Distinct Expression of CXCL8 and Its Receptors CXCR1 and CXCR2 and Their Association With Vessel Density and Aggressiveness in Malignant Melanoma

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Key Words: Interleukin-8/CXCL8; CXCR1; CXCR2; Angiogenesis; Metastasis

Abstract

We examined the expression of CXCL8 (interleukin-8), its receptors, CXCR1 and CXCR2, and vessel density in human melanoma by immunohistochemical analysis of tumors from different Clark levels, depths, and thicknesses. Expression of CXCL8 and CXCR2 was lower in Clark level I and II specimens than in level III through V specimens and metastases. CXCR1 expression was observed ubiquitously in the majority of human melanoma tumor specimens irrespective of disease state, with the highest intensity in Clark level III specimens. We observed a significant difference in CXCL8 and CXCR2 expression between thin (≤0.75 mm) and thick (>0.75 mm) melanomas and between thin and metastatic lesions. Positive correlations were observed between Clark level and CXCL8 or CXCR2 and between thickness and CXCR2 expression. We found no correlation between vessel density and Clark level or thickness. Our data suggest that expression of CXCL8 and CXCR2 contributes to aggressive growth and metastasis in human malignant melanoma. Consistent with the transition from radial to vertical growth phase melanoma, expression of CXCL8 and its receptor, CXCR2, may be key in the switch to an aggressive, more metastatic phenotype.

The incidence and mortality of melanoma is rising faster than any other cancer in men and second only to lung cancer in women,1,4 and the American Cancer Society predicts there will be about 7,770 deaths due to melanoma during 2005.5 The chance of developing melanoma increases with age, but it affects all age groups and is one of the most common cancers in young adults. Most melanomas progress through an initial radial growth phase, or in situ melanoma, to a more aggressive vertical growth phase that exhibits growth in the mesenchyme and in the epithelium. Clark6,7 described the initial steps of primary neoplasia as levels I through III, and invasive neoplasms are categorized as levels IV and V. Similarly in 1970, Breslow8 related the importance of thickness and its impact on survival. Today the Clark classification and overall thickness are important prognostic factors.

Melanoma specimens and cell lines derived from them have been to shown to express a variety of chemokines, including CXCL8 (interleukin-8), CXCL1 through CXCL3 (MGSA/Groα-γ), CCL2 (monocyte chemotactic protein-1), and CCL5 (RANTES).9 Thus, there are implications for these factors in the control of the progression of malignant disease and secondary organ metastasis. CXCL8 can influence the processes of tumor progression and metastasis because it has been shown to be an autocrine growth factor,10 to induce angiogenesis,11,12 and to influence migration of melanoma cells13 through the binding and activation of its receptors. Reports from our laboratory and others suggest that metastasis of cutaneous malignant melanoma is associated with constitutive expression of CXCL8.14-18

Two receptors for CXCL8, the type A CXCL8 receptor (CXCL8RI or CXCR1) and type B CXCL8 receptor (CXCL8RII or CXCR2) have been shown to bind CXCL8...
with high affinity.\textsuperscript{19-21} CXCR1 is selective for CXCL8, whereas CXCR2 also interacts with other chemokines.\textsuperscript{22,23} CXCR1 and CXCR2 are expressed on keratinocytes, fibroblasts, and endothelial and melanoma cells\textsuperscript{24,25} and also have been implicated in the angiogenic response and in the migration of neutrophils and lymphocytes.\textsuperscript{23,24,26-29} Norgauer et al\textsuperscript{30} found low expression of CXCR2 on normal human melanocytes, which was up-regulated after treatment with tumor necrosis factor $\alpha$, with subsequent enhancement of proliferation in response to CXCL8, whereas CXCR1 expression was not detectable.

CXCL8 has been shown to be an important angiogenic factor, and the CXCR2 receptor has been demonstrated to be the putative angiogenic receptor.\textsuperscript{23} In addition, expression of CXCR1 and CXCR2 has been demonstrated on endothelial cells.\textsuperscript{31-34} However, the association of CXCL8 and its receptors with the progression of melanoma remains uncertain. To determine the functional role of CXCL8 and to elucidate the individual role of each receptor in the progression of malignant melanoma, expression of CXCL8 and its receptors, CXCR1 and CXCR2, on malignant melanoma cells was examined in a sequential manner.

