Down-regulation of CXCL5 Inhibits Squamous Carcinogenesis

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Abstract

We report a novel role for the CXC-chemokine, CXCL5, in the proliferation and invasion of head and neck squamous cell carcinoma (HNSCC). Previously, we reported transcriptional up-regulation of CXCL5 in metastatic cells. In this study, we provide biological validation of these findings and show that CXCL5 is intimately involved in tumor cell proliferation, migration, and invasion. Cells derived from a lymph node metastasis, but not from a synchronous primary tumor, secreted CXCL5 as judged by Western blotting of conditioned media. We used RNA interference to generate cell lines (shL5) in which CXCL5 expression was greatly reduced, and tested whether this modulated the cell phenotype. shL5 cells showed decreased proliferation compared with cells harboring non-targeting control sequences. In addition, we found that the ability of shL5 cells to migrate and invade in vitro through a basement membrane substitute was greatly impaired compared with control cells. Finally, whereas control cells were highly tumorigenic in nude mice, the tumorigenic potential in vivo of shL5 cells was found to be ablated. Taken together, these data suggest that CXCL5 production contributes to both enhanced proliferation and invasion of squamous cell carcinomas and that targeting of chemokine pathways may represent a potential therapeutic modality for these lesions. (Cancer Res 2006; 66(8): 4279-84)

Introduction

Epithelial growth, development, and differentiation require a balance between growth promotion and growth inhibition, mediated through the activation and inactivation of specific biochemical pathways. During squamous cell tumor progression, these control mechanisms become perturbed and result in tumor cells acquiring properties, such as deregulated proliferation, altered responses to growth factors, enhanced survival, and increased motility and adhesion. This last attribute facilitates both the migration of tumor cells from the site of the primary lesion and their survival at distant sites, leading to formation of metastatic tumor deposits that frequently result in death.

Head and neck squamous cell carcinoma (HNSCC), encompassing oral, pharyngeal, and laryngeal cancer, is the sixth most common malignancy in the developed world and is a major world health problem. In spite of aggressive therapeutic combinations, the combined 5-year survival rate for these tumors is in the region of 50% (1). Although new treatment modalities are becoming available, including improved chemotherapeutic agents and molecular therapy, current management is both costly and disfiguring. Death from HNSCC generally occurs as a result of local invasion coupled with regional and/or distant metastatic spread of tumor cells from the primary tumor site. Five-year survival is extremely low for patients with late-stage disease and may be as low as 4% for stage IV hypopharyngeal lesions (1). To combat disease progression more effectively, it is critical that we understand the biochemical mechanisms that underpin the biological properties of invasive cells, so that rational therapies can be developed to control their behavior, leading to improved survival and quality of life for cancer patients.

Epithelial tumor cell metastasis is a complex, multistep process involving interactions between tumor cells, stromal cells, extracellular matrix, and vasculature. To migrate from the site of the primary lesion, tumor cells have to degrade basement membrane, migrate through surrounding structures, and intravasate into lymphatic or blood vessels. In addition, they must exit from the circulatory system, as well as survive and proliferate at a distant site. Thus, it is not surprising that multiple biochemical mediators, including growth factors, such as epidermal growth factor (EGF) and hepatocyte growth factor, cytokines, such as transforming growth factor β, and enzymes, such as matrix metalloproteinases, are thought to regulate these processes [reviewed by Fidler (2)].

Among these potential mediators of tumor spread, chemokines have begun to attract considerable interest (3). The chemokine superfamily comprises a large number of small, secreted proteins, all but two of which can be grouped into two classes: the CXC- and CC-chemokines (4, 5). These molecules function as immune modulators and may act as chemoattractants and/or activators of lymphocytes. However, they are also able to regulate other cell types. For example, the CC-chemokine macrophage inflammatory protein 1α can act as a negative regulator of keratinocyte growth (6). Chemokines signal through G-protein coupled receptors, and again, these can be categorized as CXCR or CCR in relation to the class of chemokine to which they bind. Stimulation of various chemokine receptors has been reported to activate signal transduction through a number of intracellular pathways, including Ras/extracellular signal–regulated kinase (7, 8) and phosphatidylinositol-3-OH kinase (8–10), as well as the small GTPases Rac, Rap, and cdc42 (11, 12), which are key mediators of actin reorganization and motility signaling (13).

