

**ANTINEOPLASTIC DRUGS MODULATE CXCR4 AND CD26 CELL-SURFACE
EXPRESSION AND FUNCTION ON COLORECTAL CARCINOMA CELLS**

by

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ABSTRACT

CXCR4 is the cell-surface receptor for the chemokine CXCL12; CXCR4-bearing cells tend to localize in CXCL12-secreting tissues, a process that is essential for normal cellular trafficking in contexts such as hematopoiesis. An increase in CXCR4 expression has been observed in a range of cancers, and correlates with poor clinical outcome as this facilitates tumour cell migration to and/or expansion at CXCL12-rich metastatic sites. We show that treating cells with each of several anticancer drugs (5-fluorouracil, cisplatin, vinblastine or methotrexate) down-regulates cell-surface CXCR4 protein, leading to a corresponding decrease in cellular migration toward CXCL12.

CD26 is another cell-surface molecule that has been implicated in the metastatic process. CD26 is important for cell-to-cell adhesion and binding of adenosine deaminase (ADA). Furthermore, CD26 has dipeptidyl peptidase IV (DPPIV) activity which cleaves certain chemokines, including CXCL12, thus reducing the migratory potential of CXCR4-expressing cells. CD26 is often down-regulated in cancer and a decline in CD26 has been correlated with increased invasion, migration, and enhanced morbidity in rodent tumor models. We show that treatment of cells with anticancer drugs leads to an up-regulation of CD26 at the cell-surface and an increase in DPPIV activity and ADA-binding capacity *in vitro*. These drugs also up-regulate CD26 expression in an *in vivo* orthotopic model of colorectal carcinoma.

Therefore, treatment of colorectal carcinoma cells with diverse anticancer drugs causes changes in the expression of CXCR4 and CD26 that would correspond to a reduced metastatic potential of the tumor cells. This suggests that it may be possible to use anticancer drugs to reduce metastatic spread.

LIST OF ABBREVIATIONS AND SYMBOLS USED

°C	degrees Celsius
15dPGJ ₂	15-deoxy- $\Delta^{12,14}$ -prostaglandin J ₂
5-FU	5-fluorouracil
ADA	adenosine deaminase
ADAbp	adenosine deaminase binding protein
ADCP	adenosine deaminase-complexing protein
AK	autologous killer
ANOVA	analysis of variance
AP-1	activator protein-1
ATM	ataxia telangiectasia mutated
ATR	ATM-Rad3-related
BRCA	breast cancer
BSA	bovine serum albumin
C	cysteine
CD	cluster of differentiation antigen
cDNA	complementary DNA
CFTR	cystic fibrosis transmembrane conductance regulator
ChIP	chromatin immunoprecipitation
CHK	checkpoint kinase
CIS	cisplatin
cpm	counts per minute
CTR	copper transporter
CYP	cytochrome P450
DMEM	Dulbecco's modified Eagle's medium
DNA	deoxyribonucleic acid
DPPIV	dipeptidyl peptidase IV
DR	death receptor
dT	deoxythymidine

DTT	dithiothreitol
dUTP	deoxyuridine triphosphate
EC ₅₀	concentration producing half-maximal effect
ECM	extracellular matrix
EDTA	ethylenediamine tetraacetic acid
EGFR	epidermal growth factor receptor
ELISA	enzyme-linked immunosorbent assay
ER	endoplasmic reticulum
FACS	fluorescence activated cell scanning (flow cytofluorimetry)
FdUMP	fluorodeoxyuridine monophosphate
FdUTP	fluorodeoxyuridine triphosphate
FITC	fluorescein isothiocyanate
FOLFIRI	5-fluorouracil, folinic acid, irinotecan
FOLFOX	5-fluorouracil, folinic acid, oxaliplatin
FUTP	fluorouridine triphosphate
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
Gly-Pro- <i>p</i> NA	glycine-proline- <i>p</i> -nitroaniline <i>p</i> -toluene sulfate salt
GPCR	G protein-coupled receptor
h	hour
H2AX	histone 2AX
HEK	human embryonic kidney
HIF	hypoxia-inducible factor
HIV	human immunodeficiency virus
HRE	hypoxia response element
HUVEC	human umbilical vein endothelial cell
i.p.	intraperitoneal
ICAM	intercellular adhesion molecule
IFN	interferon
Ig	immunoglobulin

IKK	I κ B kinase
IL	interleukin
JNK	c-Jun N-terminal kinase
KDEL	lysine-aspartate-glutamate-leucine
L	ligand
LPS	lipopolysaccharide
MAPK	mitogen-activated protein kinase
MDM	mouse double minute
MEF	mouse endothelial fibroblast
MHC	major histocompatibility complex
min	minute
MMC	mitomycin C
M-MLV	Moloney murine leukemia virus
mRNA	messenger RNA
MTX	methotrexate
NaOH	sodium hydroxide
NBS	nibrin
NCS	newborn calf serum
NEMO	NF- κ B essential modulator
NF- κ B	nuclear factor- κ B
NH ₃ OH	ammonium hydroxide
NK	natural killer
p53	tumour suppressor protein 53
PAK	p21-activated kinase
PBS	phosphate-buffered saline
PG	prostaglandin
Pgp	P glycoprotein
PIDD	p53-inducible death-domain containing
pNA	<i>p</i> -nitroaniline

PPAR	peroxisome proliferator-activated receptor
PTEN	phosphatase and tensin homolog
pVHL	von Hippel-Lindau tumour suppressor protein
PYLL	potential years of life lost
R	receptor
RNA	ribonucleic acid
ROS	reactive oxygen species
RT-PCR	reverse transcriptase-polymerase chain reaction
s.c.	subcutaneous
sCD26	soluble CD26
SCID	severe combined immunodeficiency
SDF	stromal cell-derived factor
SE	standard error of the mean
siRNA	small inhibitory RNA
TBST	tris-buffered saline tween-20
TGF	transforming growth factor
TNF	tumour necrosis factor
TNM	tumour invasiveness, lymph node status, presence of metastases
TRAIL	TNF- α -related apoptosis-inducing ligand
UTP	uridine triphosphate
VB	vinblastine
VEGF	vascular endothelial growth factor
WHIM	warts, hypogammaglobulinemia, immunodeficiency, and myelokathexis
X	any amino acid

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CHAPTER 1

INTRODUCTION

An Overview of Colorectal Carcinoma

Cancer is the most common cause of premature death in Canada (Figure 1.1; Canadian Cancer Society/National Cancer Institute of Canada 2007) and describes a heterogeneous group of diseases. Among these, colorectal cancer is the fourth most common in terms of incidence and is the second leading cause of cancer death, preceded only by lung cancer (Canadian Cancer Society/National Cancer Institute of Canada 2007). The early symptoms of colorectal cancer, as with most cancers, tend to be non-specific and thus easy to ignore or easy to rationalize. These symptoms include unexplained weight loss, persistent low-grade fever, nausea, diarrhea, or constipation. More specific symptoms, such as blood in the stool or abdominal pain, are present in more advanced cases and are typically the symptoms that bring patients to the clinic (Rodriguez-Bigas, Hoff *et al.* 2006).

Although there is strong evidence that early detection, as a result of regular screening, reduces colorectal cancer mortality and incidence rates (Mandel, Bond *et al.* 1993; Kronborg, Fenger *et al.* 1996; Mandel, Church *et al.* 1999), all provinces do not offer regular screening and few people request or participate in these screens (Canadian Cancer Society/National Cancer Institute of Canada 2006). As a result, many patients already have metastases to sites such as the liver at the time of diagnosis; this significantly increases the chance that they will die of this disease (Fazio, Cotterchio *et al.* 2005; McCarthy, Ngo *et al.*).

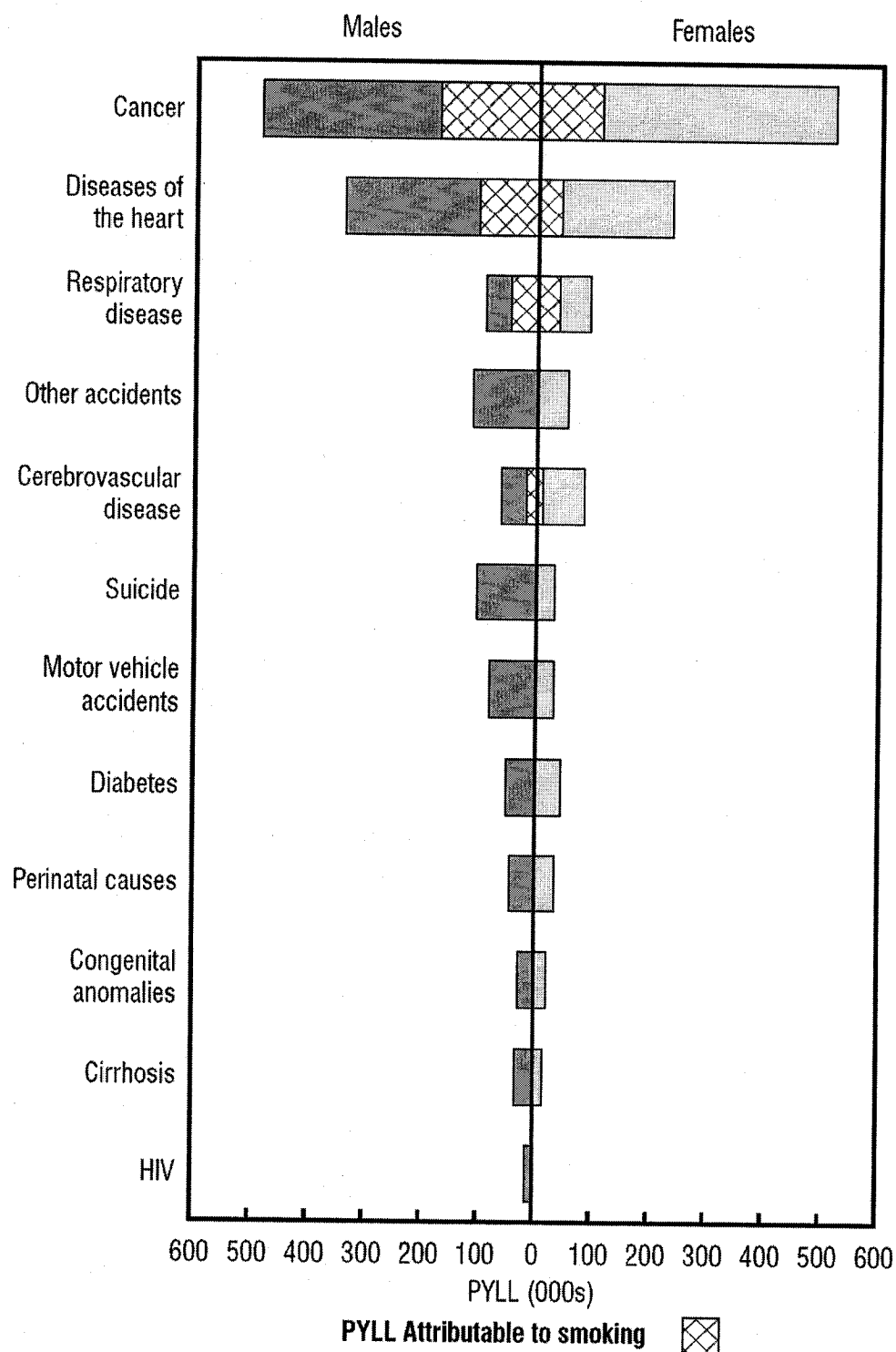


Figure 1.1. Potential years of life lost by Canadian males and females, ranked by cause of death. Left: Deaths among males. Right: Deaths among females. Hatched bars: deaths attributable to smoking (from Canadian Cancer Society/National Cancer Institute of Canada 2007).

Normal Colorectal Anatomy

The large intestine consists of the ascending, transverse, descending, and sigmoid colon, as well as the rectum and cecum. A mucosal surface borders the lumen of the large intestine and epithelial cells, organized in crypts and supported by the lamina propria, produce mucus to lubricate and protect the colon. Beneath the lamina propria lies the muscularis mucosa followed by the submucosa, which is an innervated supportive layer of connective tissue containing blood and lymphatic vessels. The muscular layers, which control peristalsis, are next and consist of an inner circular muscle layer and an outer longitudinal muscle layer. The serosa makes up the outer layer of the colon, which is a fibrous connective tissue layer covered by mesothelium. The intestines are surrounded by the mesentery, a layer of fatty tissue which contains the lymph nodes (Figure 1.2; Junqueira and Carneiro 2007).

Cancer Development and Progression

The vast majority of colorectal tumours are adenocarcinomas; tumours which have developed, over a period of a decade or more (Rodriguez-Bigas, Hoff *et al.* 2006), from the glandular epithelium of the colon lumen. Most tumours arise spontaneously as a result of genetic change within one cell. This change renders a cell more capable of growth, thereby leading to its monoclonal expansion. The neoplasm may undergo further genetic changes, each one enhancing the tumour's proliferative capacity. One such change can lead to tumour angiogenesis, or blood vessel development, as a result of growth factor production by the tumour cells (Fearon and Vogelstein 1990). An adenoma at this stage of development may be visible in the colon as a polyp, however, not all polyps are malignant adenocarcinomas (Muto, Bussey *et al.* 1975).

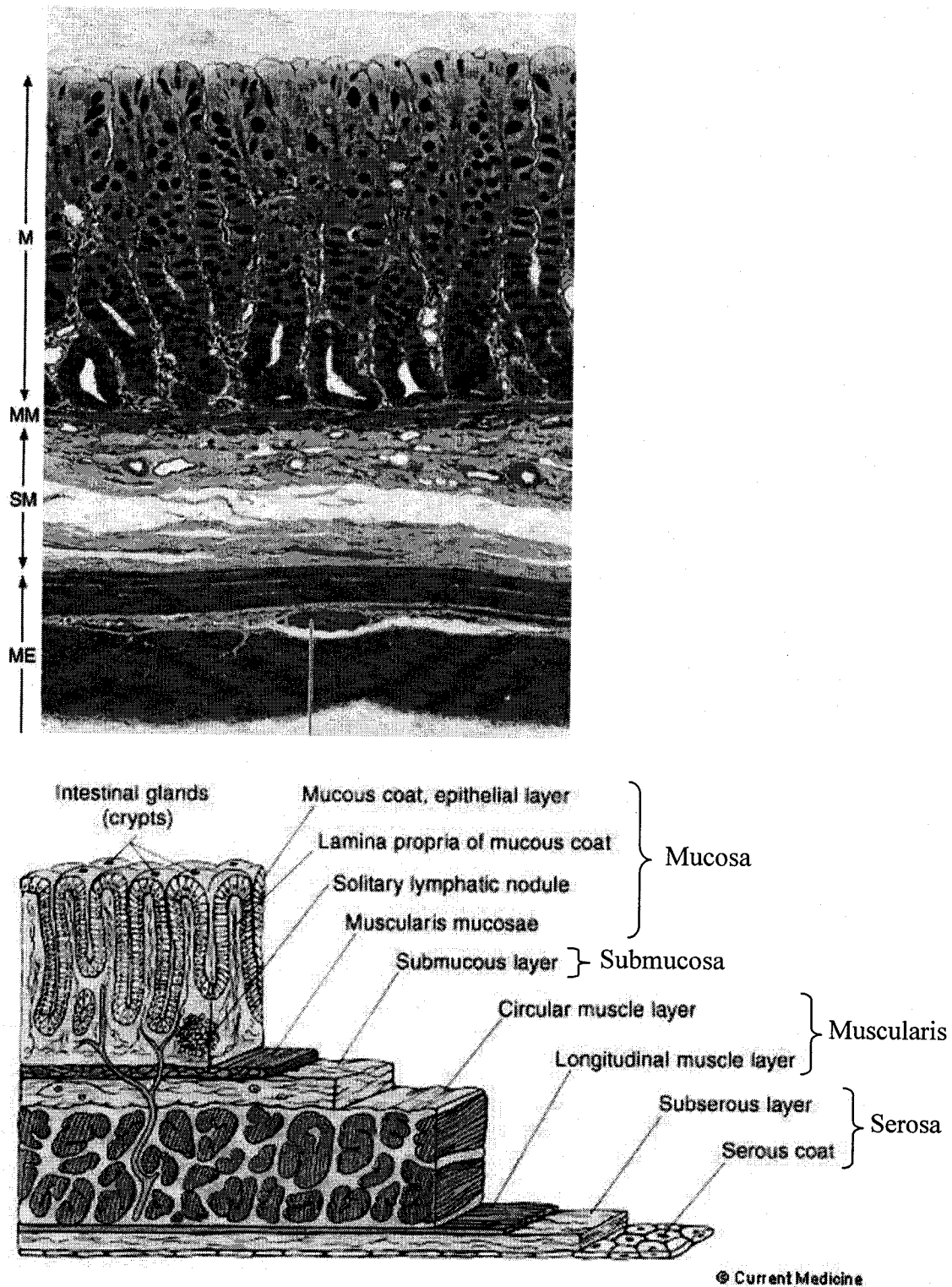


Figure 1.2. Histologic section and schematic of the colon wall. Top: A section of the wall of the large intestine, showing the epithelial mucosa (M), the muscularis mucosae (MM), the submucosa (SM) and the muscularis (ME) layers (from Junqueira and Carneiro 2007). Bottom: A drawing depicting these layers (adapted from AstraZeneca 2007).

If identified during a routine screen at this stage, the polyp can be removed easily with little risk of further harm to the patient. If a malignant polyp is not identified, the tumour will continue to expand and penetrate the wall of the colon. As the tumour grows and invades local blood and lymphatic vessels, malignant cells may break off and metastasize to different parts of the body (Figure 1.3; Fearon and Vogelstein 1990).

Often, metastases appear first in the lymph nodes adjacent to the primary tumour; in more advanced cases they will also be found in regional lymph nodes and then distant organs.

Interestingly, most cancers will metastasize to specific organs. For example, the most common sites of breast cancer metastasis include bone, lung, liver, or brain. A large study, involving the analysis of necropsy data from 1541 colorectal carcinoma patients between 1944 and 1984, demonstrated the typical patterns of metastasis in colorectal carcinoma. This study showed that 53% of colorectal carcinoma patients had metastatic spread to regional lymph nodes, 44% to the liver, 21% to lung tissue, and 23% had metastases in other tissues, including the adrenal glands and bone marrow, at the time of their death (Weiss, Grundmann *et al.* 1986).

Upon diagnosis, the tumour is often resected; this ameliorates symptoms and allows determination of tumour stage and grade. The grade describes the cells that make up the tumour and how aggressive these cells are. Tumours are graded from I – IV based on their level of cellular differentiation. Normal progenitor cells differentiate into specific mature cell types; however, as cancer cells develop a more aggressive phenotype, they tend to de-differentiate, becoming less normal. The less differentiated (that is, corresponding to a less normal mature phenotype) the cells of a certain tumour are, the

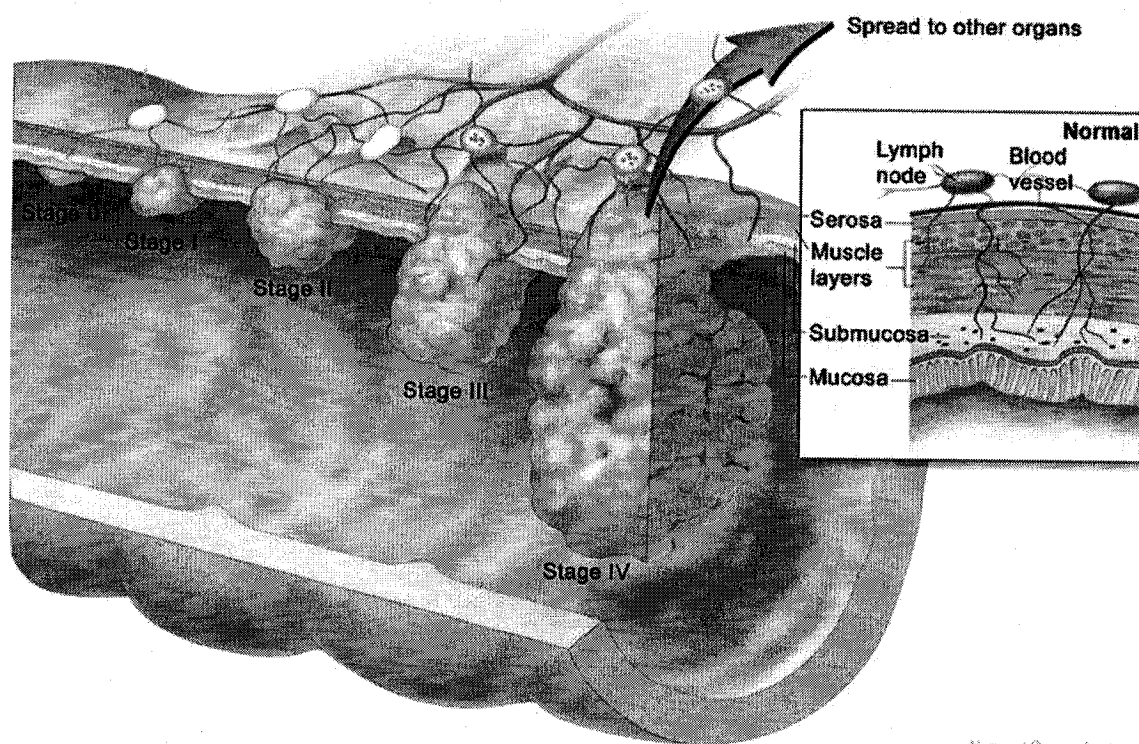


Figure 1.3. Depiction of colorectal carcinoma progression from stage 0 through stage IV disease. Inset: Cross-sectional diagram of the colon wall (from National Cancer Institute 2005)

higher the tumour grade; high tumour grade correlates with poor prognosis (Muto, Bussey *et al.* 1975). The majority of colorectal tumours are identified as grade I or II, well- or moderately well-differentiated, adenocarcinomas after resection (Newland, Chapuis *et al.* 1981).

The most important predictor of patient outcome is the extent and severity of tumour progression, or the tumour stage (Newland, Chapuis *et al.* 1981). Stage is determined by extensive imaging of the colon and other organs, and often cannot be defined until the tumour is examined surgically. Stages 0 - IV are described by the TNM system, based on tumour invasiveness, extent of lymph node involvement, and the presence or absence of metastases (International Union against Cancer 2002).

Stage 0 (*in situ*) colorectal cancer describes a superficial non-invasive tumour (using the TNM system, it is described as “Tis”). In stage I colorectal cancer, the tumour has invaded the submucosa (T1) or the muscularis propria (T2) of the colon. In both cases, there is no lymph node involvement (N0) and no distant metastases (M0). Patients with stage II disease have a tumour that has invaded the subserosa (T3), nearby organs, or the peritoneum (T4). There is no lymph node involvement (N0) and no metastases (M0). Stage III colorectal cancer describes a tumour that has spread to local lymph nodes. If fewer than four lymph nodes are involved, the tumour is described as N1; if four or more lymph nodes are involved it is described as N2. The tumour may or may not have penetrated the colon wall (T1-4) and there are no distant metastases (M0). With stage IV colorectal cancer, patients have distant metastases, most often to the liver or lungs (T1-4, N1-2, M1; International Union against Cancer 2002).

It is important to stage tumours because the stage at diagnosis is one determinant of treatment modality and it correlates with prognosis (Newland, Chapuis *et al.* 1981). Unfortunately, fewer than 10% of colorectal cancers are detected at stage 0, with the remaining 90% of cases fairly evenly split between stages I through IV. A full 20% of patients present with metastatic (stage IV) disease (McCarthy, Ngo *et al.*), and this proportion increases in patients under age 45, in patients of non-white ethnicity, or in those living in rural communities (Fazio, Cotterchio *et al.* 2005). The five-year survival rate for patients with stage 0 or stage I disease is greater than 90%, while that for patients with metastatic stage IV disease is less than 5% (Mandel, Bond *et al.* 1993). These statistics point to the importance of screening to identify colorectal cancer at an early stage and the dire need for treatments that are effective against not only the primary tumour but also against metastatic disease.

Colorectal Cancer Treatment

As described above, colorectal cancers are diverse; two people with colorectal cancer may have very different prognoses and therefore would receive very different treatments. Treatment, like prognosis, varies depending on the stage and grade of the disease as well as the patient's overall health. Furthermore, treatment recommendations differ from country to country and even province to province within Canada. Consequently, each province and/or country mandates guidelines for treatment using evidence-based medicine as a foundation. These guidelines are stratified based upon the stage of colorectal cancer at diagnosis; however, these are only guidelines, not rules. If a patient fails to respond to the first-line therapy, or is uncomfortable with surgery or

chemotherapy, other treatments may be offered. Outlined below are the guidelines described by Cancer Care Ontario for colorectal carcinoma treatment (Cancer Care Ontario 2000):

Stage 0 or I Colorectal Carcinoma

These patients are treated surgically, either with a simple polypectomy, if the neoplasia is a small stage 0 polypoid growth, or a partial or complete bowel resection if it is a large stage 0 or any size stage I tumour. The tumour, as well as a margin of healthy tissue, is excised and examined to confirm stage and ensure that all the malignant tissue was removed. Chemotherapy is not usually necessary in these patients.

Stage II Colorectal Carcinoma

These patients receive either surgery alone or surgery and chemotherapy. Whether patients receive chemotherapy depends on the risk of tumour recurrence. Patients with high risk of recurrence are those with a high grade tumour, T4 disease, or tumours that have either blocked or perforated the colon. The standard chemotherapy regimen in these cases is FOLFOX: 5-fluorouracil (5-FU), folinic acid, and oxaliplatin. If patients do not tolerate 5-FU, they may be offered capecitabine.

Stage III Colorectal Carcinoma

Surgery is the primary first-line treatment for patients with stage III colorectal cancer, followed by FOLFOX chemotherapy.

Stage IV Colorectal Carcinoma

If the extent of metastasis is limited, surgical resection of the primary and metastatic lesions, followed by chemotherapy, is considered. If metastatic spread is extensive, removal of the primary tumour or partial removal of the metastases may be required for symptom palliation, particularly if the primary tumour is blocking the intestine or causing bleeding or pain. Most stage IV colorectal cancer patients receive chemotherapy to reduce the size and slow the growth of their tumour, prolonging life and relieving symptoms. Chemotherapy regimens include either FOLFOX or FOLFIRI (5-FU, folinic acid, and irinotecan) as first-line therapy combined with bevacizumab. Bevacizumab is a monoclonal antibody that targets vascular endothelial growth factor (VEGF) and inhibits tumour angiogenesis.

If the tumour becomes resistant to either FOLFOX or FOLFIRI, the other is offered and may be combined with cetuximab if bevacizumab was ineffective. Cetuximab is another monoclonal antibody which blocks tumour growth by targeting the epidermal growth factor receptor (EGFR). Other drugs, such as the antifolate raltitrexed, may also be offered. Radiation therapy is rarely used in colorectal cancer treatment unless the primary tumour or its metastases are inoperable and are causing pain, such as is the case in metastasis to the bone.

Chemotherapeutic Agents for the Treatment of Malignant Disease

There are many classes of chemotherapeutic agents currently in clinical use. These include the purine and pyrimidine analogs, folate antagonists, mitotic spindle poisons, alkylating agents and platinum compounds, and topoisomerase poisons (Table 1.1).

Table 1.1. Classes of cytotoxic agents used in cancer treatment. Outlined are examples of each, their mechanisms of cytotoxicity, and their indications (adapted from Von Hoff and Hanauske 2006).

Drug Class	Mechanism of Cytotoxicity	Example	Cancer Indications
Purine and pyrimidine analogs	Inhibit DNA and RNA synthesis by mimicking purine and pyrimidine nucleosides	5-Fluorouracil	Colon, rectal, breast, stomach, pancreatic, and basal cell carcinomas; solar keratoses
Folate antagonists	Inhibit DNA, RNA and protein synthesis by inhibiting folate production	Methotrexate	Acute lymphocytic leukemia and meningeal leukemia; breast, head and neck, and lung carcinomas; gestational choriocarcinoma; non-Hodgkin's lymphoma; osteosarcoma; others
Mitotic spindle poisons	Inhibit mitosis by inhibiting microtubule dynamics	Vinblastine	Hodgkin's disease; lymphocytic and histiocytic lymphomas; testicular and breast carcinoma; Kaposi sarcoma; choriocarcinoma
Alkylating agents and platinum compounds	Inhibit DNA and RNA synthesis by cross-linking DNA	Cisplatin	Testicular, ovarian, and bladder carcinoma
Topoisomerase poisons	Inhibit DNA and RNA synthesis by intercalating DNA and/or binding topoisomerase I or II	Irinotecan	Colon or rectal carcinoma

Purine and Pyrimidine Analogs

The purine and pyrimidine analogs are a class of antimetabolite drugs that inhibit DNA and RNA synthesis. These drugs were rationally designed with the theory that this inhibition should slow the growth of rapidly dividing tumour cells while having minimal effects on normal cells that cycle less frequently. One of the most commonly used chemotherapeutic agents for the treatment of colorectal cancer is the pyrimidine analog 5-FU.

As one of the first rationally designed drugs, 5-FU has been a mainstay in cancer treatment for nearly 50 years. Therapeutic steady state plasma levels of 5-FU reach 1-5 μM (Seifert, Baker *et al.* 1975) and 5-FU enters cells by passive diffusion (Nakamura, Horimoto *et al.* 2003). Once in cells, it is converted to its active metabolites, fluorodeoxyuridine monophosphate (FdUMP), fluorodeoxyuridine triphosphate (FdUTP), and fluorouridine triphosphate (FUTP). The metabolite FdUMP inhibits thymidylate synthase, the enzyme responsible for thymidylate synthesis; decreased thymidylate production leads to decreased thymidine triphosphate. Cells deficient in this essential nucleotide undergo apoptosis as a result of DNA synthesis inhibition (Santi, McHenry *et al.* 1974). In addition to this, FdUTP may be incorporated into DNA, leading to error-prone repair or cell death in cancer cells lacking normal DNA repair mechanisms (Dusenbury, Davis *et al.* 1991). Finally, FUTP is incorporated into RNA as efficiently as uridine triphosphate (UTP); however, the exact mechanism by which this incorporation leads to cell death is not clear (Glazer and Lloyd 1982). 5-Fluorouracil is administered intravenously and often, it is not tolerated well by patients; as a result some are offered the orally available 5-FU prodrug capecitabine.

Folate Antagonists

Like 5-FU, folate antagonists, or antifolates, are antimetabolites which are relatively specific for the S phase of the cell cycle. These drugs were rationally designed following the observation that folate administration to leukemia patients accelerated disease progression (Farber, Cutler *et al.* 1947). Methotrexate (MTX) is an antifolate which has been in clinical use for cancer treatment since the 1950s and remains a commonly used drug today. Methotrexate is a broad-spectrum drug used not only for many types of cancer treatment but also as an anti-inflammatory agent for rheumatoid arthritis (Yamanaka, Inoue *et al.* 2007), asthma (Aaron, Dales *et al.* 1998), psoriasis (Eskicirak, Zemheri *et al.* 2006), and as an immunosuppressant (Genestier, Paillot *et al.* 1998).

Reduced folates are important co-factors in many enzymatic cellular processes. Methotrexate and its polyglutamated metabolites inhibit the enzyme dihydrofolate reductase, which reduces dihydrofolate to folate (Osborn, Freeman *et al.* 1958; Jolivet and Chabner 1983). By binding this enzyme with more than 1000-fold greater affinity than dihydrofolate, MTX effectively inhibits its action, leading to decreased levels of folate in cells. This primarily inhibits purine biosynthesis, leading to inhibition of DNA, RNA, and protein synthesis (Hryniuk 1972). Polyglutamated MTX also inhibits the folate-requiring enzyme thymidylate synthase, leading to cell death through reduced DNA synthesis in the same way as 5-FU (Allegra, Chabner *et al.* 1985). Methotrexate reaches plasma concentrations of 0.1 – 1 μ M (Henderson, Adamson *et al.* 1965; Evans, Pratt *et al.* 1979) and enters cells by either the reduced folate carrier or the membrane folate binding protein (Jansen, Schornagel *et al.* 1990).

Mitotic Spindle Poisons

In contrast to rationally designed drugs such as 5-FU and MTX, the vinca alkaloids and the taxanes are two classes of natural or semi-synthetic mitotic spindle poisons originally derived from the pink periwinkle plant (*Catharensis roseus*; Johnson, Armstrong *et al.* 1963) and the pacific yew tree (*Taxus brevifolia*; Wani, Taylor *et al.* 1971). The mitotic spindle, necessary for the segregation of chromosomes into daughter cells, is made from microtubules. Microtubules are cellular structures that are important for cellular transport, movement, and cell division. These are dynamic structures formed from filaments consisting of tubulin dimers. Microtubules are constantly elongating and shrinking; this dynamic interaction can be inhibited by either stabilizing or destabilizing tubulin polymerization with the taxanes or vinca alkaloids, respectively. These two processes result in the same net effect of mitosis inhibition and cell death (Jordan, Toso *et al.* 1993; Jordan, Wendell *et al.* 1996; Panda, Jordan *et al.* 1996).

Vinblastine (VB) is an example of a vinca alkaloid commonly used to treat a variety of malignancies. It reaches peak plasma concentrations of approximately 0.4 μM (Rowinsky 2006) and most likely enters cells by passive diffusion (Zhou, Placidi *et al.* 1994). Another vinca alkaloid, vincristine, is identical in structure to VB with the exception of the single substitution of a methyl group for a formyl group; however, their antitumour spectra and toxicities are very different (Ferguson, Phillips *et al.* 1984).

Alkylating Agents and Platinum Compounds

The alkylating agents contain highly reactive alkyl groups and act by forming covalent bonds with cellular nucleophiles: DNA, RNA, and proteins. Their main mechanism of cytotoxicity is their DNA-crosslinking activity (Brookes and Lawley

1961). The first use of the alkylating agent, mustard gas, was as a chemical weapon in World War I. When its suppressive effects on blood cells were identified, sulfur mustards and other alkylating agents were examined for activity against cancer cells (Rhoads 1978). In contrast to the many chemotherapy agents that target certain stages of the cell cycle, this class of drug targets cells at any stage of the cell cycle. Cyclophosphamide is an example of a commonly used alkylating agent. With the exception of hormones, these were the first successful anticancer drugs (Colvin 2006).

