[Frontiers in Bioscience 10, 853-865, January 1, 2005]

# THE ROLE OF INTERLEUKIN-8 IN CANCER CELLS AND MICROENVIRONMENT INTERACTION

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## 1. ABSTRACT

Interleukin (IL)-8, a cytokine of the CXC chemokine family that was originally classified as a neutrophil chemoattractant, is now reported to play an important role in tumor progression and metastasis in a variety of human cancers, including lung cancers. IL-8 biologic activity in tumors and the tumor microenvironment may contribute to tumor progression through its potential function in the regulation of angiogenesis, cancer cell growth and survival, tumor cell motion, leukocyte infiltration and modification of immune responses. Recently, infiltrating macrophages in tumor stroma have been considered to be able to stimulate cancer growth, enhance angiogenesis and promote metastasis, and has prognostic significance in several human cancers. Accumulating evidence also shows that cancer cells and stromal cell interaction can stimulate cancer cells, as well as stromal cells in the expression of IL-8 and other growth factors. Here, we summarize current information about IL-8 biology in human lung cancers and focus on its effect on tumor angiogenesis, regulation of IL-8 expression in tumors, its prognostic significances, the role of tumor infiltrating macrophages in the production of IL-8 in cancer cells and the tumor microenvironment, gene expression profiles after cancer cell-stromal cell interaction, and the effect of a variety anti-inflammatory drugs on the modification of IL-8 and other gene expressions in cancer cells and the tumor microenvironment in lung cancers.

## 2. INTRODUCTION

Interleukin (IL)-8 was first purified and molecularly cloned as a neutrophil chemotactic factor in the supernatant of lipopolysaccharide-stimulated human mononuclear cells (1, 2). IL-8 belongs to a super family of chemokines that has chemotactic activity for neutrophils, esosinophils, basophils, monocytes, mast cells, dendric cells, nature killer (NK) cells, and T and B lymphocytes (3). The chemokine are divided into four subgroups, CXC, CC, CX3C and C chemokines, in which C indicates NH2-terminal cysteines and X indicates intervening amino acid, respectively (4). IL-8 is a member of the CXC chemokine family and is now classified and referred to as CXCL8 according to the new nomenclature systems (4). CXC chemokines can be further subclassified into Glu-Leu-Arg $(ELR)^+$ and $ELR^-$ CXC chemokines, based on the presence or absence of tripeptide motif ELR of the NH2 terminal before the first cysteine. $ELR^+$ CXC chemokines and $ELR^-$ CXC chemokines have different biological functions (5).

The gene encoding cytokine IL-8/CXCL8 is found on human chromosome 4, q12-21, and consists of four exons and three introns (6, 7). The 5'-flanking region of IL-8 contains "CCAAT" and "TATA" box-like structures and has a number of binding sites for transcriptional factors (8). IL-8 cDNA encodes a 99 amino acid precursor protein, which is cleaved to yield 77 or 72-residue mature proteins (2). IL-8 can be produced by leukocytic cells and non-leukocytic somatic cells, including endothelial cells, fibroblasts and epithelial cells (9-12). Under most physiologic conditions, IL-8 expression is not constitutive, but inducible by proinflammatory cytokines and other stimuli (2).

In terms of chemokine receptors, the following chemokine receptors have been described: about six CXCRs, 10 CCRs, one CR and one CX3CR (13). IL-8 binds to two distinct receptors, CXCR1 and CXCR2, with a similar high affinity (14, 15). CXCR1 and CXCR2 have been shown to be expressed in a variety of cells, such as leukocytes, endothelial cells and malignant cells (13).

The biologic effect of IL-8 originally described includes chemotaxis for a variety of leukocytes, facilitating leukocyte transmigration into the tissue by inducing adhesion molecule expression and promoting neutrophil adhesion to extracellular matrix, activating various functions of neutrophil, including degranulation and the release of

leukotrienes B4 and platelet-activating factors (10, 16). Recent evidence has revealed that IL-8 has several biologic functions not related to leukocyte chemotaxis and migration and may play an important role in cancer progression.

Tumor associated IL-8 expression was first found in malignant melanoma cell lines, and its expression was considered to play a role in regulating the growth and metastasis of melanoma (17). Endogenous expression of IL-8 has since been found in various human cancers, including AML (18), B-cell CLL (19), breast cancer (20), colon cancer (21), cervical cancer (22), gastric cancer (23), Hodgkin's lymphoma (24), and ovarian, prostate and lung cancers (25-27). These tumors are characterized by disregulated IL-8 production, as well as abnormal IL-8 receptor expression and signaling in tumor cells and/or microenvironmental stromal cells. Tumor associated IL-8 is thought to play at least five roles in the biology of primary and metastatic cancers, including the following: (1) control leukocyte infiltrate into tumor tissue, (2) modification of tumor immune response, (3) regulation of angiogenesis, (4) autocrine or paracrine regulation of tumor growth and survivals, and (5) promoting tumor cell migration (28).

Evidence shows that IL-8 biologic activity in tumors and the microenvironment may contribute to cancer progression, and in other circumstances to host anti-tumor response, and this biologic response may be different in different types of human cancers. In this article, we focus on discussion of current information about IL-8 biology in human lung cancers, especially non-small cell lung cancers. This will help develop novel biological therapy for lung cancer in the future.

