

Host CXCR2-Dependent Regulation of Melanoma Growth, Angiogenesis, and Experimental Lung Metastasis

Seema Singh, Michelle Varney, and Rakesh K. Singh

Department of Pathology and Microbiology, University of Nebraska Medical Center, Omaha, Nebraska

Abstract

Crucial steps in tumor growth and metastasis are proliferation, survival, and neovascularization. Previously, we have shown that receptors for CXCL-8, CXCR1, and CXCR2 are expressed on endothelial cells and CXCR2 has been shown to be a putative receptor for angiogenic chemokines. In this report, we examined whether tumor angiogenesis and growth of CXCL-8-expressing human melanoma cells are regulated *in vivo* by a host CXCR2-dependent mechanism. We generated mCXCR2^{-/-}, mCXCR2^{+/-}, and wild-type nude mice following crosses between BALB/c mice heterozygous for nude^{+/-} and heterozygous for mCXCR2^{+/-}. We observed a significant inhibition of human melanoma tumor growth and experimental lung metastasis in mCXCR2^{-/-} mice as compared with wild-type nude mice. Inhibition in tumor growth and metastasis was associated with a decrease in melanoma cell proliferation, survival, inflammatory response, and angiogenesis. Together, these studies show the importance of host CXCR2-dependent CXCL-8-mediated angiogenesis in the regulation of melanoma growth and metastasis. [Cancer Res 2009;69(2):411-5]

Introduction

Although melanoma is a disease that affects only 4% of persons afflicted with skin cancer, it is one of the leading causes of malignancy-related mortality in the United States. In 2008, it is estimated that 62,480 new cases of melanoma will be diagnosed in the United States, and 8,420 people will die due to this devastating disease (1). Due to the high mortality associated with this disease, management requires an in-depth understanding of the biology of this complex disease.

The expression of CXCL-8 in melanoma has been shown to correlate positively with disease progression (2, 3). The aggressiveness of malignant melanoma is attributed, in part, to the expression of CXCL-8 and its receptors CXCR1 and CXCR2 (4). In addition to melanoma cells, both CXCR1 and CXCR2 are expressed on endothelial cells and neutrophils (5, 6). Analysis of CXCR2 in human melanoma specimens showed that CXCR2 is expressed predominantly by higher grade melanoma tumors and metastases, suggesting an association between expression of CXCL-8 and CXCR2 in advanced lesions and metastases (4). Previous reports from our laboratory and others have shown that CXCR1 and CXCR2 are expressed on endothelial cells (7-9), and CXCR2 has been shown to play a role in angiogenesis, as it has been shown to be a putative receptor for angiogenic chemokines (5, 10). In this

report, we hypothesized that tumor angiogenesis and growth in CXCL-8-expressing melanoma cells are regulated *in vivo* by a host mCXCR2-dependent mechanism. We observed a significant inhibition of human melanoma tumor growth and experimental lung metastasis in mCXCR2^{-/-} mice as compared with wild-type mice. Inhibition in tumor growth and metastasis was associated with a decrease in melanoma cell proliferation, survival, inflammatory response, and angiogenesis.

Materials and Methods

Animals. BALB/c mice heterozygous for nude^{+/-} or heterozygous for mCXCR2^{+/-} were obtained from Jackson Laboratory. Mice that lack an intact *mIL-8Rh* (mouse homologue of human interleukin 8 receptor/mCXCR2) gene, were originally developed by gene targeting with a vector constructed by deleting the single exon containing the 350-amino acid open reading frame of the murine IL-8 receptor [which has 68% and 71% amino acid identity with human IL-8 receptors A (CXCR1) and B (CXCR2); ref. 11]. We generated mCXCR2^{-/-} nude mice following crosses between BALB/c mice heterozygous for nude^{+/-} and heterozygous for mCXCR2^{+/-}. Mice were housed and handled according to protocols approved by the University of Nebraska Medical Center Institutional Animal Care and Use Committee.