Considerable interest has arisen from the fact that chemokines are key regulators of leukocyte trafficking and recruitment, leading to the hypothesis that tumor cells might use chemokine-dependent mechanisms for targeting to specific secondary sites. Indeed, in a landmark study, Müller et al. (14) showed specific up-regulation of the chemokine receptors CXCR4 and CCR7 in breast cancer cells and that their activation could induce actin polymerization and migration and invasion both in vitro and in vivo. Furthermore, this study identified expression of ligands for these receptors in organs...
that represent the primary sites for breast cancer metastasis, suggesting that this type of ligand-receptor "homing" mechanism functions in vivo to target tumor cells to secondary sites. Additional studies have reported up-regulation of chemokine receptors in breast (15), ovarian (7), and epidermoid carcinomas (16). Interestingly, a recent report indicated that alterations in expression of the chemokine receptors CCR6 and CCR7 occur in some HNSCCs (17). Specifically, this study showed loss of CCR6 expression in metastatic lesions with concomitant elevation of CCR7 and that ligands for CCR7 could induce migration of metastatic cells in vitro, suggesting that this latter event might play a role in targeting tumor cells to sites of secondary growth in vivo.

Our recent studies have used a model system of cell lines derived from synchronous primary HNSCC and nodal metastatic tumors to investigate growth factor signal transduction (18) and gene expression patterns (19) in cellular invasion. We identified groups of genes of which the expression difference between primary and metastatic cells, and found that metastatic cells expressed relatively high levels of the CXC-chemokine, CXCL5, compared with primary tumor cells. In the present study, we have investigated the biological consequences of CXCL5 overexpression for tumor cell proliferation and motility.

Materials and Methods

Plasmids. Targeting sequences for construction of CXCL5-short-hairpin RNA (shRNA) plasmids were designed using a web-based software program (siRNA Target Finder, Ambion, Austin, TX)5 and the sequences for sense/antisense oligos are indicated in Table 1. A further two complementary oligonucleotides were used to generate a nontargeting "scrambled" control (NTC; Table 1). Each complementary oligonucleotide pair, containing 5′ oligonucleotides were used to generate a nontargeting "scrambled" control antisense oligos are indicated in Table 1. A further two complementary vectors were similarly transfected into HN12 cells and individual clones were selected in 400 μg/mL G418. shRNA and nontargeting control plasmid were carried out in accordance with local Institutional Animal Care and Use Committee guidelines. Cells cultured to 70% confluence were trypsinized directly ligated into the pCEFL plasmid vector, which directs synthesis from the EF1 promoter. All plasmid constructions were verified by nucleotide sequence analysis.

Generation of cell lines that express CXCL5 and shRNA constructs. HN4 cells derived from a primary tongue squamous cell carcinoma and HN12 cells derived from a synchronous nodal metastasis were cultured as previously described (19). HN22 cells derived from a primary SCC and HN8 cells derived from a lymph node metastasis in the same patient were similarly cultured. Recombinant human EGF was purchased from Austral Biologicals (San Ramon, CA). Recombinant human CXCL5 (ENA-78, Santa Cruz Biotechnology, Santa Cruz, CA) was purchased from R&D Systems (Minneapolis, MN).

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Cell lines and growth factors. HN4 cells were detached from a primary tongue squamous cell carcinoma and HN12 cells derived from a synchronous nodal metastasis were cultured as previously described (19). HN22 cells derived from a primary SCC and HN8 cells derived from a lymph node metastasis in the same patient were similarly cultured. Recombinant human EGF was obtained from Austral Biologicals (San Ramon, CA). Recombinant human CXCL5 (ENA-78) was purchased from R&D Systems (Minneapolis, MN).