## 3. IL-8 AND TUMOR ANGIOGENESIS

Koch *et al.* was one of the first to demonstrate that IL-8 derived from macrophages/monocytes had an angiogenic effect in rat cornea models and could induce proliferation and chemotaxis of human umbilical vein endothelial cells (29). The angiogenic activity in condition medium of cultured macrophages isolated from rheumatoid synovial tissue can be blocked by either IL-8 neutralizing antibodies or IL-8 antisense oligonucleotide. The involvement of IL-8 in tumor angiogenesis was first demonstrated by Smith *et al*, who showed that IL-8 was overexpressed in the cancer cells of bronchogenic carcinoma. The angiogenic activity of tissue homogenate of tumors in an *in vitro* endothelial cell chemotaxis model and an *in vivo* corneal neovascularization model can be attenuated by neutralizing antiserum to IL-8 (27, 30). The angiogenic activity of macrophage derived IL-8 and tumor cell derived IL-8 was later confirmed in several studies (31-38). Richard *et al.* showed that IL-8 receptor expression (IL-8RA and IL8-RB) was strongly expressed in endothelial cells, with IL-8RB expression being high in microvessels, and IL-8RA expression high in large vessels (39, 40). The interaction of IL-8 and IL-8R can promote angiogenesis through the induction of endothelial cell migration, proliferation and chemotaxis (41).

Recent studies have shown that IL-8 was constitutively expressed in several human cancer cell lines derived from astrocytoma, hepatoma, transitional cell carcinoma, and melanoma (42-45), and is associated with angiogenesis and metastatic potential in human melanoma cell lines in a nude mice model (45, 46). Arenberg and coworkers (30) showed that the anti-IL-8 antibody could reduce tumor growth and intratumoral microvessel density in xenografts of the A549 cell line (lung adenocarcinoma) in severe combined immunodeficient (SCID) mice. Several studies have demonstrated in xenograft models that IL-8 overexpression is directly correlated with angiogenesis, tumorigenesis and metastatic potential in some cancer cell lines, such as human melanoma, pancreatic cancer, prostate cancer, gastric cancer, and transitional cell carcinoma (24, 45, 47).

IL-8 overexpression has also been demonstrated in tumor specimens of several human solid cancers, such as squamous cell carcinoma of the head and neck (HNSCC) (48), colorectal cancer (49), glioblastoma (50), melanoma (51) and lung cancer (52). Yatsunami *et al.* showed that IL-8 immunoreactivity was positive in 36 of 56 (64%) NSCLC specimens, but negative in most small cell carcinoma (52). In terms of its relationship to tumor angiogenesis, Kitadai *et al.* showed that in tumor tissue the IL-8 expressed at higher levels than in corresponding normal mucosa, correlating strongly with vascularization of human gastric carcinoma (53). Haraguchi *et al.* (54) showed that in colorectal cancer, the IL-8 levels in the tumor tissue and the serum IL-8 levels were significantly correlated with the microvessel density, and serum IL-8 levels were higher in Dukes' C colorectal cancer with hepatic metastasis than in Dukes' A and B cancer, and Dukes' C cancer without hepatic metastasis. They concluded that elevated IL-8 levels in resectable Dukes' C colorectal cancer indicates a high risk for developing hepatic metastasis. Tahara *et al.* (55) showed that IL-8, IL-8RA and IL-8RB are expressed in the majority of gastric carcinomas, and IL-8 increases expression of EGF receptor, VEGF and IL-8 itself by tumor cells themselves, whereas IL-8 decreases expression of E-Cadherin and increases MMP-9 expression and activity. These results suggest that IL-8 produced by gastric cancer cells is used to sustain angiogenesis and tissue invasion and metastasis via autocrine/paracrine mechanisms.

We evaluated IL-8 mRNA expression, using real-time quantitative reverse-transcription polymerase chain reaction (RTQ RT-PCR) in 58 NSCLC surgical specimens, and correlated tumoral IL-8 mRNA expression with clinicopathologic variables, intratumoral MVC and patient outcome (56). The results showed that high IL-8 mRNA expression is associated with tumors in advanced stages, distant lymph node metastasis, high tumor MVC, short patient survival and early disease relapse. Multivariate analysis revealed that IL-8 mRNA expression and intratumoral MVC were the most important predictors of patient survival and disease relapse. This study indicates that IL-8 expression is strongly associated with tumor angiogenesis, tumor progression and patient outcome in NSCLC, which also highlights the potential of targeting IL-8 in cancer therapy in the future.

In addition to IL-8, several other chemokines may also regulate angiogenesis in the epithelial tumor microenvironment. CXC chemokines containing the three amino acids of glutamine-leucine-arginine (ELR) motif, including IL-8, CXCL1, CXCL5 (ENA-78), CXCL6 (GCP-2) and CXCL7 (NAP-2), promote angiogenesis (57). In contrast, CXC chemokines, such as CXCL9 and CXCL10, which lack the ELR motif, are often anti-angiogenic (57). CXCL10 levels in human lung cancer has been found to be inversely correlated with tumor progression (57).