Genotyping using allele-specific PCR and mRNA analysis. Each mouse was genotyped (a representative gel picture shown in Fig. 1A) using mouse tail DNA and amplified for CXCR2^{1m1Mwm} using CXCR2 wild-type primers (GGT CGT ACT GCG TAT CCT GCC TCA G and TAG CCA TGA TCT TGA GAA GTC CAT G), which amplify a 360 bp fragment of the wild-type allele and for the inserted neomycin gene using primers (CTT GGG TGG AGA GGC TAT TC and AGG TGA GAT GAC AGG AGA TC), which amplify a 280 bp fragment. Furthermore, to confirm the nude background, DNA was amplified for Foxn1tm using primers (GGC CCA GCA GGC AGC CCA AG and AGG GAT CTC CTC AAA GGC TTC), which amplified a 168 bp fragment of the wild-type and mutant alleles. Subsequently, 10 μ L of the amplified product was digested in a 20 μ L volume with *Bse*DI, which digests the Foxn1tm allele to 110 and 58 bp products and the wild-type allele to 90, 53, and 20 bp. Absence of a band at 110 bp indicated a wild-type genotype and the presence of bands at 110 and 90 bp indicated a heterozygous genotype (Fig. 1A). Average size and weight of mCXCR2^{-/-} nude mice were lower as compared with the age-matched wild-type and mCXCR2^{+/-} nude mice (Fig. 1C).

mCXCR1 and mCXCR2 mRNA expression in skin was determined in all three types of mice by semiquantitative reverse transcription-PCR as described earlier (12) using the following primers: mCXCR1, 5'-AAT CTG TTG TGG CTT CAC CCA-3' (forward) and 5'-GCT ATC TTC CGC CAG GCA TAT-3' (reverse); mCXCR2, 5'-GCT GTC GTC CTT GTC TTC C-3' (forward) and 5'-GCC TTG TCA ATG TCA TCG C-3' (reverse); glyceraldehyde-3-phosphate dehydrogenase, 5'-CGC ATT TGG TCG TAT TGG G-3' (forward), and 5'-TGA TTT TGG AGG GAT CTC GC-3' (reverse; Fig. 1B). PCR conditions were as follows: 95°C for 10 min; 35 cycles of 95°C for 45 s, 60°C for 1 min and 72°C for 1 min. PCR fragments were separated on a 2% agarose gel containing ethidium bromide (0.25 μ g/mL) and visualized and analyzed using the Alpha Imager gel documentation system (Alpha Innotech).

Cell culture and reagents. A375SM, a highly metastatic human melanoma cell line was maintained in culture in DMEM supplemented with fetal bovine serum, 1% L-glutamine, 1% vitamin solution (MediaTech), and gentamicin (Invitrogen). Cultures were maintained for no longer than 4 weeks after recovery from frozen stocks.

Requests for reprints: Rakesh K. Singh, Department of Pathology and Microbiology, University of Nebraska Medical Center, 985845 Nebraska Medical Center, Omaha, NE 68198-5845. Phone: 402-559-9949; Fax: 402-559-4077; E-mail: rsingh@unmc.edu.

©2009 American Association for Cancer Research.
doi:10.1158/0008-5472.CAN-08-3378