Like many other scientific discoveries, the effectiveness of the platinum compounds against tumour cells was discovered serendipitously; Rosenberg and colleagues were studying the effects of electric current on bacterial growth using a platinum electrode. When they found that the growth inhibitory effect was attributable to the platinum released from the electrode into the medium, rather than the electric current itself (Rosenberg, Vancamp *et al.* 1965), they examined the effects of various platinum compounds on rodent tumour models and found they exhibited great efficacy (Rosenberg, VanCamp *et al.* 1969).

Forty years later, the exact mechanism of action of the platinum compounds remains unknown; although they seem to act as alkylating agents by covalently binding nucleophilic molecules (Dedon and Borch 1987), they do not contain alkyl groups. Cisplatin (CIS) reaches steady state plasma levels of 2-3 μM (Campbell, Howell *et al.* 1983) and enters cells through the use of copper transporters (Ishida, Lee *et al.* 2002; Lin, Okuda *et al.* 2002). Oxaliplatin is an example of another platinum compound which, in contrast to CIS, has activity against colorectal carcinoma (Rixe, Ortuzar *et al.* 1996).

Topoisomerase Poisons

The final class of anticancer drugs discussed in this thesis includes those that target DNA topoisomerase I, II α , and II β . Topoisomerases are required to facilitate DNA uncoiling, allowing the replication and transcription machinery access to the DNA. Topoisomerase I creates single-strand DNA nicks and is necessary for both replication and transcription (Brill, DiNardo *et al.* 1987), while topoisomerases II α and II β create double-strand nicks and are required only for replication (DiNardo, Voelkel *et al.* 1984). There are also topoisomerases III α and III β ; however, their exact role in DNA maintenance, and whether they are targets of topoisomerase poisons, is unclear.

Like the mitotic spindle poisons, the topoisomerase poisons are derived from natural sources. The topoisomerase II poisons, doxorubicin and daunorubicin, are anthracycline antibiotics produced by certain species of *Streptomyces*. Although these drugs were originally identified for their capacity to create free radicals and intercalate DNA (Sinha, Katki *et al.* 1987a; Sinha, Katki *et al.* 1987b), their primary mechanism of cytotoxic action is attributable to their topoisomerase II inhibiting-activity (Tewey, Rowe *et al.* 1984). The topoisomerase I poison, irinotecan, is a semi-synthetic analog of camptothecin, an alkaloid produced by the Chinese tree *Camptotheca acuminata*. Irinotecan reaches plasma concentrations of up to 43.4 $\mu\text{g/L}$ (Kuppens, Dansin *et al.* 2006) and enters cells by both active (Yamamoto, Verweij *et al.* 2001) and passive (Kobayashi, Bouscarel *et al.* 1999) transport. Irinotecan is an important drug used in colorectal cancer therapy, particularly in recurrent or metastatic disease.

The Scheduling of Chemotherapy

The ideal outcome of chemotherapy is cure; at a minimum, chemotherapy aims to stabilize disease, ameliorate symptoms, and ultimately prolong life. In order to achieve this by causing maximal tumour cell death, chemotherapy agents are typically administered at the highest doses that patients can tolerate while experiencing acceptable levels of toxicity (Frei and Eder 2006). To target as many tumour cells as possible while limiting this toxicity, chemotherapy is usually given in cycles. The FOLFOX regimen, recommended for stages II-IV colorectal cancer, consists of a two day cycle. On the first day of the cycle, oxaliplatin, 5-FU and folinic acid are given intravenously. On the second day, patients receive only 5-FU and folinic acid. This protocol is repeated every two weeks for twelve cycles (Cancer Care Ontario 2000). A major dose-limiting toxicity of many cytotoxic agents is myelosuppression; however, bone marrow cells tend to recover more quickly than tumour cells following a high-dose chemotherapy regimen. This allows a subsequent cycle of treatment leading to an increasing effect on tumour cell kill but little risk of cumulative myelosuppression (Clarkson, Ohkita *et al.* 1967; Clarkson, Fried *et al.* 1970; Clarkson, Strife *et al.* 1970).

A common benchmark by which most chemotherapy drugs are measured is in their ability to reduce tumour cell burden or tumour size. While this has been a useful outcome to measure, it is becoming apparent that this is not the only parameter of importance. If any tumour cells remain following treatment completion, they will eventually form a new tumour. This recurrent tumour, whether it presents at the primary site or as a metastatic lesion, has survived the first round of chemotherapy. As a result, it tends to be more aggressive and is more likely to be resistant to treatment. Some groups

have suggested controlling the tumour, rather than eradicating it, as a more effective long-term goal.

In support of this theory, there has been some evidence that low doses of certain cytotoxic drugs, such as 5-FU or cyclophosphamide, administered continuously rather than in cycles leads to reduced local and metastatic tumour recurrence compared to that observed following conventional drug regimens (O'Connell, Martenson *et al.* 1994; Colleoni, Rocca *et al.* 2002; Orlando, Cardillo *et al.* 2006). This cannot be attributed to tumour cell death caused by these agents, but may be due, at least in part, to endothelial cell death. Endothelial cells are more sensitive to these agents than tumour cells, so doses that are too low to kill the cancer cells may still lead to endothelial cell loss. This in turn inhibits tumour angiogenesis, cutting off tumour blood supply and preventing the tumour from growing any larger (Klement, Baruchel *et al.* 2000; Shaked, Emmenegger *et al.* 2005; Emmenegger, Morton *et al.* 2006).

The Effects of Cytotoxic Drugs on Transcription Factors

There is substantial evidence that cytotoxic drugs affect the expression of a variety of cellular proteins. Johnston and colleagues performed complementary DNA (cDNA) microarray analysis on 5-FU-treated and untreated human MCF-7 breast cancer cells and found that 5-FU treatment led to a more than 3-fold change in the expression levels of over 600 genes, which was the equivalent to more than 25% of the genes examined (Maxwell, Longley *et al.* 2003). Two similar studies by the same group showed that 5-FU or oxaliplatin treatment led to altered (≥ 2 -fold) expression of 855 or 1233 genes, respectively, in human HCT-116 colorectal carcinoma cells (Boyer, Allen *et al.*

2006) and 5-FU altered the expression of 619 genes more than 3-fold in human MCF-7 breast carcinoma cells (Boyer, Maxwell *et al.* 2004). This massive change in the transcriptional program of cells following DNA damage suggests that the cellular response to genotoxic stress is very complex.

Although the physiological relevance of many of these microarray-identified changes in expression remains to be shown, many of the proven targets of anticancer drugs relate to their cytotoxicity. For example, it has been well-established that genotoxic agents activate the ataxia telangiectasia mutated (ATM) and/or ATM-Rad3-related (ATR) proteins, which phosphorylate downstream targets. These include proteins involved in apoptosis or cell-cycle blockade, such as p53 (Kastan, Zhan *et al.* 1992; Canman, Lim *et al.* 1998; Khanna, Keating *et al.* 1998; Lakin, Hann *et al.* 1999), checkpoint kinase-1 (CHK1; Liu, Guntuku *et al.* 2000) and CHK2 (Matsuoka, Huang *et al.* 1998), histone 2AX (H2AX; Burma, Chen *et al.* 2001; Ward and Chen 2001), and mouse double minute-2 (MDM2; Khosravi, Maya *et al.* 1999), as well as those involved in DNA repair, such as nuclear factor- κ B (NF- κ B) essential modulator (NEMO; Janssens, Tinel *et al.* 2005), breast cancer-1 (BRCA1; Cortez, Wang *et al.* 1999) and nibrin (NBS1; Gatei, Young *et al.* 2000). Of these, the most widely studied is the tumour suppressor p53.

The phosphorylation of p53 leads to its stabilization and enhanced p53-mediated gene expression; adding to the complexity described above, the level of cellular stress can determine the transcriptional program activated by p53. Mayo and colleagues showed that low- and high-level stress, mediated by different doses of etoposide, led to a different profile of phosphorylation sites within p53 (Mayo, Seo *et al.* 2005). This resulted in

different transcriptional programs in human U2OS osteosarcoma cells: low-level stress led to p53-dependent activation of MDM2, resulting in DNA repair and cell survival; high-level stress led to p53-dependent activation of phosphatase and tensin homolog (PTEN) followed by apoptosis. Other groups have shown that p53 activation by high levels of genotoxic stress leads to up-regulation of CD95 (APO-1/Fas), facilitating CD95L-mediated cell death (Müller, Strand *et al.* 1997; Müller, Wilder *et al.* 1998; Wu, Mizutani *et al.* 2000).

Many cancer cells lack p53 or express mutant p53. Cells lacking or expressing a mutant copy of this tumour suppressor protein tend to be more resistant to genotoxic stress; however, they are still susceptible to apoptosis or cell cycle arrest through p53-independent routes. Some agents, such as CIS, activate p38 mitogen-activated protein kinase (MAPK) and c-Jun N-terminal kinase (JNK) signaling leading to increased expression of the transcription factors activator protein-1 (AP-1) and NF- κ B; this results in enhanced expression of CD95L and apoptosis (Kasibhatla, Brunner *et al.* 1998). Microtubule-targeting agents also activate p38 MAPK as well as p21-activated kinase (PAK), leading to competing cell death and survival signals (Deacon, Mistry *et al.* 2003). Like p53, phospho-p38 MAPK phosphorylates and activates many transcription factors that regulate apoptosis and growth inhibition, including p53 itself (Bulavin, Saito *et al.* 1999; Hildebrandt, Reutter *et al.* 2000) and p73 (Sanchez-Prieto, Sanchez-Arevalo *et al.* 2002). These are not the only targets of p38 MAPK, however. Many other transcription factors unrelated to p53 can be activated by p38 MAPK phosphorylation (Raingeaud, Whitmarsh *et al.* 1996; Wang and Ron 1996; Han, Jiang *et al.* 1997; van der Houven van Oordt, Diaz-Meco *et al.* 2000; Johnson and Lapadat 2002).

As previously mentioned, ATM phosphorylates NEMO following DNA damage, resulting in enhanced I κ B kinase (IKK) activation and NF- κ B activity. This enhanced NF- κ B activation occurs following treatment with topoisomerase I (Piret, Schoonbroodt *et al.* 1999) and topoisomerase II (Boland, Fitzgerald *et al.* 2000) poisons and the anthracycline doxorubicin (Panta, Kaur *et al.* 2004). DNA damage has also been shown to activate NF- κ B independently of ATM, instead occurring through p53-inducible death-domain containing (PIDD)-mediated NEMO phosphorylation (Janssens, Tinel *et al.* 2005). These signals through NF- κ B tend to drive the cell toward repair of damaged DNA and survival; however, PIDD can also mediate cell death signals by activating caspase-2 (Janssens, Tinel *et al.* 2005).

Overall, the effects of cytotoxic drugs on cellular proteins as well as the factors that drive the cellular response in the direction of survival or death are very complex and remain poorly understood. Many of the processes that are understood, such as p53, NF- κ B, and p38 MAPK activation, result in altered transcriptional programs that lead to changes in protein expression. It is interesting to note that the vast majority of proteins known to be affected by cytotoxic drugs are cytoplasmic or nuclear; very few cell surface proteins have been shown to be altered by treatment with these agents.

The Effects of Cytotoxic Drugs on Cell-Surface Protein Expression

The cell-surface proteins shown to be regulated by cytotoxic drug treatment fall into three broad groups: cell death and cell survival proteins, transporter proteins, and adhesion molecules and T cell antigens. The most studied of these groups is the first;

within this group, the cell-surface death receptor CD95 has been examined in the most detail.

Cytotoxic Drugs Up-Regulate Cell Death and Survival Proteins

Galle and colleagues first identified CD95 regulation by genotoxic stress following treatment of hepatoma cells with either the antitumour antibiotic bleomycin, the platinum agent CIS, or the antifolate MTX (Müller, Strand *et al.* 1997). Using reverse transcriptase-polymerase chain reaction (RT-PCR) and flow cytofluorimetry they found that each of these drugs up-regulated both CD95 messenger RNA (mRNA) and cell-surface protein, which led to enhanced CD95L-mediated cell death. This up-regulation was shown to be dependent, at least in part, upon p53 because p53 mutant or null hepatoma cells showed reduced up-regulation of CD95 following treatment with CIS or MTX, and no up-regulation at all following treatment with bleomycin, as compared to cells with wild-type p53. They confirmed this observation in a subsequent publication, showing that 5-FU, MTX, mitomycin C (MMC), CIS, cyclophosphamide, mitoxantrone, doxorubicin, etoposide, and bleomycin all up-regulated CD95 mRNA and protein, which was dependent on the p53 status of the cells. In contrast, CD95L was up-regulated by these drugs independent of p53 status (Müller, Wilder *et al.* 1998).

Around the same time, Hoskin and coworkers showed that CIS or etoposide treatment also led to an up-regulation of both CD95 mRNA and cell-surface protein in murine P815 mastocytoma cells. This sensitized the cells to autologous killer (AK)-T cell-mediated lysis, suggesting that these drugs could be used to increase patient responsiveness to immunotherapy in cancer treatment (Williams, Makrigiannis *et al.* 1997). Subsequently, similar results were shown in renal cell carcinoma cells treated with

doxorubicin or etoposide (Wu, Mizutani *et al.* 2000), breast carcinoma cells treated with etoposide (Morgan, Williams *et al.* 2002), and colorectal carcinoma cells treated with CIS (van Geelen, de Vries *et al.* 2003), 5-FU, oxaliplatin, irinotecan, or tomudex (McDermott, Longley *et al.* 2005).

The levels of death receptors other than CD95 have also been shown to be modulated at the cell surface by cytotoxic drug treatment. el-Deiry and colleagues identified death receptor (DR) 5 as a target of doxorubicin- or ionizing radiation-mediated genotoxic stress in breast carcinoma, myeloid leukemia, lung carcinoma, and colon carcinoma cells, as its mRNA levels increased following treatment with either agent (Sheikh, Burns *et al.* 1998).

Johnson and coworkers expanded on this finding, showing that etoposide induced the cell-surface expression of DR5 and tumour necrosis factor (TNF)- α -related apoptosis-inducing ligand (TRAIL) in primary human mammary and lung epithelial cells. This was dependent upon NF- κ B, as cells containing the dominant negative I κ B α , which inhibits NF- κ B activation, failed to up-regulate DR5 or TRAIL in response to etoposide treatment (Spalding, Jotte *et al.* 2002). Finally, Repasky and colleagues showed that this up-regulation of DR5 is physiologically relevant: using patient-derived colon tumours implanted subcutaneously (s.c.) into severe combined immunodeficiency (SCID) mice, irinotecan led to an increase in DR5 immunoreactivity on the surface of tumour cells (Naka, Sugamura *et al.* 2002).

The effects of chemotherapeutics on DR4 have been more controversial. Hoskin and colleagues found that etoposide and doxorubicin increased DR4 expression on breast carcinoma cells (Morgan, Williams *et al.* 2002), while Johnson and colleagues found that

etoposide reduced DR4 in lung carcinoma cells (Spalding, Jotte *et al.* 2002). A recent report examining the effects of doxorubicin on renal cell carcinoma cells shows that it up-regulates surface DR4 dramatically. As measured by flow cytometry, 93.5% of doxorubicin-treated cells, compared to only 5.3% of control-treated cells, were measured as being positive for DR4 (Jin, Wu *et al.* 2007).

A recent publication by Winograd-Katz and Levitzki demonstrates that CIS causes p38 MAPK-dependent phosphorylation and internalization EGFR in MDA-MB-468 cells (Winograd-Katz and Levitzki 2006). This internalization was independent of EGFR activation and the authors suggested that this CIS-mediated EGFR internalization shifts the cells from proliferation signaling to survival signaling.

Cytotoxic Drugs Modulate the Levels of Cell-Surface Transporters

Hamilton's group has evaluated the effects of chemotherapy agents on the drug efflux pump, P-glycoprotein (Pgp), and cystic fibrosis transmembrane conductance regulator (CFTR), which are both members of the ATP-binding cassette (ABC) family of transport proteins. This was first published in 1997, when they showed that the DNA crosslinkers, MMC and CIS, decreased both Pgp mRNA and cell-surface expression in breast and colon carcinoma, hepatoma, neuroblastoma, and leukemia cell lines (Ihnat, Lariviere *et al.* 1997). Pre-treatment of breast carcinoma or hepatoma cell lines with MMC led to enhanced toxicity of doxorubicin, a Pgp substrate. Interestingly, neither co-treatment with MMC and doxorubicin, nor pre-treatment with MMC followed by treatment with CIS (which is not a Pgp substrate), led to increased toxicity compared to either drug alone. This suggests that the enhanced toxicity of doxorubicin following MMC pre-treatment was caused by decreased efflux secondary to MMC-mediated Pgp

down-regulation (Ihnat, Lariviere *et al.* 1997). This group subsequently showed that when mice harbouring MDA-MB-435 breast carcinoma xenografts were pre-treated with MMC, they showed reduced Pgp protein expression. Furthermore, MMC pre-treatment, followed by doxorubicin or paclitaxel (but not CIS), led to an enhanced reduction in tumour growth rate, compared to treatment with any agent alone (Ihnat, Nervi *et al.* 1999).

In 2001, Hamilton's group examined the regulation of Pgp by MMC and doxorubicin in greater detail and found that MMC led to a reduction in Pgp mRNA at all time points tested. Interestingly, despite the reduction in mRNA levels, total protein remained unchanged and cell-surface protein actually increased at the 24 h time point, eventually going down to below control levels by 48 h, suggesting at least two independent mechanisms of regulation of Pgp by MMC (Maitra, Halpin *et al.* 2001). Doxorubicin, which is a Pgp substrate, led to enhanced mRNA and protein expression of Pgp at all time points (Ihnat, Lariviere *et al.* 1997; Maitra, Halpin *et al.* 2001), which has been independently confirmed by a second group (Günthert, Gründker *et al.* 2004). This is not surprising, as substrate-mediated enhancement of drug efflux pumps is a major reason for treatment failure in cancer patients (Dixon, Bell *et al.* 1992; Koh, Chung *et al.* 1992).

Finally, this group examined the expression of CFTR following treatment with MMC in HT-29 and T84 colorectal carcinoma cells (Maitra, Shaw *et al.* 2001a) as well as the effect of doxorubicin on CFTR and $\Delta F508$ -CFTR expression in T84 cells (Maitra, Shaw *et al.* 2001b). The $\Delta F508$ -CFTR mutant is the most common CFTR mutation in cystic fibrosis. The mutation leads to the chloride channel's retention in the ER, resulting

in the pathology of cystic fibrosis (Ward, Omura *et al.* 1995). In each case, Hamilton and colleagues found that drug treatment led to an up-regulation of CFTR and the $\Delta F508$ -CFTR mutant at the cell surface. They showed that this regulation occurred post-transcriptionally and suggested that doxorubicin analogs with reduced toxicity may be useful for cystic fibrosis therapy (Maitra, Shaw *et al.* 2001b).

Cisplatin treatment leads to the down-regulation of a different cell-surface transporter. Cisplatin enters cells primarily through the use of the copper influx transporter, copper transporter 1 (CTR1; Ishida, Lee *et al.* 2002; Lin, Okuda *et al.* 2002; Holzer, Samimi *et al.* 2004; Song, Savaraj *et al.* 2004). Clinically relevant concentrations of CIS down-regulate the cell-surface expression of CTR1 through rapid macropinocytosis of the receptor to beneath the level of detection by Western blot or immunofluorescence. CTR1 protein levels returned to normal in about 30 min following removal of CIS (Holzer and Howell 2006).

Cytotoxic Drugs Modulate the Levels of Cellular Adhesion Molecules and T Cell Antigens

Adhesion molecules make up the third group of cell-surface proteins shown to have altered expression following cytotoxic drug treatment. Ishikawa and colleagues first showed this in 1995 when they treated primary squamous cell carcinoma cells with low doses of 5-FU, CIS, or carboplatin. They found that treated cells had increased cell-surface expression of intercellular adhesion molecule-1 (ICAM-1; CD54) and major histocompatibility complex (MHC) class I compared to control cells. These cells had enhanced susceptibility to AK-T cell-mediated lysis (Matsuoka, Eura *et al.* 1995).

In contrast to this, Perico and associates found that the microtubule-targeting agent colchicine led to a decrease in cell-surface L-selectin (CD62L) on human T cells and also inhibited interleukin (IL)-1 β -mediated increases in ICAM-1 and E-selectin on human umbilical vein endothelial cells (HUVECs). This resulted in reduced T cell activation, as measured by IL-2R induction following stimulation with anti-CD3 alone or in combination with anti-CD4 or anti-L-selectin antibodies (Perico, Ostermann *et al.* 1996). A second group showed that 5-FU treatment also decreased L-selectin as well as $\alpha_L\beta_2$ integrin (CD11a; LFA-1) cell-surface expression on hematopoietic progenitor cells (Noach, Ausema *et al.* 2003).

Hoskin's group has examined the effect of microtubule-targeting cytotoxic drugs on cellular adhesion molecules in some detail. In 2002 they reported that both paclitaxel and VB treatment led to reduced surface expression of $\alpha_L\beta_2$ integrin and ICAM-1 on murine P815 mastocytoma and Yac-1 lymphoma cells (Zhao, Morgan *et al.* 2003). Using RT-PCR, they showed that this change in protein by these agents was regulated at the mRNA level. This down-regulation of $\alpha_L\beta_2$ integrin and ICAM-1 led to reduced AK-T cell-mediated lysis, which was in direct contrast to their findings that CIS and etoposide up-regulated CD95, resulting in enhanced T-cell-mediated lysis of P815 mastocytoma cells (Williams, Makrigiannis *et al.* 1997). This is an important finding because it suggests that some drugs, such as CIS and etoposide, may be good choices for combination with immunotherapy, while other drugs, such as paclitaxel and VB, may be poor choices for combination therapy.

In 2003, they followed this work with a publication describing the role of paclitaxel in mediating changes in integrin expression. They showed that low-dose

paclitaxel treatment, equivalent to the EC₂₅, reduced the expression of α_L , α_4 , α_5 , and β_7 integrins by Jurkat T lymphocytes. This resulted in decreased T cell adhesion to monolayers of MDA-MB-435 cells (Bhan, Mader *et al.* 2004). They showed similar results describing reduced natural killer (NK)-like YT cell binding to K562 leukemia cells following paclitaxel treatment as a result of reduced adhesion molecule expression (Loubani and Hoskin 2005).

The mechanism for low-dose paclitaxel-mediated down-regulation of ICAM-1 was described in 2005. Hoskin and associates showed that paclitaxel reduced cell-surface ICAM-1 expression by MDA-MB-435 breast carcinoma cells and that this was at least partially dependent upon the generation of reactive oxygen species (ROS) and activation of caspase-3. They suggested that doses of paclitaxel, below those required to kill cells, led to ROS-dependent activation of caspase-3, followed by caspase-3 degradation of the p65 NF- κ B subunit and a reduction in NF- κ B-mediated ICAM-1 transcription (Fawcett, Mader *et al.* 2005).

Interestingly, bone marrow progenitor cells treated with 5-FU demonstrated reduced expression of the chemokine receptor CXCR4 (Noach, Ausema *et al.* 2003). Unlike all the other proteins described above, CXCR4 is primarily involved in cellular chemotaxis. Therefore, it does not fit neatly into any of the categories of proteins known to be affected by genotoxic stimuli.

Chemokines and Chemokine Receptors

Chemokines are small, bioactive peptides that bind to G protein-coupled receptors (GPCRs; Premack and Schall 1996; Baggiolini, Dewald *et al.* 1997; Yoshie, Imai *et al.*

1997; Luster 1998; Zlotnik, Morales *et al.* 1999). Activation of these receptors by ligand binding leads to adenylate cyclase inhibition and calcium mobilization; this typically causes cell polarization, chemotaxis, and/or proliferation (Myers, Wong *et al.* 1995; Shyamala, Khoja *et al.* 1998; Hall, Beresford *et al.* 1999). There are four classes of chemokines, the nomenclature of which is based upon the location of conserved cysteine residues within their amino acid sequence: C, CC, CXC, and CX₃C, where “C” identifies cysteine and “X” identifies any other amino acid. Following the cysteine designation, there is an “L” or an “R”, signifying that the molecule is either a ligand (chemokine) or receptor (Murphy, Baggiolini *et al.* 2000). More than 40 chemokines have been identified (including CCL1 through 27, CXCL1 through 14, XCRL1 and 2, and CX₃CL1) as well as at least 19 different chemokine receptors (including CCR1 through 10, CXCR1 through 7, XCR1, and CX3CR1; Table 1.2; Murphy, Baggiolini *et al.* 2000; Murphy 2002). Many chemokine/receptor interactions are promiscuous: most chemokine receptors can bind more than one chemokine, and most chemokines can act on a variety of receptors. For example, CCR2 acts as a receptor for CCL2, 7, 8, and 13; CCL13 can bind to CCR1, 2, and 3 (Murphy, Baggiolini *et al.* 2000).

Many of these interactions are important in immune system processes such as inflammation and lymphocyte trafficking, as well as in wound healing, angiogenesis, and organogenesis. Early publications showed that CCL5 and CCL2 are chemoattractive for T lymphocytes and monocytes (Furutani, Nomura *et al.* 1989; Schall, Bacon *et al.* 1990; Carr, Roth *et al.* 1994). Subsequently, a 1996 report described the release of CXCL8, CCL2, and CCL5 by human peritoneal mesothelial cells following stimulation with heat-killed *E. coli* (Kinnaert, De Wilde *et al.* 1996), suggesting that this chemokine release

may be important in the recruitment of leukocytes to the site of infection. Since this time, many other publications have described the roles of each of the other chemokines in attracting immune cells to sites of assault, resulting in a complex picture of cellular migration (Table 1.3). Butcher and colleagues examined this complexity directly by culturing leukocytes in agarose (Foxman, Campbell *et al.* 1997). They simultaneously exposed the leukocytes to two chemokines (for which the leukocytes expressed receptors) at varying concentrations and in different combinations, and examined cellular migration. They found that leukocyte chemotaxis is a multistep process, where cells respond sequentially to chemokines. They suggested that the net result of chemokine binding on a given cell depends on its profile of chemokine receptors and the sequence of chemokines it encounters (Foxman, Campbell *et al.* 1997).

The CXCR4/CXCL12 Axis

The chemokine/receptor pair, CXCR4 and CXCL12, is different from other chemokine and receptor pairs in a number of ways. In contrast to the promiscuity of other chemokines and receptors and the multiplicity of their functions (Murphy, Baggiolini *et al.* 2000), CXCR4 can bind only CXCL12 and CXCL12 binds only CXCR4 or CXCR7 (Nagasawa, Hirota *et al.* 1996; Ma, Jones *et al.* 1998; Balabanian, Lagane *et al.* 2005), although the importance of CXCR7 binding has yet to be elucidated. Although the CXCR4-CXCL12 axis is important for immune system function, by regulating B lymphocyte maturation (Nagasawa, Hirota *et al.* 1996) and chemoattracting T cells, monocytes and hematopoietic progenitor cells (Bleul, Fuhlbrigge *et al.* 1996; Aiuti, Webb *et al.* 1997), this axis plays important roles in early development as well. CXCR4

Table 1.3. The roles of chemokines in immune cell function. Chemokine/receptor interacting partners and their immune system functions are indicated (adapted from Murphy, Baggiolini *et al.* 2000; Rossi and Zlotnik 2000; Murphy 2002).

Receptor	Chemokine	Function
CXCR1	CXCL8	Neutrophil migration; innate immunity; acute inflammation
CXCR2	CXCL1-3, 5-8	Neutrophil migration; innate immunity; acute inflammation; angiogenesis
CXCR3	CXCL9-11	T cell migration; adaptive immunity; Th ₁ inflammation
CXCR4	CXCL12	B cell lymphopoiesis; bone marrow myelopoiesis; central nervous system and vascular development; HIV infection
CXCR5	CXCL13	B cell trafficking; lymphoid development
CXCR6	CXCL16	T cell migration
CCR1	CCL3, 5, 7, 8, 13-16, 23	T cell and monocyte migration; innate and adaptive immunity; inflammation
CCR2	CCL2, 7, 8, 13	T cell and monocyte migration; innate and adaptive immunity; Th ₁ inflammation
CCR3	CCL5, 7, 8, 11, 13, 15, 24, 26	Eosinophil, basophil, and T cell migration; allergic inflammation
CCR4	CCL17, 22	T cell and monocyte migration; allergic inflammation
CCR5	CCL3-5, 7, 8, 11, 13, 14	T cell and monocyte migration; innate and adaptive immunity; HIV infection
CCR6	CCL20	Dendritic cell migration
CCR7	CCL19, 21	T cell and dendritic cell migration; lymphoid development; primary immune response
CCR8	CCL1, 4, 17	T cell trafficking
CCR9	CCL25	T cell homing to gut
CCR10	CCL26-28	T cell homing to skin
XCR1	XCL1, 2	T cell trafficking
CX ₃ CR1	X ₃ CL1	T cell and NK cell trafficking and adhesion; innate and adaptive immunity; Th ₁ inflammation

and CXCL12 are in fact so important in early development that knock-out animals for either gene die in late embryogenesis or shortly after birth because of severe defects in heart, brain, and gastrointestinal tract development (Nagasawa, Hirota *et al.* 1996; Ma, Jones *et al.* 1998; Tachibana, Hirota *et al.* 1998; Ma, Jones *et al.* 1999). This suggests an important role for CXCL12 signaling through CXCR4 in many cellular processes, including hematopoiesis, organ vascularization, and neurogenesis.

There are no reports of CXCR4 and CXCL12 deficiency in humans, most likely as a result of embryonic lethality. There are reports, however, of patients with a very rare disease caused by a truncating mutation in CXCR4, leading to enhanced receptor activity (Hernandez, Gorlin *et al.* 2003; Kawai, Choi *et al.* 2005). This leads to hematopoietic progenitor cell retention in the bone marrow resulting in warts, hypogammaglobulinemia, immunodeficiency, and myelokathexis (WHIM) syndrome.

Finally, a very important disease relating to CXCR4 expression on the surface of T cells is human immunodeficiency virus (HIV)-1. Long before CXCR4 was shown to have a role in cancer it was known to be a co-receptor for T-tropic (X4) HIV-1 (Bleul, Farzan *et al.* 1996; Oberlin, Amara *et al.* 1996). Many CXCR4 inhibitors, such as AMD3100, that have been suggested for use in cancer were originally developed with the goal of reducing HIV-1 progression to T-tropic disease (Hendrix, Flexner *et al.* 2000).

The CXCR4/CXCL12 Axis in Cancer

The past decade has seen reports describing the role of CXCR4 and CXCL12 in more than 25 different malignancies (Table 1.4; Balkwill 2004). One of the first reports identified increased CXCR4 expression in glioblastoma cells compared to normal brain

tissue. In this study, Murphy and colleagues used cDNA microarray technology to identify genes that were differentially expressed in glioblastoma multiforme tumour tissue and cell lines compared to normal brain tissue (Sehgal, Keener *et al.* 1998). They found that CXCR4 was required for the proliferation of glioblastoma cells and reported an up-regulation of CXCR4 mRNA in breast cancer tissue and cell lines compared to normal breast tissue. This important publication was followed by a study exploring the expression of CXCR4 in colorectal, esophageal, and gastric cancers. Interestingly, this group found there was no difference in CXCR4 mRNA expression in any of these malignant tissues compared to control tissues (Mitra, Shibuta *et al.* 1999).

In 2001, Zlotnik and colleagues published a landmark paper demonstrating the importance of CXCR4 and CXCL12 in the site-directed metastasis of breast cancer (Müller, Homey *et al.* 2001). While CXCR4 was absent from normal breast cells and tissues, it was present in primary breast cancer cells, breast cancer cell lines, and cancerous breast tissue. Interestingly, tissues toward which breast cancer cells metastasize, such as the lymph nodes, lungs, and liver, expressed high levels of CXCL12; tissues representing sites to which breast cancer rarely metastasizes, such as the kidney, skin, and muscle, expressed very low levels of CXCL12. Importantly, following tail-vein injection or orthotopic breast tumour implantation, mice treated with a neutralizing CXCR4 antibody showed reduced lung and lymph node metastases (Müller, Homey *et al.* 2001). Based on these results, this group postulated that CXCL12 is important for determining the site-specific migration of cancer cells.

In contrast to this, another group found that CXCL12 was important for the site-specific proliferation of cancer cells (Zeelenberg, Ruuls-Van Stalle *et al.* 2003). In this

Table 1.4. The CXCR4-CXCL12 axis is involved in many malignancies. Listed are select cancers that have increases in CXCR4 expression compared to normal tissues, as well as the relevance of these changes to prognosis, disease progression, and/or survival.