### Materials and Methods

#### Tumor Specimens

Paraffin-embedded tumor tissue samples of cutaneous malignant melanoma from the archives of the Department of Pathology and Microbiology, University of Nebraska Medical Center, Omaha, were studied. All primary melanoma samples were classified by a pathologist (S.L.J.) according to thickness and depth of invasion, using previously defined parameters.\textsuperscript{6-8} \textbf{Table 1}. Similarly, metastatic lesions were categorized according to organ site \textbf{Table 2}. After an initial review of the H&E-stained slides, serial sections were recut from the tumors to include adjacent uninvolved tissue.

<table>
<thead>
<tr>
<th>Clark Level</th>
<th></th>
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</thead>
<tbody>
<tr>
<td>I</td>
<td>II</td>
</tr>
<tr>
<td>Invasion</td>
<td></td>
</tr>
<tr>
<td>Radial</td>
<td>7</td>
</tr>
<tr>
<td>Vertical</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>7</td>
</tr>
<tr>
<td>Thickness (mm)</td>
<td></td>
</tr>
<tr>
<td>≤0.75</td>
<td>7</td>
</tr>
<tr>
<td>&gt;0.75</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>7</td>
</tr>
</tbody>
</table>

\textbf{Table 2}

Metastatic Melanomas by Organ Site

<table>
<thead>
<tr>
<th>Site</th>
<th>No. of Cases</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain</td>
<td>3</td>
</tr>
<tr>
<td>Lymph node</td>
<td>7</td>
</tr>
<tr>
<td>Meninges</td>
<td>1</td>
</tr>
<tr>
<td>Skin</td>
<td>3</td>
</tr>
<tr>
<td>Spinal cord</td>
<td>2</td>
</tr>
<tr>
<td>Total</td>
<td>16</td>
</tr>
</tbody>
</table>

#### Immunohistochemical Analysis

CXCL8, CXCR1, and CXCR2 expression and neovascularization were analyzed by immunohistochemical techniques as described earlier.\textsuperscript{18,35} Briefly, 6-µm-thick sections of archival paraffin blocks were deparaffinized by incubation in EZ-Dewax (BioGenex, San Ramon, CA) and rinsed in deionized water to remove residual EZ-Dewax. The slides were processed for antigen retrieval in citrate buffer with microwave treatment. After rinsing, endogenous peroxidase was blocked by incubation in 3% hydrogen peroxide in water for 5 minutes. Nonspecific binding was blocked by incubation in 10% horse serum. Slides then were incubated with primary antibody (anti-CXCL8 antibody, dilution 1:200, Endogen, Woburn, MA; anti-CXCR1 antibody, dilution 1:100, BD Pharmingen, San Diego, CA; anti-CXCR2 antibody, dilution 1:100, BD Pharmingen; or anti-CD34 antibody, dilution 1:50, BioGenex) overnight at 4°C. The slides were rinsed; antimouse/antirabbit IgG biotinylated secondary antibody, dilution 1:500, was added; and slides were incubated for 1 hour at room temperature. Immunoreactivity was detected by using the ABC Elite kit and diaminobenzidine substrate (Vector Laboratories, Burlingame, CA) according to the manufacturer’s instructions. A reddish brown precipitate in the cytoplasm indicated a positive reaction. Negative control experiments used all reagents except the primary antibody.

Intensity of staining for CXCL8, CXCR1, and CXCR2 and CD34 expression was graded on a scale of 0 to 3+, with 0 representing no detectable staining and 3+ representing the strongest staining. Two independent observers (R.K.S. and...
Expression of CXCL8 in Human Malignant Melanoma

Expression of CXCL8 was evaluated in archival paraffin specimens from patients treated at the University of Nebraska Medical Center to further elucidate the association of CXCL8 and its role in melanoma progression, angiogenesis, and metastasis. Staining was evaluated as described in the “Materials and Methods” section. CXCL8 staining was observed predominantly in tumor cells. In specimens with a low Clark level, the overall positivity for CXCL8 staining was lower. Correlation analysis revealed a positive correlation between the Clark level and CXCL8 staining intensity with the Clark level (Figure 1).

Correlation analysis of CXCL8 staining intensity with the Clark level revealed a positive correlation ($r = 0.251; P < .05$). Correspondingly, Mann-Whitney analysis showed a significant difference between CXCL8 staining in thick (≥0.75 mm) and thin (>0.75 mm) ($P < .01$) melanomas and a statistically significant difference between thin melanomas and metastatic lesions ($P < .05$). In addition, a positive correlation between melanoma thickness and CXCL8 staining was detected ($r = 0.195; P < .05$; data not shown).