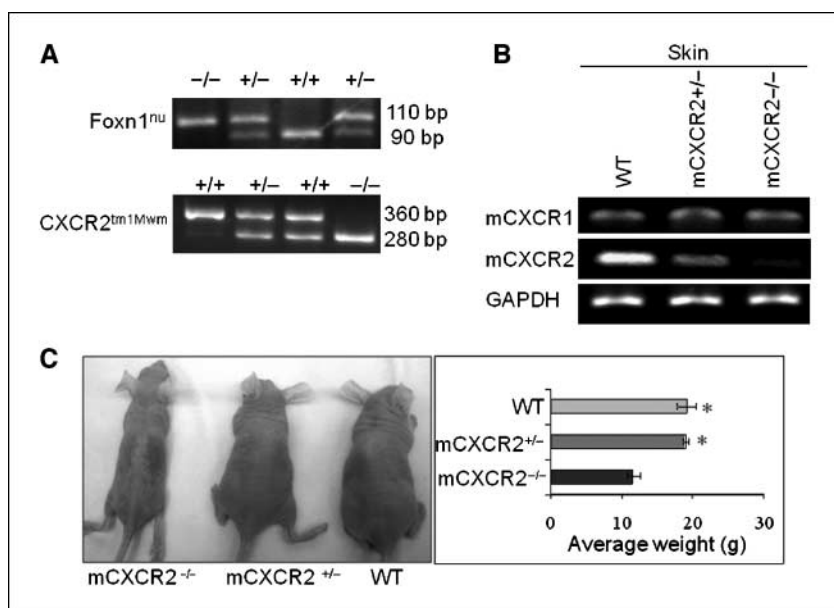


Figure 1. Generation of CXCR2 knockout mice. Genotyping of nude CXCR2 mice by reverse transcription-PCR analysis (A). *Top*, for confirmation of the nude background, F2 progeny DNA was amplified for Foxn1^{nu} as described in Materials and Methods. *Bottom*, the 360 bp PCR product represents the wild-type allele (lane 1), 360 and 280 bp fragments represent heterozygous alleles (lanes 2 and 3), and the 280 bp product represents the knockout allele (lane 4). B, expression of mCXCR1 at the mRNA level was consistent in wild-type (WT), mCXCR2^{+/-}, and mCXCR2^{-/-} nude mice whereas expression mCXCR2 was reduced in mCXCR2^{+/-} and completely knocked down in mCXCR2^{-/-} nude mice. C, average size and weight of mCXCR2^{-/-} mice were lower when compared with age-matched mCXCR2^{+/-} and wild-type nude mice.

In vivo tumor growth and experimental lung metastasis. Tumor growth was examined by s.c. injection of A375SM cells (1×10^6 cells/0.1 mL of HBSS) into wild-type, mCXCR2^{+/-}, or mCXCR2^{-/-} nude mice. Tumor growth was monitored twice a week, and tumor volume was calculated by the following formula: volume = $\pi/6 \times (\text{smaller diameter})^2 \times (\text{larger diameter})$. Animals were sacrificed and tumor tissues were harvested and processed for histochemical analysis. To examine experimental lung metastasis, A375SM cells (1×10^6 cells/0.1 mL of HBSS) were injected i.v. into wild-type, mCXCR2^{+/-}, or mCXCR2^{-/-} nude mice. Mice were sacrificed 8 weeks following tumor cell injection and their lungs harvested and fixed in Bouin's solution. Lung metastatic nodules were counted under a dissecting microscope.

Immunohistochemical analysis. Immunostaining was performed as described previously (4) using antibodies: mouse monoclonal anti-proliferating cell nuclear antigen (PCNA; 1:40; Santa Cruz Biotechnology), rat anti-mouse GR-1 (1:50; Southern Biotechnology Associates, Inc.), and mouse biotinylated GS-IB4 (isolectin from *Griffonia simplicifolia*; 1:50; Vector Laboratories). Tumor cell apoptosis was measured using terminal deoxynucleotidyl transferase biotin-dUTP nick-end labeling (TUNEL). Briefly, tumor sections were deparaffinized by incubation in EZ-Dewax (Biogenex) and rinsed in distilled water to remove residual EZ-Dewax. Sections were blocked for 30 min and incubated with primary antibodies overnight at 4°C. Subsequently, the sections were incubated with the respective biotinylated secondary antibodies (1:500 in PBS) for 45 min, except for GS-IB4, at room temperature. Immunoreactivity was visualized by incubation with avidin-biotin complex and diaminobenzidine tetrahydrochloride substrate (Vector Laboratories). The sections were observed and stained cells and vessels were counted microscopically (Nikon E400 microscope) using a 5 × 5 reticle grid (Klarmann Rulings).

Statistical analysis. All the values are expressed as mean ± SE. Differences between the groups were compared using unpaired two-tailed *t* test using SPSS software (SPSS, Inc.). *In vivo* analysis was performed using Mann-Whitney *U* test for significance. $P \leq 0.05$ was considered statistically significant.

Results

Knockdown of mCXCR2 decreased human melanoma growth and experimental lung metastasis. To examine the role of host CXCR2 in melanoma growth, nude mice (wild-type, mCXCR2^{+/-}, or mCXCR2^{-/-}) were s.c. injected with A375SM cells. Tumor size was measured over a 6-week period. We observed a

significant inhibition in melanoma growth in mCXCR2^{-/-} and mCXCR2^{+/-} mice as compared with the wild-type mice (Fig. 2A). mCXCR2^{+/-} mice showed lower melanoma growth kinetics as compared with wild-type mice and higher as compared with mCXCR2^{-/-} mice, suggesting that the host mCXCR2 levels may be critical in melanoma growth.