Malignancy	Relevant to prognosis, progression, or survival?	References
Acute lymphoblastic leukemia	Yes	(Crazzolara, Kreczy <i>et al.</i> 2001)
Brain cancer	Unknown	(Sehgal, Keener <i>et al.</i> 1998; Barbero, Bajetto <i>et al.</i> 2002; Zhou, Larsen <i>et al.</i> 2002; Rubin, Kung <i>et al.</i> 2003)
Breast cancer	Yes	(Müller, Homey <i>et al.</i> 2001; Kato, Kitayama <i>et al.</i> 2003; Cabioglu, Yazici <i>et al.</i> 2005)
Cervical cancer	Yes	(Majka, Drukala <i>et al.</i> 2006; Kodama, Hasengaowa <i>et al.</i> 2007)
Chronic lymphocytic leukemia	Yes	(Möhle, Bautz <i>et al.</i> 1998; Möhle, Failenschmid <i>et al.</i> 1999; Ishibe, Albitar <i>et al.</i> 2002)
Colorectal cancer	Yes	(Zeelenberg, Ruuls-Van Stalle <i>et al.</i> 2003; Kim, Takeuchi <i>et al.</i> 2005; Ottaiano, di Palma <i>et al.</i> 2005; Schimanski, Schwald <i>et al.</i> 2005; Ottaiano, Franco <i>et al.</i> 2006)
Endometrial cancer	Unknown	(Mizokami, Kajiyama <i>et al.</i> 2004)
Esophageal cancer	Yes	(Gockel, Schimanski <i>et al.</i> 2006; Koishi, Yoshikawa <i>et al.</i> 2006)
Gastric cancer	Yes	(Yasumoto, Koizumi <i>et al.</i> 2006)
Head and neck squamous cell cancer	Yes	(Katayama, Ogino <i>et al.</i> 2005)
Hemangioblastoma	Unknown	(Zagzag, Krishnamachary <i>et al.</i> 2005)
Hepatocellular cancer	Yes	(Schimanski, Bahre <i>et al.</i> 2006)
Melanoma	Yes	(Robledo, Bartolome <i>et al.</i> 2001; Cardones, Murakami <i>et al.</i> 2003; Bartolomé, Molina-Ortiz <i>et al.</i> 2006; Scala, Giuliano <i>et al.</i> 2006)
Multiple myeloma	Yes	(Sanz-Rodríguez, Hidalgo <i>et al.</i> 2001; Van de Broek, Leleu <i>et al.</i> 2006)

Malignancy	Relevant to prognosis, progression, or survival?	References
Nasopharyngeal cancer	Yes	(Hu, Deng <i>et al.</i> 2005; Wang, Wu <i>et al.</i> 2005)
Neuroblastoma	Yes	(Geminder, Sagi-Assif <i>et al.</i> 2001; Russell, Hicks <i>et al.</i> 2004)
Non-Hodgkin's lymphoma	Unknown	(Bertolini, Dell'Agnola <i>et al.</i> 2002)
Non-small cell lung cancer	Yes	(Su, Zhang <i>et al.</i> 2005)
Osteosarcoma	Yes	(Laverdière, Hoang <i>et al.</i> 2005; Laverdière and Gorlick 2006)
Ovarian cancer	Yes	(Scotton, Wilson <i>et al.</i> 2001; Scotton, Wilson <i>et al.</i> 2002; Jiang, Wu <i>et al.</i> 2006; Oda, Ohishi <i>et al.</i> 2007)
Pancreatic cancer	Yes	(Koshiba, Hosotani <i>et al.</i> 2000; Marchesi, Monti <i>et al.</i> 2004; Wehler, Wolfert <i>et al.</i> 2006)
Prostate cancer	Yes	(Taichman, Cooper <i>et al.</i> 2002; Mochizuki, Matsubara <i>et al.</i> 2004; Singh, Singh <i>et al.</i> 2004)
Renal cell cancer	Yes	(Schrader, Lechner <i>et al.</i> 2002; Staller, Sulitkova <i>et al.</i> 2003)
Rhabdomyosarcoma	Unknown	(Libura, Drukala <i>et al.</i> 2002)
Small cell lung cancer	Unknown	(Kijima, Maulik <i>et al.</i> 2002)
Thyroid cancer	Unknown	(Hwang, Hwang <i>et al.</i> 2003; Castellone, Guarino <i>et al.</i> 2004)

body of work, an intrakine approach was used to block cell-surface expression of CXCR4. A lysine-aspartate-glutamate-leucine (KDEL) sequence was fused to CXCL12 so that it would be retained intracellularly by binding to the KDEL receptor in the endoplasmic reticulum (ER). CXCR4 bound this intracellular CXCL12, causing it to be retained in the ER as well, effectively preventing its cell-surface expression.

Intrasplenic injection of colorectal cancer cells typically results in liver and lung metastases. When CXCL12-KDEL-transfected cells were injected intrasplenically, they showed reduced macroscopic metastatic lesions compared to control cells. However, upon microscopic examination, there was evidence of tumour cells in these organs, which simply failed to develop into pathologically important metastases. Therefore, this group concluded that CXCL12 is required for the tissue-specific expansion, rather than the directed migration, of CXCR4 expressing colorectal cancer cells (Zeelenberg, Ruuls-Van Stalle *et al.* 2003).

Since the publication of these two important papers, there have been reports of CXCR4 demonstrating altered expression in more than 25 cancers. Importantly, high expression of CXCR4 predicts poor prognosis or reduced survival in more than 15 of these malignancies (Table 1.4).

CD26-Dependent Regulation of CXCL12

As described above, the levels of CXCR4 expressed by tumour cells are very important in regulating their metastasis to and/or proliferation at sites that are rich in CXCL12. It logically follows that the levels of CXCL12 encountered by these cells will also be important in determining their site-specific spread and growth. The most

important regulator of CXCL12 activity is a cell-surface molecule termed CD26 (EC.3.4.14.5; Shioda, Kato *et al.* 1998; Lambeir, Proost *et al.* 2001; Busso, Wagtmann *et al.* 2005).

CD26 has a variety of functions, and as such has been referred to as a “moonlighting” protein (Figure 1.4; Boonacker and Van Noorden 2003). CD26 was first described in 1966 by Hopsu-Havu and Glenner for its enzyme activity and was termed glycylproline naphthylamidase (Hopsu-Havu and Glenner 1966). This group was interested in isolating the enzyme which was responsible for hydrolyzing the chromogenic substrate glycyl-DL-prolyl- β -naphthylamide. Using a series of fractionation steps, they isolated glycylproline naphthylamidase (CD26) from a commercially available enzyme preparation as well as rat liver and kidney, and showed that the enzyme was highly active in both tissues. Since then, the proteolytic activity of CD26 has been described in depth. Specifically, CD26 has intrinsic dipeptidyl peptidase IV (DPPIV) activity; it cleaves dipeptides from proteins or peptides containing proline or alanine in the penultimate NH₂-terminal position (Kenny, Booth *et al.* 1976; Püschel, Mentlein *et al.* 1982; Tanaka, Camerini *et al.* 1992). Many active molecules, such as hormones, cytokines, and chemokines, contain DPPIV-sensitive cleavage sites (reviewed by De Meester, Korom *et al.* 1999). Some examples include members of the glucagon family (Bongers, Lambros *et al.* 1992; Mentlein, Gallwitz *et al.* 1993; Kieffer, McIntosh *et al.* 1995), substance P (Ahmad, Wang *et al.* 1992), TNF- α (Bauvois, Sancéau *et al.* 1992), prolactin (Nausch, Mentlein *et al.* 1990), and the CXCR4 ligand, CXCL12 (Shioda, Kato *et al.* 1998; Lambeir, Proost *et al.* 2001; Busso, Wagtmann *et al.* 2005).

In 1998, Nagai and colleagues discovered that CXCL12 is a substrate for CD26

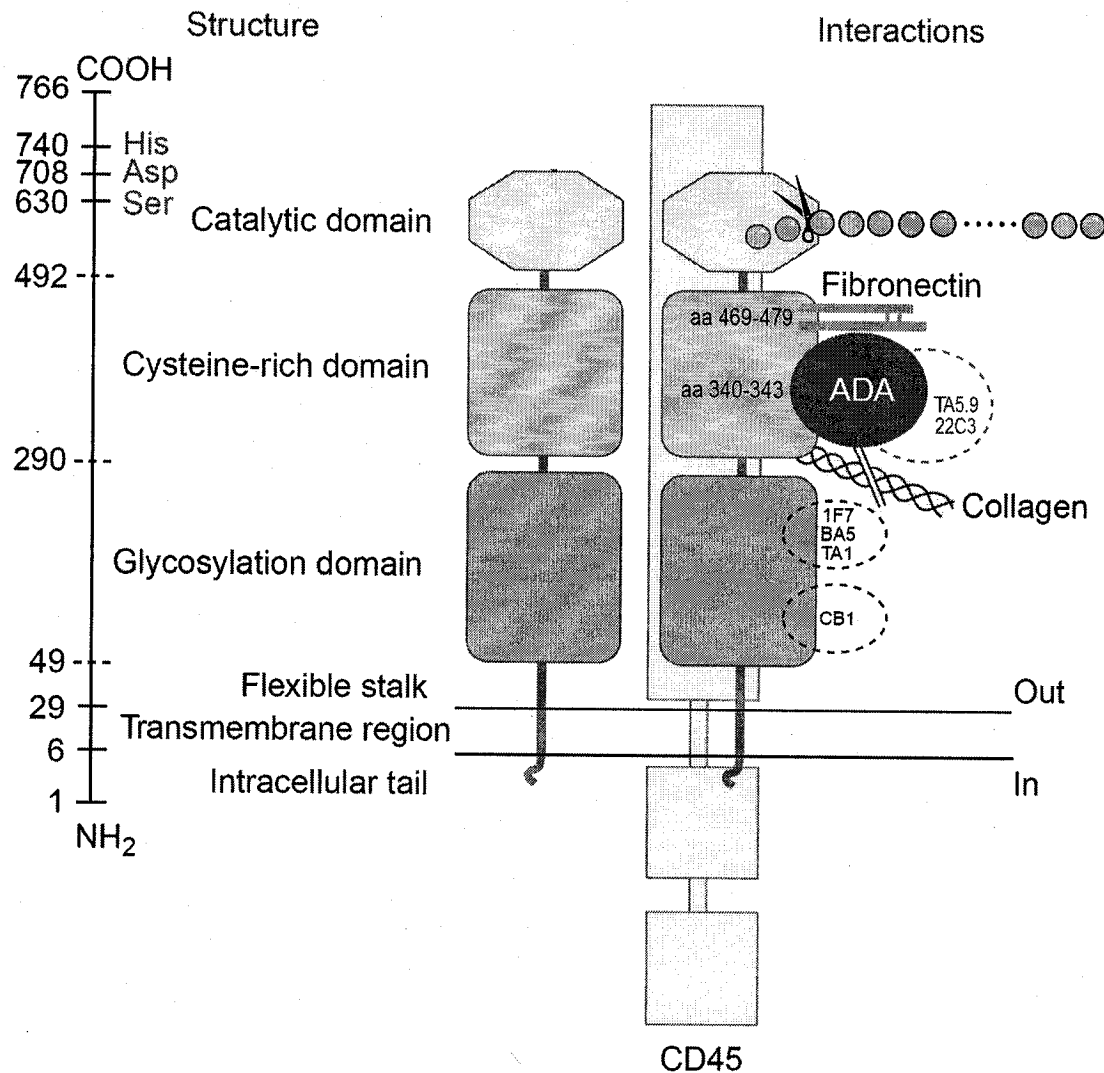


Figure 1.4. CD26 is a multifunctional or “moonlighting” protein. The dipeptidase activity and adenosine deaminase and extracellular matrix protein binding capacity of CD26, as well as its homodimerization and CD45 interaction are shown in this diagram (from De Meester, Korom *et al.* 1999).

(Shioda, Kato *et al.* 1998). They examined the ability of CXCL12 to block HIV-1 entry into cultured T cells that expressed the co-receptor CXCR4. They found that anti-CXCR4 antibodies were capable of blocking entry in both MT4 and H9 T cell lines, but interestingly, CXCL12 blocked entry into MT4 but not H9 cells. They examined the chemotactic capacity of CXCL12 for these cells and similarly found it was reduced in H9 cells compared to MT4 cells. They performed a Western blot for CXCL12 on the culture supernatant of each cell line and sequenced the bands to show that CXCL12 was cleaved in the presence of H9 but not MT4 cells. They correlated this increased HIV-1 infectivity and reduced chemotaxis with increased levels of CD26 on the surface of the H9 cells and showed that addition of diprotin A, a specific inhibitor of DPPIV activity, restored the anti-viral activity of CXCL12 in these same cells (Figure 1.5; Shioda, Kato *et al.* 1998).

De Meester and colleagues expanded on this knowledge in 2001 by examining the kinetics of DPPIV-mediated cleavage of some of the most common CD26 substrates (Lambeir, Proost *et al.* 2001). They incubated purified peptides with soluble CD26 in a cell-free assay and measured the ratios of intact and cleaved products on a mass spectrometer, in the presence or absence of DPPIV inhibitors. They found that of these, CXCL12 was cleaved with the greatest efficiency and specificity, demonstrating a catalytic rate constant (k_{cat}) of 12 s^{-1} and k_{cat}/K_m of $5 \times 10^{-6} \text{ M}^{-1}\text{s}^{-1}$. The half-life of CXCL12 in this system (in which the concentration of CD26 was estimated to be similar to that of plasma) was less than one min (Lambeir, Proost *et al.* 2001). In 2005, Grouzman and co-workers examined the circulating CXCL12 levels in CD26^{-/-} mice. They found that CXCL12 levels were much higher in the deficient animals compared to

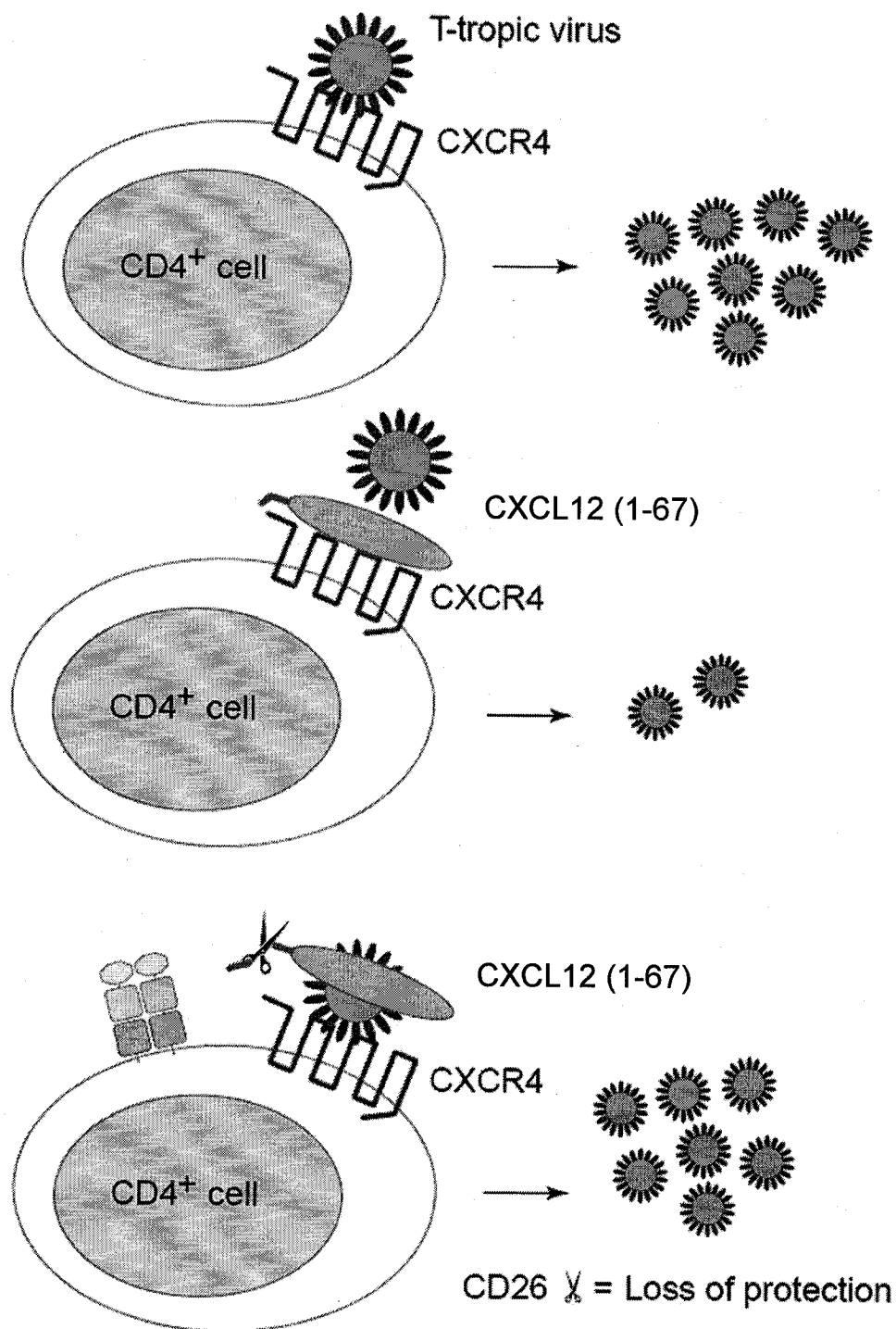


Figure 1.5. CD26 expression by T cells inhibits CXCL12-mediated inhibition of T-tropic HIV-1 infection (adapted from De Meester, Korom *et al.* 1999).

age-matched CD26^{+/+} controls, suggesting that CD26 controls the *in vivo* half-life of CXCL12 as well (Busso, Wagtmann *et al.* 2005).

CD26 Binds Adenosine Deaminase

A second important role for CD26 is that of a receptor for adenosine deaminase (ADA); CD26 is identical to the ADA-complexing protein (ADCP), or ADA-binding protein (ADAbp; Kameoka, Tanaka *et al.* 1993; Morrison, Vijayasaradhi *et al.* 1993) first described in 1978 by Schrader and Pollara (Schrader and Pollara 1978), and characterized in 1982 by Schrader and Bryer (Schrader and Bryer 1982). Schrader and Bryer showed that ADCP (CD26) exists in two forms, soluble and insoluble, and suggested that these were products of the same gene. Furthermore, they described the expression of CD26 by kidney brush-border epithelial cells (Schrader and Bryer 1982).

Adenosine deaminase catalyzes the conversion of extracellular adenosine to inosine. Adenosine is expressed at increased levels in tumours and is known to enhance tumour cell growth, proliferation, migration, and CXCR4 expression (Mujoomdar, Hoskin *et al.* 2003; Richard, Tan *et al.* 2006). In addition, adenosine reduces CD26 expression and ADA-binding capacity, thereby driving a feedback loop which limits its own degradation (Tan, Mujoomdar *et al.* 2004). In opposition to this, the ADA/CD26 complex reduces local levels of adenosine, therefore limiting its tumour-promoting capacity (Tan, Mujoomdar *et al.* 2004). This is also important in terms of immune system function: patients with an inherited autosomal recessive ADA-deficiency disorder, SCID, demonstrate decreased T and B cell activation as a consequence of increased local

concentrations of adenosine (Giblett, Anderson *et al.* 1972; Trotta, Smithwick *et al.* 1976).

CD26 Regulates Immune Cell Function

CD26 plays a second role in immune system function: it acts as a costimulatory molecule for T cell activation and IL-2 production. In fact, during the time that protein biologists were studying DPPIV or ADCP for its enzyme activity or binding properties, immunologists were studying a molecule, termed CD26, as a marker of T cell activation and differentiation. In particular, stimulation of CD26 with a specific monoclonal antibody leads to IL-2 production as well as CD26 association with CD45, resulting in tyrosine phosphorylation of a variety of intracellular mediators involved in T cell activation, proliferation, and differentiation into the T helper cell subset (Dang, Torimoto *et al.* 1990a; Dang, Torimoto *et al.* 1990b; Dang, Torimoto *et al.* 1990c). Fleischer and colleagues first suggested that DPPIV and CD26 may be the same molecule, based on antibody binding studies (Hegen, Niedobitek *et al.* 1990). This was subsequently confirmed in a study by Flad and colleagues (Ulmer, Mattern *et al.* 1990). This group immobilized purified DPPIV and measured anti-CD26 antibody binding in an enzyme-linked immunosorbent assay (ELISA). A different antibody, directed against CD26, bound purified DPPIV; this binding was competitively inhibited by anti-DPPIV antibody. Finally, Morimoto and associates described the binding of ADA to CD26 (Kameoka, Tanaka *et al.* 1993); around the same time, Houghton and colleagues purified and sequenced ADCP and found it to be identical to CD26 (Morrison, Vijayasaradhi *et al.*

1993). As well, Trugnan and coworkers sequenced DPPIV and also confirmed its identity with CD26 (Darmoul, Lacasa *et al.* 1992).

CD26 Binds Extracellular Matrix Proteins

A final, important function of CD26 is as an adhesion molecule for the extracellular matrix (ECM) proteins collagen and fibronectin. Immunohistochemical studies have shown that CD26 often colocalizes with collagenous fibers and is concentrated at sites of cell-ECM contact (Hartel, Gossrau *et al.* 1988). Subsequent studies have shown that CD26 antiserum reduced cell spreading on collagen-coated plates (Hanski, Huhle *et al.* 1988) and soluble CD26 blocked the adhesion of fibroblasts to a collagen matrix (Bauvois 1988). Similarly, CD26 overexpression enhanced binding to both collagen- and fibronectin-coated plates (Kikkawa, Kajiyama *et al.* 2003), and CD26 binding to fibronectin occurs with greater affinity than does binding to collagen (Piazza, Callanan *et al.* 1989). Finally, breast carcinoma cells covered in cell surface fibronectin are capable of binding to CD26-expressing lung endothelial cells and peptides containing the CD26-binding site of fibronectin blocked pulmonary metastasis in a rodent model (Cheng, Abdel-Ghany *et al.* 2003).

Normal CD26 Expression

CD26 is expressed by a variety of tissues and cell types (Table 1.5). As previously mentioned, it is expressed on T and B cells, in a highly regulated fashion (Giblett, Anderson *et al.* 1972; Trotta, Smithwick *et al.* 1976; Dang, Torimoto *et al.* 1990a; Dang, Torimoto *et al.* 1990b; Dang, Torimoto *et al.* 1990c). In contrast, CD26 is expressed

constitutively on the surface of kidney, lung, liver, spleen, and intestinal epithelium (Schrader and Stacy 1979; Dinjens, ten Kate *et al.* 1989a). Bosman and colleagues (Dinjens, ten Kate *et al.* 1989a) examined over 20 different human tissues and cell types using a radioimmunoassay and found that all cells expressing CD26 showed immunoreactivity in both the cytosolic and membrane fractions. CD26 expression was higher in the membrane fraction in all cases. Further examination using immunohistochemistry showed that this expression of CD26 was predominantly expressed at the apical surface of absorptive (brush border) epithelia and exocrine glandular cells (Dinjens, ten Kate *et al.* 1989a). This group also examined CD26 in the mouse and rat and found a similar pattern of distribution as that observed in human tissues (Dinjens, ten Kate *et al.* 1989b).

CD26 also exists in a soluble form (sCD26), lacking both the intracellular tail and the transmembrane region (Schrader and Stacy 1979; Schrader, Woodward *et al.* 1979; Schrader and West 1985). This CD26 species is fully functional in terms of binding capabilities and dipeptidase activities and is found in plasma, serum, saliva, tears, cerebrospinal fluid, semen, and in small amounts in urine (Schrader and Stacy 1979; Schrader, Woodward *et al.* 1979; Thompson, Piper *et al.* 1985; Dinjens, ten Kate *et al.* 1989a; Cejková, Zvárová *et al.* 2004; Narikawa, Misu *et al.* 2006). Increases or decreases in serum sCD26 have been studied for use as markers of disease in a number of pathophysiological processes (reviewed by Gorrell, Gysbers *et al.* 2001; Boonacker and Van Noorden 2003). For example, Raus and colleagues identified a correlation between severe depression and decreased sCD26 (Maes, De Meester *et al.* 1991), while Szalay

Table 1.5. CD26 expression by select human tissues or cells (unless otherwise indicated, from Dinjens, ten Kate *et al.* 1989a).

Tissue or Cell Type	Is CD26 Expressed?
Bile ducts	No
Cecum	Yes
Cervix	Yes
Colon	Yes
Duodenum	Yes
Endometrium	Yes
Erythrocytes	No
Gallbladder	Yes
Hair follicles	No
Ileum	Yes
Islets of Langerhans	No
Jejunum	Yes
Kidney	Yes
Liver	Yes
Lung	Yes
Mammary gland	Yes
Melanocytes	Yes (Houghton, Albino <i>et al.</i> 1988)
Mucinous glands	No
Ovary	Yes (Kajiyama, Kikkawa <i>et al.</i> 2003)
Pancreas	Yes
Placenta	Yes
Prostate	Yes
Rectum	Yes
Sebaceous glands	No
Seminal vesicles	No
Serous glands	Yes
Skin	Yes
Spleen	Yes
Stomach	No
Testis	No
Thymus	Yes
Thyroid	No
Trachea	Yes
Urinary bladder	No

and associates found that liver cirrhosis was correlated with elevated levels of sCD26 (Lakatos, Firneisz *et al.* 1999).

CD26 Expression in Cancer

Changes in CD26 expression have been identified in an array of tumour types (Table 1.6), with the most striking differences occurring in melanoma; malignant melanoma cells often demonstrate a complete loss of CD26 expression compared to normal melanocytes (Houghton, Albino *et al.* 1988; Morrison, Vijayasaradhi *et al.* 1993). This was first identified in 1988 when Houghton and colleagues examined 51 cultured melanocyte and 102 cultured melanoma cell lines, derived from either primary or metastatic lesions (Houghton, Albino *et al.* 1988). They found high expression of CD26 in normal melanocytes and absent expression in melanoma cells. Interestingly, this loss of CD26 expression was evident in all melanoma cells examined, regardless of disease stage or cell proliferation, suggesting that CD26 down-regulation is associated with malignant transformation in melanoma rather than disease progression. Eisinger and associates examined this directly by transforming normal melanocytes *in vitro* and examining the parental and transformed cells for CD26 expression. As predicted, they found that malignant transformation was associated with loss of CD26 expression (Houghton, Albino *et al.* 1988).

A key paper published in 1999 confirmed that CD26 down-regulation is an early event in the malignant transformation of melanocytes (Wesley, Albino *et al.* 1999). Re-introduction of CD26 into melanoma cells drastically inhibited their malignant phenotype (including loss of growth-factor independence, loss of anchorage-independent growth,

Table 1.6. CD26 expression in cancer. Listed are select cancers in which CD26 has been implicated. Whether expression is increased or decreased in each malignancy is indicated.

Malignancy	CD26 Expression Compared to Normal Tissue	References
Anaplastic large cell lymphoma	Increased	(Carbone, Cozzi <i>et al.</i> 1994)
Colon carcinoma	Variable	(ten Kate, Wijnen <i>et al.</i> 1984; ten Kate, Wijnen <i>et al.</i> 1985; ten Kate, van den Ingh <i>et al.</i> 1986)
Endometrial carcinoma	Decreased	(Khin, Kikkawa <i>et al.</i> 2003)
Hepatocellular carcinoma	Variable	(Stecca, Nardo <i>et al.</i> 1997)
Hodgkin's lymphoma	Increased	(Kameoka, Ichinohasama <i>et al.</i> 2006)
Melanoma	Decreased	(Houghton, Albino <i>et al.</i> 1988)
Non-small cell lung carcinoma	Decreased	(Wesley, Tiwari <i>et al.</i> 2004)
Ovarian carcinoma	Decreased	(Kajiyama, Kikkawa <i>et al.</i> 2002; Kajiyama, Kikkawa <i>et al.</i> 2003)
Prostate carcinoma	Decreased	(Wesley, McGroarty <i>et al.</i> 2005)
Renal cell carcinoma	Increased	(Inamoto, Yamochi <i>et al.</i> 2006)
Thyroid carcinoma	Increased	(Kotani, Aratake <i>et al.</i> 1991)

and morphology suggesting enhanced differentiation). Furthermore, CD26 expression rendered melanoma cells non-tumourigenic in mice. Interestingly, melanoma cells engineered to express mutant CD26 (lacking enzyme activity) demonstrated a moderate level of tumourigenicity, suggesting that reversal of the malignant phenotype depended, at least in part, on functional DPPIV activity (Wesley, Albino *et al.* 1999).

Not all cancers demonstrate a decrease in CD26 compared to normal tissue. For example, thyroid cancers demonstrate a dramatic up-regulation of CD26 expression. In 1991, Ohtaki and colleagues (Kotani, Aratake *et al.* 1991) examined the DPPIV activity in thyroid tissues and found that while all five normal tissues were negative for enzyme activity, all papillary and follicular thyroid carcinomas were positive. Other types of thyroid cancers showed variable DPPIV activity. In thyroid aspiration biopsy samples from 55 patients, this group identified a strong correlation between DPPIV activity and malignant disease (Aratake, Kotani *et al.* 1991). All 14 examples of carcinoma stained positive while 40 of 41 examples of benign disease (including follicular adenoma, adenomatous goiter, and Hashimoto's thyroiditis) showed very low or absent enzyme activity.

This group continued studying the relationship between CD26 and thyroid malignancy throughout the 1990s and published an interesting paper in 1999, suggesting that CD26 may be a better marker for diagnosing follicular thyroid carcinoma than traditional cytological techniques (Hirai, Kotani *et al.* 1999). In the authors' experience, follicular thyroid carcinoma is difficult to diagnose; it is not uncommon for patients to receive an incorrect diagnosis of benign neoplasia and subsequently present with distant metastases, indicating that the disease was in fact malignant. A retrospective examination

of thyroid biopsies, from patients whom had received the wrong diagnoses, showed that in 7 of 10 cases CD26 staining was positive. In contrast, 28 of 29 biopsies, from patients whom had received the proper diagnosis of benign disease, were CD26-negative (Hirai, Kotani *et al.* 1999).

In 2004, Noguchi and colleagues directly examined the diagnostic utility of CD26 in follicular thyroid carcinoma (Maruta, Hashimoto *et al.* 2004). They found that CD26 activity was a better marker for malignant disease than was tumour size, serum thyroglobulin, ultrasonography, or frozen-section pathology. Dipeptidyl peptidase IV activity as a marker demonstrated significantly better sensitivity (97% of malignant tumours were assessed as positive) than any of the other methods, and better specificity (95% of benign tumours were assessed as negative) than any other method except frozen-section pathology (85%), which did not reach statistical significance (Maruta, Hashimoto *et al.* 2004).

Changes in both CXCR4 and CD26 expression have been identified in numerous malignancies. CXCR4 expression is nearly always increased in cancerous tissues, while CD26 is sometimes increased and sometimes decreased. Considering the multifunctional nature of CD26 and the plethora of DPPIV substrates, it may not come as a surprise that the changes in CD26 expression are not always consistent from one cancer type to the next.

Cytotoxic drugs are used to treat most types of malignant disease. Furthermore, CXCR4 and CD26 have both been implicated in the proliferation and spread of cancer cells throughout the body. We chose to examine the relationship between cytotoxic drug

treatment and the expression of CXCR4 and CD26 on colorectal carcinoma cells with the following general objectives and hypotheses:

Objectives

1. To determine if cytotoxic drugs regulate the expression of CXCR4 and/or CD26 on the surface of human colorectal carcinoma cells.
2. To determine if any changes observed in CXCR4 or CD26 levels lead to corresponding increases or decreases in the functions of these molecules.
3. To determine if these molecules are regulated on tumour cells *in vivo* by systemic cytotoxic drug treatment.

Hypotheses

1. Cytotoxic drugs, at concentrations achieved in patients, will regulate the expression of CXCR4 and CD26 on human colorectal carcinoma cells *in vitro*, so as to oppose metastasis.
2. CXCR4-directed migration will decrease and CD26 dipeptidyl peptidase IV activity and adenosine deaminase-binding capacity will increase.
3. These changes will be evident in an orthotopic mouse model of colorectal carcinoma.

CHAPTER 2

CYTOTOXIC DRUGS DOWN-REGULATE THE CELL-SURFACE EXPRESSION OF CXCR4 ON HUMAN HT-29 COLORECTAL CARCINOMA CELLS

INTRODUCTION

The Role of CXCR4 in Metastasis

In most cancer cases, it is not the primary tumour itself but rather the resulting metastatic lesions that ultimately lead to the death of the patient. In some cases, death occurs for obvious reasons, such as organ compression or hemorrhage. In other cases, the cause of death is less apparent or unknown, but may result from cachexia or opportunistic infection (Lichtenstein 2005). Consequently, many groups are focusing their research efforts on understanding the process and regulation of metastasis so as to develop treatments to prevent or slow cancer spread.

The process of metastasis is complex and involves a number of steps including detachment from the primary cell mass, invasion into and movement through the blood stream or lymphatic system, and extravasation followed by seeding and growth at a secondary site within the body. Cell detachment from the primary tumour mass is a frequent event; tumours may shed millions of cells into the blood stream or lymphatic system each day (Butler and Gullino 1975; Glaves and Mayhew 1984). It is interesting, however, that few of these cells lead to clinically relevant metastatic disease. Although this may be partially attributable to the harsh environment of the blood stream, where cancer cells will need to survive shear stress and avoid anoikis, it is more likely a consequence of the inefficiency of organ colonization. Studies have shown that over 87% of melanoma cells injected into the mesenteric vein arrested in the liver within 90 min

of melanoma cells injected into the mesenteric vein arrested in the liver within 90 min (Luzzi, MacDonald *et al.* 1998). Although most of these cells extravasated into the liver and were present there after 3 days, very few formed micrometastases and only 0.02% of the initially injected cells formed life-threatening metastatic lesions (Luzzi, MacDonald *et al.* 1998).

It is interesting to note that not all organs are equal in terms of their colonization efficiency by metastatic cancer cells. In fact, many tumours exhibit specific patterns of metastases, spreading to predictable locations throughout the body. For example, breast cancer most often spreads to the lymph nodes, lungs, liver, and bone (Paget 1889), while colorectal cancer metastasizes primarily to the lymph nodes, liver, and lungs (Weiss, Grundmann *et al.* 1986). Paget first described patterns of breast cancer metastasis in 1889 together with his “seed and soil” hypothesis. He postulated that the cancer cells (the “seeds”) depend on the secondary organ (the “soil”) for growth (Paget 1889). Ewing countered this argument, suggesting that blood-flow patterns, which would carry tumour cells to other organs, were sufficient to account for the locations of secondary tumours (Ewing 1928).

More recent evidence shows that these two theories may both be correct. For example, the liver is the first site of blood flow from the colon, followed by the lungs. For that reason, it makes sense that the primary site of colorectal cancer metastasis is the liver and the second is the lung tissue. Likewise, breast cancer most often metastasizes to the lungs, which would be the first organ encountered by circulating tumour cells. However, this blood flow theory does not account for the relatively high incidence of breast and colorectal cancer metastases to the adrenal gland, for example. Furthermore, we would

expect to see a higher incidence of metastasis from the breast or the colon to the skin or skeletal muscle, if blood flow was the only determining factor (Weiss 1992). This suggests that some other mechanism must be in place to regulate the organ-specific metastasis of these, and many other, cancers.

Recent evidence suggests that chemokines and their receptors are such regulators. First in breast cancer (Müller, Homey *et al.* 2001), followed by colorectal cancer (Zeelenberg, Ruuls-Van Stalle *et al.* 2003), and subsequently more than 25 malignancies (Table 1.4), this site-specific metastasis was shown to be regulated in part by the interaction of chemokine receptors, expressed on the surface of cancer cells, with chemokines, present in favoured metastatic locations. The interaction of CXCL12 with its receptor, CXCR4, is perhaps the most prevalent, and certainly the most studied, chemokine/chemokine receptor pair in terms of metastasis regulation.

The Regulation of CXCR4 Expression

Many groups have been searching for the factor(s) which are responsible for up-regulating CXCR4 on the surface of such a wide variety of cancer cells. One of the earliest identified positive regulators of CXCR4 in cancer cells was found to be TNF- α , which is highly expressed in a number of malignancies (Saarinen, Koskela *et al.* 1990; Nakano, Kobayashi *et al.* 1999). To identify this, Benveniste and colleagues examined the expression of CXCR4 by astrogloma cell lines following treatment with a variety of stimuli (Oh, Drabik *et al.* 2001). Importantly, they found that TNF- α treatment led to increased CXCR4 mRNA and protein expression in these cells, which resulted in enhanced calcium mobilization following CXCL12 treatment.

