly shown that several types of cancers express various chemokine receptors, and that these receptors may be implicated in the process of distant metastasis and even organ specific metastasis. In addition, chemokines have been demonstrated to deliver growth signals to tumor cells and therefore may be directly involved in their survival and progression. As a consequence, chemokines and their receptors are now regarded a valuable molecular target for the treatment of malignant tumors. Indeed, disrupting the interaction between the receptor and its ligand chemokine may prove to be a useful approach for treating cancers.

Several drugs targeting the chemokine system are being developed and actively investigated in inflammatory and autoimmune diseases and could be useful to treat cancer patients. In several pre-clinical studies, these molecules have shown activity both as inhibitors of leukocyte recruitment, as well as inhibitors of metastatic spreading. These studies provide a scientific rationale for the use of anti-chemokine or anti-receptor agents in human cancer (81, 83, 91, 315, 316).

Some of these drugs are chemokine antagonists and prevent the binding and/or the signalling of the right ligand through its specific receptor. Recent pre-clinical studies have reported anti-cancer activity of chemokine receptor specific antibodies, in several murine cancer models. For instance, an anti-CXCR4 monoclonal antibody significantly

inhibits the metastasis of human breast carcinoma cells to the lymph node of SCID mice (81). Pretreatment of non-Hodgkin lymphoma cells with anti-CXCR4 antibody also inhibits subsequent growth of the cells in immunodeficient mice (317). In a model of non-small-cell lung cancer, immunodeficient mice inoculated with CXCR4-positive human NSCLC had lower organ metastasis if mice were injected with antibodies to CXCL12 (318). However, whether the inhibitory effects observed with these antibodies are caused only by the inhibition of chemotaxis remains unclear, because antibody-bound tumor cells are likely to be subject to Fc-mediated trapping by the liver and/or lung and to Fc-mediated killing by macrophages.

Other studies have focused on selective inhibitors, including the CXCR4 antagonist AMD3100, originally tested in HIV patients and shown to have a good safety profile (296, 297); these studies have provided the scientific rationale for the clinical evaluation of AMD3100 in malignant brain tumors (298, 299). We interestingly found that AMD3100 inhibits the CXCL12-stimulated proliferation of CXCR4-positive pancreatic tumour cell lines. Consistently with our results, recently, Saur et al found out that AMD3100 effectively blocks in vivo the enhanced metastatic potential of CXCR4-expressing pancreatic cancer cells (319). Another CXCR4 inhibitor, the synthetic polypeptide TN14003, was recently demonstrated to block breast cancer metastasis in an animal model (320), as well as the CXCR4 inhibitor TC114012 strongly counteracted lymphoma development in SCID mice with lymphomas (321, 322).

Murine breast cancer cells express also the chemokine CCL5 and the leukocyte infiltrate bears the corresponding receptors CCR1 and CCR5 (212). In a recent work, treatment of mice with Met-CCL5, a CCR1/CCR5 receptor antagonist, significantly reduced the volume and weight of tumors. Furthermore, this treatment strongly

decreased the total number of tumor-infiltrating macrophages, supporting the hypothesis that macrophages contribute to tumor development (212).

An original strategy to inhibit chemokine and receptor binding is to prevent their cell surface expression. Cells transfected with a modified CXCL12 bearing an endoplasmic reticulum (ER) sequence (KDEL), retained both ligand and receptor (CXCR4) in the ER, thus preventing CXCR4 from reaching the cell surface (323). When a T cell hybridoma was transfected, the CXCR4-negative cells failed to disseminate to multiple organs upon intravenous injection, indicating a decisive role for CXCR4 in the dissemination of haematopoietic malignancies (323). Another example comes from colon carcinoma cells transfected with the ER-retention sequence. As a matter of fact, CXCR4-deficient cells did colonize the lungs to the same extent as control cells, however, they proliferated significantly less and did not expand (324). Once more, these results underline the important role of CXCL12 in the survival and proliferation of cancer cells *in vivo*.

Recently, RNA interference technology has also been used to prevent tumorigenesis in different mouse models. In mice injected with breast cancer cells expressing CXCR4, a reduction in lung metastases was observed, by silencing CXCR4, either by transfecting breast cancer cells with interfering RNA molecules or weekly injecting them in the animals (325). In other works, RNAi reduced expression of CXCR4 in a murine highly metastatic mammary cancer cell line, thus limiting the growth of orthotopically transplanted breast cancer cells (326) and in nasopharyngeal human carcinoma cell lines injected in mice, limiting metastasis to the lungs (327).