IL-8 is also a well-known chemoattractant factor for leukocytes, and leukocytes infiltration is frequently seen in some types of human cancers, including NSCLC. These recruited inflammatory cells can enhance angiogenesis by secreting several cytokines, such as tumor necrotic factor (TNF)-α. Thus, in addition to its direct angiogenic effect, IL-8 secreted by tumor cells might also indirectly induce angiogenesis by recruiting inflammatory cells to the tumor tissues (56).

IL-8 has also been reported to have an autonomous and mitogenic effect on tumor cells that produce IL-8 themselves in certain types of human cancers. Schadendorf *et al.* was one of the first who demonstrated that endogenously produced IL-8 could act as an important growth factor for human melanoma cells (57). Antisense oligonucleotides against human IL-8 mRNA can inhibit IL-8 protein expression and the subsequent tumor cell proliferation and colony formation in soft agar in a dose-dependent fashion in melanoma cell lines. The mitogenic effect of IL-8 on tumor cells that secrete it has been further supported by the subsequent identification of IL-8 receptors (IL-8Rα and IL-8Rβ) in melanoma cell lines, colorectal carcinoma cells and pancreatic cancer (58-60). However, this autocrine growth effect of IL-8 on tumor cells has not been proven in NSCLC yet. The addition of a neutralizing anti-IL-8 antibody to the culture medium did not alter the proliferation of several NSCLC cell lines *in vitro* (30), and IL-8 receptors have not been reported to be expressed in lung cancer cells. The role of IL-8 as an autocrine growth factor in NSCLC requires further clarification.

Although many reports have demonstrated that tumoral IL-8 overexpression is associated with tumor angiogenesis, *in vitro*, *in vivo* or in clinical studies, several studies have demonstrated no correlation between IL-8 expression and tumor angiogenesis in some melanoma and pancreatic cancer cell lines (61, 62). This may result from a different origin of cancer cell lines, use of different xenograft models, and *in vitro* and *in vivo* selection of cell lines in prolonged culture and xenograft growth.

## 4. REGULATION OF IL-8 EXPRESSION IN TUMORS

IL-8 expression can be induced by various stimuli, such as lipopolysaccharides, cytokines (IL-1, TNF-α), and bacterial or viral products, while IL-8 is also constitutively expressed in many human cancers (42-51). Cellular stress, such as hypoxia and acidosis, nitric oxide (NO) and cell density was also recently found to have significant influence on IL-8 expression in human cancers (50, 63).

In a number of studies, it was found that a sequence spanning nucleotide from -1 to -133 within 5' flanking region of the IL-8 gene is essential and sufficient for transcriptional regulation of IL-8 gene expression (64, 65). As demonstrated by site directed mutagenesis analysis, this promoter element contains the binding sides for activating protein (AP)-1, nuclear factor (NF)-κB and CAAT/enhancer-binding protein (C/EBP) (also called NF-IL-6-like factor) (66, 67). The NF-κB site is essential for the induction of IL-8 expression, while the NF-κB and AP-1 sites have been shown to be mainly responsible for constitutive expression of IL-8 in certain human cancers. The maximal IL-8 expression has been reported to be generated by a combination of three different mechanisms: (1) transcriptional activation of genes by NF-κB and JUN-N-terminal protein kinase pathway, (2) derepression of IL-8 gene promoter by histone acetylation and (3) mRNA stabilization by p38 mitogen-activated protein kinase pathway (66).

The growth of tumors is dependent on neovascularization. However, although angiogenesis activity increases in the majority of solid tumors, a lot of cancer cells are still exposed in a hypoxic environment, either due to immature and impaired functional tumor microvasculature or due to too rapid a proliferation of cancer cells. A local decrease in oxygen tension may cause the induction of many angiogenic factors, including IL-8. Immunohistochemical studies have shown that IL-8 is predominately expressed in the tumor cells surrounding necrotic areas of human cancer lesions in glioblastoma, malignant melanoma and pancreatic cancer (50, 68-70). Desbaillets *et al.* (50) demonstrated that expression of a human glioblastoma cell line to anoxic stress might cause upregulation of both IL-8 and VEGF mRNA, but with different time courses. This upregulation of IL-8 mRNA and protein expression is also associated with increased protein binding to the AP-1 site on IL-8 promotors. Kunz *et al.* (70) showed that in melanoma cell lines, anoxia might induce IL-8 expression in highly aggressive/metastatic cell lines, but not in poorly aggressive cell lines (70). Kepin *et al.* also showed that hypoxia increases the transcriptional rate of the IL-8 gene and stability of IL-8 transcript in pancreatic cancer (71). A decrease in extracellular pH also promotes hypoxia-mediated IL-8 upregulation and causes rapid IL-8 expression in colon cancer and pancreatic cancer cell lines (72). Mild acidosis (pH=6.9~7.4) can activate AP-1 and NF-κB activity (73).

Other stimuli can also regulate constitutive IL-8 expression in tumors. IL-8 expression has been shown to be upregulated by inflammatory cytokines (74) and ultraviolet light (46) in melanoma, and downregulated by interferon (INF)-α and INF-β (75). Paclitaxel and all-trans retinoic acid enhances IL-8 protein release in ovarian cancer cell lines (76, 77). Cell density has also been shown to directly influence IL-8 expression in culture conditions (73).