Wilson and coworkers published a report in 2005 describing the role of TNF- α in regulating CXCR4 expression in ovarian carcinoma (Kulbe, Hagemann *et al.* 2005). They first examined CXCR4 and TNF- α protein expression levels in primary ovarian cancer cells, ovarian cancer cell lines, and clinical ovarian cancer samples, where they found a positive correlative relationship between these two proteins. Furthermore, when they treated these cell lines with TNF- α , they observed a substantial increase in cell-surface CXCR4 expression, which led to enhanced migration to CXCL12 in Boyden chamber assays. They also showed that endogenous TNF- α played a role in maintaining CXCR4 levels in these cells: when endogenous TNF- α was inhibited with a blocking antibody, they found a decrease in cell-surface CXCR4 levels (Kulbe, Hagemann *et al.* 2005).

Vascular endothelial growth factor is another soluble mediator that increases CXCR4 expression on cancer cells. Mercurio and colleagues were the first to demonstrate this when they found that inhibition of VEGF with antisense technology led to reduced CXCR4 expression in MDA-MB-231 breast carcinoma cells; addition of recombinant VEGF rescued CXCR4 expression (Bachelder, Wendt *et al.* 2002). They further explored this and showed that VEGF enhanced CXCL12-directed migration. A similar regulatory pathway was subsequently identified in glioblastoma (Zagzag, Lukyanov *et al.* 2006) and glioma (Hong, Jiang *et al.* 2006).

Hypoxia is a very important regulator of CXCR4 expression. As a result of their abnormal vasculature, solid tumours contain regions with very low levels of oxygen (Vaupel 2004). Hypoxia regulates gene expression by relieving von Hippel-Lindau tumour suppressor protein (pVHL)-mediated suppression of the transcription factor

hypoxia-inducible factor (HIF)-1 α (Maxwell, Wiesener *et al.* 1999). Thus hypoxia frees HIF-1 α to interact with DNA and modulate the transcription of its targets.

Krek and colleagues were the first group to identify CXCR4 as a target of HIF-1 α ; they performed a microarray study comparing gene expression by cells with pVHL to those without (Staller, Sulitkova *et al.* 2003). CXCR4 was identified as being strongly expressed in cells lacking pVHL and reintroduction of pVHL into these cells strongly suppressed CXCR4 mRNA and protein expression. Culturing human embryonic kidney (HEK) cells or primary human proximal renal tubular epithelial cells in hypoxia led to enhanced CXCR4 expression, and a hypoxia response element (HRE) was identified in the CXCR4 promoter (Staller, Sulitkova *et al.* 2003).

Sica and associates examined hypoxia-mediated regulation of CXCR4 and showed that hypoxia up-regulated CXCR4 in monocytes and macrophages as well as HUVEC, CAOV3 ovarian carcinoma, and MCF-7 breast carcinoma cell lines (Schioppa, Uranchimeg *et al.* 2003). They found that when HIF-1 α expression was blocked in mouse endothelial fibroblasts (MEFs), hypoxia was no longer an effective mediator of CXCR4 up-regulation. Finally, they used chromatin immunoprecipitation (ChIP) assays to show that HIF-1 α binds the CXCR4 promoter directly (Schioppa, Uranchimeg *et al.* 2003).

The CXCR4 promoter region contains binding sites for other transcription factors, such as NF- κ B, which also up-regulates CXCR4 expression. NF- κ B binds the promoter of CXCR4 through binding to a non-classical NF- κ B binding sequence (Helbig, Christopherson *et al.* 2003); NF- κ B is often constitutively activated in tumour cells, which may contribute to the enhanced CXCR4 expression in many malignancies (Helbig, Christopherson *et al.* 2003; Kukreja, Abdel-Mageed *et al.* 2005).

Finally, as a typical GPCR, CXCR4 is subject to desensitization and internalization, two different but related processes. Receptor desensitization is an example of a classical negative feedback loop and is a mechanism for rapidly dampening a signal, even in the continued presence of an agonist. Heterologous desensitization occurs following activation of second messengers such as protein kinase A or C. Once activated, these kinases phosphorylate residues within the cytosolic regions of many GPCRs, including CXCR4, and inhibit G protein binding. The GPCRs which are desensitized are often independent of those being bound by agonist and activating second messengers (Maudsley, Martin *et al.* 2005).

Homologous desensitization describes a mechanism by which only agonist-bound receptors become desensitized. G protein-coupled receptor kinases (GRKs) phosphorylate the activated form of the receptor, which promotes the binding of β -arrestin. The presence of β -arrestin blocks G proteins from binding the receptor and signaling. G proteins themselves can be targets of desensitization through the binding of regulator of G protein signaling (RGS) proteins. These act as GTPase activating proteins, promoting the hydrolysis of GTP to GDP, thus dampening the agonist signal (Pierce, Premont *et al.* 2002).

Receptor internalization can occur by several different pathways, most notably through receptor interactions with β -arrestin, as is the case for CXCR4. Not only can β -arrestin bind GPCRs, as described above, it can also bind components of clathrin-coated pits. As a result, β -arrestin targets GPCRs for endocytosis in these pits which can lead to either endosomal receptor resensitization and recycling back to the cell-surface or lysosomal receptor degradation (Claing, Laporte *et al.* 2002).

CXCR4 Targeting as Cancer Therapy

It is becoming increasingly evident that CXCR4 is an attractive target for the treatment of many cancers. Before its identification as an important receptor in cancer growth and metastasis, it was identified as “fusin” and found to be a co-receptor for HIV-1 entry into cells (Bleul, Farzan *et al.* 1996; Feng, Broder *et al.* 1996; Oberlin, Amara *et al.* 1996). Consequently, a number of CXCR4 antagonists and neutralizing antibodies have been developed and tested, both in animals and humans, for efficacy in reducing CXCR4-mediated HIV-1 entry (Hendrix, Flexner *et al.* 2000; Doranz, Fillion *et al.* 2001; Hendrix, Collier *et al.* 2004; Hatse, Princen *et al.* 2005). Although not very successful for reducing HIV-1 viral load, some of these agents have been tested for anticancer activity in pre-clinical tumour models with very promising results. For example, systemic administration of the small-molecule non-competitive CXCR4 antagonist AMD3100 in mice with glioblastoma or medulloblastoma xenografts led to increased tumour cell death and decreased proliferation, with no evidence of toxicity (Rubin, Kung *et al.* 2003).

Other strategies, such as administration of neutralizing antibodies, peptide inhibitors, or gene therapy using CXCR4-targeted small inhibitory RNA (siRNA) molecules have also had success in animal models. For example, Zlotnik and colleagues found that administration of a blocking antibody to mice harbouring MDA-MB-231 cells prevented metastasis (Müller, Homey *et al.* 2001). A similar strategy was used to reduce the growth of non-Hodgkin's lymphoma cells in mice (Bertolini, Dell'Agnola *et al.* 2002). Omata and coworkers found that a neutralizing anti-CXCR4 antibody was capable of inhibiting the growth of subcutaneous pancreatic cell tumours in mice, despite these tumour cells lacking CXCR4 expression. They determined that this was because of CXCR4 blockade on tumour vasculature, which resulted in angiogenesis inhibition

(Guleng, Tateishi *et al.* 2005). The CXCR4 peptide antagonist T22 led to decreased pulmonary metastasis in mice injected with CXCR4-expressing B16 murine melanoma cells (Murakami, Maki *et al.* 2002). Another antagonist, 4F-benzoyl-TN14003, inhibited pulmonary metastasis of MDA-MB-231 breast cancer cells (Tamamura, Hori *et al.* 2003) and B16-BL6 melanoma cells (Takenaga, Tamamura *et al.* 2004).

Finally, CXCR4 gene therapy using siRNA technology has shown some promise in preclinical studies. For example, Shim and colleagues transfected MDA-MB-231 cells with siRNA targeting CXCR4 and injected these cells into the tail veins of immunodeficient mice (Liang, Yoon *et al.* 2005). Inhibition of CXCR4 gene expression was maintained by administering naked siRNA twice weekly to these mice. As expected, mice receiving transfected cells as well as twice weekly injections of siRNA molecules showed greatly reduced rates of lung tumour development, compared to control animals. Surprisingly, a group of mice injected with control cells (that is, cells with high levels of CXCR4), who were treated biweekly with the CXCR4-targeted siRNA oligonucleotides, showed reduced lung tumour formation as well. Although this effect was not as substantial as that observed in mice with CXCR4 siRNA-transfected cells, it showed that naked CXCR4 siRNA delivery can have some benefit (Liang, Yoon *et al.* 2005)

The vast majority of patients with invasive or metastatic colorectal carcinoma are treated with cytotoxic drugs. We were interested in determining the effects of these agents on cell-surface CXCR4 expression on human colorectal carcinoma cells. We show here that cytotoxic drugs, commonly used to treat malignant disease, reduce the level of cell-surface CXCR4 expressed by human HT-29 colorectal carcinoma cells.

METHODS

Materials and Animals

HT-29 human colorectal carcinoma cells were obtained from the American Type Culture Collection (Manassas, VA) and culture vessels (Nunc) were from VWR (Mississauga, ON). 5-Fluorouracil, CIS, MTX, and irinotecan were from Mayne Pharma (Montreal, Quebec, Canada), and VB, cytarabine, and doxorubicin were from Faulding (Montreal, Quebec, Canada). Oxaliplatin, vincristine, type V collagen, primers, Mayer's hematoxylin solution, bovine serum albumin, ultrapure water, NaOH, and sodium azide were from Sigma Chemical Co. (St. Louis, MO). Mouse anti-human CXCR4 monoclonal antibody [clone 12G5] and mouse IgG_{2a} isotype-matched control antibody [clone G155-178]) were from BD Pharmingen (San Diego, CA), and secondary [¹²⁵I]-labeled goat anti-mouse IgG fragment was obtained from PerkinElmer Life Sciences (NEN, Boston, MA). Alexa Fluor[®] 488 goat anti-mouse IgG, sera, TRIzol reagent, oligo(dT)₁₂₋₁₈, deoxynucleotide triphosphate (dNTP) mix, Moloney murine leukemia virus (M-MLV), RT, dithiothreitol (DTT), and 5x first strand buffer were from Invitrogen Canada (Burlington, ON). Brilliant SYBR[®] Green kits were from Stratagene (Cedar Creek, TX). Transwell[®] culture inserts were from Corning Inc. (Corning, NY) and recombinant human CXCL12 was from Chemicon International (Temecula, CA). Ketamine/xylazine was from CDMV (St. Hyacinthe, QC), buprenorphine was from McGill University (Montreal, QC), and Ethicon[®] polypropylene sutures were from Johnson & Johnson (Halifax, NS). CD1 nu/nu mice were from Charles River (Wilmington, MA).

Cell Culture

HT-29 colorectal carcinoma cells were cultured in 80-cm² flasks containing Dulbecco's modified Eagle's medium (DMEM) with 5% (v/v) newborn calf serum (NCS) and were kept in an undifferentiated state. Cultures were maintained at 37°C in a humidified atmosphere of 90% air and 10% CO₂. Cells were routinely sub-cultured by brief exposure to 0.05% (w/v) trypsin/0.53 mM ethylenediamine tetraacetic acid (EDTA).

Cells were seeded at 100,000 cells per ml of DMEM containing 10% (v/v) NCS, unless otherwise stated. For binding assays, cells were seeded at a density of 50,000 cells per well in 48-well plates; for real-time quantitative RT-PCR, 200,000 cells per well in 6-well plates; for migration assay pre-treatments or Western blots, 1,000,000 cells per 10 cm dish. Cells were allowed to adapt to culture for 48 h, at which time the medium was replaced with DMEM containing 1% (v/v) NCS. In most situations drugs or vehicle controls were added after a subsequent 48 h and assays were performed at the indicated times.

Radioantibody Binding Assays

Binding assays on monolayer cultures were performed as previously described (Tan, Mujoomdar *et al.* 2004; Richard, Tan *et al.* 2006). All steps were carried out at 4°C on HT-29 cells grown in monolayer culture. Cells in 48-well plates were washed with ice-cold phosphate-buffered saline (PBS; 137 mM NaCl, 5 mM KCl, 24.8 mM Tris, 0.7 mM Na₂HPO₄, 0.5 mM MgSO₄, 1 mM CaCl₂; pH 7.2) containing 0.2% (w/v) bovine serum albumin (BSA). Cells were incubated for 1 h with 125 µl PBS containing 1% (w/v) BSA and 1 µg/ml mouse anti-human CXCR4 or isotype control antibodies. Cells were washed twice with PBS containing 0.2% BSA and incubated for 1 h with 125 µl PBS

containing 1% (w/v) BSA and 1 $\mu\text{Ci/ml}$ [^{125}I]-labeled goat anti-mouse IgG. Following a final three washes, cells were solubilized in 500 μl 0.5 M NaOH, followed by counting of radioactivity. Cells cultured and treated in parallel were counted using a Coulter[®] Model ZM30383 particle counter (Beckman Coulter, Mississauga, ON).

Real-Time Quantitative RT-PCR

Cells in 6-well plates were dissolved in 1 ml TRIzol[®] per well; frozen tissues were homogenized on ice in 1 ml TRIzol[®] and centrifuged at 12,000 x g for 10 min; the pellet was discarded. In both cases, RNA was subsequently isolated by following the manufacturer's directions. Briefly, 200 μl chloroform was added to each sample, the aqueous phase was saved, and 500 μl of isopropanol was added. The precipitated RNA was washed with 1 ml of 75% ethanol, dried briefly, and resuspended in ultrapure water. Two micrograms of total RNA was reverse transcribed to cDNA using M-MLV reverse transcriptase and oligo(dT)₁₂₋₁₈ primers. The major CXCR4 transcript variant, encoding CXCR4 isoform b (Gupta and Pillarisetti 1999), or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was amplified using a Stratagene Mx3000P system (Cedar Creek, Texas, USA) from 1 μl total cDNA using Brilliant SYBR[®] Green mastermix and the following primer sets:

CXCR4: 5'-GCCTGAGTGCTCCAGTAGCC-3'

5'-TGGAGTCATAGTCCCCT-3'

GAPDH: 5'-CATGAGAAGTATGACAACAGCCT-3'

5'-AGTCCTTCCACGATACC-3'

CXCR4 gene expression was analyzed using the Stratagene MxPro software. CXCR4 expression was standardized to GAPDH expression and normalized to control expression at 0 h using the $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen 2001).

Migration Assay

Cells grown in 10 cm dishes were pre-treated with cytotoxic drugs or vehicle control for 48 h. Pre-treated cells were suspended in serum-free DMEM containing 0.1% (w/v) BSA following brief exposure to 0.05% (w/v) trypsin/0.53 mM EDTA. Vehicle- or CXCL12-containing DMEM with 0.1% (w/v) BSA was added to the wells of a 24-well plate and porous Transwell® culture inserts (8 µm pores), which had been coated overnight at 37°C with 3 µg/ml type V collagen in serum-free DMEM, were inserted into each well. Inserts were seeded with 250,000 pre-treated cells and were incubated in the wells of the plates at 37°C for 18 h. The porous filters were scraped with a cotton-tipped applicator to remove cells from the upper surface, then the filters were removed from the inserts, fixed with ethanol, stained with Mayer's hematoxylin, and mounted on slides. Slide identifiers were obscured by a colleague and cells that had migrated to the lower surface of the filters were visualized at 400X magnification and counted.

Statistics

Figures show representative results from at least three independent experiments. Data were analyzed using analysis of variance or two-tailed Student's *t*-test for unpaired data using Graphpad Prism software.

RESULTS

5-Fluorouracil Decreases CXCR4 Cell-Surface Expression in HT-29 Cells

Since 5-FU is a commonly prescribed cytotoxic drug with a long history in colorectal carcinoma treatment, particularly in the metastatic setting, we tested its effect on cell-surface CXCR4 expression in the HT-29 colorectal cancer cell line. Previous reports and work in this laboratory have shown that HT-29 colorectal carcinoma cells express CXCR4 protein at the cell surface and that the cell-surface pool can be selectively measured on viable cells using cell-impermeable antibodies (Tan, Mujoomdar *et al.* 2004; Richard, Tan *et al.* 2006). We quantified cell-surface CXCR4 using a radioantibody binding assay and corrected all values for non-specific antibody binding and cell number to account for spurious binding and possible toxic effects of treatment.

We found an average maximal reduction in HT-29 cell number of 41.7% at high doses of 5-FU with an overall EC_{50} of 2.47 $\mu\text{g/ml}$ (Figure 2.1A, Table 2.1). With these same doses of 5-FU, we found an $80.8 \pm 7.4\%$ average maximal reduction in cell-surface CXCR4 on surviving viable cells, which was nearly twice the reduction observed in cell number (Figure 2.1B, Table 2.1). The EC_{50} for 5-FU-mediated CXCR4 reduction was $0.78 \pm 0.25 \mu\text{g/ml}$, which was not significantly different from the EC_{50} for cell number reduction ($2.47 \pm 1.32 \mu\text{g/ml}$; Table 2.1). We tested doses lower than 0.2 $\mu\text{g/ml}$ and showed that this was the lowest dose of 5-FU that caused a significant decrease in CXCR4 expression in the HT-29 cell line (Figure 2.1C). We attempted to confirm these results using flow cytometry; however, we were unable to detect CXCR4 expression by HT-29 cells reproducibly using this less sensitive assay. This made it

difficult to observe the expected decrease in CXCR4 expression following 5-FU treatment, so we used the more sensitive radioantibody binding assay for all subsequent experiments examining cell-surface CXCR4 protein expression.

Many Cytotoxic Drugs Decrease HT-29 CXCR4 Cell-Surface Expression

Next, we wanted to determine if this decrease in CXCR4 following treatment was specific to 5-FU or if it was a universal property of cytotoxic drugs. We examined other drugs commonly used in colorectal cancer treatment (irinotecan, oxaliplatin) as well as drugs used to treat other types of cancer (CIS, VB, MTX, doxorubicin, vincristine, and cytarabine; Von Hoff and Hanauske 2006). Despite the diverse mechanisms of action of these various drugs (Table 1.1), we found that in each case they led to a significant and substantial (62% to 94% maximum) decrease in CXCR4 expression (Figure 2.2; Table 2.1). We chose to focus on four drugs, 5-FU, CIS, VB, and MTX, representative of some of the different mechanistic classes of chemotherapy agents (5-FU, pyrimidine analog; CIS, DNA-crosslinker; VB, microtubule destabilizer; MTX, folate antagonist), for subsequent experiments.

Non-Specific Cellular Damage Does Not Decrease Cell-Surface CXCR4 Expression

As such a wide variety of cytotoxic agents caused a decrease in CXCR4 at the cell-surface, we questioned whether this was an effect specific to cytotoxic drugs or if it was a more general result of cellular damage. We tested three different cell stressors: alkaline pH, high salt concentrations, and the bile acid deoxycholate. In each case we

found there was no change in CXCR4 cell-surface expression following 48 h of treatment (Figure 2.3).

Cytotoxic Drugs Demonstrate Different Time Courses of Cell-Surface CXCR4 Reduction

Next we determined the time course of the effect of these four drugs on CXCR4 expression at the surface of HT-29 cells. We found that VB (2 $\mu\text{g/ml}$) reduced CXCR4 the most rapidly, with a significant and nearly complete (93%) decrease identified at the earliest time point tested, 24 h. In fact, VB consistently showed the most dramatic reduction in CXCR4 expression of any agent tested. Cisplatin (20 $\mu\text{g/ml}$) and MTX (2 $\mu\text{g/ml}$) led to a significant decrease (26% and 60%, respectively) in CXCR4 expression by 36 h, while we did not observe an effect of 5-FU (20 $\mu\text{g/ml}$) until 60 h after treatment, at which time there was a 32% reduction in cell-surface CXCR4 expression. The decreases in CXCR4 expression remained evident until at least 72 h (Figure 2.4).

We determined the minimum time of cellular exposure to 5-FU, CIS, VB, or MTX that was required to induce a down-regulation in cell-surface CXCR4 expression at 48 h. We treated cells with each cytotoxic agent and washed the drug away after 5 min, 1 h, 6 h, or 24 h; we measured cell-surface CXCR4 at a constant time point of 48 h. We found that exposure to 20 $\mu\text{g/ml}$ 5-FU for 24 h was sufficient to yield a significant reduction (58%) in CXCR4 at 48 h after treatment. HT-29 cells required 6 h of exposure to 20 $\mu\text{g/ml}$ CIS or MTX to observe a down-regulation of CXCR4 (56% and 62%, respectively) at 48 h, while a 1 h exposure time to 0.1 $\mu\text{g/ml}$ VB was required to cause a 64% reduction in cell-surface CXCR4 at 48 h (Figure 2.5).

Cytotoxic Drugs Differentially Affect CXCR4 mRNA Expression

Next we examined changes in CXCR4 mRNA levels in HT-29 cells following treatment with cytotoxic drugs. Only VB was capable of reproducibly decreasing CXCR4 mRNA at the time points tested, while 5-FU, CIS, and MTX showed variable results at these time points, occasionally reaching statistical significance in HT-29 cells. Treatment with 2 µg/ml VB led to a 44% decrease in CXCR4 mRNA by 3 h, which was sustained until 12 h, as detected by real-time quantitative RT-PCR (Figure 2.6). CXCR4 mRNA returned to control levels by 24 h (data not shown).

Pre-treatment with Cytotoxic Drugs Decreases CXCL12-Mediated Chemotaxis

To confirm that this down-regulation of CXCR4 at the cell surface was functionally important, we performed migration assays using Transwell® inserts coated with collagen. We found that control-treated HT-29 cells showed a more than 2-fold increase in migration across the Transwell® (average 115% increase over control) when CXCL12, the ligand for CXCR4, was added to the lower chamber. When cells were pre-treated with any of 5-FU, CIS, VB, or MTX, migration across the Transwell® toward CXCL12 was reduced to control levels, either completely (5-FU, CIS, VB) or partially (MTX, 61% reduction in CXCL12-mediated chemotaxis), consistent with the reduction in cell-surface CXCR4 expression (Figure 2.7).

Cytotoxic Drugs Demonstrate Variable Effects on CXCR4 Expression by Other Cell Lines

We tested a number of other cell lines (SW480, SW620, T84, HRT-18, and SH-SY5Y) and found that most had very low cell-surface CXCR4 expression as detected by

the anti-CXCR4 antibody (12G5 clone). This finding suggests that HT-29 cells are more representative of tumour tissue, in terms of CXCR4 levels (Table 1.4). As a result of this very low baseline in these other cell lines, it was not possible to detect any reproducible change in CXCR4 protein following treatment (data not shown).

Combination Treatment of HT-29 Cells with 5-Fluorouracil and 15-deoxy- $\Delta^{12,14}$ -prostaglandin J_2

Recent work in this laboratory has shown that 15-deoxy- $\Delta^{12,14}$ -prostaglandin J_2 (15dPGJ₂) also reduces cell-surface CXCR4 expression in HT-29 cells (Richard, Tan *et al.* 2006). We combined 5-FU and 15dPGJ₂ at various concentrations to determine if treatment with one agent could enhance the effect of the other on CXCR4 reduction. Indeed we found that treatment with 5-FU led to a reduction in the EC₅₀ for 15dPGJ₂-mediated CXCR4 down-regulation by 79% (Figure 2.8). Interestingly, although both 15dPGJ₂ and 5-FU were toxic to cells, this enhanced reduction in CXCR4 was not accompanied by increased toxicity when the two drugs were combined (data not shown).

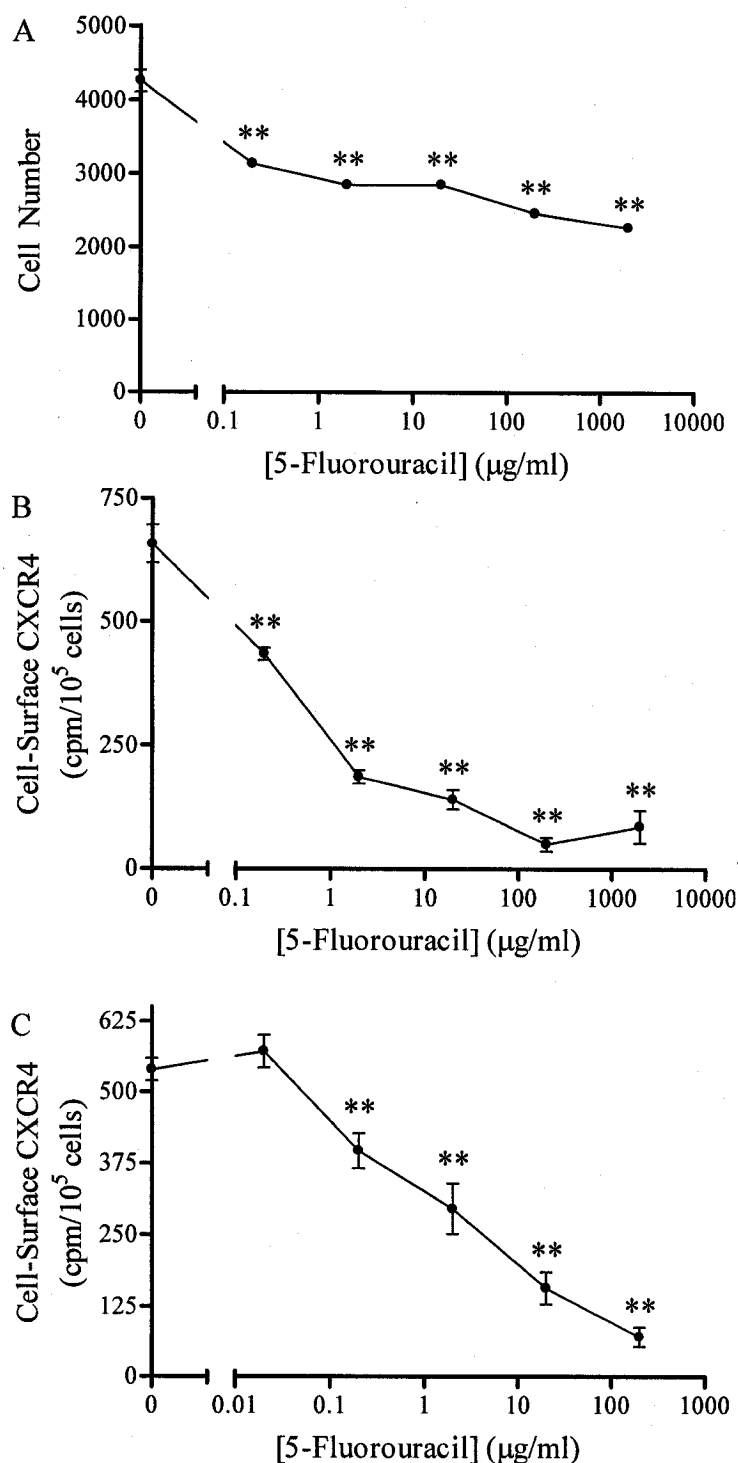


Figure 2.1. 5-Fluorouracil decreases HT-29 cell-surface CXCR4. Cells were treated with 5-FU at the indicated doses and assayed 48 h later. (A) 5-Fluorouracil effects on cell number; (B and C) 5-FU effects on cell-surface CXCR4. Data are represented as mean values \pm SE ($n = 4$ or 6) and are representative of 15 independent experiments. One-way analysis of variance $p < 0.0001$; **, significant change compared to control, Dunnett's multiple comparison post-test $p < 0.01$.

Table 2.1. Cytotoxic drugs decrease HT-29 cell-surface CXCR4 and cell number. HT-29 cells were treated with various doses of cytotoxic drugs and assayed 48 h later. Data are represented as mean values \pm SEM ($n = 4$ or 6) and are representative of the indicated number of independent experiments. Significant change compared to control, Student's t-test $p < 0.05$.

Cytotoxic Agent	CXCR4 (max % reduction/ 10^5 cells; mean \pm SE)	Cell number (max % reduction; mean \pm SE)	P value	EC ₅₀ for CXCR4 reduction/ 10^5 cells (μ g/ml; mean \pm SE)	EC ₅₀ for cell number reduction (μ g/ml; mean \pm SE)	P value	Number of independent experiments (n = 4 or 6)
5-Fluorouracil	80.8 \pm 7.4	41.7 \pm 4.5	< 0.0001	0.78 \pm 0.25	2.47 \pm 1.32	0.3054	11-16
Cisplatin	89.3 \pm 9.5	48.9 \pm 4.8	0.0009	1.64 \pm 0.32	2.03 \pm 0.64	0.6397	7-10
Vinblastine	87.3 \pm 9.2	48.8 \pm 3.3	0.0002	0.0044 \pm 0.0014	0.0026 \pm 0.00076	0.2364	9-13
Methotrexate	62.2 \pm 6.3	29.2 \pm 2.3	< 0.0001	6.42 \pm 6.37	0.12 \pm 0.08	0.1946	5-9
Doxorubicin	82.3 \pm 12.6	41.4 \pm 6.2	0.0244	0.47 \pm 0.17	0.064 \pm 0.019	0.0371	3-4
Irinotecan	76.3 \pm 5.5	51.0 \pm 3.6	0.0049	9.78 \pm 2.03	11.03 \pm 4.70	0.8133	5
Oxaliplatin	69.9 \pm 7.3	31.7 \pm 5.2	0.013	1.00 \pm 0.16	0.23 \pm 0.12	0.0183	3
Cytarabine	43.4	24.4	n/a	0.29	0.0060	n/a	1
Vincristine	94.0	44.6	n/a	0.020	0.0018	n/a	1

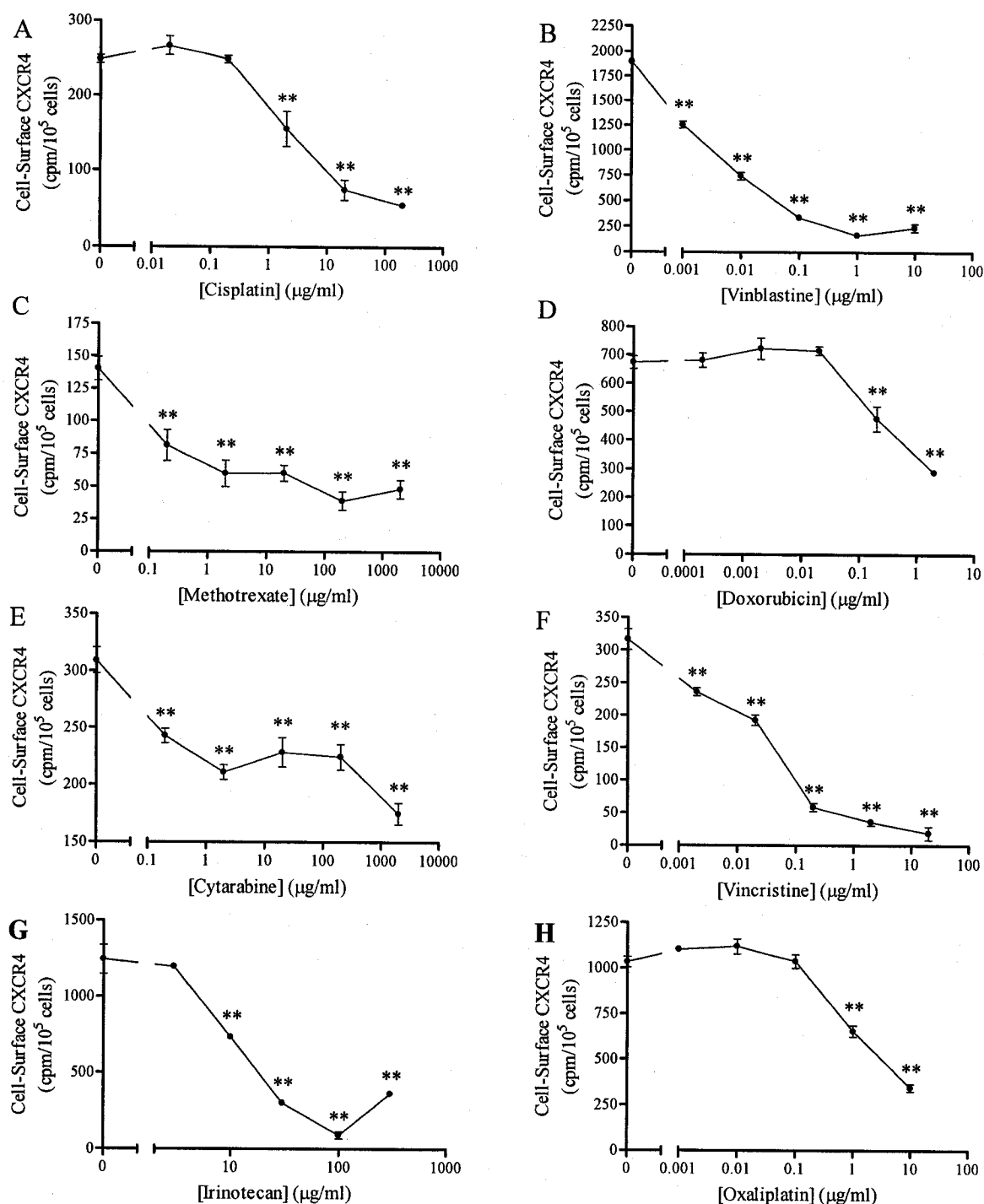


Figure 2.2. Various cytotoxic drugs decrease HT-29 cell-surface CXCR4. Cells were treated with (A) CIS; (B) VB; (C) MTX; (D) doxorubicin; (E) cytarabine; (F) vincristine; (G) irinotecan; or (H) oxaliplatin at the indicated doses and assayed 48 h later. Data are represented as mean values \pm SE ($n = 4$) and are representative of between one and eight independent experiments. One-way analysis of variance $p < 0.0001$; **, significant change compared to control, Dunnett's multiple comparison post-test $p < 0.01$.