Finally, viral proteins could represent promising molecules for the chemokine

system targeting. Such proteins, used as immune escape mechanism by the virus, in some instances can bind and inhibit chemokine activity. Among others, the protein M3 has been described to bind several human and murine CC chemokines (including CCL2, CCL13, CCL5, and CCL21), some CXC chemokines, such as CXCL1 and CXCL10 and also Fractalkine. Binding of chemokines is associated with inhibition of their activity (328-330) Finally, more recently, the vaccinia virus soluble protein 35K, which binds to and inactivates a broad spectrum of CC-chemokines, has been shown to inhibit macrophage recruitment and atherosclerosis in ApoE-/- mice (161, 162).

These and other strategies based on emerging mechanistic data represent some of the promising new directions in the therapy of metastatic disease. However, some problems need to be faced: although CXCR4 has been found to be the most expressed chemokine receptor in cancer cells from different histological types, tumors display heterogeneity in respect to chemokine receptor expression and selective inhibitors might be required to treat different cancers. In this perspective, gene-array analyses are revealing important tools in defining tumour subsets based on specific marker heterogeneity (331).

Another important consequence of the use of chemokine inhibitors to treat cancer arises from the complexity of the chemokine system and their role in normal physiology; disrupting this system could bear important clinical side effects. However, a peculiarity of the complex system of chemokines and their receptors is their redundancy, which guarantees the chemokine system performance, even if alterations affecting individual components occur.

It is more probable that chemokine-receptor-based agents could contribute significantly to the control of tumor cell invasion and metastasis, making cancer clinically manageable, when used in conjunction with other therapeutic regimens. In

fact, in many tumor types, including pancreatic cancer, classical surgical intervention is often impossible or not successful, due to the persistence of micrometastatic lesions and chemokines, used as adjuvant therapy, might be useful to limit tumor dissemination.

This could be even more important in the case of pancreatic adenocarcinoma nerve invasion, which is a source of severe pain in tumour patients; indeed, if the CX3CR1 receptor is involved, as supported here, blocking it could represent an alternative, less aggressive procedure, targeting the interaction of tumour cells with nerves.

7.3 Future plans

A number of questions arose from this thesis and we are now planning further experiments necessary to investigate in detail the role of chemokine and receptors in human pancreatic adenocarcinoma.

Specifically, some tasks we would like to achieve in the future:

- localisation of CXCR4 in vivo, by immunohistochemistry on surgical sections. It would be interesting to analyze different pancreatic cancer stages and investigating if a correlation exists between the progression of the disease and CXCR4 tumor expression This would give us a confirmation of the role of CXCR4 in human pancreatic adenocarcinoma dissemination *in vivo*.
- assess the therapeutic benefits of CXCR4 antagonists; we have already used AMD3100 in vitro and to assess its effect in *in vivo* models could be an interesting task. We have several pancreatic adenocarcinoma cell lines that will grow as a xenograft in nude mice. The effect of AMD3100 or other CXCR4 antagonists on tumour growth could be assessed.
- functional *in vitro* characterization of CX3CR1-GFP MiaPaCa2, to verify if the CX3CR1 receptor confers pancreatic tumor cells with an enhanced capability to spread to nerves. To this aim, adhesion assays and other functional activities will be tested.
- investigate if proliferation and survival of CX3CR1 positive pancreatic cancer cell lines are affected by the receptor expression. To this aim, the proliferation rate of the infected cell line, upon stimulation with the ligand CX3CL1, compared to the uninfected cell line will be assessed, to test whether a possible autocrine loop exists.
- optimize the model of sciatic nerve crush and perform *in vivo* experiments with the CX3CR1-GFP MiaPACa2 cell line

- perform other *in vivo* experiments; CX3CR1-transfected tumor cells will be inoculated orthotopically in nude mice and their growth and metastatic potential will be characterized and compared to parental cells.

- assess the therapeutic benefits of CX3CR1 antagonists. As up to now there are no selective CX3CR1 inhibitors; we could plan to silence the receptor with the available tool of RNA interference.

This work has contributed to the following publications (reprints are enclosed at the end of the thesis)

Marchesi F, Monti P, Leone BE, Zerbi A, Vecchi A, Piemonti L, Mantovani A, Allavena A. Increased survival, proliferation and migration in metastatic human pancreatic tumor cells expressing functional CXCR4. **Cancer Res. 2004; 64: 8420-8427.**

Monti P, **Marchesi F**, Reni M, Mercalli A, Sordi V, Zerbi A, Balzano G, Di Carlo V, Allavena P, Piemonti L. A comprehensive in vitro characterization of pancreatic ductal carcinoma cell line biological behavior and its correlation with the structural and genetic profile. **Virchows Arch. 2004 Sep;445(3):236-47.**