Tumor oncogenes and suppressor genes have been reported to play important roles in carcinogenesis, tumor progression, and in the metastatic process of many human cancers. In terms of NSCLC, oncogene, such as c-

myc and Ras activation, and tumor suppressor genes, such as p53 gene inactivation (mutation or aberrant expression) are important in cancer development. Whether oncogene activation or tumor suppressor gene inactivation in cancer cells can influence the constitutive IL-8 expression in NSCLC remains unclear. We evaluated aberrant p53 expression in 65 NSCLC surgical specimens and investigated the correlation between aberrant p53 expression and IL-8 and VEGF mRNAs expression, and intratumoral MVC and patient survival (78). The results showed that tumors with high aberrant p53 expression show significantly higher VEGF and IL-8 mRNA expression and higher MVC than those with low aberrant p53 expression. Survival and postoperative relapse time were significantly shorter in patients with high aberrant p53 expression and high IL-8 mRNA expression in tumors. This result indicates that p53, in addition to its role as a tumor suppressor and apoptosis inducer, might also inhibit angiogenesis by negatively regulating VEGF and IL-8 mRNA in NSCLC. The mechanism involved in regulation of IL-8 expression by p53 proteins in cancers is still unclear. Several studies have shown a reverse association between wild-type p53 expression and IL-8 expression in inflammatory diseases (79, 80). Michel *et al.* found that antipsoriatic agent Tacrolimus (FK506) causes decreased expression of IL-8 mRNA in cultured primary keratinocytes, with concomitant increased wild-type p53 mRNA and protein expressions (79). Mirmohammadsadegh *et al.* showed that in epidermal cells, N-(trifluoromethyl phenyl)-2-cyano- 3-hydroxy-crotonic acid amide caused a dose-dependant reduction in IL-8RA mRNA expression and upregulation of wild-type p53 expression *in vitro* (80). Whether wild-type p53, as a transcriptional factor, binds to the TATA box or other unknown p53 binding site in IL-8 gene promoter, and whether it represses IL-8 mRNA transcription, needs to be clarified by further studies.

## 5. TUMORAL IL-8 EXPRESSION AND CLINICAL OUTCOME

Accumulating evidence generated in clinical studies shows increased tumoral IL-8 expression correlates with adverse patient prognosis in a variety of human cancers. High tumoral IL-8 expression has been shown to be associated with progressive and recurrent disease in breast cancer (81), associated with poorly differentiated tumors and liver and lung metastasis in colon cancer (49), associated with superficial spreading of melanoma and a worsening prognosis (82), and with an increased prostate cancer stage (83). Other studies have shown that IL-8 expression is associated with a poor prognosis in epithelial ovarian cancer patients (84), as a marker of advanced disease in gastrointestinal non-Hodgkin's lymphoma (85), and is associated with a higher TNM stage, more recurrence, and shorter disease-free survival in head and neck squamous cell carcinoma (86). Serum IL-8 levels have been shown to be correlated with an increasing prostate cancer stage (83) and with metastatic melanoma (87). In addition, IL-8 mRNA is up-regulated in the peripheral blood leukocyte (PBN) of patients with metastatic prostate cancers (83). In contrast, a few studies have shown a lack of correlation between IL-8 expression in tumors and patient outcome. Inoue *et al.* (88) evaluated the prognostic value of several angiogenic factor expressions for predicting recurrence and metastasis of bladder cancer after neoadjuvant chemotherapy and cystectomy, and they found that among bFGF, VEGF, IL-8 and microvessel density (MVD), only the bFGF, VEGF and MVD identifies patients who are at high risk of developing metastasis.

## 6. CANCER CELL AND MICROENVIRONMENT STROMAL CELL INTERACTION

Cancer progression is a complex multi-step process that consists of transformation, tumor growth, invasion and metastasis. Recent evidence shows that stromal extracellular matrix and stromal cells (including fibroblast, inflammatory cells and endothelial cells etc) play an important role in promoting tumor progression. Reactive stroma in tumor enhances tumorigenesis by supporting cancer cell survival, proliferation, migration and by inducing angiogenesis (89). Cancer cells can secrete a variety of growth factors that act on tumor cells themselves and increase proliferation, migration and decrease apoptosis of cancer cells (cell-autonomous effect) (90). In addition, genetically changed epithelial cells can change the stromal compartment so as to establish a permissive and supportive environment for growth, de-differentiation, invasion and ectopic survival of cancer cells. This is also called a "landscaping effect" (90).

Therefore, cancer progression is not exclusively regulated by the disruption of oncogene and tumor suppression genes in cancer cells, but also depends on the stromal compartment to create a more tumor promoting microenvironment. The interaction between cancer cells and the stromal cells has been shown recently to promote tumorigenesis and might be a new target for cancer therapy in the future.

The stroma was initially considered as a passive supportive structure that contributes little to the biological function of tissues. Increasing evidence has indicated that stroma is a dynamic environment directly affecting epithelial cell behavior. The stromal compartment consists of stromal cells and an extracellular matrix containing a variety of growth factors, regulatory molecules and remodeling enzymes (91). Modification in stromal cell phenotype, extracellular matrix composition has been reported in several types of human cancers, including breast and colon cancers (91, 92). The reactive stroma (also referred to as desmoplasia) generates a new microenvironment that affects cancer progression, and this stroma reaction includes stromal cell phenotype switching, extracellular matrix remodeling, increased growth factor bioavailability, increased protease activity, increased angiogenesis and influx of inflammatory cells (89). TGF-β expression from cancer cells and the myofibroblast phenotype switching are well-known examples of the cross-talk between cancer cells and the stroma, and can increase cancer cell invasion by stimulating degradation of ECM, enhancing migration of cancer cells, disturbing of adhesion and inducing angiogenesis and lymphangiogenesis (93). This molecular cross-talk between cancer cells and stroma has been demonstrated in colon cancer (94).