Expression of CXCR1 and CXCR2 in Human Malignant Melanoma

CXCL8 binds to 2 receptors with high affinity. To assess the functional significance of CXCL8 expression, consecutive sections were stained for expression of CXCR1 and CXCR2, the receptors for CXCL8. Both CXCR1 and CXCR2 were expressed predominantly in tumor cells. Tumor endothelial cells also were positive for CXCR1 and CXCR2 (data not shown).

The pattern of immunostaining for CXCR1 demonstrated that the majority of melanoma specimens analyzed expressed CXCR1, regardless of the Clark level. Correlation analysis revealed a positive correlation between CXCR2 staining (Table 4). In addition, Mann-Whitney analysis of the data revealed a statistically significant difference between CXCR2 staining in thin and thick melanomas ($P < .001$) and between thin melanomas and metastatic lesions ($P < .001$).

Vessel Density in Malignant Melanoma and Its Association With Expression of CXCL8, CXCR1, or CXCR2 and Aggressiveness

Because CXCL8 can act as an angiogenic factor, we examined the level of neovascularization in our melanoma specimens. To quantitate neovascularization, vessel density, as detected by CD34 immunostaining, was counted using a reticle grid, in addition to evaluating overall staining intensity. We
found no difference in the vessel density among different Clark levels or metastases (Table 4). Similarly, we did not observe any correlation between neovascularization and CXCL8, CXCR1, or CXCR2 expression (Table 4).

Discussion

In the present study, we extended our earlier observation using more tumor specimens; we have shown that human malignant melanomas from different Clark levels with varying thicknesses and metastatic potential express diverse levels of CXCL8 and CXCR2 but not CXCR1. In our analysis, the majority of tumor cells in metastatic samples were CXCL8+, whereas we observed a few CXCL8+ cells in melanomas with a low Clark level. Maximum expression was reached in Clark level III lesions, which may be related to the transition from radial to vertical growth phase melanoma.

CXCL8 has been demonstrated to serve as an essential autocrine growth factor for melanoma cells and to stimulate the migration of melanoma cells. In addition, CXCL8 has been shown to regulate angiogenesis and the expression of matrix metalloproteinases and to inhibit tumor infiltration by lymphocytes.

Our previous report suggests that CXCL8 might act as an autocrine and/or paracrine growth factor for melanoma through binding to CXCR1 and CXCR2. Previously we demonstrated that neutralization of CXCR1 and CXCR2 inhibited the proliferation of melanoma cells expressing CXCL8; thus, CXCL8 can exert its function in an autocrine manner by binding with CXCR1 and CXCR2. In addition to autocrine growth signaling, our data suggest that the CXCL8-CXCR2...
Figure 1: Expression of CXCL8 (interleukin [IL]-8), CXCR1, and CXCR2 and vessel density in malignant melanoma with different aggressive behavior. Intensity of immunostaining for CXCL8 (A), CXCR1 (B), CXCR2 (C), and vascularization as determined by CD34+ staining (D) in melanoma specimens was examined by 2 independent observers. Intensity of staining was graded on a scale of 0 to 3+, with 0 representing no detectable staining and 3+ representing the strongest staining. Vessel density was quantitated microscopically with a 5 × 5 reticle grid (Klarmann Rulings, Litchfield, NH) and a 40× objective (250 µm total area). *Significantly different from Clark level I.

Table 4
Correlation Between Expression of CXCL8, CXCR1, CXCR2, and Neoangiogenesis With Aggressiveness in Melanoma