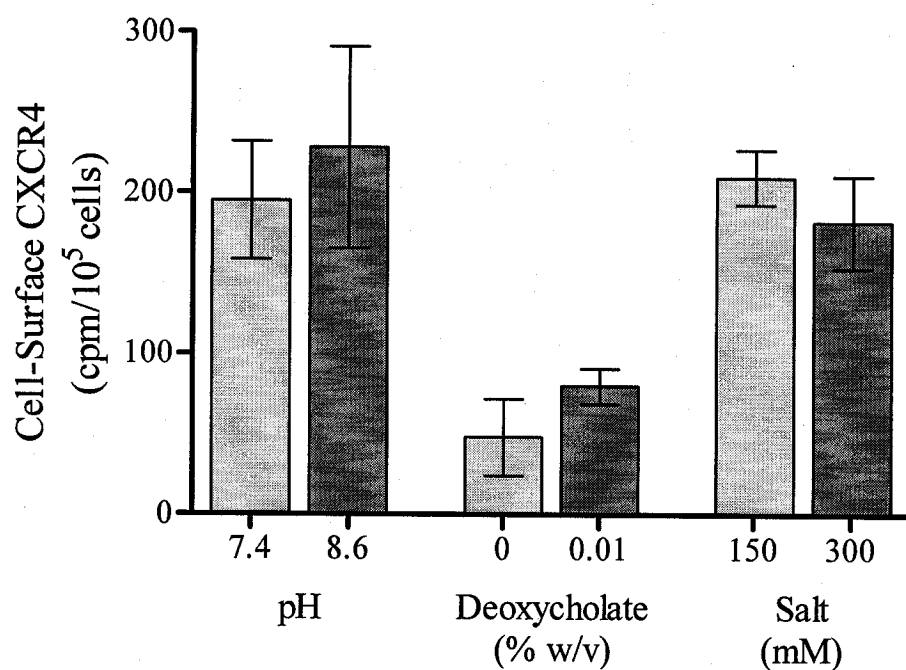


Figure 2.3. Non-specific cellular damage does not affect cell-surface CXCR4 expression. Cells were exposed to vehicle control (light bars) or each damaging agent (dark bars) for 1 h at 37°C at the indicated doses, washed, and assayed 48 h later. Data are represented as mean values \pm SE ($n = 4$) and are representative of 3 independent experiments.

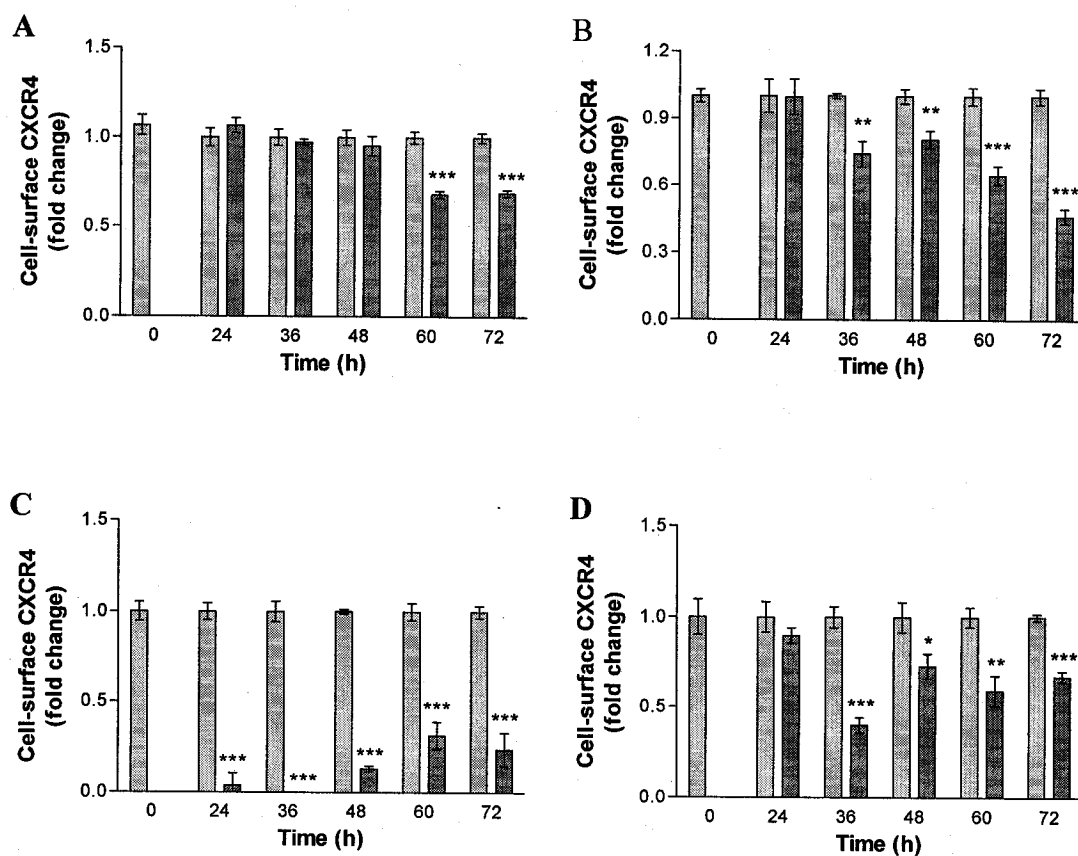


Figure 2.4. CXCR4 expression on HT-29 cells decreases over time with cytotoxic drug treatment. Cells were treated with vehicle (light bars) or (A) 20 µg/ml 5-FU; (B) 20 µg/ml CIS; (C) 2 µg/ml VB; or (D) 2 µg/ml MTX (dark bars) and assayed at the indicated times. Data are represented as mean values \pm SE ($n = 4$). Two-way analysis of variance $p < 0.0001$; *, significant change compared to control, Bonferroni post-test $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

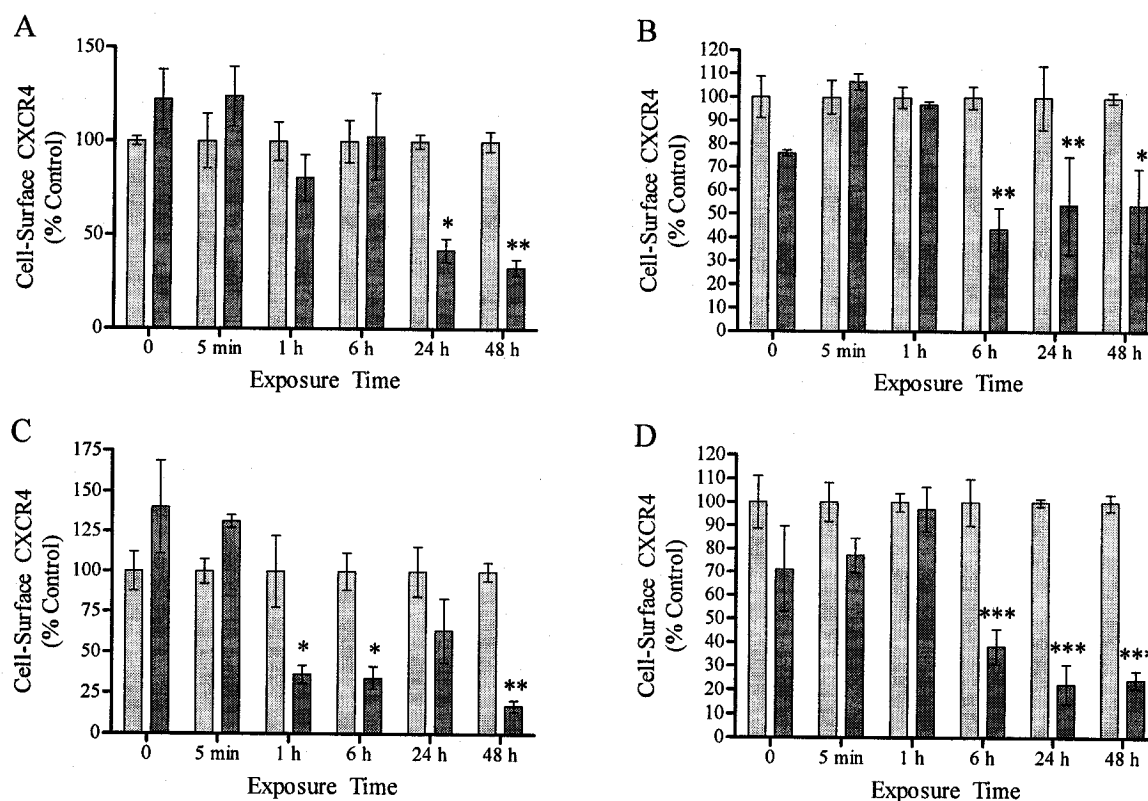


Figure 2.5. CXCR4 expression on HT-29 cells decreases with short exposure to cytotoxic drugs. Cells were treated with vehicle (light bars) or (A) 20 μ g/ml 5-FU; (B) 20 μ g/ml CIS; (C) 0.1 μ g/ml VB; or (D) 20 μ g/ml MTX (dark bars) and washed at the indicated times. Fresh medium was added to the cells and they were assayed 48 h following initial exposure to each agent. Data are represented as mean values \pm SE ($n = 4$). Two-way analysis of variance $p < 0.0001$; *, significant change compared to control, Bonferroni post-test $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

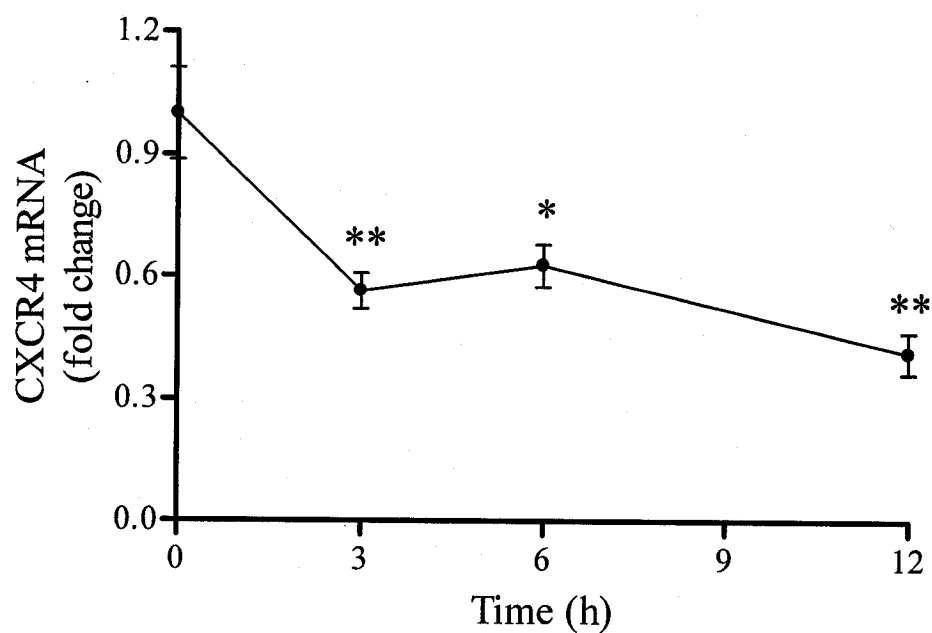


Figure 2.6. Vinblastine reduces HT-29 CXCR4 mRNA levels. Cells were treated with vehicle or 2 µg/ml VB and assayed at the indicated times. Data are represented as mean values \pm SE ($n = 3$). One-way analysis of variance $p < 0.01$; *, significant change compared to control, Dunnett's multiple comparison post-test $p < 0.05$; ** $p < 0.01$.

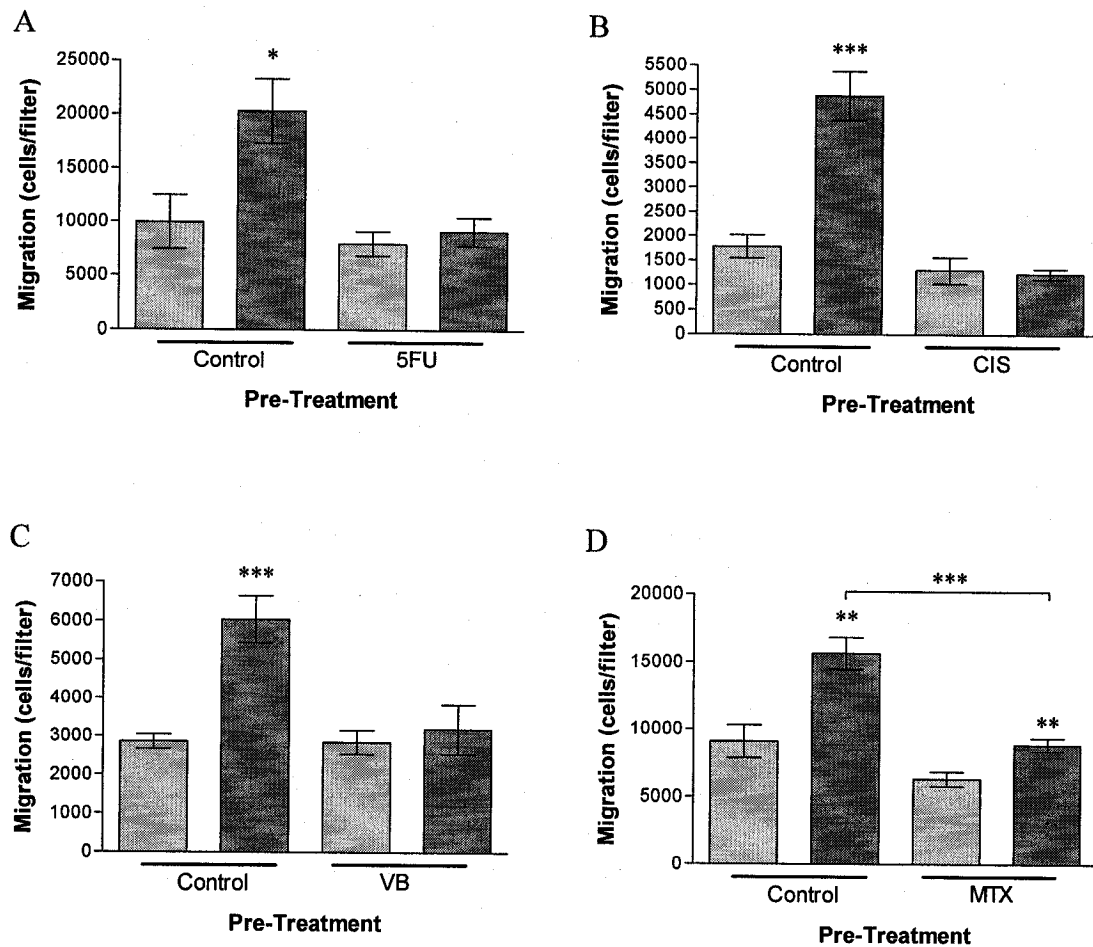


Figure 2.7. Cytotoxic drug pre-treatment decreases HT-29 cell chemotaxis toward CXCL12. Cells were pre-treated with vehicle control or (A) 5-FU; (B) CIS; (C) VB; or (D) MTX for 48 h then submitted to a chemotaxis assay in the presence (dark bars) or absence (light bars) of CXCL12. Data are represented as mean values \pm SE ($n = 6$). *, significant increase in migration, Student's t-test $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

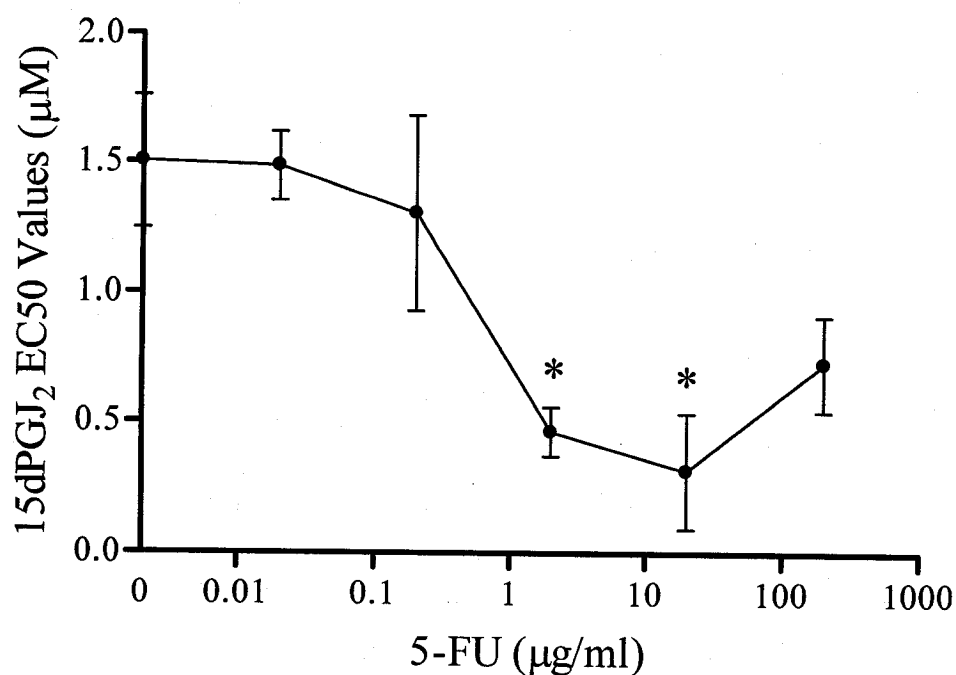


Figure 2.8. Co-treatment of HT-29 cells with 5-fluorouracil reduces the 15-deoxy- $\Delta^{12,14}$ -prostaglandin J₂ EC₅₀ values for CXCR4 reduction. Cells were treated with vehicle control or 0.02, 0.2, 2, 20, or 200 $\mu\text{g/ml}$ 5-FU and 0.1, 0.3, 1, 3, or 10 μM 15dPGJ₂ in all possible permutations. After 48 h cell-surface CXCR4 was measured using a radioantibody binding assay and 15dPGJ₂ EC₅₀ values, in the presence or absence of 5-FU, were calculated. Data are represented as mean values \pm SE (averaged across three independent experiments; $n = 6$). One-way analysis of variance $p < 0.01$; *, significant change compared to control, Dunnett's multiple comparison post-test $p < 0.05$.

DISCUSSION

The chemokine receptor CXCR4 has been strongly implicated in cancer progression, metastasis, and growth (Müller, Homey *et al.* 2001; Zeelenberg, Ruuls-Van Stalle *et al.* 2003; Scala, Ottaiano *et al.* 2005; Schimanski, Schwald *et al.* 2005). CXCR4 expression by cancer cells has been examined at different stages of tumour development in humans (Kim, Takeuchi *et al.* 2005; Schimanski, Schwald *et al.* 2005) and its expression has been correlated with poor prognosis (Table 1.4). Despite its important role in tumourigenesis, there has been only a handful of studies examining CXCR4 expression following chemotherapy (Noach, Ausema *et al.* 2003; Gazitt and Akay 2004; Muller, Sonkoly *et al.* 2006).

Nearly all patients diagnosed with cancer are treated with cytotoxic agents and, in most cases, these drugs are administered at the maximally tolerated doses; these doses are toxic for both normal and cancerous cells. The rationale for treating tumours with high doses of these agents is strong and based on pharmacological principles and modeling studies (Goldin, Venditti *et al.* 1956; Skipper, Schabel *et al.* 1964; Simpson-Herren and Lloyd 1970), as well as fifty years of evidence-based medicine. Despite this, low-dose treatment with cytotoxic agents has been an effective therapy for some cancer patients, in some cases even for those who have failed on maximally tolerated doses of the same drugs (Fennelly, Aghajanian *et al.* 1997; Greco 1999; Burstein, Manola *et al.* 2000; Glode, Barqawi *et al.* 2003; Gilewski and Norton 2006). This beneficial effect does not seem to be a direct result of tumour cell death; it is, at least in part, a consequence of decreased tumour angiogenesis attributable to the differential sensitivity of endothelial cells and cancer cells to chemotherapy (Bocci, Nicolaou *et al.* 2002; Man, Bocci *et al.*

2002; Yap, Veliceasa *et al.* 2005). This means that doses of these agents that are too low to kill tumour cells are sufficient to kill endothelial cells, thus inhibiting tumour angiogenesis and, thus, tumour growth.

Furthermore, unlike normal cells, cancer cells are genetically unstable (Stoler, Chen *et al.* 1999), which allows them to develop resistance to cytotoxic agents. Consequently, doses of drugs that are unable to kill cancer cells may have other effects that are beneficial to treatment. These include inhibition of angiogenesis through direct actions on tumour cells rather than, or in addition to, toxic effects on endothelial cells. For example, there have been numerous studies linking CXCR4 expression and activation with tumour angiogenesis; moreover, mice lacking CXCR4 die embryonically as a result of massive vascular defects (Tachibana, Hirota *et al.* 1998). CXCR4 was shown to be up-regulated by the pro-angiogenic molecule VEGF in an autocrine manner (Bachelder, Wendt *et al.* 2002) and CXCR4 neutralization, using an anti-CXCR4 antibody, blocked tumour growth through angiogenesis inhibition (Guleng, Tateishi *et al.* 2005). Furthermore, prostate cancer cells with CXCR4 siRNA developed smaller tumours with decreased *in vivo* angiogenesis compared to controls (Wang, Wang *et al.* 2005). *In vivo* angiogenesis using primary ovarian cancer cell lines could not be induced by either CXCL12 or VEGF alone, but angiogenesis could be induced when these molecules were used in combination (Kryczek, Lange *et al.* 2005). Clearly, the chemokine receptor, CXCR4, and its ligand, CXCL12, play an important role in tumour angiogenesis and perturbation of this axis may be a determinant of the successfulness of low-dose chemotherapy.

Although there is little doubt that angiogenesis inhibition is one mechanism by which low-dose therapy benefits patients, it is not necessarily the only mechanism involved. For example, Kerbel and colleagues found that low-dose chemotherapy inhibited the growth of micrometastases, but not the primary tumour, in a rodent model of breast cancer (Munoz, Man *et al.* 2006). We therefore sought to determine if a range of doses of these chemotherapy agents, commonly used to treat a variety of malignancies, affect CXCR4 expression by colorectal carcinoma cells.

We used a radioactive antibody binding assay to measure *in vitro* changes in cell-surface CXCR4 expression following drug treatment. We have found this to be a very sensitive assay which can specifically quantify CXCR4 expression below the limits of detection by flow cytofluorimetry. In fact, we cannot reproducibly detect CXCR4 at the surface of HT-29 cells by means of flow cytofluorimetry using several different approaches. We have shown that this binding assay specifically detects cell-surface CXCR4 (Richard, Tan *et al.* 2006) and all values are expressed as radioactive counts per viable cell, thus accounting for any effects of treatment on cell proliferation or toxicity. We are confident that any decreases observed reflect a true decrease in cell-surface CXCR4.

Other groups have used a similar assay involving radiolabeled CXCL12, the CXCR4 ligand; however, Balabanian and colleagues recently showed that CXCL12 does not specifically bind to only CXCR4, as previously believed, and can in fact bind to CXCR7 (RDC1) as well, a former orphan GPCR. This group showed that the primary antibody (clone 12G5) used in our binding assays does not bind to CXCR7 (Balabanian, Lagane *et al.* 2005).

Cytotoxic Drugs Decrease Cell-Surface CXCR4 on HT-29 Colorectal Carcinoma Cells

We used the radioantibody binding assay described above to assess a number of cytotoxic drugs for their effect on cell-surface CXCR4 expression in human HT-29 colorectal carcinoma cells. The drugs tested are commonly used to treat various malignancies and include: the pyrimidine analogs, 5-FU and cytarabine; the platinum compounds, CIS and oxaliplatin; the mitotic spindle poisons, VB and vincristine; the folate antagonist, MTX; the topoisomerase I inhibitor, irinotecan; and the DNA intercalator and topoisomerase II inhibitor, doxorubicin (Table 1.1). Of these, 5-FU, oxaliplatin, and irinotecan are routinely used in the treatment of colorectal carcinoma (Von Hoff and Hanauske 2006). We found that each of these chemotherapeutics caused a decrease in CXCR4 expression at the cell-surface of HT-29 cells which was detectable at 48 h following treatment (Figure 2.1; 2.2). The percent maximal reduction in cell-surface CXCR4 per 100,000 cells ranged from 43.4% (cytarabine) to 94.0% (vincristine), with a median of $82.3 \pm 12.6\%$ (doxorubicin; Table 2.1).

The only drugs tested that did not affect CXCR4 cell-surface expression following a 48 h incubation period with HT-29 colorectal cancer cells were the 5-FU prodrug, capecitabine, and the alkylating agent, cyclophosphamide (data not shown). This lack of effect is not surprising as these are both prodrugs that require metabolism by either hepatic carboxylesterase (capecitabine; Tabata, Katoh *et al.* 2004) or the cytochrome p450 (CYP) enzyme, CYP2B6 (cyclophosphamide; Chang, Weber *et al.* 1993). Indeed, the carboxylesterase activity in HT-29 cell microsomes was shown to be less than 2 pmol per min per mg protein compared to the 40 μ mol per min per mg protein observed in liver microsomes (Pavillard, Agostini *et al.* 2002) and HT-29 cells do not express CYP2B6 (Jounaidi and Waxman 2004). Additionally, a range of doses of these

drugs did not affect HT-29 cell number after a 48 h incubation period, which is another indication that these drugs were inactive in HT-29 cells.

Since the down-regulation of CXCR4 occurred with each active drug tested, we speculated that CXCR4 down-regulation may be a general cellular response to cytotoxic stress. To test this, we incubated HT-29 cells with non-specific cell stressors, including alkaline pH, the bile acid deoxycholate, or excessive concentrations of sodium chloride. These treatments led to a similar extent of cell number reduction compared to that observed with cytotoxic drug treatment, yet had no effect on cell-surface CXCR4 expression (Figure 2.3). This lends strength to the argument that this reduction in CXCR4 is a specific consequence of drug treatment and is not a general cellular response to stress.

5-Fluorouracil

5-Fluorouracil is a pyrimidine analog used to treat a number of tumours, particularly primary and metastatic colorectal carcinoma. 5-Fluorouracil caused an $80.8 \pm 7.4\%$ average maximal reduction in cell-surface CXCR4 and exhibited an EC_{50} of $0.78 \pm 0.25 \mu\text{g/ml}$ ($0.006 \mu\text{M}$; Table 2.1; Figure 2.1B and C). Interestingly, this is a nearly 1000-fold lower concentration than the $1\text{--}5 \mu\text{M}$ steady state plasma concentration observed in patients (Seifert, Baker *et al.* 1975).

This reduction in CXCR4 was relatively slow, becoming evident after 36–60 h of culture in the presence of 5-FU (Figure 2.4A). Consistent with this slow time course, we found there were no reproducibly significant changes in CXCR4 mRNA expression following treatment with 5-FU, as measured by real-time quantitative RT-PCR (data not shown). Interestingly, we found that although we did not observe a decrease in CXCR4

until approximately 48 h after treatment, if we washed 5-FU from the cells after a minimum of 24 h we still found this decrease at the cell-surface at 48 h (Figure 2.5A). This suggests that 5-FU exerts its effect on CXCR4 at least 24 h before we can observe a decrease at the surface of the cell. CXCR4 is a GPCR that is efficiently recycled to and from the cell-surface and it has been shown that the vast majority of CXCR4 expressed by cells is present intracellularly, rather than at the cell surface (Zhang, Foudi *et al.* 2004). Consequently, it is likely that the effect of 5-FU on CXCR4 reduction requires an additional 24-36 h to become evident as a decrease in CXCR4 at the cell-surface, as intracellular pools diminish.

Cisplatin

The second drug we examined in detail was the platinum compound, CIS, which acts by covalently binding to nucleic acids and cellular proteins. Cisplatin led to an average maximum reduction in cell-surface CXCR4 of $89.3 \pm 9.5\%$, and demonstrated an EC_{50} of $1.64 \pm 0.32 \mu\text{g/ml}$ (Table 2.1; Figure 2.2A). Cisplatin led to a more rapid decrease in cell-surface CXCR4 expression than did 5-FU; in each of four independent experiments a statistically significant reduction was evident by 36 h and in one experiment a significant reduction occurred as early as 12 h following treatment (Figure 2.4B). In this case, if we washed CIS from the cells after as little as 6 h, there was nearly as substantial a reduction in CXCR4 expression 42 h later at the 48 h point, compared to cells incubated with CIS for the entire 48 h (Figure 2.5B).

This similarity between the 6 and 48 h CIS exposure time is not likely a result of the cells metabolizing and eliminating CIS by 6 h, essentially rendering these two time points the same. The half-life of CIS in culture is 360-390 min (Bruinink and Birchler

1993); CIS would have only undergone one half-life by 6 h, before being washed away. However, incubations with CIS of six and 48 h led to similar levels of cell death, supporting the possibility that all free CIS was sequestered within the cells by 6 h, limiting the effectiveness of our washes. Analogous to our 5-FU findings, CIS demonstrated variable effects on CXCR4 mRNA levels (data not shown).

There are two reasons that the response, in terms of reduced CXCR4 expression, may be more rapid with CIS treatment compared to 5-FU treatment. Firstly, CIS undergoes active transport into cells through the action of CTR1 (Holzer and Howell 2006), whereas 5-FU depends on passive diffusion to enter cells (Nakamura, Horimoto *et al.* 2003), suggesting that CIS will enter cells more rapidly, allowing it to act sooner than 5-FU. Secondly, while CIS is administered in its active form, 5-FU is a prodrug and must undergo metabolism to its active compounds, FdUMP, FUTP, and FUDP, once it enters cells. In cultures of HCT-116 colorectal carcinoma cells, intracellular concentrations of these metabolites, particularly FdUMP, are not increased to any great extent until 24 h following treatment with 5-FU (Grem and Fischer 1986).

Methotrexate

Next we examined the antifolate, MTX. Methotrexate exhibited an average maximal CXCR4 reduction of $62.2 \pm 6.3\%$ and an EC_{50} of $6.42 \pm 6.37 \mu\text{g/ml}$ (Table 2.1; Figure 2.2C). Despite their very different mechanisms of action, the effects of MTX on CXCR4 expression were very similar to those of CIS. Like CIS, MTX is actively transported into cells, in this case through either the reduced folate carrier or the membrane folate binding protein (Jansen, Schornagel *et al.* 1990). Furthermore, although intracellular polyglutamation of MTX increases its activity, the parent compound is still

active in terms of dihydrofolate reductase inhibition (Hryniuk 1972). Therefore, not surprisingly, the time course of MTX action paralleled that of CIS action: MTX reduced CXCR4 protein by 36 h (Figure 2.4D) and had no reproducible effect on CXCR4 mRNA expression (data not shown). Like CIS, only a 6 h MTX exposure time was necessary to see a significant decrease in CXCR4 at 48 h (Figure 2.5D).

Vinblastine

Finally, we identified the effects of the mitotic spindle poison, VB, on CXCR4 expression. Of all the drugs tested, VB reduced CXCR4 expression the most rapidly and with the greatest potency. It induced an average $87.3 \pm 9.2\%$ maximal reduction in CXCR4 and had an EC_{50} value of 0.0044 ± 0.0014 $\mu\text{g/ml}$ (Table 2.1; Figure 2.2B). Vinblastine led to a nearly complete loss (96% reduction) of CXCR4 protein at the cell-surface at the earliest time point tested, 24 h (Figure 2.4C). Remarkably, a 1 h exposure time to VB was sufficient to cause a reduction in CXCR4 expression evident at 48 h (Figure 2.5C). Interestingly, VB was the only drug tested which reproducibly reduced CXCR4 mRNA levels in HT-29 cells, demonstrating a 44% reduction in CXCR4 mRNA at 3 h, which was sustained until at least 12 h (Figure 2.6). This could explain the more dramatic reduction in cell-surface CXCR4 expression following treatment with VB, compared to treatment with 5-FU, CIS, or MTX.

Furthermore, the inhibitory effects of VB on microtubule dynamics can explain, at least in part, this rapid and potent effect on CXCR4 expression. By targeting microtubules (Johnson, Armstrong *et al.* 1963), VB inhibits intracellular transport and as such can affect receptor export to the cell surface (Seybold, Bieger *et al.* 1975). As described above, the majority of CXCR4 is found in intracellular pools (Zhang, Foudi *et*

al. 2004), ready to be transported to the cell-surface as needed. Although not confirmative, this data suggests that VB inhibits both *de novo* CXCR4 synthesis as well as transport of intracellular CXCR4 to the cell surface.

Having established these data in HT-29 cells, we examined other cell lines for effects of cytotoxic drugs on CXCR4 expression. We examined the human SW480, SW620, HRT-18, and T84 colorectal carcinoma cell lines and the human SH-SY5Y neuroblastoma cell line. Although we found some expression of CXCR4 in each of these cell lines, only the SH-SY5Y neuroblastoma cells consistently expressed detectable levels of CXCR4. Of the four drugs tested, only VB caused a reproducible response in any of these cell lines, causing a decrease in CXCR4 expression in SW480 colorectal carcinoma cells and an increase in the neuroblastoma SH-SY5Y cells (data not shown), suggesting that a decline in CXCR4 may not be the only possible outcome in all cancers.

Interestingly, the p53 tumour suppressor protein is mutated in HT-29 and SW480 colorectal carcinoma cells (Rodrigues, Rowan *et al.* 1990), but wild-type in SH-SY5Y neuroblastoma cells (Davidoff, Pence *et al.* 1992). A recent report described the role of p53 in regulating CXCR4 expression (Mehta, Christopherson *et al.* 2006) led us to question if this differential response to cytotoxic drugs, in terms of CXCR4 expression, was a consequence of the p53 status of these cell lines. To directly assess this, we obtained HT-29 cells into which wild-type p53 had been introduced (Bras-Gonçalves, Rosty *et al.* 2000); however, we could not detect CXCR4 cell-surface expression in either of two HT-29 wild-type p53 clones nor in the control-transfected parental HT-29 cell line. Furthermore, there were no consistent changes in CXCR4 following treatment with a range of doses of 5-FU, CIS, VB, or MTX (data not shown). We examined these cells

more closely and found them to be infected with *Mycoplasma* (data not shown), which could account for the lack of CXCR4 expression in these cells. Other groups have reported changes in CXCR4 expression following bacterial infection (Hoshino, Tse *et al.* 2004; Lei, Wu *et al.* 2005). Furthermore, bacterial lipopolysaccharide (LPS) down-regulates cell-surface CXCR4 expression (Verani, Sironi *et al.* 2002).

Cytotoxic Drugs Decrease Cellular Migration to CXCL12

One of the most important consequences of increased CXCR4 expression on cancer cells *in vivo* is enhanced migration to and/or proliferation at CXCL12-rich sites (Müller, Homey *et al.* 2001; Zeelenberg, Ruuls-Van Stalle *et al.* 2003). Previous work from our laboratory and others has shown that CXCL12 causes HT-29 cell proliferation and migration; perturbation of cell-surface CXCR4 levels leads to changes in these functional activities (Brand, Dambacher *et al.* 2005; Richard, Tan *et al.* 2006). We therefore speculated that the cytotoxic drug-mediated decrease in CXCR4 cell-surface expression would correlate with decreased cellular migration toward CXCL12.