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Western blot analysis. Cells were cultured in complete growth media to 80% to 90% confluence, then media were replaced with serum-free DMEM containing 0.1% bovine serum albumin and incubated for a further 48 hours. Conditioned media samples, harvested from a defined number of cells as determined by cell counting, were concentrated 10-fold using a centrifugal microfilter apparatus (Microcon YM-3, Millipore, Bedford, MA) according to the protocol of the manufacturer. Volumes of conditioned media proportional to equivalent cell numbers were resolved in 20% SDS-PAGE gels and blotted to polyvinylidene difluoride membrane as previously described (21). CXCL5 was detected by incubation with a specific antibody (ENA-78, Santa Cruz Biotechnology, Santa Cruz, CA) followed by incubation with horseradish peroxidase (HRP)--conjugated secondary antibody and detection by enhanced chemiluminescence (ECL Plus, Amersham Biosciences, Piscataway, NJ).

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Table 1. Oligonucleotide sequences for siRNA plasmid construction and quantitative real-time PCR

<table>
<thead>
<tr>
<th>Oligonucleotide Sequence</th>
<th>Function</th>
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<tbody>
<tr>
<td>shL5-S, 5′-GATCCGTTGTTAGCTTCCGAGTTTCACAGGATCC-3′</td>
<td>ShRNA Target Finder, Ambion, Austin, TX</td>
</tr>
<tr>
<td>shL5-AS, 5′-AATTTCCTGAAGAAAAAAGAATTCGCTTCCGTAAGT-3′</td>
<td>ShRNA Target Finder, Ambion, Austin, TX</td>
</tr>
<tr>
<td>NTC-S, 5′-GATCCGGGAGGCTTATACCTGAGTTTCTCTCAGGTC-3′</td>
<td>Nontargeting &quot;scrambled&quot; control</td>
</tr>
<tr>
<td>NTC-AS, 5′-AATTTCCTGAAGAAAAAAGAATTCGCTTCCGTAAGT-3′</td>
<td>Nontargeting &quot;scrambled&quot; control</td>
</tr>
<tr>
<td>qCXCL5-S, 5′-GAGAGCTTGGTTCGTTGTTG-3′</td>
<td>CXCL5 antisense oligo</td>
</tr>
<tr>
<td>qCXCL5-AS, 5′-TTCTTTGTTTCCACCGTCA-3′</td>
<td>CXCL5 antisense oligo</td>
</tr>
</tbody>
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6 http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi.
CXCL5 in Squamous Cell Carcinoma Tumor Progression

CXCL5 is overexpressed in metastatic HNSCC cells. Previously, we investigated global gene expression patterns in primary and metastatic cells using a microarray approach (19) and identified several genes which showed dramatically altered patterns of expression. One of these was the gene encoding CXCL5, a ligand belonging to the CXC family of chemokines, which was highly up-regulated in metastatic HN12 cells compared with the HN4 primary tumor cells as judged by microarray (19). Western blot analysis of conditioned media samples collected from HN4 and HN12 cells indicated that the metastatic cells secrete readily detectable levels of CXCL5 polypeptide (Fig. 1A). We further confirmed expression of CXCL5 in the HN4-HN12 cell line pair and in an additional primary-metastasis cell line pair (HN22-HN8) using quantitative real-time PCR. As shown in Fig. 1B, both HN12 and HN8 cell lines, derived from metastatic tumors, express higher levels of CXCL5 compared with the respective primary tumor cell lines, HN4 and HN22. These observations suggested that elevated CXCL5 expression represents a clear difference between these primary and metastatic HNSCC cells which, potentially, might underpin key alterations in their biology.