In the next set of experiments, we examined the role of host mCXCR2 in melanoma metastasis. For experimental lung metastasis, A375SM cells were injected i.v. into wild-type, mCXCR2^{+/-}, or mCXCR2^{-/-} nude mice. We found a significantly lower median number and size of metastatic lung nodules in mCXCR2^{-/-} as compared with mCXCR2^{+/-} and wild-type mice (Fig. 2B). We also confirmed the levels of mCXCR2 in tumors obtained from all three groups of mice (Fig. 2C).

Knockdown of host mCXCR2 inhibits human melanoma proliferation, survival, and angiogenesis. To further assess the mechanism for reduced melanoma growth and metastasis in mCXCR2^{-/-} mice, we next examined cellular proliferation, survival, and angiogenesis in tumors from mCXCR2^{-/-}, mCXCR2^{+/-}, and wild-type nude mice. Cellular proliferation was analyzed by PCNA immunostaining of tumor sections which showed a significant decrease in PCNA-positive cells in tumors obtained from mCXCR2^{-/-} and mCXCR2^{+/-} nude mice as compared with wild-type nude mice (Fig. 3A). To examine apoptosis in tumors, TUNEL immunostaining of tumor sections was performed. We observed a significant increase in TUNEL-positive cells in tumors from mCXCR2^{-/-} and mCXCR2^{+/-} nude mice as compared with tumors in wild-type mice (Fig. 3B). Microscopic examination of tumor tissue immunostained for blood vessels showed significantly lower numbers of microvessels in tumors from mCXCR2^{-/-} and mCXCR2^{+/-} mice as compared with tumors from wild-type nude mice (Fig. 4A). On average, tumors from mCXCR2^{-/-} and mCXCR2^{+/-} mice showed a 64.3% and 35.7% reduction, respectively, in the number of blood vessels as compared with the tumors from wild-type nude mice (Fig. 4B). Together, these data show that host CXCR2 is necessary for malignant cell proliferation, survival, and neovascularization.

Reduced tumor-induced inflammatory response in mCXCR2^{-/-} nude mice. CXCR2-deficient mice have been shown

to have a deficiency in neutrophil recruitment following inflammation (13). We examined the importance of host CXCR2 on tumor-induced inflammatory response by examining neutrophil infiltration in tumor tissue using GR-1 immunohistochemistry. Neutrophil infiltration in tumors from mCXCR2^{-/-} nude mice was greatly reduced (Fig. 4B) as compared with the tumors from wild-type nude mice. Quantification of neutrophil infiltration showed a significant (3.0-fold to 4.3-fold decrease) difference in the neutrophil population in tumors from mCXCR2^{-/-} and mCXCR2^{+/-} nude mice as compared with tumors from wild-type nude mice (Fig. 4D).