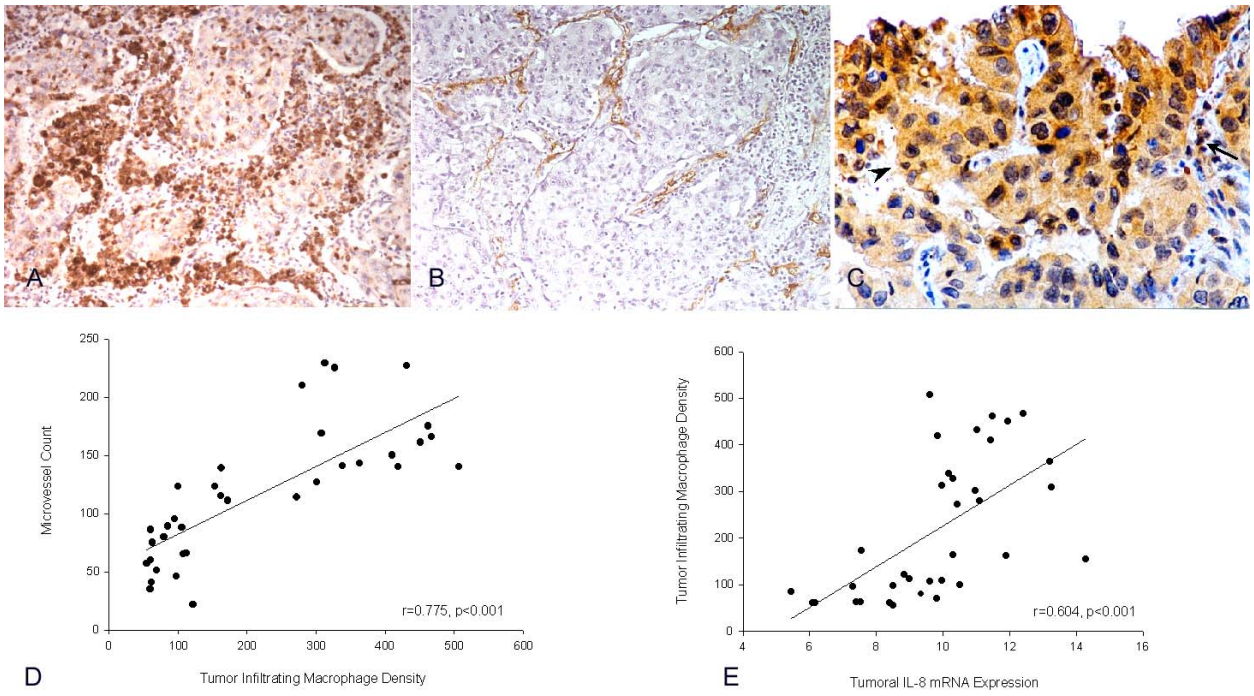


**Figure 1.** Immunohistochemical staining of tumor infiltrating macrophages (A), microvessel counts (B) and IL-8 protein expression (C) in a NSCLC. The arrowhead in (C) indicates IL-8 protein expression in lung cancer cells and the arrow indicates IL-8 protein expression in tumor infiltrating macrophages. The tumor infiltrating macrophage density was correlated with MVC and IL-8 expression (D and E). Macrophages, microvessel and IL-8 protein expression were stained using mouse monoclonal anti-CD68 antibody, mouse polyclonal anti-CD34 antibody and mouse monoclonal anti-IL-8 antibody respectively as the primary antibody. The microvessel counts, tumor infiltrating macrophage counts, and the detail procedures of immunohistochemical staining are described in the previous reports (56, 107, 109).

The interaction between tumor cells and surrounding stromal elements may promote the release of angiogenic factors. Anderson *et al.* has shown that NSCLC/fibroblast interaction can promote the release of bFGF from stromal fibroblast (95). NSCLC cancer cells and pulmonary fibroblast co-culture also induce IL-8 mRNA and protein expression in both fibroblast and NSCLC cell lines (96). Tumor/stromal interaction may influence the production of other angiogenic factors, such as VEGF and hepatocyte growth factor (HGF) (97, 98). Fukumura *et al.* has shown that the VEGF promoter is strongly activated in tumor-associated fibroblast in a transgenic mice model (97). Nakamura *et al.* also showed the induction of hepatocyte growth factor expression in fibroblasts co-cultured with tumor cells, also showing the induction of HGF affecting the invasiveness of tumor cells of human NSCLC, small cell lung cancer, cholangiocellular carcinoma cells and epidermoid carcinoma cells (98). They further demonstrated that the IL-1, bFGF and platelet-derived growth factors (PDGF) derived from tumor cells play a role in inducing HGF expression in stromal fibroblasts, and the HGF from the fibroblast, in turn, leads to invasive growth in carcinoma cells (98).