Although basal HT-29 cell migration toward vehicle-containing medium was fairly low, we consistently detected a statistically significant increase in migration toward CXCL12-containing medium. Importantly, when we pre-incubated cells with 5-FU, CIS, or VB, there was a reduction in CXCL12-mediated migration to control levels (Figure 2.7A-C). In contrast, MTX did not completely inhibit migration toward CXCL12; however, this reduction in migration was statistically significant (Figure 2.7D). As described above, MTX had the smallest maximal effect on CXCR4, on average reducing its cell-surface expression by $62.2 \pm 6.3\%$, compared to $80.8 \pm 7.4\%$, $89.3 \pm 9.5\%$, and

87.3 ± 9.2% for 5-FU, CIS, and VB, respectively (Table 2.1). This could account for the lack of a complete block in migration. Furthermore, it is possible that if the dose of MTX was increased we would see an increase in its capacity to block CXCL12-mediated migration.

Interestingly, pre-treatment with cytotoxic drugs did not affect baseline migration of these cells, in the absence of CXCL12 (Figure 2.7). This implies that these drugs, at the administered doses, do not perturb other processes involved in cell motility. From these migration experiments, we can conclude that CXCR4 is functional in HT-29 cells, as previously described, and cytotoxic drug treatment reduces the ability of HT-29 cells to migrate toward CXCL12, presumably through their reduction of CXCR4 protein expression at the cell-surface.

Co-Treatment with Cytotoxic Agents and Eicosanoids Enhances CXCR4 Reduction

Previous work in this laboratory has shown that eicosanoids can reduce CXCR4 expression at both the mRNA and protein level in HT-29 cells (Richard, Tan *et al.* 2006). Eicosanoids are naturally present within the tumour microenvironment (Rigas, Goldman *et al.* 1993; Qiao, Kozoni *et al.* 1995) and some have been shown to stimulate tumour growth (prostaglandin E₂; PGE₂; Pai, Soreghan *et al.* 2002) while others have been shown to inhibit tumour growth (PGD₂ and its derivatives delta-12,14-PGD₂ and 15dPGJ₂; Yoshida, Ohki *et al.* 1998; Kitamura, Miyazaki *et al.* 1999; Shimada, Kojima *et al.* 2002). We hypothesized that CXCR4 expression may be reduced, either additively or synergistically, by combining eicosanoids (which are commonly present within tumours) with antineoplastic drugs (which are commonly administered to patients).

Indeed, we found that treatment with 5-FU reduced the EC_{50} for CXCR4 reduction by the eicosanoid 15dPGJ₂ (Figure 2.8). Although this study was not robust enough to distinguish synergy from additivity, these results suggest that when cancer cells, in the presence of 15dPGJ₂, are treated with 5-FU, the reduction in CXCR4 would be greater than the reduction observed following treatment with either agent alone.

CONCLUSION

The data summarized in this chapter describe the effect of cytotoxic drug treatment on CXCR4 expression on the surface of HT-29 colorectal carcinoma cells. We have clearly shown that a variety of drugs, with different mechanisms of cytotoxic action, decrease cell-surface CXCR4 protein expression. This effect is retained even after drugs are removed from the culture medium and is enhanced by co-treatment with eicosanoids which are normally present within the tumour microenvironment. Non-specific cellular stressors do not affect CXCR4 protein expression, suggesting that this reduction by anticancer drugs is not a general response to cell death. This decline in CXCR4 expression is reflected functionally because cytotoxic drug-mediated CXCR4 reduction leads to a concomitant reduction in HT-29 cellular migration toward the CXCR4 ligand, CXCL12. These data demonstrate a mechanism of action of these agents that until now has not been described: Cytotoxic agents down-regulate HT-29 cell-surface CXCR4, which leads to a reduced migratory capacity of these cells.

CHAPTER 3

CYTOTOXIC DRUGS UP-REGULATE THE CELL-SURFACE EXPRESSION OF CD26 ON HUMAN HT-29 COLORECTAL CARCINOMA CELLS *IN VITRO* AND *IN VIVO*

INTRODUCTION

CD26 is a cell-surface protein involved in a number of physiologic processes. It binds ADA, therefore enhancing adenosine metabolism (Kameoka, Tanaka *et al.* 1993; Morrison, Vijayasaradhi *et al.* 1993); it binds the ECM proteins, collagen and fibronectin, therefore facilitating cell-to-cell and cell-to-substrate adhesion (Bauvois 1988; Hanski, Huhle *et al.* 1988; Piazza, Callanan *et al.* 1989; Cheng, Abdel-Ghany *et al.* 1998); and it cleaves many bioactive molecules, therefore controlling immune system responses and cellular migration (De Meester, Korom *et al.* 1999). Importantly, CD26 cleaves the CXCR4 ligand, CXCL12, rendering it inactive (Herrera, Morimoto *et al.* 2001; Christopherson, Hangoc *et al.* 2002; Mizokami, Kajiyama *et al.* 2004).

The Role of CD26 in Regulating CXCL12 Bioactivity

In the case of DPPIV-mediated CXCL12 cleavage, and indeed the cleavage of most other DPPIV substrates, the bioactivity of the cleaved molecule is reduced by DPPIV processing. In fact, there have been a number of reports describing a role for CD26 in regulating the bioactivity of CXCL12 (Herrera, Morimoto *et al.* 2001; Christopherson, Hangoc *et al.* 2002; Mizokami, Kajiyama *et al.* 2004). For example, Van Damme and associates showed that CD26 can regulate both the chemotactic and anti-HIV-1 properties of CXCL12 (Figure 1.5; Proost, Struyf *et al.* 1998), while Broxmeyer and colleagues demonstrated that DPPIV-mediated CXCL12 cleavage reduced bone

marrow homing of hematopoietic progenitor cells (Christopherson, Hangoc *et al.* 2004). It is possible that CD26 down-regulation in malignancy may facilitate tumour cell migration through reduced cleavage of CXCL12. This theory has been directly examined by two different groups.

The first of these groups, Mizutani and associates (Mizokami, Kajiyama *et al.* 2004), examined the role of CD26 in regulating CXCL12-mediated cellular proliferation in endometrial adenocarcinoma. These cells and tissues expressed both CXCL12 and CXCR4 and showed reduced CD26 expression compared to normal endometrial cells. When they transfected endometrial adenocarcinomas cells with CD26, CXCR4 expression remained constant; however CXCL12 production in the cell-culture supernatant was decreased in CD26 transfectants and exogenously added CXCL12 had a reduced effect on cellular proliferation in these cells compared to those that were mock-transfected. This work suggested an important role for CD26 in regulating cancer cell behaviour through its effects on CXCL12 (Mizokami, Kajiyama *et al.* 2004).

The second of these groups to link CD26 and CXCR4 in cancer, Russo and colleagues (Narducci, Scala *et al.* 2006), studied the regulation of CXCL12 by CD26 in Sézary syndrome, which is a rare form of cutaneous T cell lymphoma (Narducci, Scala *et al.* 2006). Sézary syndrome is characterized by a loss of CD26 expression and demonstrates high levels of metastasis to the skin (Scala, Narducci *et al.* 2002). Russo and coworkers hypothesized that this directed metastasis was a result of decreased DPPIV-mediated cleavage of CXCL12 in the skin. Indeed, they found that CXCR4 is expressed at a high level on the surface of circulating and skin-infiltrating Sézary cells and, furthermore, CXCL12 was highly expressed in the skin of these patients. They

confirmed that CD26 expression is lost on the surface of Sézary cells and plasma sCD26 was reduced in these patients compared to healthy control subjects. Finally, they showed that when Sézary cells were incubated with sCD26 *in vitro*, these cells exhibited reduced migration to CXCL12. The authors concluded that reduced CD26 expression on the surface of Sézary cells, as well as reduced plasma sCD26, controls the metastasis of these cells to the skin through regulation of the CXCR4-CXCL12 axis (Narducci, Scala *et al.* 2006).

The Regulation of CD26

Two independent groups solved the complete coding sequence of CD26 in 1992 (Darmoul, Lacasa *et al.* 1992; Tanaka, Camerini *et al.* 1992). One of these groups identified changes in CD26 mRNA expression in HT-29 and Caco-2 colorectal carcinoma cells which correlated with cellular differentiation (Darmoul, Lacasa *et al.* 1992). This observation has since been explored by several independent groups, showing that CD26 expression increases with increasing cellular differentiation (Imai, Maeda *et al.* 1992; Ruiz, Hao *et al.* 1997; Sedo, Malik *et al.* 1998; Sato, Fujiwara *et al.* 2002).

Several groups have examined the regulation of CD26 by cytokines, in the contexts of cellular differentiation in immune responses or dedifferentiation in tumourigenesis. For example, Fujiwara and colleagues found that TNF- α and IL-1 α were each capable of enhancing cell-surface CD26 expression at the surface of human luteinizing granulosa cells (Fujiwara, Fukuoka *et al.* 1994). In contrast, Erickson and colleagues (Erickson, Lai *et al.* 2000) found that TNF- α and transforming growth factor (TGF)- β 1 treatment reduced cell-surface CD26, while IL-4, IL-13, and interferon (IFN)- γ

each led to an increase in its cell-surface expression in both renal cell carcinoma and renal tubular epithelial cell lines. Of these mediators, only IL-4 and IL-13 affected DPPIV activity, increasing it in each case.

Cordero and coworkers examined CD26 regulation on human lymphocytes and showed that in their system, TNF- α suppressed and IL-12 enhanced cell-surface CD26 expression. The IL-12-mediated increase was independent of mRNA transcription yet depended, at least in part, on *de novo* CD26 translation; the authors suggested that enhanced trafficking to the cell-surface may also be involved (Salgado, Vela *et al.* 2000).

In support of Mori's findings (Fujiwara, Fukuoka *et al.* 1994), but in contrast to Kim's (Erickson, Lai *et al.* 2000) and Cordero's (Salgado, Vela *et al.* 2000) findings, Gonzalez-Gronow and colleagues found that patients treated with anti-TNF- α antibodies for the treatment rheumatoid arthritis showed reduced serum sCD26 (Mavropoulos, Cuchacovich *et al.* 2005), suggesting that TNF- α is a positive regulator of CD26 expression.

Few other mediators of CD26 expression have been identified, with the exception of the tumour mitogen adenosine. Previous work in the Blay laboratory has shown that the nucleoside adenosine, at concentrations present within the tumour microenvironment, is capable of down-regulating CD26 mRNA and cell-surface protein in HT-29 colorectal carcinoma cells. This correlates with reduced DPPIV activity and ADA-binding capacity by these cells (Tan, Mujoomdar *et al.* 2004; Tan, Richard *et al.* 2006). This is an important finding because adenosine has also been shown to enhance tumour cell proliferation, migration, and cell-surface CXCR4 expression. This suggests that certain

stimuli may be capable of simultaneously altering CD26 and CXCR4 expression on cancer cells, both in directions that correlate with enhanced tumour aggressiveness.

Since CXCR4 expression was decreased in HT-29 colorectal carcinoma cells following treatment with cytotoxic drugs, and CXCR4 and CD26 are closely related in terms of tumourigenesis, we sought to determine if these same drugs could alter CD26 expression. We show here that cytotoxic agents, at physiologically relevant concentrations, enhance cell-surface CD26 expression, enzyme activity, and ADA-binding capacity in HT-29 colorectal carcinoma cells. Furthermore, drugs commonly used in the treatment of colorectal carcinoma enhance CD26 expression by HT-29 cells grown as orthotopic colorectal tumours *in vivo*.

METHODS

Materials

Materials were as described in Chapter 2. In addition, T84, HRT-18, SW480, and SW620 cells were from the American Type Culture Collection (Manassas, VA). Mouse anti-human CD26 (clone M-A261) and mouse IgG₁ (clone W3/25) isotype control antibodies were from Cedarlane Laboratories Ltd. (Hornby, Ontario, Canada). Rat anti-bovine ADA was from Alpha Diagnostic International (San Antonio, TX) and donkey anti-rabbit [¹²⁵I]-labeled IgG, F(ab')₂ fragment was from Amersham Biosciences Inc. (Baie d'Urfé, QC). Rat anti-mouse IgG fluorescein isothiocyanate (FITC)-conjugated monoclonal antibody was from PharMingen (San Diego, CA). Gly-Pro-*p*-nitroaniline *p*-

toluene sulfate salt (Gly-Pro-pNA), low molecular weight agarose, and calf spleen ADA was from Sigma Chemical Co. (St. Louis, MO).

Cell Culture

HT-29 cells were cultured as described in Chapter 2. T84 and HRT-18 cells were cultured in DMEM supplemented with 5% (v/v) NCS; SW480 and SW620 cells were cultured in DMEM supplemented with 10% (v/v) NCS. All cells were maintained as stocks in 80-cm² flasks at 37°C in a humidified atmosphere of 90% air/10% CO₂ and were routinely passaged by brief exposure to 0.05% (w/v) trypsin/0.53 mM EDTA.

For monolayer experiments, cells were seeded at 100,000 cells per ml of DMEM containing 10% (v/v) NCS, unless otherwise stated. For binding assays and enzyme activity assays, cells were seeded at a density of 50,000 cells per well in 48-well plates; for real-time quantitative RT-PCR, 200,000 cells per well in 6-well plates; for flow cytometry, 1,000,000 cells per 10 cm dish. Cells were allowed to adapt to culture for 48 h, at which time the medium was replaced with DMEM containing 1% (v/v) NCS. In most situations drugs or vehicle controls were added after a subsequent 48 h and assays were performed at the indicated times.

For seeding spheroid cell cultures, each well of a 96-well plate was first coated with 50 µl of 0.5% (w/v) agarose in low-glucose (1 g/L) DMEM containing 10% (v/v) NCS, heated to 80°C. Once solidified, we seeded 50,000 cells in a volume of 100 µl on top of the agarose layer. Spheroids were allowed to form for seven days before drug or vehicle control additions performed. Spheroids were assayed at the indicated time points.

Radioantibody Binding Assay

Monolayer Cultures

We quantified CD26 protein in monolayer and spheroid cultures as described in Chapter 2 for CXCR4 protein. Briefly, cells were incubated with 1 µg/ml mouse anti-human CD26 primary antibody followed by 1 µCi/ml [¹²⁵I]-labeled goat anti-mouse IgG. Radioactive counts were corrected for both non-specific binding of an isotype control antibody as well as cell number.

To detect ADA-binding capacity, cells were treated for 60 min at 37°C with 10 µg/ml calf spleen ADA. We then proceeded with the binding assay as described above and in Chapter 2 using 1 µg/ml rabbit anti-bovine ADA primary antibody followed by 0.5 µCi/ml [¹²⁵I]-labeled donkey anti-rabbit secondary antibody F(ab')₂ fragment. Counts were corrected by subtracting the values obtained from non-ADA treated cells as well as final cell number.

Spheroid Cultures

For cells grown in spheroid culture, six spheroids were pooled per replicate. Spheroids were dissociated by incubation in 0.05% (w/v) trypsin/0.53 mM EDTA at 37°C for 15 min followed by gentle pipetting. The cell suspensions were transferred to a 96-well V-bottom plate which was centrifuged at 300 x g for 5 min on an IEC Centra CL3R centrifuge (Fisher Scientific, Whitby, ON) with a plate-rotor attached. Cells were then washed and incubated with primary and secondary antibodies as described above for monolayer cell cultures, with the exception that each wash and incubation was followed by a centrifugation step. To determine cell number, six spheroids were pooled and

dissociated with 0.05% (w/v) trypsin/0.53 mM EDTA, followed by counting as described above.

Dipeptidyl Peptidase IV Enzyme Activity Assay

Cells grown in 48-well plates were placed on ice and washed with ice-cold PBS. They were incubated with 2 mM Gly-Pro-pNA for 60 min at 37°C, at which time 100 μ l of the supernatant was transferred to a 96-well flat-bottomed microtiter plate. The absorbance, attributable to pNA release following DPPIV-mediated cleavage, was measured spectrophotometrically at 405 nm. The absorbance of cell-free controls was also analyzed and values were subtracted to determine DPPIV enzyme activity.

Flow Cytofluorimetry

HT-29 cells were released from 10 cm dishes by brief exposure to 0.05% (w/v) trypsin/0.53 mM EDTA. One million cells were suspended in PBS containing 2.5% (w/v) BSA and 0.2% (w/v) sodium azide (fluorescence-activated cell scanning (FACS) buffer), washed twice, and incubated for 45 min at 4°C with 200 μ l FACS buffer containing 1 μ g mouse anti-human CD26. We washed the cells twice with FACS buffer and then incubated with either a FITC-conjugated rat anti-mouse IgG antibody (5-FU-treated cells; 1 μ g/ 10^6 cells) or an Alexa Fluor[®] 488-conjugated goat anti-mouse IgG antibody (VB-treated cells; 0.2 μ g/ 10^6 cells) for 40 min at 4°C in the dark. After three further washes the cells were fixed in 1% paraformaldehyde and stored in the dark at 4°C until analyzed. Flow cytofluorimetry was carried out with a FACScan (BD Immunocytometry Systems, Mountain View, CA) flow cytometer equipped with a 15-mW argon laser operating at a

wavelength of 488 nm and detection at 680 nm. Data were analyzed using Lysis II software.

Orthotopic Tumour Model

One or both flanks of CD1 nu/nu mice were injected s.c. with 5,000,000 HT-29 cells in 100 μ l serum-free DMEM and tumours were allowed to grow for 18-20 days, until they were no greater than 1 cm in diameter. These mice were euthanized under anesthesia, tumours were harvested aseptically, and all observable non-tumour tissue was dissected away. The tumours were washed in ice-cold saline and cut into 1 mm³ pieces to prepare for tumour transplantation. One s.c. tumour was sufficient to generate orthotopic tumours in three mice.

A second group of CD1 nu/nu mice (six mice per treatment group) were anesthetized with 70 mg/kg ketamine and 14 mg/kg xylazine, intraperitoneally (i.p.), and were administered 0.3 mg/kg i.p. buprenorphine for post-surgical analgesia. A 1 cm abdominal incision was made to the right of midline and the cecum was exteriorized. The proximal end of the ascending colon was identified and abraded gently with the wooden end of a cotton-tipped applicator. Three 1 mm³ tissue pieces were sutured onto the muscularis of the proximal ascending colon, while taking care not to pierce the colon wall (Figure 3.1). The cecum was interiorized and the incision was sutured. Mice were observed daily for signs of morbidity.

Twenty-six and twenty-eight days following surgery, mice were weighed and injected i.p. with drugs or vehicle control (saline). Two days after the second dose, they were weighed again and euthanized. Tumours were harvested and all visible non-tumour

tissue was dissected away. Tumours were weighed and snap-frozen in liquid nitrogen. Tumours were maintained at -70°C until analyzed for protein or mRNA expression.

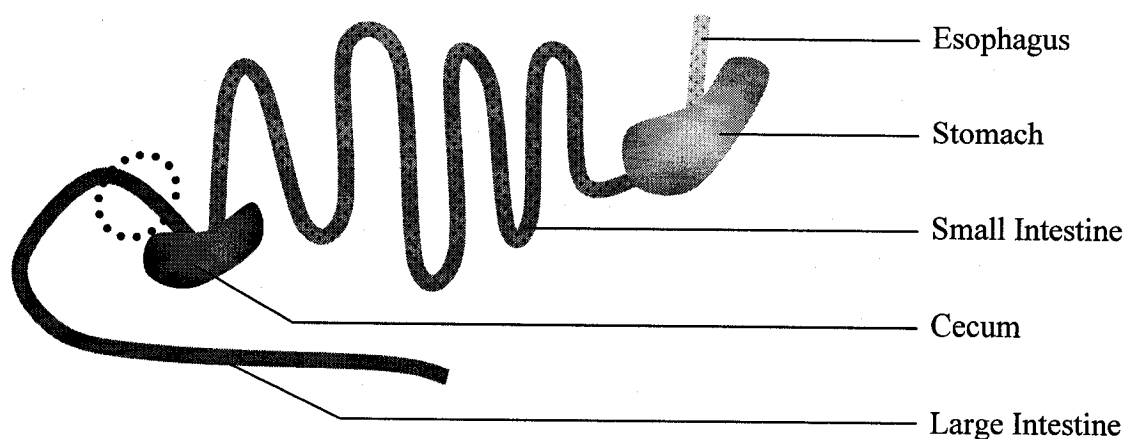


Figure 3.1. The murine gastrointestinal tract. The site of tumour transplantation is circled.

Real-Time Quantitative RT-PCR

We quantified CD26 mRNA as described for CXCR4 in Chapter 2. Briefly, RNA was isolated with TRIzol[®], reverse transcribed to cDNA and quantified using real-time quantitative RT-PCR. Expression of CD26 mRNA was standardized to GAPDH and normalized to expression at 13 days using the $2^{-\Delta\Delta\text{CT}}$ method (Livak and Schmittgen 2001). The following primer sets were used for cDNA amplification:

CD26: 5'-TACATGGGTCTCCCAACTCC-3'

5'-TGAAGTGGCTCATGTGGGTA-3'

GAPDH: 5'-CATGAGAAGTATGACAACAGCCT-3'

5'-AGTCCTTCCACGATACC-3'

Sectioning and Immunohistochemistry

Tumours were frozen in OCT[®] and sectioned at a thickness of 8 μ m with a Leica CM 3050S cryostat. Sections were mounted on slides and maintained at -20°C. For immunohistochemistry, all steps were carried out at 4°C, unless otherwise described. Sections were thawed briefly, rinsed with PBS containing 1 mg/ml BSA and 0.1% Tween 20 (PBS/BSA/Tween), blocked with 3% goat serum in PBS/BSA/Tween for 30 min, then incubated with 25 μ l of PBS/BSA/Tween containing 5 μ g/ml mouse anti-human CD26 primary antibody for 2 h in a humidified chamber. Sections were washed three times with PBS/BSA/Tween, then incubated with 25 μ l of PBS/BSA/Tween containing 2 μ g/ml of an Alexa Fluor[®] 488-conjugated goat anti-mouse IgG secondary antibody for 2 h in a humidified chamber in the dark. Slides were washed a further three times, post-fixed with PBS containing 10% formaldehyde for 10 min at room temperature, and rinsed with distilled water. Coverslips were mounted on sections using low-fade Gel/mount[®] and fluorescence was observed using Leica DM 2000 fluorescence microscope and analyzed with Q Imaging software. The average fluorescence intensity of each slide was measured in an area of constant size containing tumour cells, as determined by a blinded observer.

Statistics

As described in Chapter 2.

RESULTS

Cytotoxic Drugs Increase HT-29 Cell-Surface CD26

CD26 is a multi-functional cell-surface protein that has dipeptidase activity and cleaves the CXCR4 ligand CXCL12, rendering it inactive (Herrera, Morimoto *et al.* 2001; Christopherson, Hangoc *et al.* 2002; Mizokami, Kajiyama *et al.* 2004). As a result of our finding that cytotoxic drugs decrease CXCR4 cell-surface protein, we chose to screen some of these drugs for their activity against CD26. We began by screening a number of cytotoxic agents, including 5-FU, CIS, VB, MTX, doxorubicin, irinotecan, daunorubicin, and oxaliplatin, and measured changes in cell-surface CD26 expression using a radioantibody binding assay. Of these, 5-FU, irinotecan, and oxaliplatin are indicated for the treatment of colorectal carcinoma (Von Hoff and Hanauske 2006). We corrected all values for both non-specific antibody binding and cell number to account for treatment toxicity. In each case, cytotoxic drug treatment led to a significant increase (the direction which would oppose metastatic spread) in cell-surface CD26 expression (Figure 3.2).

The different cytotoxic drugs had distinct abilities in their effects on CD26. While some drugs could more than double the amount of cell-surface CD26 protein (CIS, $72.1 \pm 26.0\%$; doxorubicin, $51.7 \pm 3.7\%$; and daunorubicin, 60.1%), other drugs had a more modest effect on protein expression (5-FU, $30.0 \pm 5.8\%$; VB, $41.5 \pm 10.2\%$; MTX, $26.4 \pm 6.4\%$; irinotecan, 40.0% ; and oxaliplatin, $22.4 \pm 2.8\%$; Table 3.1). Based on these results we chose to continue our focus from the previous chapter on the following four cytotoxic agents: 5-FU, CIS, VB, and MTX.

Cytotoxic Drug Treatment Leads to a Rapid and Prolonged Increase in Cell-Surface CD26 Expression

We found that 5-FU treatment led to an increase in cell-surface CD26 by 24 h while CIS, VB, and MTX increased CD26 as early as 12 h, the earliest time point tested (Figure 3.3). Each of the drugs maintained an increase in CD26 until at least 72 h. Interestingly, we found that these drugs did not have to be present in culture medium for more than 5 min (VB), 1 h (5-FU), or 6 h (CIS, MTX) to mediate this increase, as measured 48 h, 47 h, or 42 h later, respectively (Figure 3.4). At 48 h after a 5 min exposure time to VB we observed a 25% increase in cell-surface CD26 expression. Similarly, a 1 h exposure time to 5-FU resulted in a 39% increase in CD26 at the surface of HT-29 cells at 48 h, and 6 h of exposure to CIS or MTX led to 36% and 28% increases in cell-surface CD26 42 h later, at 48 h.

Non-Specific Cellular Damage Does Not Affect CD26 Cell-Surface Expression

As for our studies with CXCR4, we tested whether the effect of these drugs on CD26 expression could simply be a standard cellular response to damage. We found that treatment with non-specific cell stressors, such as alkaline pH, high salt, or deoxycholate, had no effect on cell-surface CD26 expression by HT-29 cells (Figure 3.5), indicating that this is not a general cellular response to stress.

This Increase in CD26 is at the Cell Surface

Since CD26 is known to interact with the extracellular matrix proteins and can be shed in small membrane vesicles, we wanted to ensure that the increase we were seeing following cytotoxic drug treatments was a bona fide increase in CD26 at the surface of

HT-29 cells rather than accumulation of soluble CD26 on the ECM over time. We therefore stripped HT-29 cell monolayers from their extracellular matrix on cell culture plates using three different methods: treatment with 0.1 M NH_3OH for 30 min; treatment with 5 mM EDTA for 60 min; or treatment with 5 mM EDTA and 1 mM DTT for 60 min. We found that in each case CD26 immunoreactivity was reduced to levels similar to background (Figure 3.6).

CD26 is known to undergo efficient sorting to the apical surface of intestinal epithelial cells (Slimane, Lenoir *et al.* 2000; Alfalah, Jacob *et al.* 2002). To confirm that CD26 was truly increasing over the entire cell rather than just at the apical surface, we analyzed the protein expression using flow cytometry. We found that the increase with CD26 identified using binding assays was reproducible, and in fact larger, when measured using flow cytometry (Figure 3.7).

Cytotoxic Drugs Increase CD26 in HT-29 Cells Grown in Three Dimensions

Sometimes cytotoxic drugs have different effects on cells grown in monolayer compared those grown in three-dimensions (Lowthers, Richard *et al.* 2003). To test if these agents could modulate CD26 expression when cells were grown as multicellular tumour spheroids we first validated an adaptation of the radioantibody binding assay for use with cells in suspension. We tested different numbers of untreated HT-29 cells for CD26 immunoreactivity and found that a minimum of approximately 80,000 HT-29 cells were required for efficient CD26 detection (Figure 3.8). We then cultured HT-29 cells as spheroids and found that mature 5-FU-treated spheroids contained approximately 25,000

cells each (data not shown), indicating that at least four spheroids should be pooled to obtain a sufficient number of cells for CD26 detection.

Having validated this approach, we then tested the effect of 5-FU on HT-29 cells grown in multicellular tumour spheroid culture and found that CD26 increased dramatically with treatment, up to 3.6-fold compared to control-treated cells (Figure 3.9). In fact, this increase was 8.5-fold greater than the 5-FU-mediated increase in CD26 on the surface of cells grown in monolayer culture (Table 3.1; Figure 3.2).

Cytotoxic Drugs Increase Cell-Surface CD26 on Many Colorectal Carcinoma Cell Lines

We tested a number of colorectal carcinoma cell lines (HT-29, T84, HRT-18, SW480, and SW620) and found that, although there was variable CD26 baseline expression in these lines, in each case cytotoxic drug treatment increased CD26 cell-surface expression. HT-29 cells expressed the highest baseline CD26 expression, followed by T84, HRT-18, SW480, and SW620 cell lines (Table 3.2).

CD26 Up-Regulation Correlates with Increased Adenosine Deaminase Binding Capacity and Dipeptidyl Peptidase IV Activity

To determine if cytotoxic-drug mediated induction of CD26 expression at the cell surface is functionally relevant we tested both CD26 dipeptidase activity and ADA-binding capacity following drug treatment. We found that each of 5-FU, CIS, VB and MTX increased both of these CD26 functions to an extent that was similar to the increase in CD26 protein at the cell surface (Figure 3.10). Following treatment with each cytotoxic agent, we observed the expected CD26 protein increase. The DPPIV activity of

CD26, as measured spectrophotometrically through *p*NA release from the CD26 substrate Gly-Pro-*p*NA, increased along with CD26 protein, as did the binding of ADA, as measured by a radioactive antibody binding assay.

Cytotoxic Drug-Mediated CD26 Up-Regulation Occurs In Vivo

We employed an orthotopic model of colorectal cancer to determine if cytotoxic drugs modulate CD26 expression *in vivo*. We first determined that CD26 could be detected in tumour samples using quantitative RT-PCR or immunofluorescence. We transplanted HT-29 tumours onto the colons of CD1 nu/nu mice and allowed the tumours to grow for different periods of time (13, 20, 25, and 30 days). We found that both CD26 mRNA and protein were strongly expressed by HT-29 tumours following a 30 day growth period (Figure 3.11A and B). We therefore examined the effects of drug treatments on CD26 expression at this time.

On the 26th and 28th days following tumour implantation, the mice were administered i.p. saline, 5-FU, irinotecan, or oxaliplatin; on the 30th day the mice were euthanized under anesthesia. When we analyzed sections from these tumours using fluorescence microscopy, we found that in each case, drug treatment enhanced CD26 immunoreactivity. Objective quantification was carried out by a blinded observer using dedicated software. Treatment with 10 mg/kg irinotecan demonstrated a 30% increase in fluorescence intensity (indicative of CD26 expression) over saline treatment (Figure 3.13), 5 mg/kg 5-FU caused a 48% increase over saline (Figure 3.12), and 0.5 mg/kg oxaliplatin resulted in a 58% increase over that observed in saline-treated animals (Figure 3.14).

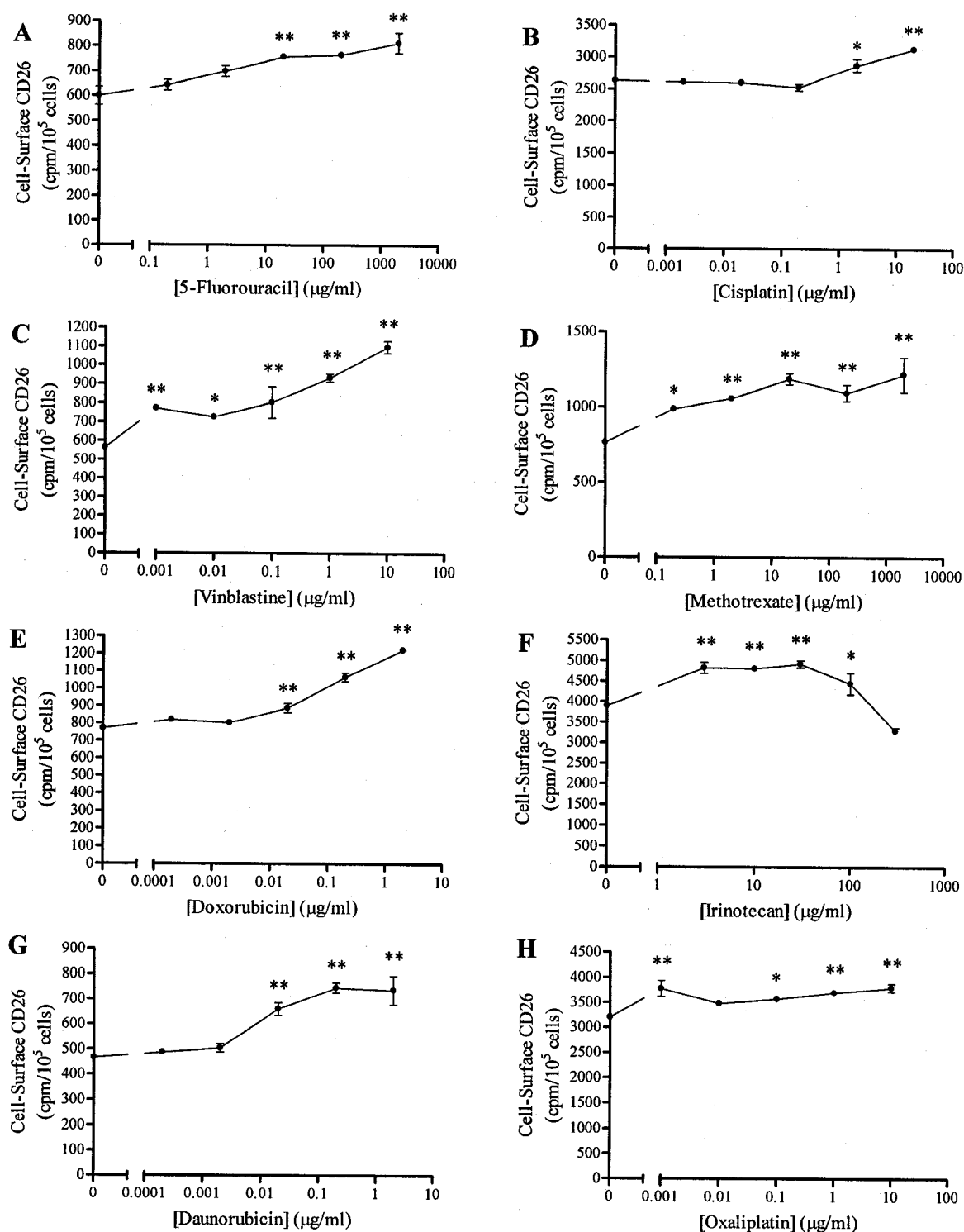


Figure 3.2. Cytotoxic drugs increase cell-surface CD26. Cells were treated with (A) 5-FU; (B) CIS; (C) VB; (D) MTX; (E) doxorubicin; (F) irinotecan; (G) daunorubicin; or (H) oxaliplatin at the indicated doses and assayed 48 h later. Data are represented as mean values \pm SE ($n = 4$) and are representative of between 1 and 16 independent experiments. One-way analysis of variance $p < 0.0001$; *, significant change compared to control, Dunnett's multiple comparison post-test $p < 0.05$; **, $p < 0.01$.