Discussion

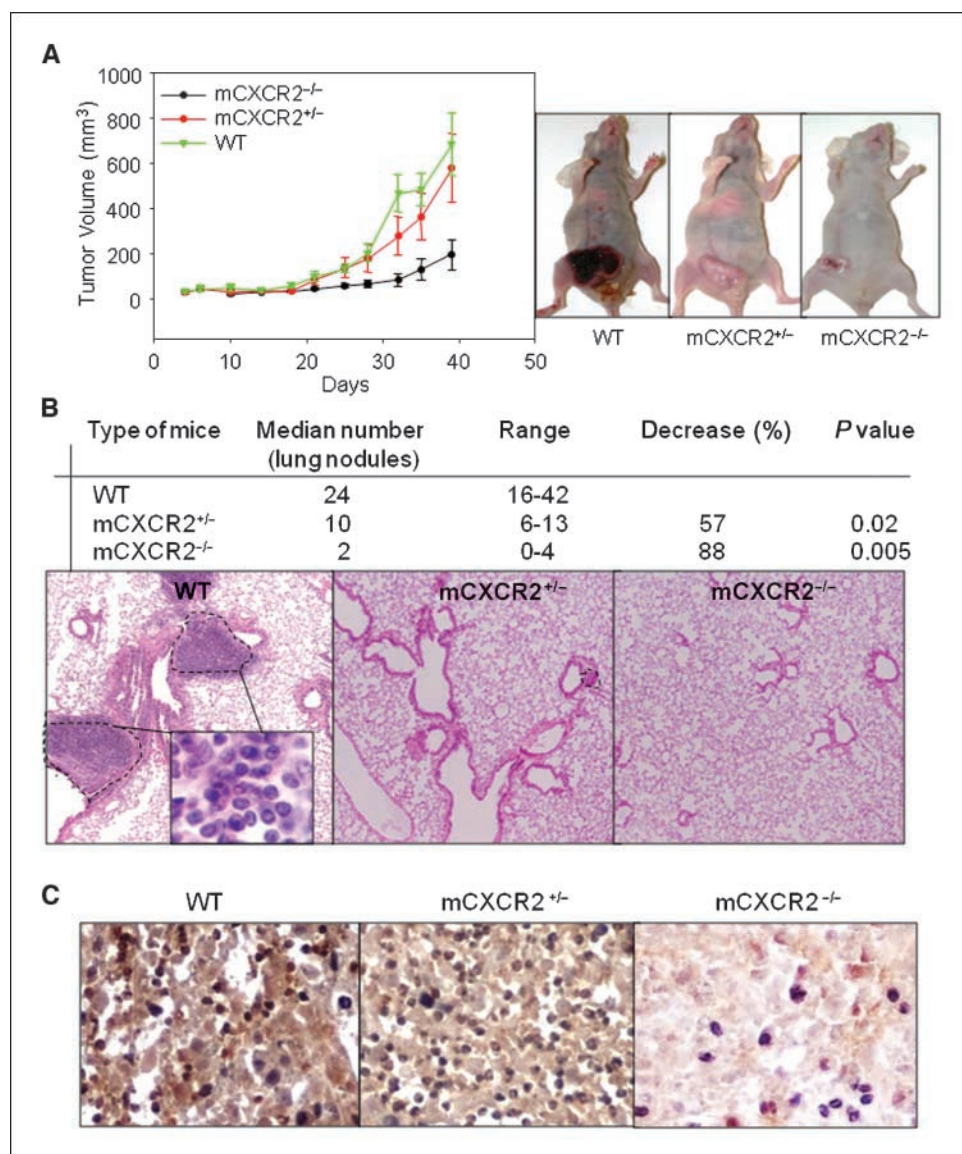
In this report, we show that host mCXCR2 plays an important role in melanoma growth and metastasis. The decrease in tumor growth and experimental lung metastasis in mice that lack mCXCR2 expression was associated with a decrease in melanoma cell proliferation, survival, inflammation, and angiogenesis.

CXCR2 is expressed by vascular endothelial cells and is the primary functional chemokine receptor in mediating endothelial

cell chemotaxis in response to CXCL-8 (5). We have shown that CXCL-8, a CXCR2 ligand, induces endothelial cell tube formation and inhibits apoptosis in endothelial cells via CXCR1 and CXCR2 (8). Therefore, the fact that tumor growth was inhibited in mCXCR2^{-/-} mice suggests the importance of host CXCR2 as a key determinant of melanoma angiogenesis, growth, and experimental lung metastasis. Additional support is provided by the observation that tumors in mCXCR2^{-/-} mice had decreased tumor cell proliferation and survival, suggesting the importance of host CXCR2 expression in the regulation of phenotypes associated with melanoma growth and experimental lung metastasis.