<table>
<thead>
<tr>
<th></th>
<th>CXCR1</th>
<th>CXCR2</th>
<th>CXCL8</th>
<th>Vessel Density</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clark level</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Correlation coefficient</td>
<td>0.090</td>
<td>0.613</td>
<td>0.251</td>
<td>0.011</td>
</tr>
<tr>
<td>Significance (2-tailed)</td>
<td>.297</td>
<td>.001†</td>
<td>.011†</td>
<td>.938</td>
</tr>
<tr>
<td>CXCR1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Correlation coefficient</td>
<td>—</td>
<td>0.140</td>
<td>0.060</td>
<td>0.059</td>
</tr>
<tr>
<td>Significance (2-tailed)</td>
<td>—</td>
<td>.125</td>
<td>.608</td>
<td>.728</td>
</tr>
<tr>
<td>CXCR2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Correlation coefficient</td>
<td>0.140</td>
<td>—</td>
<td>0.392</td>
<td>—</td>
</tr>
<tr>
<td>Significance (2-tailed)</td>
<td>.125</td>
<td>—</td>
<td>.001†</td>
<td>—</td>
</tr>
<tr>
<td>CXCL8</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Correlation coefficient</td>
<td>0.062</td>
<td>0.392</td>
<td>—</td>
<td>0.020</td>
</tr>
<tr>
<td>Significance (2-tailed)</td>
<td>.608</td>
<td>.001†</td>
<td>—</td>
<td>.924</td>
</tr>
<tr>
<td>Vessel density</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Correlation coefficient</td>
<td>0.059</td>
<td>—</td>
<td>0.020</td>
<td>—</td>
</tr>
<tr>
<td>Significance (2-tailed)</td>
<td>.728</td>
<td>.475</td>
<td>.924</td>
<td>—</td>
</tr>
</tbody>
</table>

*Bivariate correlation analysis was performed using the Spearman p correlation coefficient. Correlation measures how variables are related. Correlation coefficients range from −1 (a perfect negative relationship) to +1 (a perfect positive relationship). A value of 0 indicates no linear relationship.
†Significant correlation between groups.
Expression of CXCR1 and CXCR2 in human melanoma tumors from different Clark levels examined by immunohistochemical analysis. CXCR1 immunoreactivity in Clark level I (A), level III (C), and level V (E). CXCR2 immunoreactivity in Clark level I (B), level III (D), and level V (F) (A-F, ×100).
pathway also may be involved in regulating the invasive potential of human melanoma cells, as a previous report from our group suggests that CXCL8 up-regulates matrix metalloproteinase-2 expression in melanoma cells.38

CXCR1 and CXCR2 are high-affinity receptors for CXCL8 and share a high degree of sequence similarity within the membrane-spanning domain but differ within the extracellular and intracellular loops and the NH2- and COOH-terminal domains.39 The role of these receptors in modulating cellular phenotypes associated with melanoma cell proliferation, invasion, and angiogenesis is hitherto unclear. Previously, we observed a differential effect on melanoma cell proliferation and migration37 in response to antibody neutralization of CXCR1 and CXCR2, suggesting that binding of CXCL8 to CXCR1 and CXCR2 can initiate diverse cellular responses. The present analysis of CXCR1 in human melanoma specimens from different Clark levels demonstrates that CXCR1 is expressed ubiquitously in all Clark levels. In contrast, CXCR2 is expressed predominantly by higher grade melanoma tumors and metastases. An earlier report by Norgauer et al30 suggested that human melanocytes and melanoma cells in vitro express CXCR2. In the present study, the expression of CXCR2 correlated with the Clark levels and metastases. In addition, we observed significant differences in the expression of CXCR2 in thin and thick melanomas, and thickness is an important prognostic indicator for melanoma.4

These reports, in combination with our present data, suggest diverse roles for CXCR1 and CXCR2 in vivo.

CXCR1 and CXCR2 have been implicated in the angiogenic response and in the haptotactic migration/chemotaxis of melanoma cells.23,28,29 Despite similar affinities for CXCL8 and similar receptor numbers, chemotaxis of neutrophils is mediated primarily by CXCR1.22,40 Similarly, CXCL8 expression by endothelial cells was shown to elicit a haptotactic response from melanoma cells through the CXCR1 receptor.41 A report from Addison et al23 described the CXCR2 receptor as the putative receptor for mediating glutamic acid–leucine–arginine containing CXC chemokine-induced angiogenesis, further confirming the diverse roles of CXCR1 and CXCR2 in modulating an aggressive malignant phenotype.

The importance of angiogenesis in the progression of malignant melanoma is controversial and unclear.42–44 We have shown the expression of CXCR1 and CXCR2 receptors on endothelial cells, suggesting that CXCL8 might act as a paracrine angiogenic factor.31,32 Because CXCR2 has been described as a key receptor involved in angiogenesis,23 we analyzed vessel density in our specimens. In the present study, we found no relationship between vessel density and melanoma progression and metastasis. This observation could be because malignant melanomas have been shown to co-opt existing vessels45,46 and to participate in vasculo-genic mimicry.47–49

Our data suggest an association between expression of CXCL8 and CXCR2 but not CXCR1 and vessel density in melanoma progression and metastasis. In addition to its prognostic significance, inhibition of CXCL8 production and/or activity might be an ideal target for the management of malignant melanoma.

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