Allavena P, **Marchesi F**, Mantovani A. Role of chemokines and their receptors in tumor progression and invasion: potential new targets of biological therapy. **Current Cancer Treatment Review. 2005; 1: 81-92.**

Monti P, Leone BE, **Marchesi F**, Balzano G, Zerbi A, Scaltrini F, Pasquali C, Calori G, Pessi F, Sperti C, Di Carlo V, Allavena P, Piemonti L. The CC chemokine MCP-1/CCL2 in pancreatic cancer progression: regulation of expression and potential mechanisms of antimalignant activity. **Cancer Res. 2003 Nov 1;63(21):7451-61.**

Papers not directly related to the topic of the thesis

Borrello MG, Alberti L, Fischer A, Degl'innocenti D, Ferrario C, Gariboldi M, **Marchesi F**, Allavena P, Greco A, Collini P, Pilotti S, Cassinelli G, Bressan P, Fugazzola L, Mantovani A, Pierotti MA. Induction of a proinflammatory program in normal human thyrocytes by the RET/PTC1 oncogene. **Proc Natl Acad Sci U S A. 2005 Oct 11;102(41):14825-30**

Sordi V, Malosio ML, **Marchesi F**, Mercalli A, Melzi R, Giordano T, Belmonte N, Ferrari G, Leone BE, Bertuzzi F, Zerbini G, Allavena P, Bonifacio E, Piemonti L. Bone marrow mesenchymal stem cells express a restricted set of functionally active chemokine receptors capable of promoting migration to pancreatic islets. **Blood 2005 Jul 14;106(2): 419-427.**

Allavena P, Signorelli M, Chieppa M, Erba E, Bianchi G, **Marchesi F**, Bonardi C, Garbi A, Lissoni A, de Braud F, Jimeno J and D'Incalci M. Anti-inflammatory properties of the novel anti-tumor agent Yondelis™ (Trabectedin): inhibition of macrophage differentiation and cytokine production. **Cancer Res. 2005; 65(7): 2964-71.**

Laskarin G, Cupurdija K, Sotosek Tokmadzic V, Dorcic D, Dupor J, Juretic K, Strbo N, Bogovic Crncic T, **Marchesi F**, Allavena P, Mantovani A, Randic L, Rukavina D. The presence of functional mannose receptor on macrophages at the maternal-fetal interface. **Human Reprod. 2005; 20(4): 1057-1066.**

List of Abbreviations

(m)Ab	(monoclonal) antibody
bp	base pair
BSA	bovine serum albumin
CAM	cell-cell adhesion molecules
CCL, CXCL, XCL, CX3CL	ligands
CCR, CXCR, XCR, CX3CR	receptors
DNA	deoxyribonucleic acid
cDNA	complementary DNA
CK7	cytokeratin 7
DC	dendritic cell
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
ELISA	enzyme-linked immunosorbent assay
ELR	tripeptide motif glutamic acid-leucine-arginine
FACS	fluorescence activated cell sorter
FBS	foetal bovine serum
FGF	fibroblast growth factor
HGF	hepatocyte growth factor
HHV8	human herpes virus 8
HIF	hypoxia inducible factor
HPC	haematopoietic progenitor cell
HPF	high power field
HUVEC	human umbilical vascular endothelial cells
ICAM	intercellular adhesion molecule
IGF	insulin-like growth factor
IFN	interferon
Ig	immunoglobulin
IL	interleukin
Kb	kilobase
kDa	kilodalton
LPS	lipopolysaccharide

MAPK	mitogen activated protein kinase
MFI	mean fluorescence intensity
MHC	major histocompatibility complex
MMP	matrix metalloproteinase
MMPI	matrix metalloproteinase inhibitor
MS	multiple sclerosis
NF-kB	nuclear factor kappa B
NK	natural killer
NSCLC	non small cell lung cancer
PanIN	pancreatic intraepithelial neoplasia
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PDCL	pancreatic ductal carcinoma cell lines
PKB/C	protein kinase B/C
PLC	phospholypase C
PMA	phorbol myristyl acetate
RNA	ribonucleic acid
mRNA	messenger RNA
RT-PCR	reverse transcription polymerase chain reaction
SE	standard error
SSC	sodium chloride, sodium citrate buffer
SD	standard deviation
SDS	sodium dodecyl sulphate
TAM	tumour associated macrophage
TGF	transforming growth factor
TIMP	tissue inhibitor of matrix metalloproteinases
TNF	tumour necrosis factor
tPA	tissue plasminogen activator
uPA	urokinase-type plasminogen activator
uPAR	urokinase-type plasminogen activator receptor
VCAM	vascular cell adhesion molecule-1
VEGF	vascular endothelial growth factor

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