Our data suggest that loss of at least one allele of host CXCR2 has a greater effect on the ability of melanoma cells to metastasize than it does on tumor growth. Melanoma growth in mCXCR2^{+/-} mice was slower as compared with growth in wild-type animals, but was not significantly different. However, we observed a significant inhibition of experimental lung metastasis in mCXCR2^{+/-} animals as compared with wild-type animals. These data suggest organ site-dependent CXCL-8-CXCR2 signaling in melanoma growth and metastasis. A previous study showed that

Figure 2. Melanoma growth and experimental lung metastasis in CXCR2 knockout mice. A375SM cells (1×10^6 in 0.1 mL of HBSS) were s.c. injected into the flank of nude mice. Tumor volume was measured twice weekly with a caliper and tumor volume calculated. Tumor growth was significantly decreased ($P < 0.05$) in mCXCR2^{-/-} nude mice as compared with wild-type (WT) nude mice. Points, mean tumor volume; bars, SE (A). Median number of lung nodules of mCXCR2^{-/-} and mCXCR2^{+/-} mice were significantly lower as compared with wild-type nude mice (B). A representative picture of immunohistochemical staining for lung metastases in wild-type nude mice at $\times 200$ magnification (C).



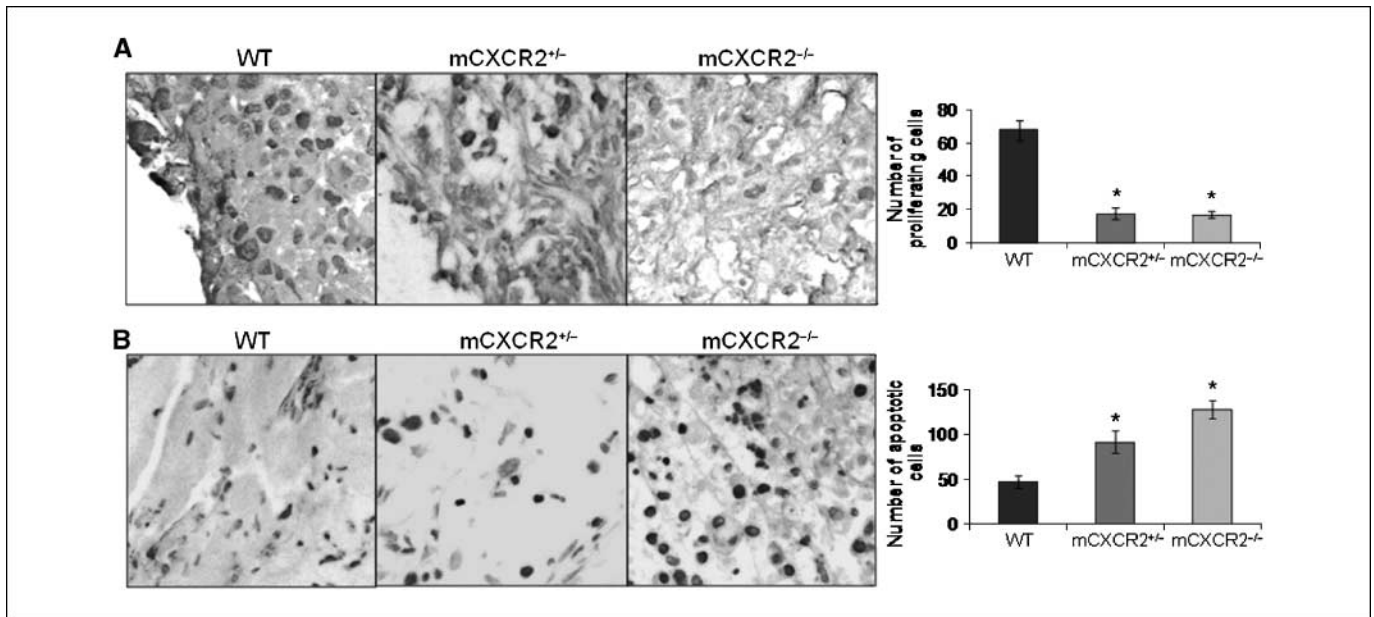


Figure 3. Decreased proliferation and survival in tumors from mCXCR knockout mice. Immunohistochemical staining for PCNA (A) and TUNEL (B) was analyzed as described in Materials and Methods. Stained cells were counted in 10 arbitrarily selected fields at $\times 200$ magnification in a double-blinded manner. Columns, average number of immunostained cells per field view (*, $P < 0.05$).

melanoma growing in the skin microenvironment expressed significantly higher levels of CXCL-8 as compared with the lung microenvironment (14), which can affect CXCL-8-mCXCR2-dependent responses. In addition, our data clearly show that loss of both alleles of host CXCR2 further inhibited melanoma growth in the skin microenvironment and experimental lung metastasis.