Recently, tumor-infiltrating macrophages (TIM) were considered as constituting an important interface between tumor cells and the immune system, and TIM may influence neoplastic growth and progression in several ways (99-101). Multiple studies have demonstrated the association between increased tumor vascularity and macrophage infiltration in several human cancers (102-104), suggesting TIM enhances the angiogenic potential of tumors. Macrophage infiltration has been shown to correlate with vessel density in endometrial, ovarian, breast and central nervous system malignancies (102-105). Furthermore, a high density of tumor-associated macrophages have been shown to be associated with adverse patient prognosis in NSCLC and breast cancers (99, 106). We measured TIM density in 35 NSCLC tumor specimens and correlated TIM density with intratumoral microvessel count, tumoral IL-8 expression and clinical outcome in patients. We found that TIM density correlated significantly and positively with tumoral IL-8 expression and intratumoral MVC, and significantly and negatively with patient survival (Figure 1) (107). The IL-8 expression was not only located in the cytoplasm of cancer cells, but also in tumor infiltrating macrophages (107). These results are consistent with those of other studies (102-105), suggesting that TIM plays an important role in increasing tumor angiogenesis, and is associated with an adverse prognosis in certain human cancers, implying that IL-8 is an important angiogenic factor secreted from tumor cells and the microenvironment (including TIM) to enhance tumor progression.

The actual mechanism of how tumor-infiltrating macrophages are associated with increased angiogenesis is currently under extensive investigation. White *et al.* examined

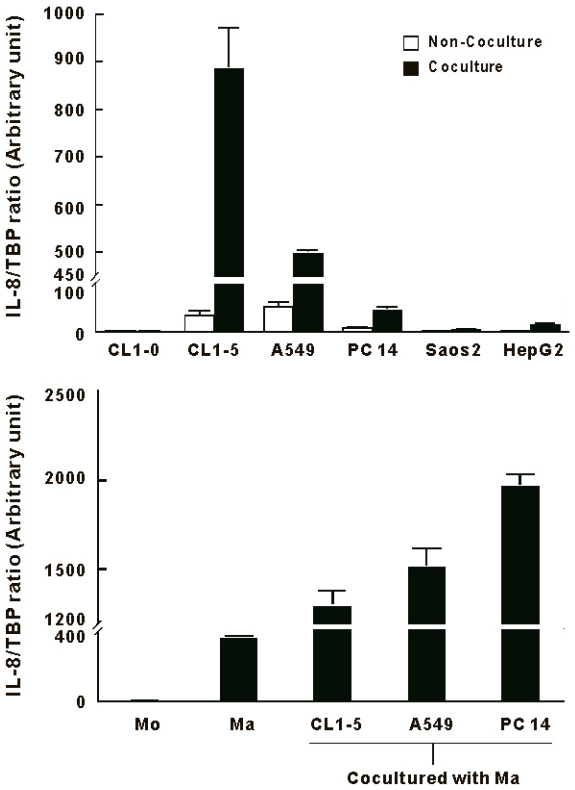


**Figure 2.** Induction of IL-8 mRNA expression in NSCLC cancer cell lines after co-culture with macrophage (upper), and in macrophage after co-culture with lung cancer cells (lower). After co-culture for 24 hours, cancer cells (or macrophages) were harvested to isolate total RNA using RNAzol B (Tel-Test, Friendswood, TX). 10ng of total RNA for each sample was used to determine IL-8 mRNA expression by real-time quantitative RT-PCR. TATA box binding protein (TBP) was quantified as an internal control. The relative amount of IL-8 cDNA standardized against the amount of TBP cDNA was measured as $-\Delta CT$(threshold cycle)$= -[CT_{IL\text{-}8} - CT_{TBP}]$. The relative ratio of IL-8 mRNA copies to TBP mRNA copies was defined as $2^{-\Delta CT} \times K$ ( K: constant).

the angiogenic potential and angiogenic factors expression in conditioned medium (CM) from coculture of peripheral blood monocot (PBM) and A549 (human bronchoalveolar cell cancers) cell lines, showing a marked increased in CXC chemokine expressions (including IL-8, ENA-78, GROα, and IP-10) and a lesser increase in VEGF in CM form PBM/A549 coculture. The neutralizing antibodies to CXC chemokines blocked the increase in endothelial cell chemotaxis. NSCLC-derived macrophage migration-inhibitor factor has been shown to be responsible for the increase of macrophage-derived angiogenic activity (108). Tumor-necrosis factor-α (TNF-α) and thymidine phosphorylase (TP), on the other hand, have been shown to play a role in TIM associated angiogenesis in breast cancer (109) and endometrial cancer (110).