Table 3.1. Cytotoxic drugs increase HT-29 cell-surface CD26. HT-29 cells were treated with various doses of cytotoxic drugs and assayed 48 h later. Data are represented as mean values \pm SE ($n = 4$ or 6) and are representative of the indicated number of independent experiments.

Cytotoxic agent	CD26 (max % increase/ 10^5 cells; mean \pm SE)	EC ₅₀ for CD26 increase/ 10^5 cells (μ g/ml; mean \pm SE)	Number of independent experiments ($n = 4$ or 6)
5-Fluorouracil	30.0 \pm 5.8	1.08 \pm 0.30	7
Cisplatin	72.1 \pm 26.0	16.8 \pm 10.4	8
Vinblastine	41.5 \pm 10.2	0.014 \pm 0.0069	7
Methotrexate	26.4 \pm 6.4	0.44 \pm 0.26	6
Doxorubicin	51.7 \pm 3.7	0.12 \pm 0.073	3
Daunorubicin	60.1	0.0094	1
Irinotecan	40.0	26.2	2
Oxaliplatin	22.4 \pm 2.8	1.47 \pm 1.46	3

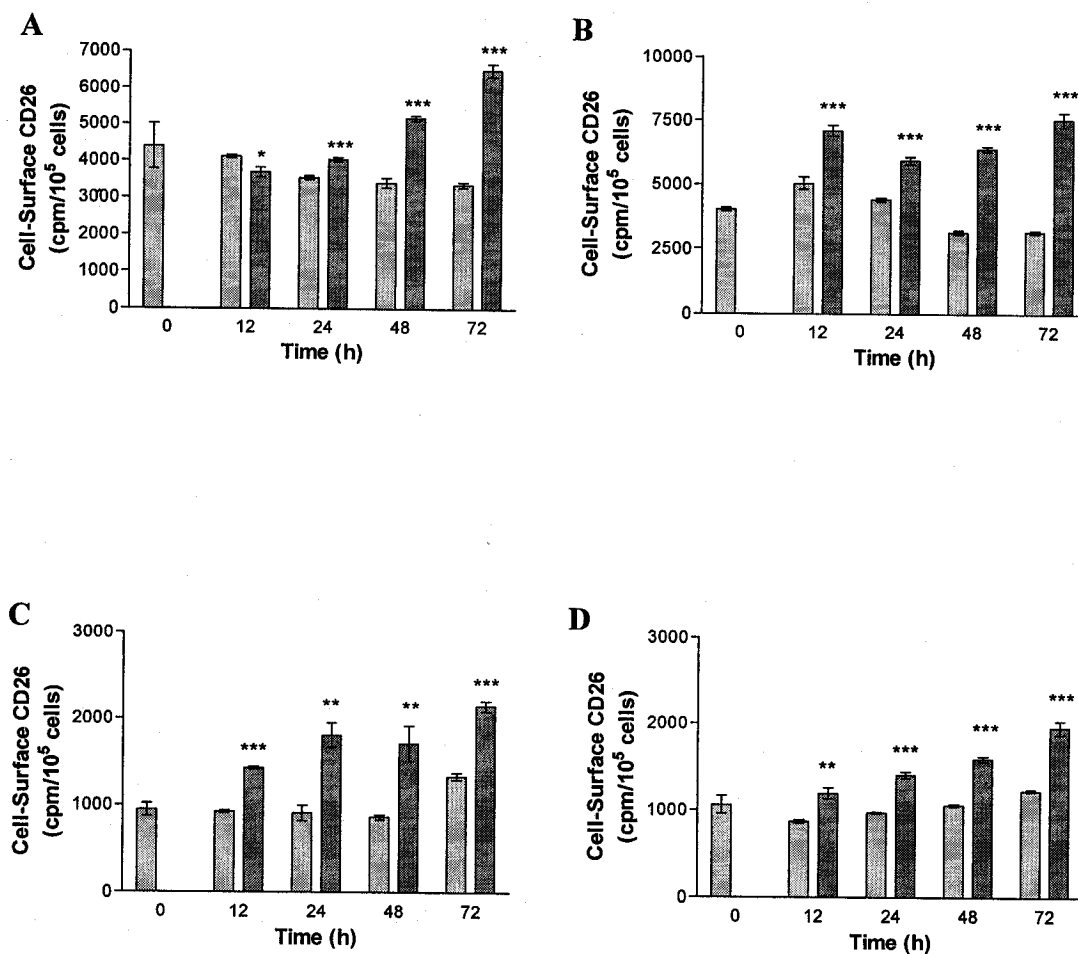


Figure 3.3. CD26 expression on HT-29 cells increases over time with cytotoxic drug treatment. Cells were treated with vehicle (light bars) or (A) 20 μ g/ml 5-FU; (B) 20 μ g/ml CIS; (C) 2 μ g/ml VB; or (D) 2 μ g/ml MTX (dark bars) and assayed at the indicated times. Data are represented as mean values \pm SE ($n = 4$). Two-way analysis of variance $p < 0.0001$; *, significant change compared to control, Bonferroni post-test $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

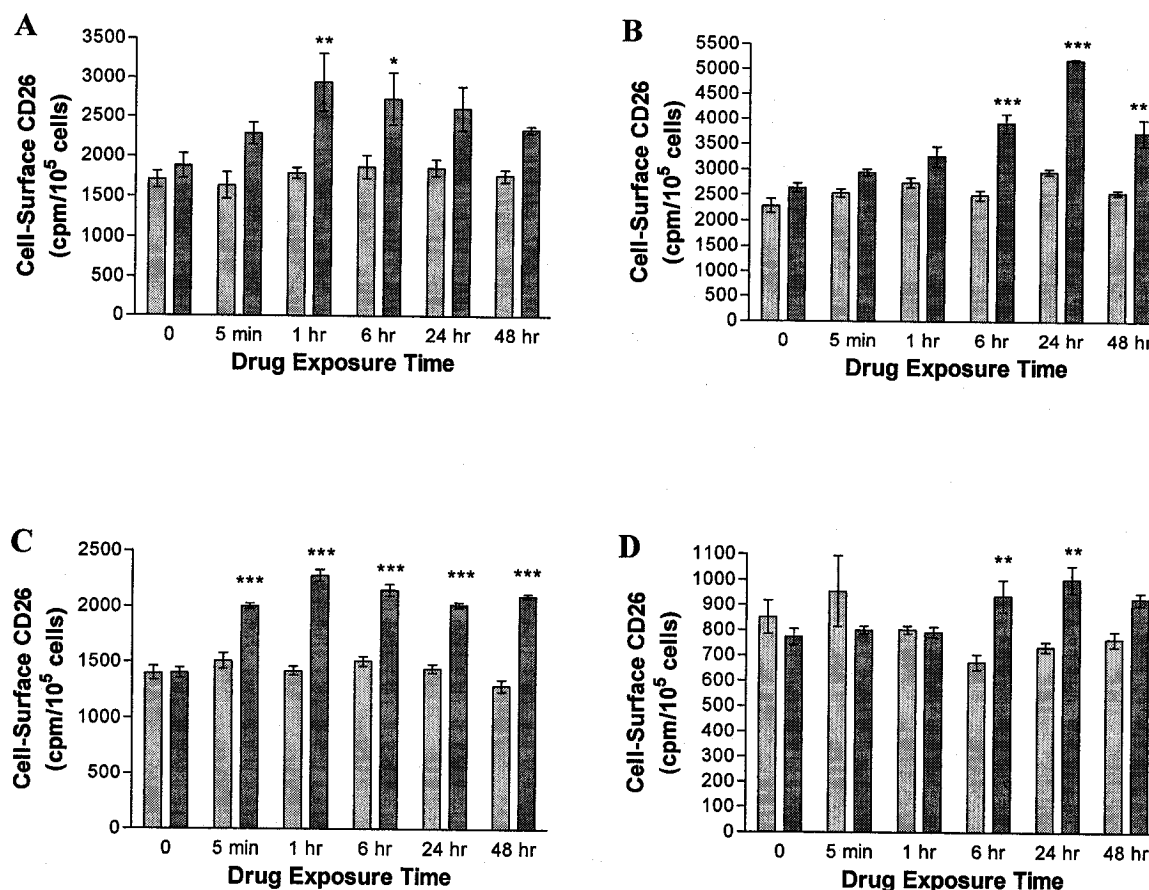


Figure 3.4. CD26 expression on HT-29 cells increases with short exposure to cytotoxic drugs. Cells were treated with vehicle (light bars) or (A) 20 µg/ml 5-FU; (B) 20 µg/ml CIS; (C) 0.1 µg/ml VB; or (D) 20 µg/ml MTX (dark bars) and washed at the indicated times. Fresh medium was added to the cells and they were assayed 48 h following initial exposure to each agent. Data are represented as mean values \pm SE ($n = 4$). Two-way analysis of variance $p < 0.0001$; *, significant change compared to control, Bonferroni post-test $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

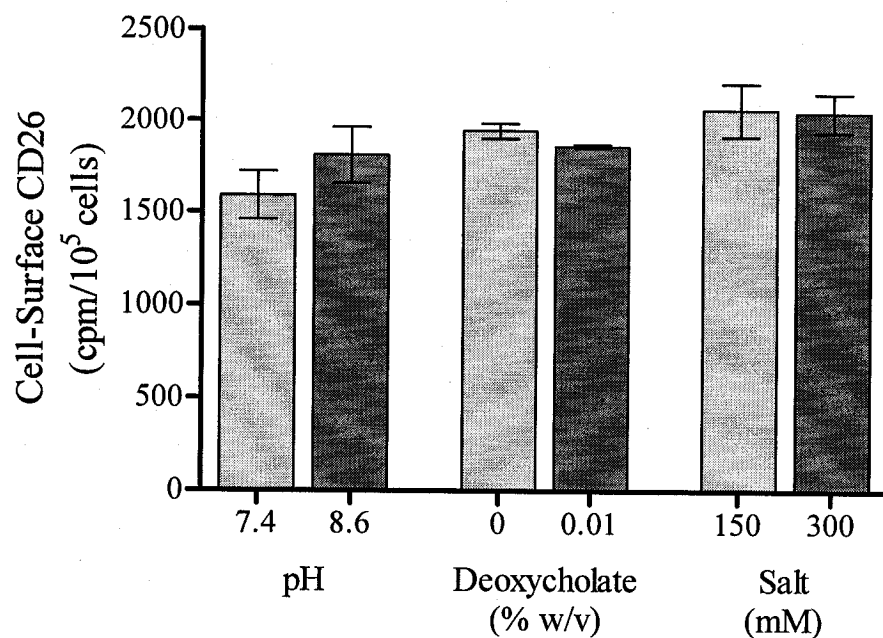


Figure 3.5. Non-specific cellular damage does not affect cell-surface CD26 expression. Cells were exposed to vehicle control (light bars) or each damaging agent (dark bars) for 1 h at 37°C at the indicated doses, washed, and assayed 48 h later. Data are represented as mean values \pm SE ($n = 4$) and are representative of 3 independent experiments.

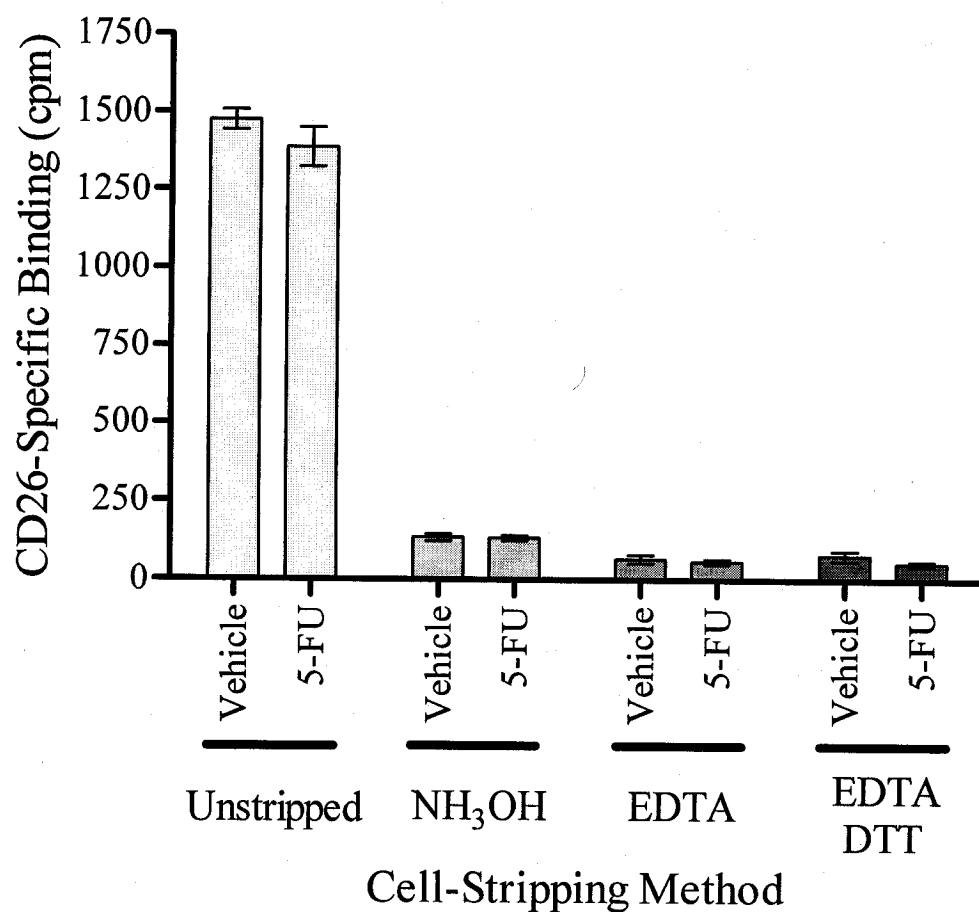


Figure 3.6. Residual CD26 immunoreactivity following cell stripping from the extracellular matrix is minimal and does not increase with 5-fluorouracil treatment. Cells were exposed to vehicle control or 200 μ g/ml 5-FU for 48 h. Before performing a radioactive binding assay, cells were stripped from the wells using the indicated chemicals. Data are represented as mean values \pm SE ($n = 4$) and are representative of 3 independent experiments.

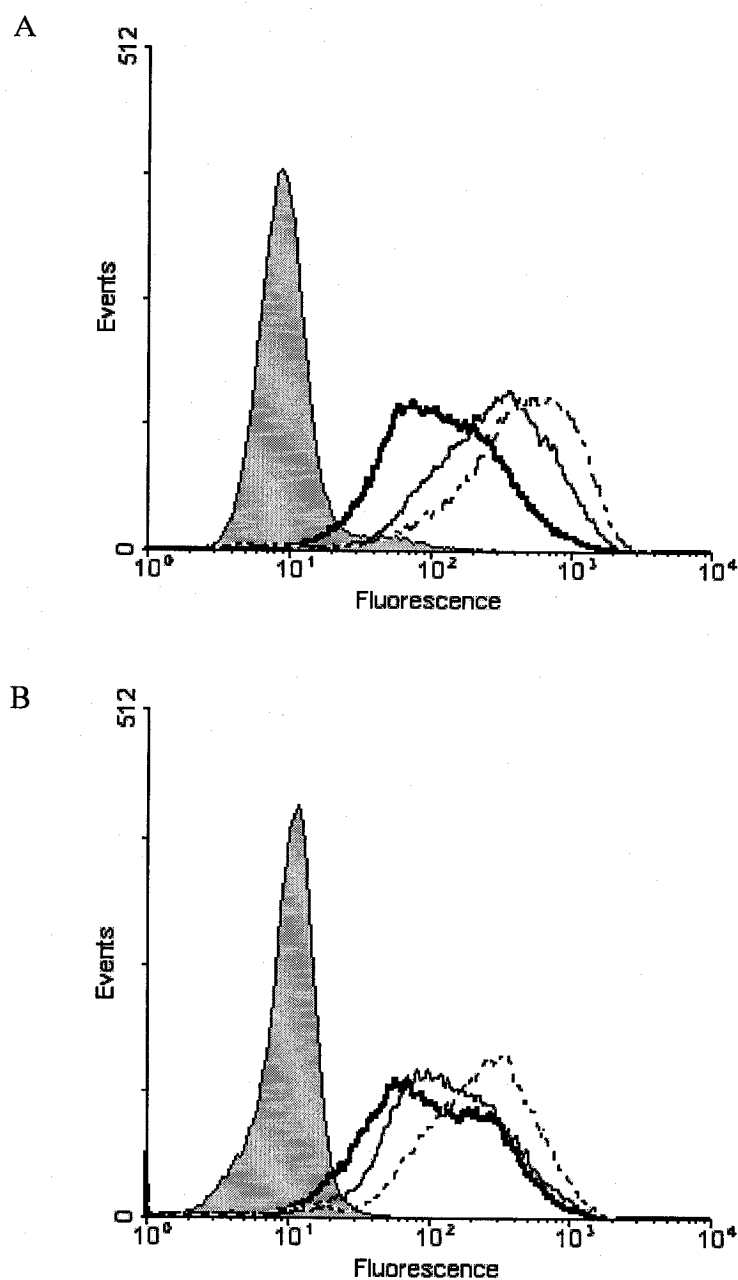


Figure 3.7. Cytotoxic drugs increase cell-surface CD26 as measured by flow cytometry. Cells were treated with vehicle (bolded lines, —) or (A) 2 µg/ml (—) or 200 µg/ml (-----) 5-FU; (B) 0.01 µg/ml (—) or 1 µg/ml (-----) VB. Shaded peaks represent isotype controls.

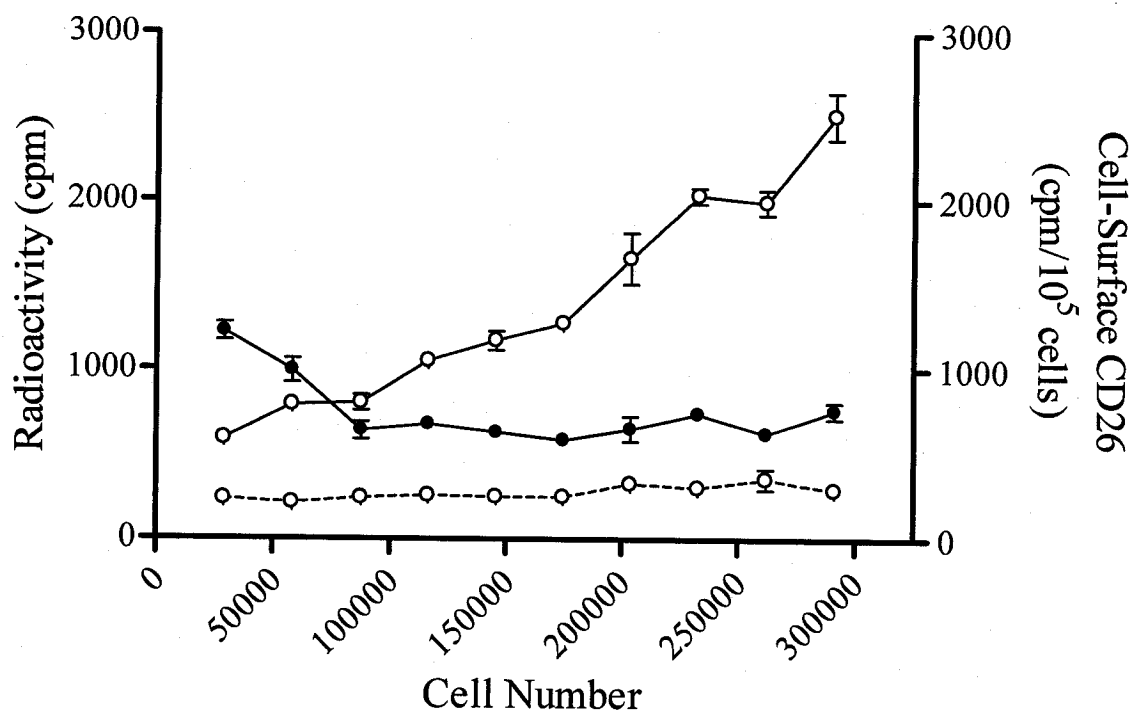


Figure 3.8. Cell-surface CD26 is detectable using a modified radioantibody binding assay on suspended cells. The indicated numbers of untreated cells were suspended and assayed for anti-CD26 antibody binding (—○—) or isotype control antibody binding (---○---; plotted on left Y axis). Counts were corrected for isotype and cell number and replotted on the right Y axis (—●—). Data are represented as mean values \pm SE ($n = 4$).

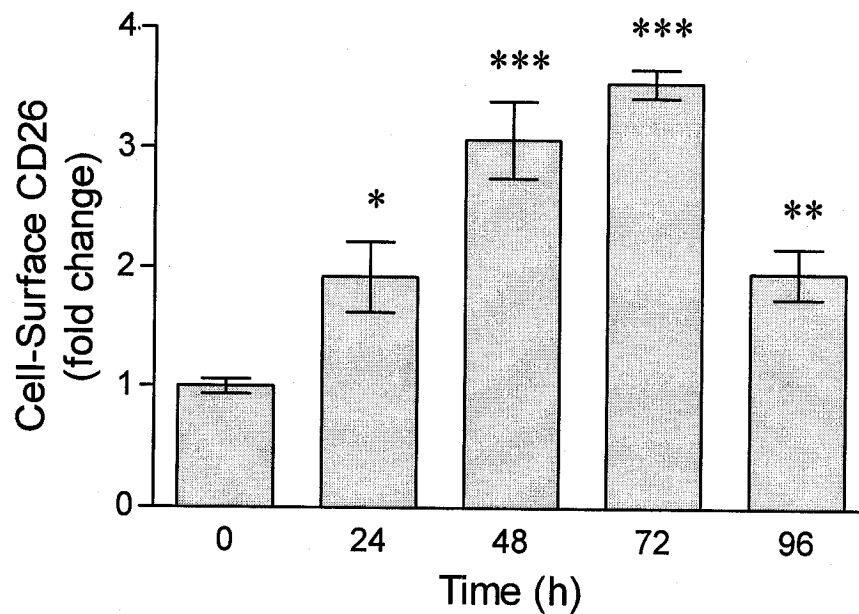


Figure 3.9. 5-Fluorouracil increases cell-surface CD26 in cells grown in three-dimensional tumour spheroids. Cells were treated with vehicle or 2 $\mu\text{g/ml}$ 5-FU and assayed at the indicated times. Data are represented as mean values \pm SE ($n = 4$). One-way analysis of variance $p < 0.0001$; *, significant change compared to control, Dunnett's multiple comparison post-test $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Table 3.2. CD26 constitutive and cytotoxic drug mediated expression in colorectal carcinoma cell lines. Cells were treated with the indicated drugs and assayed 48 h later. Constitutive expression data was taken from 12-35 independent experiments for each cell line and maximal response data was taken from 3-7 independent dose-response experiments for each drug and cell line. Data are represented as mean values \pm SE ($n = 4$). *, significant change compared to control, Student's t-test $p < 0.05$; **, $p < 0.01$; *, $p < 0.001$.

Cell Line	Basal CD26 Expression (cpm/ 10^5 cells)	5-FU	CIS	VB	MTX
<i>Increase in % above control</i>					
HT-29	1,294 \pm 135	30.0 \pm 5.8 **	72.1 \pm 26.0 ***	41.5 \pm 10.2 **	26.4 \pm 6.4 **
T84	784 \pm 69	84.3 \pm 14.0 *	60.5 \pm 42.1	77.6 \pm 6.6	73.3 \pm 42.3
HRT-18	366 \pm 64	16.6 \pm 9.1 *	19.1 \pm 9.1	23.0 \pm 11.6	17.5 \pm 9.4 **
<i>Increase in cpm/10^5 cells</i>					
SW480	55 \pm 15	75 \pm 6 *	64 \pm 20	88 \pm 8 **	210 \pm 169
SW620	26 \pm 12	36 \pm 18	6 \pm 16	74 \pm 66	102 \pm 87

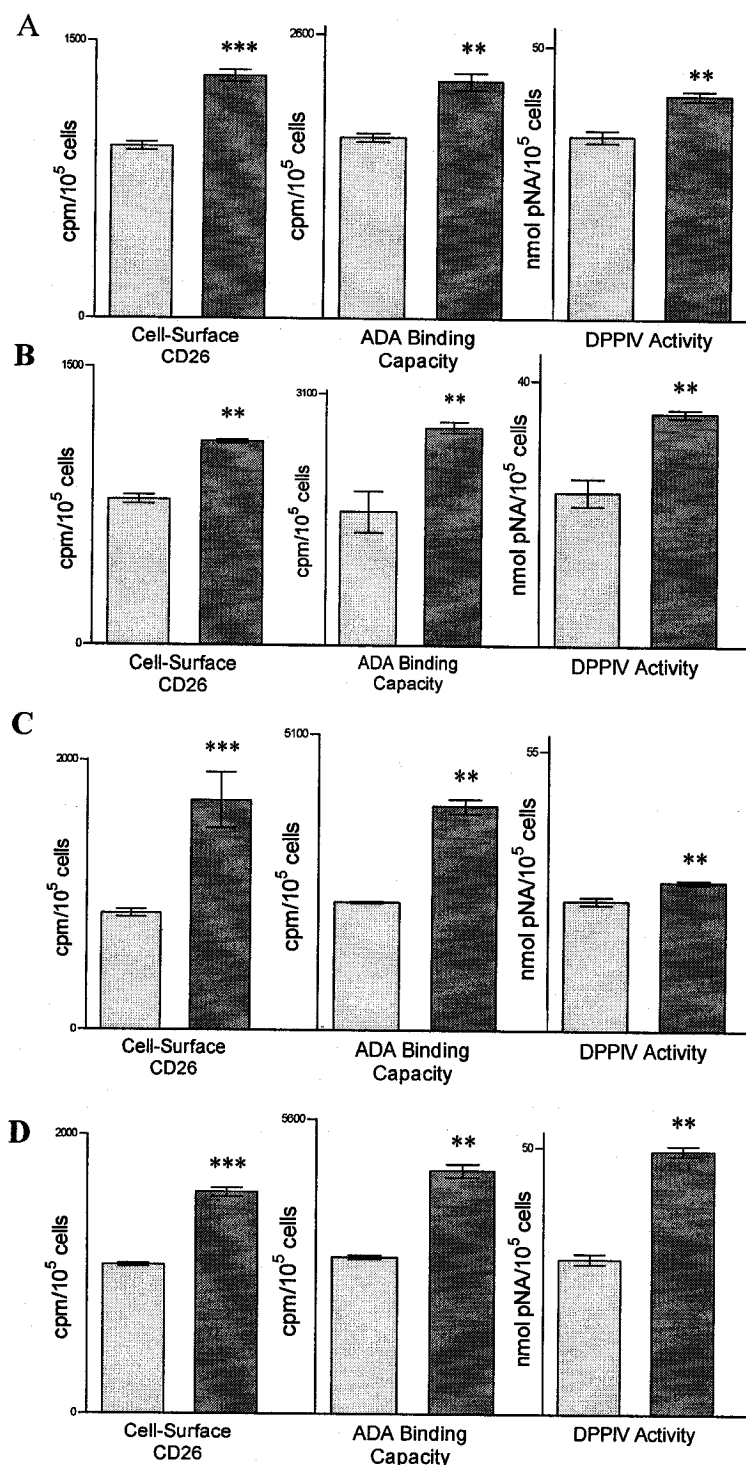


Figure 3.10. Dipeptidyl peptidase IV activity and adenosine deaminase-binding capacity increase in parallel with CD26 protein. Cells were treated with vehicle (light bars) or 2 μ g/ml (A) 5-FU; (B) CIS; (C) VB; or (D) MTX (dark bars) and assayed 48 h later for CD26 protein, ADA-binding capacity, and dipeptidase activity. Data are represented as mean values \pm SE ($n = 4$). **, significant change compared to control, Student's t-test $p < 0.01$; ***, $p < 0.001$.

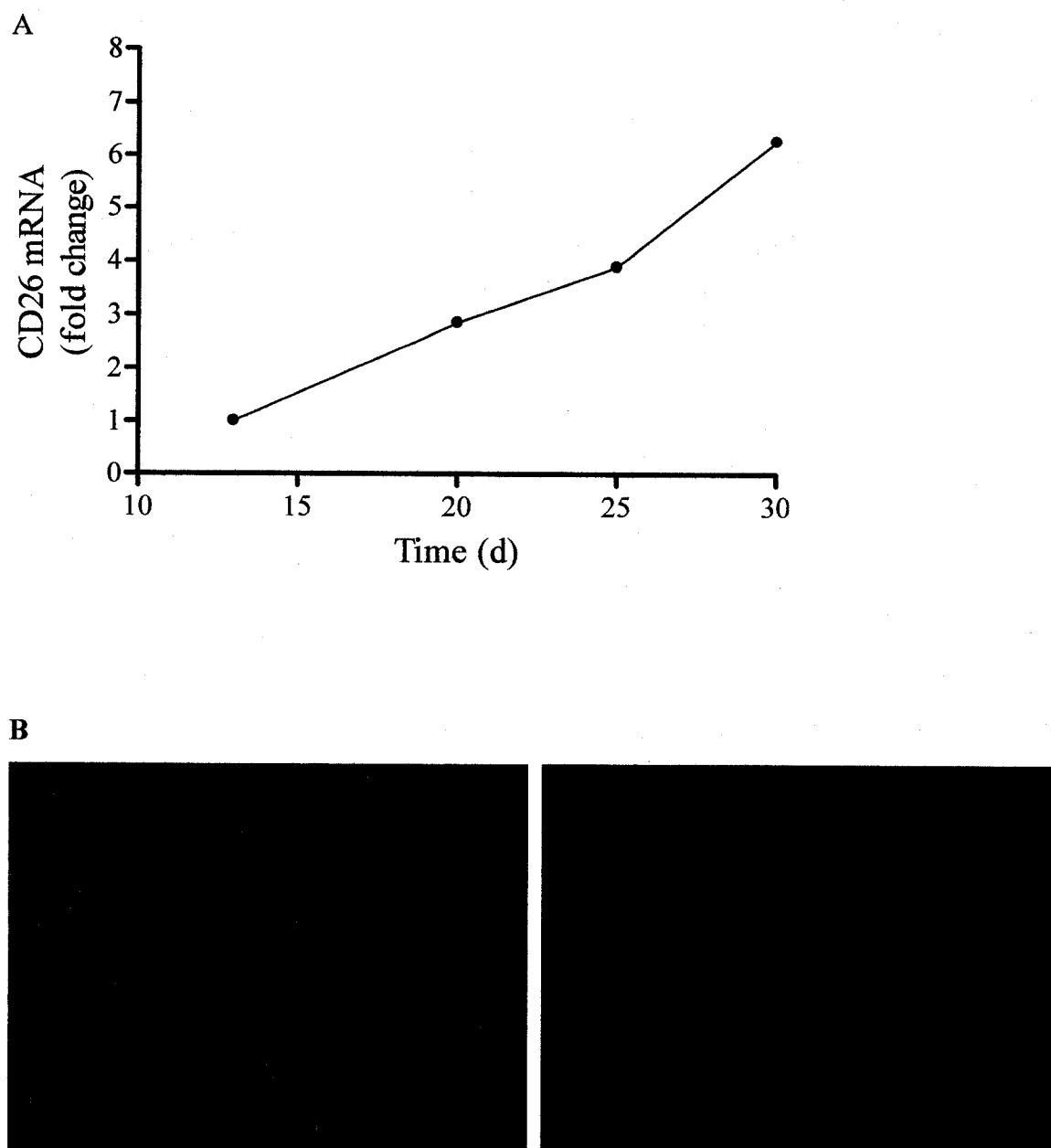


Figure 3.11. CD26 mRNA and protein expression increase with growth of HT-29 orthotopic tumours in mice. (A) CD26 mRNA expression increases with increasing time *in vivo*. (B) CD26 protein expression (left) is clearly evident above isotype control (right) after 30 days *in vivo*.

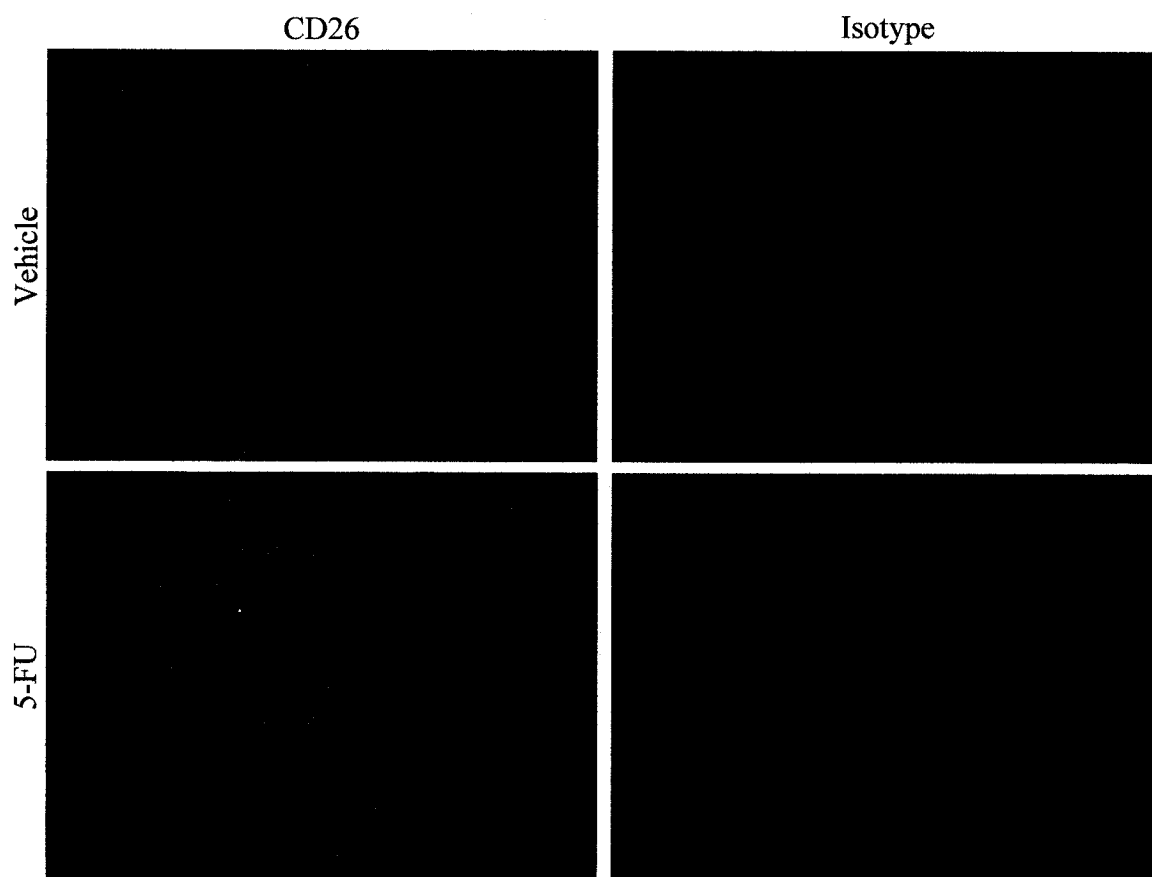


Figure 3.12. 5-Fluorouracil increases CD26 expression *in vivo*. Mice with HT-29 colorectal tumours were treated with two doses of i.p. saline (top) or 50 mg/kg 5-FU (bottom), four and two days before the tumours were harvested. Tumour sections were immunostained using anti-CD26 antibody (left) or isotype control antibody (right).