Targeted down-regulation of CXCL5 expression using shRNA. As we had observed elevated expression of CXCL5 in metastatic HNSCC cells, we were keen to determine if this played a contributory role in determining their invasive phenotype. Therefore, we used a shRNA–based approach to down-regulate CXCL5 expression in HN12 cells, as these cells showed the highest level of expression from our Western blotting and quantitative real-time PCR experiments. Appropriate shRNA and nontargeting control plasmids were constructed and transfected into HN12 cells, and puromycin-resistant colonies were expanded and tested for CXCL5 expression. As shown in Fig. 2, shRNA-transfected clones (shL5) showed inhibition of CXCL5 expression as judged by quantitative real-time PCR using CXCL5-specific primers (Fig. 2, top), whereas clones transfected with a nontargeting control vector (NTC) showed similar levels of CXCL5 expression as the parental HN12 cells. These data were further confirmed by Western blotting of conditioned media (bottom). Thus, stable targeted down-regulation of CXCL5 expression using shRNA plasmids can be achieved with a high degree of inhibition.

Inhibition of CXCL5 expression reduces cell proliferation. Next, to investigate whether CXCL5 contributes to cell growth, we compared the proliferation of shL5 and NTC control cells. Cells were seeded in quadruplicate, allowed to attach overnight, washed in serum-free media, and then incubated in serum-free media under standard culture conditions. Cell numbers were determined every 24 hours by trypsinization and counting in a hemocytometer. As shown in Fig. 3A, shL5 cells in which CXCL5 expression has been ablated showed a much decreased proliferation rate compared with nontargeting controls, with a 3- to 4-fold lower cell number over a 6-day time course. In addition, shL5 cells were cultured in the presence or absence of conditioned media from NTC cells and the proliferation rate was determined. We found that NTC conditioned media were able to rescue proliferation of shL5 cells, at least in part (Fig. 3B). Furthermore, we generated HN4 clones that overexpress CXCL5 as judged by Western blotting.

Figure 1. CXCL5 production by metastatic HNSCC cells. A, conditioned media were harvested from cultures of the indicated cell lines, concentrated, and resolved in polyacrylamide-SDS gels as described in Materials and Methods. Gels were Western blotted, probed with anti-CXCL5 antibody, and detected by ECL. rhCXCL5, 10 ng recombinant human CXCL5. B, total RNA was extracted from subconfluent cultures of the indicated cell lines and reverse transcribed as described in Materials and Methods. Equivalent amounts of cDNA were subjected to quantitative real-time PCR using CXCL5-specific primers. Expression relative to that of an internal standard, glyceraldehyde-3-phosphate dehydrogenase. Bars, SD. Representative of at least three independent experiments.

Figure 2. Inhibition of CXCL5 expression in HNSCC cells. Total RNA was extracted from subconfluent cultures of the indicated cell lines and reverse transcribed as described in Materials and Methods. Equivalent amounts of cDNA were subjected to quantitative real-time PCR using CXCL5-specific primers. As shown in Fig. 1A, both HN12 and HN8 cell lines, derived from metastatic tumors, express higher levels of CXCL5 compared with the respective primary tumor cell lines, HN4 and HN22. These observations suggested that elevated CXCL5 expression represents a clear difference between these primary and metastatic HNSCC cells which, potentially, might underpin key alterations in their biology.

Results

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Overexpression of CXCL5 in these cells was found to enhance proliferation relative to an empty vector control (Fig. 3C, bottom). Taken together, these data indicate that CXCL5 likely plays a role in potentiating HNSCC cell proliferation.