We used the coculture system to evaluate IL-8 mRNA expression (by RTQ RT-PCR) in four NSCLC cancer cell lines and human macrophages (THP-1, ATCC TIB202; ATCC, Manassas, VA) before and after cancer cells-macrophage interaction (105). The results (Figure 2) show that after cancer cell-macrophage interaction, the IL-8 mRNA expression in NSCLC cell lines increased up to 270 fold compared to that expressed in cancer cells before co-culture with macrophages. The IL-8 mRNA expression was also upregulated in macrophages after their interaction with NSCLC cells, but the IL-8 mRNA expression increased, to a less degree, up to 4.5 fold as compared to that in macrophages before coculture with cancer cells (Figure 2). IL-8 protein secretion increased (3~9 folds) in co-culture medium as compared to non-coculture medium, after interaction of four NSCLC cell lines with human macrophages. We further evaluated the role of NF-κB in upregulating IL-8 expression in cancer cells after co-culture with macrophages using Western blotting and transcriptional regulation assays (electrophoresis motility shift assay [EMSA] and NF-κB binding domain luciferase activity assay) of nucleus extract proteins. The results showed that the NF-κB protein level increased in NSCLC cancer cell lines after coculture with macrophages, and this increase was concurrent with the increase in IL-8 mRNA expression. The NF-κB transcriptional activity assay showed that the luciferase activity increased 40 fold in CL1-5 NSCLC cell lines after co-culture with macrophages. These results indicate that the increase of IL-8 expression in NSCLC cell lines is regulated in part through the NF-κB pathway.

We further evaluated the effects of six anti-inflammatory agents, including pentoxifylline, aspirin, indomethacin, dexamethasone, celecoxib and pyrrolidine dithiocarbamate (also known as a specific NF-κB inhibitor) on the upregulation of IL-8 expression and NF-κB transcriptional activity in CL1-5 NSCLC cell lines after interaction with macrophages. The results demonstrate that all six anti-inflammatory agents suppress the upregulation of IL-8 expression, in a dose-dependant manner, in the NSCLC cell lines co-cultured with macrophages, and the IL-8 mRNA and protein expression fell to about 0~8.7% of the level in the absence of anti-inflammatory agents (107). The six anti-inflammatory agents also block the increase of NF-κB protein expression and NF-κB transcriptional activity in NSCLC cancer cell co-cultured with macrophages. These results highlight the potential use of anti-inflammatory agents in suppressing IL-8 upregulation in both cancer cells and macrophages after interaction, and the subsequent tumor-associated angiogenesis in NSCLC in the future.

In addition to the paracrine regulation of IL-8 expression in NSCLC cancer cells by macrophages, we also investigated the autocrine regulation of IL-8 expression in cancer cells by co-culture of fresh lung cancer cells with macrophage sensitizing lung cancer cells (unpublished data). The results show that IL-8 mRNA expression increased up to 5 fold in the fresh CL1-5 lung cancer cell after co-culture with macrophage sensitizing CL1-5 cancer cells. This autocrine upregulation of IL-8 expression in NSCLC cancer cells was through increased NF-κB expression and was modulated by IL-1-α and TNF-α. This autocrine upregulation of IL-8 expression in cancer

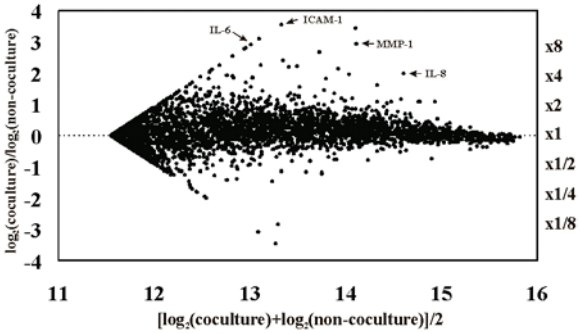


**Figure 3.** Alteration of gene expression profiles in NSCLC cancer cell lines after interaction with macrophages evaluated by a cDNA microarray containing 9600 human EST. mRNAs derived from CL1-5 cells stimulated with and without macrophage-conditioned medium were labeled with biotin during reverse transcription and then hybridized to microarrays with 9,600 putative genes to identify differentially expressed genes of CL1-5 cells between cocultured and non-cocultured with macrophages. All hybridization experiments were performed in triplicate. The details of targets preparation, hybridization, color development, image analysis, and spot quantification have been described previously (112). An MA-plot was employed to show the distribution of gene expression intensities between non-coculture and coculture. The arrows indicate genes, IL-6, ICAM-1, MMP-1, and IL-8, which had higher expression levels in CL1-5 cells cocultured with macrophages. The conventional criteria of 2-fold, 4-fold, and 8-fold differences were used to classify the significantly different genes.

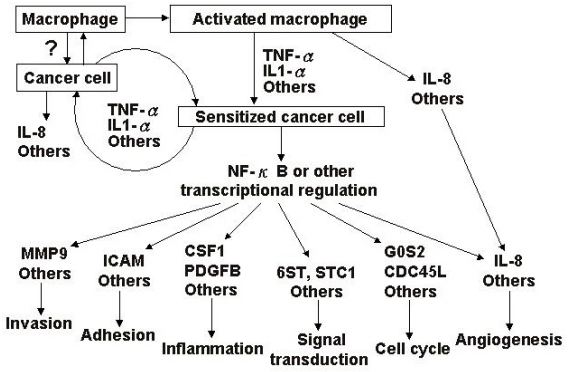


**Figure 4.** Summary of autocrine and paracrine IL-8 expression in NSCLC cancer cells and stromal cells (TIM) in the microenvironment, and the upregulated gene expression in NSCLC cancer cells after interaction of tumor infiltrating macrophages. The diagram only showed a part of genes as representatives potentially involved in NSCLC/macrophage interaction including: (1) invasion related genes such as MMP-9; (2) adhesion molecules such as ICAM-1; (3) inflammatory mediators and growth factors such as M-CSF1 and PDGFB; (4) signal transduction related genes such as IL-6ST and STC-1; (5) cell cycle regulators such as G0S2 and CDC45L; (6) angiogenesis related genes such as IL-8. Genes with multiple roles may be included in more than one category.

cells can also be suppressed by anti-inflammatory agents such as pentoxifyllines, aspirin and dexamethasone. These data indicate that IL-8 expression in NSCLC cancer cells can be upregulated by adjacent activated lung cancer cells, and this autocrine regulation of IL-8 expression in cancer cells may play an important role in substantiating tumor angiogenesis in NSCLC.