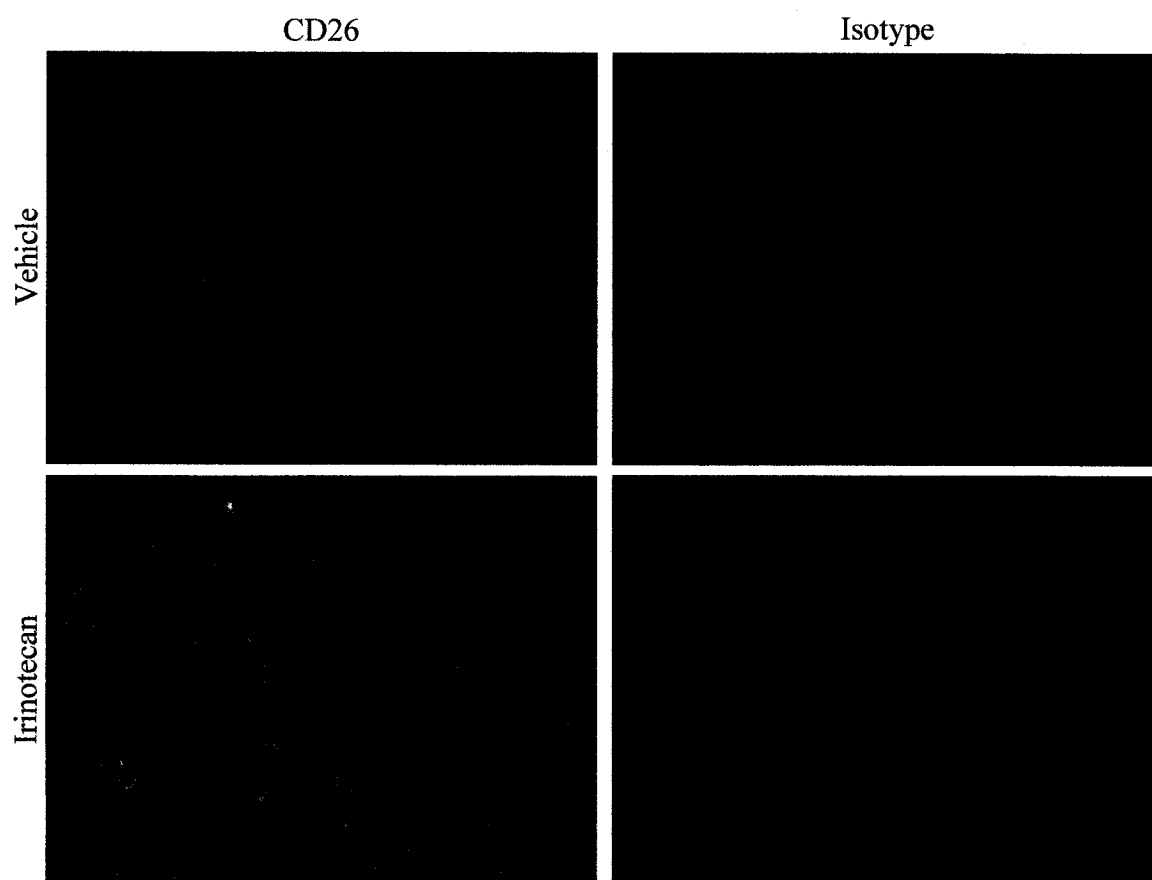


Figure 3.13. Irinotecan increases CD26 expression *in vivo*. Mice with HT-29 colorectal tumours were treated with two doses of i.p. saline (top) or 10 mg/kg irinotecan (bottom), four and two days before the tumours were harvested. Tumour sections were immunostained using anti-CD26 antibody (left) or isotype control antibody (right).

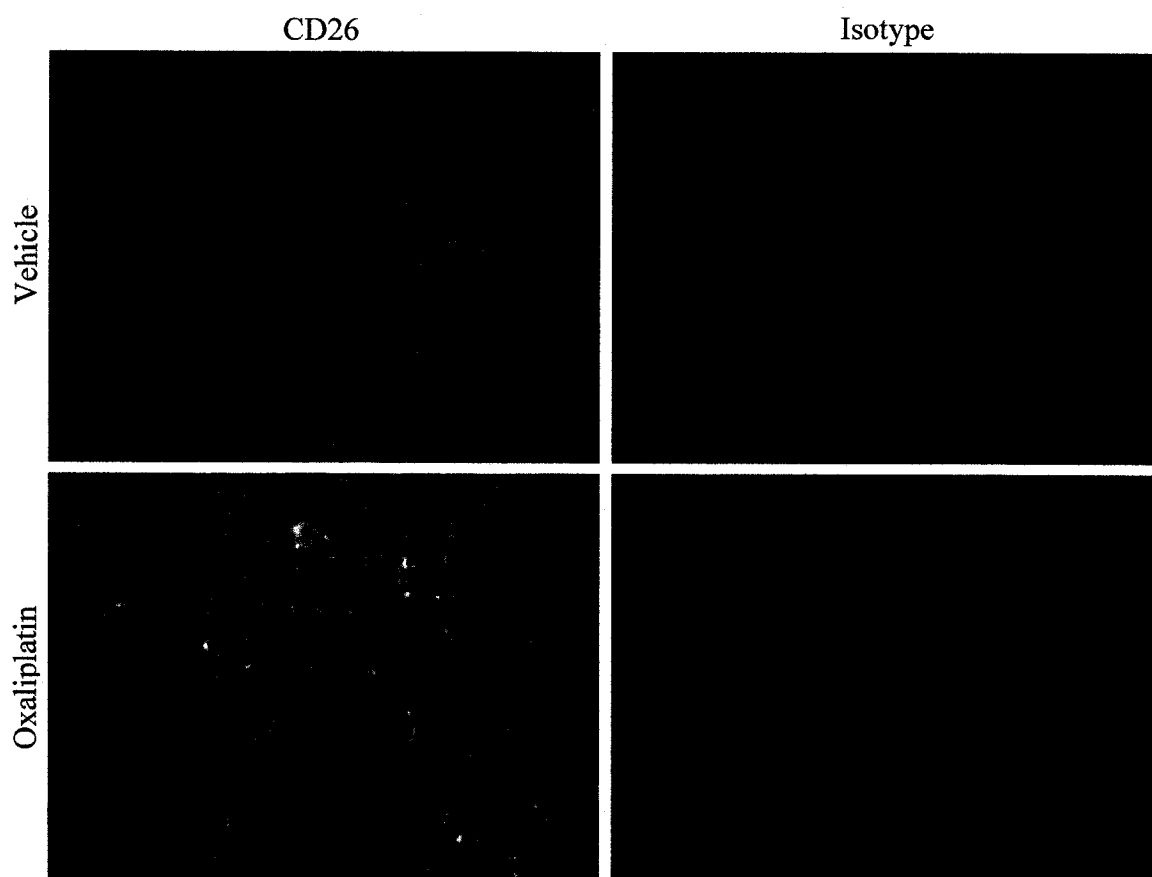


Figure 3.14. Oxaliplatin increases CD26 expression *in vivo*. Mice with HT-29 colorectal tumours were treated with two doses of i.p. saline (top) or 0.5 mg/kg oxaliplatin (bottom), four and two days before the tumours were harvested. Tumour sections were immunostained using anti-CD26 antibody (left) or isotype control antibody (right).

DISCUSSION

As was shown in Chapter 2, cytotoxic drugs modulate the levels of CXCR4 on the surface of HT-29 colorectal carcinoma cells. Like CXCR4, CD26 is implicated in the process of metastasis, but its expression is often reduced in tumours compared to normal tissues (Houghton, Albino *et al.* 1988; Morrison, Vijayasaradhi *et al.* 1993; Wesley, Albino *et al.* 1999; Kajiyama, Kikkawa *et al.* 2003; Khin, Kikkawa *et al.* 2003; Wesley, McGroarty *et al.* 2005). CD26 and CXCR4 are related through CXCL12; CXCL12 is both the ligand for CXCR4 (Bleul, Farzan *et al.* 1996; Oberlin, Amara *et al.* 1996) and is a DPPIV-sensitive substrate of CD26 (Shioda, Kato *et al.* 1998). When CD26 cleaves CXCL12 it is rendered inactive and thus is neither chemoattractive (Proost, Struyf *et al.* 1998) nor proliferative (Mizokami, Kajiyama *et al.* 2004) for cells expressing CXCR4. Therefore, a decrease in CD26 in certain tumours may enhance their metastatic capacity through decreased inactivation of CXCL12. This was shown in CXCR4-expressing endometrial carcinoma cell lines: CD26 transfection had no effect on CXCR4 levels; however, it led to a loss of CXCL12-mediated proliferation (Mizokami, Kajiyama *et al.* 2004).

After we discovered that cytotoxic drugs could decrease CXCR4 expression, a change that opposes metastasis, we speculated that they may increase CD26 expression, a change that would similarly oppose metastatic spread. Our primary assay for examining CD26 expression at the surface of cells was a radioantibody binding assay. This assay was equivalent to that described for cell-surface CXCR4 quantification, with the exception of using a primary antibody specific for CD26 rather than CXCR4. This assay has been previously validated in our lab and specifically detects cell-surface CD26 (Tan,

Mujoomdar *et al.* 2004). Again, all counts were corrected for both non-specific binding and cell number to account for any toxic effects of the treatments.

Cytotoxic Drugs Increase Cell-Surface CD26 Expression

We screened a number of cytotoxic drugs for effects on HT-29 cell-surface CD26 expression using the radioantibody binding assay described above. We tested many of the same cytotoxic agents described in Chapter 2, chosen for their diverse mechanisms of action. The drugs tested include: the pyrimidine analog, 5-FU; the platinum compounds, CIS and oxaliplatin; the mitotic spindle poison, VB; the folate antagonist, MTX; the topoisomerase I inhibitor, irinotecan; and the DNA intercalators and topoisomerase II inhibitors, doxorubicin and daunorubicin (Table 1.1). Each agent reproducibly increased CD26 expression at the cell-surface, as measured 48 h after treatment (Figure 3.2). This was not a general cellular response to cytotoxic stress, as treatment with sodium chloride, the bile acid deoxycholate, or alkaline pH had no effect on cell-surface CD26 expression (Figure 3.5).

We again focused on four representative drugs: 5-FU, CIS, MTX, and VB. These drugs represent different classes of antineoplastic agents and are commonly used to treat a variety of malignancies.

5-Fluorouracil

The pyrimidine analog, 5-FU, is commonly used to treat a number of different tumours, including primary and metastatic colorectal cancer, in the adjuvant, neo-adjuvant, and palliative settings (Von Hoff and Hanauske 2006). Treatment of HT-29 colorectal carcinoma cells with 5-FU led to an average $30.0 \pm 5.8\%$ maximal increase in

cell-surface CD26 expression. The average EC_{50} for this CD26 reduction was 1.08 ± 0.30 $\mu\text{g/ml}$, which was similar to that for CXCR4 reduction, and much lower than the average plasma concentration of 5-FU in cancer patients, which suggests that these effects may be physiologically relevant (Tables 2.1, 3.1). The time course of 5-FU-mediated CD26 up-regulation was slightly more rapid than that observed with CXCR4 down-regulation, becoming evident by 24-36 h following 5-FU treatment (Figure 3.3A), compared to 36-60 h to observe changes in CXCR4 expression (Figure 2.4A). In addition to this, cells required exposure to 5-FU for 6 h, or as little as 1 h in one experiment, to elicit a significant increase in CD26 expression at 48 h (Figure 3.4A). This data, combined with the finding that 5-FU down-regulates CXCR4 and up-regulates CD26, suggests that cytotoxic drugs modulate CXCR4 and CD26 expression through different mechanisms. Consistent with this, we found that CD26 mRNA expression was not affected at any time point tested (from 3 to 24 h) following 5-FU treatment, as tested using two different PCR primer sets in four independent experiments.

Cisplatin

Next we examined the effects of the crosslinking platinum compound, CIS. Cisplatin also increased CD26 levels at the surface of HT-29 cells; however, we were unable to calculate accurate values for its maximal effect or EC_{50} . Cisplatin seemed to enhance cell attachment and, at doses above 20 $\mu\text{g/ml}$, CIS treatment led to trypsin-resistant attachment of HT-29 cells to the cell culture substrate, most likely as a consequence of its crosslinking activity. This made it difficult to count the final cell number in these treatment groups with any confidence, and in fact seemed to skew our results in the direction of underestimating cell-surface CD26 at high CIS doses. As a

result, we could not attain the plateau for CIS, in terms of CD26 up-regulation, which is necessary to calculate maximal effect and EC_{50} values. In any case, the maximal effect we observed, using concentrations of CIS up to 20 $\mu\text{g/ml}$, was a $72.1 \pm 26.0\%$ up-regulation of CD26, with an estimated EC_{50} of $16.8 \pm 10.4 \mu\text{g/ml}$ (Table 3.1; Figure 3.2B). Cisplatin led to an up-regulation of CD26 as early as 12 h following treatment (Figure 3.3B) and cells required a minimum of 6 h exposure time to CIS for an up-regulation of CD26 to be evident by 48 h (Figure 3.4B). Cisplatin did not have any effect on CD26 mRNA expression (data not shown).

Methotrexate

The antifolate, MTX, was the next drug we examined in detail. We found that MTX led to an average $26.4 \pm 6.4\%$ up-regulation in CD26 (Table 3.1; Figure 3.2D) and this up-regulation was first evident by the earliest time point tested, 12 h (Figure 3.3D). As was the case with each drug tested, this up-regulation was sustained for at least 96 h following a single dose of drug. The EC_{50} for this effect was $0.44 \pm 0.26 \mu\text{g/ml}$ (Table 3.1). Like with CIS, cells required exposure to MTX for a minimum of 6 h to see an up-regulation in CD26 at 48 h (Figure 3.4D) and MTX treatment did not affect CD26 mRNA expression (data not shown).

Vinblastine

Finally, we tested the mitotic spindle poison, VB. Not surprisingly, we found that it also up-regulated cell-surface CD26 (Figure 3.2C), but not CD26 mRNA (data not shown). Vinblastine up-regulated cell-surface CD26 as early as 12 h following treatment (Figure 3.3C) and demonstrated an average maximal $41.5 \pm 10.2\%$ increase with an EC_{50}

of 0.014 ± 0.0069 $\mu\text{g/ml}$. Remarkably, a 5 min exposure time was reproducibly sufficient to cause a CD26 increase that was evident 48 h later (Figure 3.4C). Interestingly, VB enters cells by simple diffusion and does not reach maximal intracellular concentrations until 4 h following cell culture treatment (Zhou, Placidi *et al.* 1994), so it is unlikely that 5 min was long enough to allow a significant proportion of the applied dose to enter cells. Based on the findings by Zhou and colleagues, less than 1% of the administered VB would enter cells after only 5 min of exposure. However, VB is lipophilic and can be highly protein-bound (Rowinsky 2006), so perhaps it was bound to the cells or the cell-culture substrate in a way that prevented its removal by our washes.

It is very interesting to note that the effect of these drugs on CD26 occurs in each case approximately 24 h before the effect on CXCR4. The only exception to this is following treatment with the mitotic spindle poison, VB: both CXCR4 and CD26 cell-surface expression were affected by 12 h after treatment. This may be because of a rapid effect on vesicular transport, either preventing externalization of cytoplasmic CXCR4 or internalization of cell-surface CD26.

Cytotoxic Drug-Mediated CD26 Up-Regulation Occurs at the Cell Surface

CD26 is known to be secreted in some situations (Schrader and Stacy 1979; Schrader, Woodward *et al.* 1979; Schrader and West 1985). To confirm that the increase in immunoreactivity we were seeing was reflecting CD26 at the cell-surface, rather than that secreted and adsorbed to the cell culture substrate or bound to extracellular matrix proteins laid down by the cells, we first stripped the cells from the culture dish then performed a binding assay on the remaining plastic/extracellular matrix. Following this

procedure, there was a small amount of residual CD26 immunoreactivity, however, there was no change in residual binding following drug treatment (Figure 3.6). We stripped the cells using three different methods, so we are confident that we would have identified any change in adsorbed/bound CD26, if present.

As CD26 is generally expressed by the cell in a polarized manner, particularly at the apical surface of intestinal epithelial cells (Slimane, Lenoir *et al.* 2000; Alfalah, Jacob *et al.* 2002), we wanted to be sure that the effect we were seeing was a bona fide increase in CD26 expression, rather than an increased efficiency in its apical sorting. To reconcile these possibilities, we performed FACS analysis of cell-surface CD26 following either 5-FU or VB treatment and found a 3- to 6-fold increase in mean fluorescence intensity (Figure 3.7). This increase was in fact much larger than that observed using binding assays on cells grown in monolayer culture, suggesting we may be underestimating the effect of antineoplastic drugs on CD26 expression.

To examine this further, we grew HT-29 cells as multicellular tumour spheroids and measured CD26 expression using a modified binding assay. We first ensured that this assay could specifically detect CD26 at the cell-surface, as described in Chapter 2 for CXCR4, and found that we required approximately 80,000 cells to see CD26 levels above those of isotype control (Figure 3.8). This modification to the binding assays allows us to measure whole cell-surface CD26 on suspended cells, rather than just the apical surface that is measured in monolayer assays. Here we found that 5-FU increased CD26 expression by 3.6-fold (Figure 3.9), a result similar to that observed with FACS (Figure 3.7). This suggests that CD26 is increasing across the entire surface of HT-29 cells, rather than just the apical surface. This could be due to microenvironmental

enhancement of the cytotoxic drug effects when cells are grown in spheroids, or it could be a result of an unmasking of basolaterally increased CD26 that is not detected using binding assays on monolayer cultures.

We obtained all the above data using the human HT-29 colorectal carcinoma cell line. We wanted to determine if cytotoxic drugs could up-regulate CD26 expression in a variety of colorectal carcinoma cell lines, or if this was an effect that was unique to HT-29 cells. We tested human T84, HRT-18, SW480, and SW620 colorectal carcinoma cell lines and found variable CD26 expression on the surface of these cells (Table 3.2). HT-29 cells expressed CD26 at the highest level, followed by T84, HRT-18, SW480, and SW620 cells. We found that cytotoxic drugs increased CD26 expression in HT-29, T84, and HRT-18 cells. The SW480 and SW620 cell lines expressed CD26 at very low level, barely above the level of detection. Although there was a trend in these cell lines toward an increase in CD26 following drug treatment, this did not reach statistical significance in most cases, most likely as a result of their low baseline level of CD26 expression. This finding is important as it implicates cytotoxic drugs as important regulators of CD26 expression on a variety of colorectal carcinoma cell lines.

CD26 Up-Regulation Correlates with Increased Dipeptidyl Peptidase IV Activity and Adenosine Deaminase Binding Capacity

We predicted that increased CD26 expression would correlate with increased CD26 functionality. Two important functional CD26 activities include DPPIV enzyme activity (Kenny, Booth *et al.* 1976; Püschel, Mentlein *et al.* 1982; Tanaka, Camerini *et al.* 1992) and ADA-binding (Kameoka, Tanaka *et al.* 1993; Morrison, Vijayasradhi *et al.* 1993). Dipeptidyl peptidase IV enzyme activity is important for cleaving a number of

molecules, generally reducing or eliminating their bioactivity, although in some situations DPPIV-mediated cleavage can enhance the activity of certain peptides (De Meester, Korom *et al.* 1999). A major substrate for CD26's DPPIV activity is CXCL12, the ligand for CXCR4 (Shioda, Kato *et al.* 1998; Lambeir, Proost *et al.* 2001; Busso, Wagtmann *et al.* 2005). Dipeptidyl peptidase IV-mediated cleavage of CXCL12 renders it inactive in terms of both chemoattraction and proliferation (Herrera, Morimoto *et al.* 2001; Christopherson, Hangoc *et al.* 2002; Mizokami, Kajiyama *et al.* 2004). As a result, enhanced CD26 expression by cancer cells is an attractive target for reducing CXCR4-driven metastasis, as long as DPPIV activity increases in parallel with CD26 cell-surface expression. It is possible however, that although CD26 is increasing at the cell-surface following cytotoxic drug treatment, its DPPIV activity may remain at, or even below, control levels. We tested whether DPPIV activity increased following cytotoxic drug treatment by incubating treated or control cells with an artificial DPPIV substrate whose cleavage can be measured spectrophotometrically to quantify DPPIV activity. We tested 5-FU, CIS, MTX, and VB for effects on DPPIV activity and found in each case, as expected, there was an increase that paralleled that of the increase in cell-surface protein (Figure 3.10).

In addition to its important role as a peptide cleavage molecule, CD26 is a key binding protein for adenosine deaminase, which inactivates the tumour mitogen adenosine (Kelley, Daddona *et al.* 1977; Daddona and Kelley 1978; Kameoka, Tanaka *et al.* 1993). A decrease in CD26 in tumours could lead to decreased ADA binding and therefore increased adenosine concentrations in the extracellular fluid. Increased

adenosine concentrations, in turn, lead to enhanced proliferation (Mujoomdar, Hoskin *et al.* 2003) and increased CXCR4 expression (Richard, Tan *et al.* 2006).

Similar to the results described above for DPPIV activity, ADA-binding capacity correlated with CD26 protein levels at the cell surface (Figure 3.10). Therefore, cytotoxic drugs not only increase CD26 protein at the cell surface, but also the functionality of CD26, in terms of both DPPIV activity and ADA-binding capacity, in a direction that should oppose tumour growth and/or metastasis.

Cytotoxic Drugs Increase CD26 Expression In Vivo

Since these drugs are active *in vitro* at enhancing CD26 cell-surface protein, as well as DPPIV activity and ADA-binding capacity, we examined the effect of these drugs on CD26 expression in an orthotopic colorectal carcinoma model *in vivo*. Most cancer models in mice involve the use of heterotopic subcutaneous tumours. Although this is a straightforward, well-accepted method for measuring treatment effects on tumour growth, it is not an appropriate model for colorectal cancer. A more suitable method is to introduce tumours directly onto the colons of mice (Morikawa, Walker *et al.* 1988; Sekikawa, Arends *et al.* 1988; Flatmark, Maelandsmo *et al.* 2004). To accomplish this, we first grew HT-29 cells as subcutaneous tumours in the flanks of a first group of nude mice. Approximately 20 days later we euthanized these donor mice, excised their tumours, and sutured three 1 mm³ pieces onto the exteriorized cecum of each recipient nude mouse.

We first tested this orthotopic tumour model to ensure we could detect CD26 mRNA and protein in these tumour samples. In this pilot experiment, we introduced

orthotopic tumours and euthanized the recipient mice at various time points (13, 20, 25, and 30 days post-implantation). The tumours harboured by these mice were solid masses that invaded the intestinal wall. After excising the tumour tissue we examined CD26 expression using immunofluorescence on tissue sections. Although we could detect CD26 at the earlier time points, it was clearly evident following 30 days *in vivo* (Figure 3.11B). We then used human-specific PCR primers to detect tumour, but not murine, CD26 mRNA expression in these tumours.

Interestingly, we found that CD26 mRNA expression was enhanced over time *in vivo*, increasing at 20, 25, and 30 days after tumour implantation up to more than 6-fold compared to the 13 day time point (Figure 3.11A). Importantly, this confirmed that the PCR primers we use can detect changes in CD26 mRNA expression and the lack of change we observed following drug treatment *in vitro* is not simply a result of poor primer design. This also suggests that like CXCR4, CD26 is subject to modulation by molecules present within the *in vivo* environment.

Having validated this model we tested the effect of drug treatment on tumour CD26 expression. We chose to examine three of the drugs described above that are commonly used to treat colorectal cancer: 5-FU, irinotecan, and oxaliplatin. On days 26 and 28 following surgery, we injected each animal with one of the three drugs or saline and on day 30 we euthanized the mice and excised the tumours. These animals showed no overt signs of treatment toxicity, with the exception of the mice treated with the highest dose (50 mg/kg) of 5-FU. These animals exhibited a statistically significant weight loss (4%; 1.0 ± 0.4 g) as compared to saline-treated animals (data not shown).

Not surprisingly, and in concordance with our *in vitro* findings, we determined that these drugs had no effect on CD26 mRNA expression within the tumours (data not shown). However, we did find an increase in CD26 protein expression by tumour cells from animals treated with low doses of 5-FU (Figure 3.12), irinotecan (Figure 3.13), or oxaliplatin (Figure 3.14), as compared to animals treated with saline; each of these cytotoxic agents are commonly used to treat colorectal cancer (Rodriguez-Bigas, Hoff *et al.* 2006). These data support the theory that diverse anticancer agents may be acting to regulate the CXCR4-CXCL12 axis by enhancing CD26 protein expression. This describes a novel mechanism of action of these drugs, when administered at low, minimally toxic doses.

CONCLUSIONS

The data summarized in this chapter describe the effect of cytotoxic drugs on CD26 expression *in vitro* and *in vivo*. We have shown that cytotoxic drugs with diverse mechanisms of action all act to up-regulate CD26 cell-surface expression on a variety of colorectal carcinoma cell lines. Furthermore, these agents enhance the functionality of CD26 by increasing dipeptidyl peptidase activity and ADA-binding capacity. Finally, we determined that CD26 is up-regulated by HT-29 cells in an *in vivo* environment and is further enhanced through treatment with 5-FU, irinotecan, or oxaliplatin. These data suggest that anticancer drugs may act to reduce tumour growth and metastatic spread by enhanced ADA-mediated reduction of extracellular adenosine concentrations, as well as enhanced DPPIV cleavage of CXCL12. This is a novel mechanism of action of these agents which may be masked by their cytotoxicity when they are administered at the maximally tolerated doses.

CHAPTER 4

SUMMARY AND CONCLUSIONS

CXCR4 and CD26 are related through CXCL12

CXCR4 and CD26 are two cell-surface molecules that have been linked to one another in a number of ways. Early publications demonstrated the ability of CD26 to cleave the CXCR4 ligand, CXCL12, leading to reduced CXCL12 binding to its receptor. The importance of this cleavage and subsequent binding inhibition is several-fold. Firstly, this cleavage inhibits the chemotactic capacity of CXCL12 leading, for example, to reduced hematopoietic stem cell homing to the bone marrow (Christopherson, Hangoc *et al.* 2002; Christopherson, Hangoc *et al.* 2004). Secondly, reduced CXCL12 binding to CXCR4, because of CD26-mediated cleavage, reduces cancer cell proliferation (Mizokami, Kajiyama *et al.* 2004). A third important consequence of CXCL12 cleavage is in relation to HIV-1 entry. CXCR4 is a co-receptor for HIV-1, and CXCL12 binding to CXCR4 blocks the ability of the HIV-1 gp120 protein to bind to T cells, therefore reducing HIV-1 infectivity (Figure 1.5; Bleul, Farzan *et al.* 1996; Oberlin, Amara *et al.* 1996; Brelot, Heveker *et al.* 2000). T cells with increased CD26 expression show increased rates of HIV-1 infection, through enhanced CXCL12 cleavage and reduced antagonism of HIV-1 binding (Callebaut, Jacotot *et al.* 1998; Shioda, Kato *et al.* 1998).

CXCR4 and CD26 are directly and inversely regulated in different cell types

CXCR4 and CD26 are not only related through CXCL12; there is also evidence supporting their direct interaction in normal lymphocytes (Herrera, Morimoto *et al.*

2001). In this body of work, Herrera and colleagues showed that CXCR4 and CD26 colocalize and coimmunoprecipitate in T and B cell lines and primary lymphocytes. This conflicts with observations from our laboratory, that CXCR4 and CD26 expression seem to be oppositely linked: if a certain stimulus decreases CXCR4, this same stimulus increases CD26. For example, I have shown that cytotoxic drugs decrease CXCR4 and increase CD26; previous lab members have shown that adenosine increases CXCR4 (Richard, Tan *et al.* 2006) and decreases CD26 (Tan, Mujoomdar *et al.* 2004; Richard, Tan *et al.* 2006). However, it is possible that these two molecules may be associated with one another in lipid rafts in the cell membrane.

We have noticed that the drug-mediated changes in CD26 tend to precede the changes in CXCR4. It is therefore unlikely that the reduction in CXCR4 following drug treatment is a consequence of ligand-mediated down-regulation. This is because enhanced CD26 would limit the available CXCL12 in the cell-culture medium by means of its dipeptidase activity. In support of this theory, we found that pre-treatment of cells with the dipeptidase inhibitor, diprotin A, had no effect on drug-mediated CXCR4 reduction (data not shown).

CXCR4 and CD26 are regulated during cellular differentiation

It is interesting to note that while CXCR4 tends to be a marker of undifferentiated cells, CD26 is a marker of differentiation. In fact, differentiation of HT-29 cells results in a complete loss of CXCR4 mRNA (Jordan, Kolios *et al.* 1999) and, in contrast, an up-regulation of CD26 mRNA and protein (Darmoul, Lacasa *et al.* 1992). The role of cytotoxic drugs in HT-29 cell differentiation is not clear. In some cases, they cause

changes consistent with differentiation (Sharma, Adam *et al.* 1997; Singh, Fouladi-Nashta *et al.* 2006), while in other situations they do not (Singh, Fouladi-Nashta *et al.* 2006), even with the use of the same drugs and cell lines. It is possible that cytotoxic drug-mediated differentiation of HT-29 cells results in the modulation of CXCR4 and CD26 cell-surface expression. Another related possibility is that these drugs may target undifferentiated cells more so than their differentiated counterparts, as has been previously postulated (Schumacher, Adam *et al.* 2001). If these agents preferentially target the cells expressing high CXCR4 and low CD26 it would manifest as a decrease in relative CXCR4 expression and an increase in relative CD26 expression.

Cytotoxic drugs may not regulate CXCR4 and CD26 through the same pathway

It is also possible that these changes could occur coincidentally and may not be related at all. For example, the increase we have observed in CD26 could be a consequence of heat-shock protein activation. Heat-shock proteins are a class of cellular stress-sensing molecular chaperones. Heat-shock proteins are over-expressed by many cancerous cells and tissues (Ciocca, Clark *et al.* 1993; Kimura, Enns *et al.* 1993; Conroy, Sasieni *et al.* 1998) and respond to cellular stress by facilitating the folding of cell-survival proteins (Takayama, Reed *et al.* 2003). If CD26 is a target for Hsp90, for example, activation of Hsp90 by chemotherapy drugs would enhance CD26 folding and mature expression. In contrast, the reduction in CXCR4 could be a consequence of transcription factor activation. For example, YY1 is an important transcription factor which represses CXCR4 expression (Moriuchi, Moriuchi *et al.* 1999; Hasegawa, Yasukawa *et al.* 2001) and YY1 is known to be activated by cytotoxic drugs (Gronroos,

Terentiev *et al.* 2004). It is possible that cytotoxic drug treatment activates YY1 which, in turn, represses CXCR4 expression.

Future Studies

Further studies in the laboratory will examine how these diverse agents each achieve the same end result (a reduction in CXCR4 expression and a concomitant increase in CD26 expression). We speculate that it might be a result of a common event such as p53 activation (Kastan, Zhan *et al.* 1992; Canman, Lim *et al.* 1998; Khanna, Keating *et al.* 1998; Lakin, Hann *et al.* 1999), p38 MAPK activation (Deacon, Mistry *et al.* 2003), or HIF-1 α inhibition (Escuin, Kline *et al.* 2005; Duyndam, van Berkel *et al.* 2007), which occurs following administration of any of these drugs. Whether these two processes, CXCR4 down-regulation and CD26 up-regulation by cytotoxic drugs, are linked through heat-shock protein activation, cellular differentiation or some other mechanism, they should act in combination to reduce tumour cell proliferation and metastatic spread.

Conclusions

In conclusion, I have shown that chemotherapy agents increase CD26 expression, dipeptidase activity, and ADA-binding capacity. These effects on CD26 occur *in vivo*, suggesting that these agents may have anti-metastatic properties in cancer patients. I have also demonstrated that these drugs decrease CXCR4 expression and CXCL12-mediated migration. It is important to note that a number of companies and research labs are currently researching small molecule inhibitors of CXCR4, in an attempt to reduce

tumour cell proliferation and metastatic spread (Hendrix, Flexner *et al.* 2000; Doranz, Fillion *et al.* 2001; Hendrix, Collier *et al.* 2004; Hatse, Princen *et al.* 2005). Other groups are researching CXCR4 as a tumour-specific marker for targeted therapies (Snyder, Saenz *et al.* 2005). My findings suggest that tumour CXCR4 may be reduced as a result of the chemotherapy that nearly all patients receive. In contrast to specific CXCR4 inhibitors, these agents are widely available and inexpensive; furthermore, these data imply that CXCR4-targeted therapies will be of limited clinical use.

These findings support future studies examining the use of low-dose maintenance therapy following treatment with traditional chemotherapy regimens. This may reduce this incidence of progression or disease relapse at metastatic sites through maintaining high CD26 expression and low CXCR4 expression by remaining tumour cells.

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