Inhibition of CXCL5 expression decreases cell migration and invasion in vitro. The ability to degrade basement membrane and migrate through adjacent structures is a key feature of metastatic cells. A number of polypeptide growth factors and cytokines have been implicated in various processes related to metastatic progression, including chemokines. Previously, we showed that HN12 cells are highly invasive in vitro (18, 19). Therefore, we were keen to determine whether CXCL5 contributed to HN12 cell motility and invasion. First, we determined the ability of shL5 and NTC cells to migrate in response to EGF using standard chamber assays. As shown in Fig. 4A, shRNA-mediated inhibition of CXCL5 resulted in an ~9-fold decrease in cell migration. We extended these observations by investigating whether these cells showed differential abilities to invade through basement membrane. NTC cells consistently showed levels of invasion similar to those observed for the parental HN12 cells (Fig. 4B) whereas shL5 cells were largely noninvasive. Taken together, these data suggest that CXCL5 can contribute to both migration and invasion of HN12 metastatic HNSCC cells.

CXCL5 down-regulation abrogates tumor formation in vivo. As our previous results had shown a clear involvement of CXCL5 in tumor cell proliferation, migration, and invasion in vitro, we sought to determine whether the tumorigenic potential of HN12 cells in vivo would be affected by modulation of this chemokine. Therefore, we undertook standard xenograft experiments in athymic mice. NTC or shL5 cells were transplanted to the flanks of athymic mice and tumor formation was monitored over a 4-week period. Whereas NTC cells produced large tumors similar to the parental HN12 cells, shL5 clones seemed to have lost this tumorigenic potential (Fig. 5A and B). Furthermore, NTC tumors showed strong

**Figure 3.** CXCL5 enhances proliferation of HNSCC cells. A, the indicated cells were seeded in triplicate into 24-well culture plates at a density of $5 \times 10^3$ per well and grown for up to 6 days. Cells were counted daily following trypsinization as described in Materials and Methods. B, shL5 cells were cultured in the presence or absence of conditioned media derived from NTC cultures and counted as described above (bottom). Bars, SD. Representative of at least three independent experiments.

**Figure 4.** CXCL5 is important for migration and invasion of HN12 cells. A, the indicated cell lines were removed from culture dishes in the absence of trypsin, washed, counted, added to the upper chamber of Transwell filters, and allowed to migrate for 7 hours in the presence of EGF as described in Materials and Methods. Migrating cells were fixed, stained, and counted in 20 random high-power fields. B, cells, treated as described above, were plated on top of Matrigel-coated Transwell filters and incubated for 16 hours as described in Materials and Methods. Invading cells were fixed, stained, and counted as described above. Bars, SD. Representative of at least three independent experiments.
staining with an antibody to the proliferation marker Ki67 (Fig. 6A and B), with expression of CXCL5 being maintained by these cells (Fig. 6C and D). Taken together, these data indicate that CXCL5 expression is necessary for proliferation of HN12 cells in vivo.

Discussion

In the present study, we investigated the biological role of CXCL5 in HN12 metastatic head and neck cancer cells in which we previously found overexpression of this chemokine relative to synchronous primary tumor cells. Further, we found that an additional metastatic cell line, HN8, overexpressed CXCL5 relative to a related primary tumor-derived cell line, suggesting that this phenomenon is not an isolated observation. Indeed, it is becoming widely acknowledged that CC- and CXC-chemokines likely play multiple roles in the development and progression of a multitude of human tumor types [reviewed by Balkwill (25)]. For example, CXCL12-CXCR4 ligand-receptor interaction is documented to be involved in breast cancer metastasis (14), whereas Delibasi et al. (26) provided evidence to suggest that CXCR4 may be overexpressed in some metastatic tongue cancers. In addition, in the case of metastatic head and neck cancer, loss of expression of the chemokine receptor CCR6, together with a concomitant up-regulation of CCR7, was found in cell lines and tumor tissues (17) and was postulated as a potential mechanism to mediate migration of cells from the primary tumor site to regional lymph nodes. Furthermore, CCR7 may be involved in other aspects of tumor progression, such as enhanced survival, as it has been shown to elicit activation of the prosurvival kinase AKT, albeit in dendritic cells (27). Similarly, CXCR2, a receptor for CXCL5 and other CXC-chemokines, is likely to enhance cell survival by analogous mechanisms (28), as is CXCR3 (8). In contrast to some systems in which co-upregulation of ligand-receptor pairs occurs, we found that CXCR2 expression was similar between HN12 and HN4 cells as judged by quantitative real-time PCR and flow cytometry.7