The macrophage is the pivotal member of inflammatory cells within the tumor stroma, and may perform a multitude of functions essential for tissue remodeling, inflammation and immunity by secretion of a variety of growth factors, cytokines and proteases (111). It has became increasingly clear that interaction between cancer cells and TIM may play an important role in enhancing tumorigenesis, angiogenesis, cancer invasiveness and metastasis (111), and this is supported by the adverse patient prognosis in human cancer, which has an associated high TIM density. To explore the other possible mechanisms by which TIM increases tumor progression with the interaction between cancer cells and TIM, we further investigated the tumor invasiveness and gene expression profiles of four NSCLC cancer cell lines subsequent to their interactions with macrophages using cDNA microarray (JCO in revision). We used a cDNA microarray containing 9600 human EST to perform a genome-wide analysis of the change of the gene expression profile in NSCLC after co-culture with macrophages, and we identified about 50 genes that were upregulated more than 2-fold in cancer cells after interaction with macrophages. These upregulated genes included genes involved in angiogenesis and lymphangiogenesis, cytokine and inflammation, adhesion and protease, signal transduction, cell growth and cell cycle regulation, metabolism and unknown functions. The examples of these upregulated genes were IL-6, IL-7R, IL-8, NF-κB, ICAM-1, MMP-1, MMP-9, VEGF-A, VEGF-C, etc. (unpublished data) (Figure 3). These NSCLC cancer cell lines post-stimulated by macrophages exhibited higher invasive potential (evaluated by *in vitro* invasion assay) and increasing matrix-degradation activity (evaluated by gelatin zymography) than in un-stimulated cancer cells. These results suggest that interaction between NSCLC cancer cells and TIM might enhance tumor progression and invasion by multiple mechanisms, including increased angiogenesis, cancer migration and invasion, cancer cell proliferation and other undefined mechanisms (Figure 4). This again confirms the importance of the cross-talk between NSCLC and stromal macrophages in microenvironment in regulating tumor progression. A better understanding of the contribution of cancer cell-stromal cell interaction to cancer progression will help us identify new therapeutic targets for treatment of NSCLC in the future.

## 7. CONCLUSION

IL-8 (CXCL8), a well-known chemotatic factor for leukocytes in inflammation, is now recognized to have multiple functions and contributes to human cancer progression. Increasing *in vitro* animal studies have shown that IL-8 upregulation can induce tumor cell proliferation,

angiogenesis, cancer cell migration, and can attract inflammatory cell infiltration, which in turn, produces a variety of factors that promote tumor angiogenesis and tumor growth. Clinically, constitutive over-expression of IL-8 in cancer cells is associated with increased tumor MVC, advanced stages, distant metastasis and adverse patient prognosis in several human cancers, including breast, colon, gastric, pancreatic cancers, melanoma and NSCLC. Constitutive IL-8 expression in human cancers can be upregulated by cytokines, hypoxia and acidosis, cell density, and is possibly influenced by tumor suppressor genes, such as p53.

Recent evidence has also shown that both cancer cells and stromal cells can contribute to tumor progression and invasion by production of growth factors and angiogenic factors, enhancing ECM degradation and cancer cell migration, and disrupting cell adhesion. Cancer cell and stromal cell (including TIM and fibroblast) interaction can induce IL-8 overexpression remarkably in both cancer cells and stromal cells in NSCLC. This upregulation of IL-8 expression in cancer cells is through paracrine and autocrine mechanisms. In addition to IL-8, tumor cells and TIM interaction can also affect other gene expression in cancer cells. Cancer cell and TIM interaction can induce activation of NF-κB and other transcription regulation pathways and upregulate a variety of down-stream gene expressions that are involved in angiogenesis, cell cycle, ECM degradation and invasion, cell-cell adhesion, signal transduction and metabolism. These upregulated gene expressions in cancer cells after interaction with TIM can further enhance tumor progression and invasions of NSCLC (Figure 4). We conclude that IL-8 expression in cancer cells and the microenvironment (stroma and stromal cells) may play a critical role in enhancing tumor progression, and the interaction between cancer cells and tumor infiltrating macrophages can result in upregulation of a variety of gene expressions (including IL-8) involved in invasive tumor growth in NSCLC. The information about IL-8 expression in cancer cells and the tumor microenvironment and the effects of cancer cell-stromal cell (TIM) interaction on tumor progression might indicate new directions in cancer therapy in the future.

## 8. ACKNOWLEDGEMENT

Drs Ang Yuan, and Jeremy J.W. Chen contributed equally to this work.

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**Key Words:** IL-8, Cancer cells, Microenvironment, Stroma cell, Tumor infiltrating macrophage, Angiogenesis, Gene expressions profile, Interaction, Review

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