Using RNA interference to inhibit CXCL5 expression in our model system, we were able to show that this chemokine plays a central role in cancer cell migration and invasion. This is perhaps unsurprising, as other studies have reported that pathways involved in regulation of cell motility can be activated by CXCR1 and/or CXCR2 (11, 12). HN12 cells are highly motile and invasive in response to EGF (18) and a previous study has shown that CXCR1/2 activation leads to phosphorylation of the EGF receptor as well as stimulation of downstream signaling pathways (7). This may represent an important mechanism through which tumor cells acquire the ability to respond to multiple polypeptides available in the extracellular environment and/or cross-talk between signal transduction pathways. Such a response may be particularly useful for cells at sites distant from the tissue of origin, such as metastatic cells in a lymph node, where the complement of available growth factors may be different from that at the primary tumor site.

As well as influencing migration and invasion of HN12 cells, we found that CXCL5 also stimulated proliferation, as cells in which expression was abrogated with shRNA showed a much reduced growth rate in vitro compared with controls. Previous studies have reported that interleukin 8 (IL-8/CXCL8), a CXC-chemokine related to CXCL5, is able to induce proliferation of squamous carcinoma cells (16). However, an astonishing result from our studies was the complete inhibition of tumor cell growth in vivo when cells lacking CXCL5 were transplanted in athymic mice. Whereas this may be due at least in some part to the mitogenic effects of CXCL5 that we observed in cell culture, it may also be dependent to some extent on tumor neovascularization. CXCL5 is well recognized as a proangiogenic factor of the ELR+ family of chemokines (29, 30).

Figure 6. CXCL5 is expressed in tumors in vivo. A to D, tumors were excised and processed as described in Materials and Methods. Sections (5 μm) were incubated with normal mouse immunoglobulin as a control (A), anti-Ki67 antibody (B), goat immunoglobulin (C), or goat anti-CXCL5 antibody (D) and detected as described in Materials and Methods. Representative of two independent experiments.

Figure 5. Down-regulation of CXCL5 inhibits tumor formation in vivo. A, cells were trypsinized, counted, washed, resuspended, and transplanted to the right flank of athymic mice and tumor formation was monitored daily for 4 weeks. nd, not detectable. B, photographic comparison of representative NTC and shL5 mice 4 weeks posttransplantation.

7 Unpublished observations.
Establishment of a blood supply is a crucial event that is required to facilitate tumor growth at both primary and secondary sites. In this regard, chemokines are likely to be important proangiogenic inducers; for example, CXCL8-CXCR2 signaling has been shown to facilitate migration and proliferation of endothelial cells (31). Indeed, antiserum-mediated inhibition of CXCL5 and CXCL8 has been shown to decrease the growth of lung cancer cells (32–34) whereas the antiangiogenic action of peroxisome proliferator–activated receptor γ has been shown to be mediated by inhibition of CXCL1, 5, and 8 (35). Additionally, the ability of chemokine receptor signaling events to activate AKT may be important over and above any prosurvival effects, as this kinase is intimately involved in angiogenesis downstream of G-protein coupled receptors (36). Whereas the tumors obtained with control cells in our experiments were well vascularized, it was not possible to compare angiogenesis with CXCL5 knockdown cells as no tumor formation occurred. However, although overexpression of CXCL5 is undoubtedly important for the aggressive phenotype of HN12 cells, other factors are likely involved. In support of this, ectopic expression of CXCL5 in HN4 cells increased migration and invasive potential, but to a much lower level than observed for HN12 cells.

In summary, CXCL5 may be an important determinant of tumor development in vivo as a result of multiple biological activities that include mitogenic stimulation, angiogenesis, and migration/invasion. As such, it may be a potential target around which to develop novel therapeutic strategies for some tumors.

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References

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