Previous reports have suggested a role for the CXCL-8-CXCR2 axis in melanoma angiogenesis (4). We hypothesized that this process might be diminished in mCXCR2^{-/-} mice. Our results

show that tumor angiogenesis was decreased in mCXCR2^{-/-} and mCXCR2^{+/-} mice compared with wild-type nude mice. These results are in agreement with another report in which the absence of CXCR2 on host endothelial cells within the tumor, tumor-associated angiogenesis, was markedly reduced (15). Similarly, attenuation of CXCR2 activity has been shown to inhibit angiogenesis and tumor growth (16).

Our data showed a diminished inflammatory response as observed by decreased neutrophil infiltration in tumors in

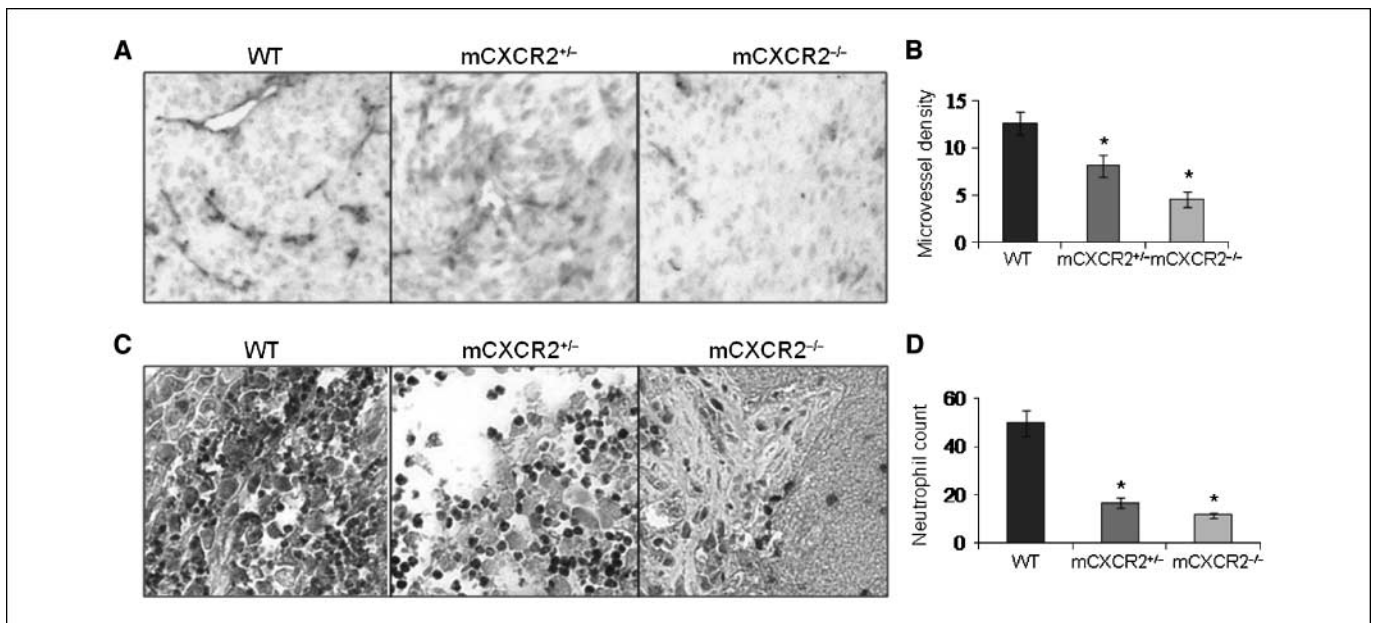


Figure 4. Inhibition in neovascularization and neutrophil infiltration in tumors from mCXCR2 knockout mice. Immunohistochemical staining for microvessels (A) using GS-IB4 and neutrophils (C) using mouse GR-1 was analyzed as described in Materials and Methods. Microvessel density (B) and neutrophils (D) were quantitated microscopically with a 5×5 reticle grid at $\times 400$ magnification. Columns, average number of immunostained positive cells; bars, SE (*, $P < 0.05$). Original magnification, $\times 200$.

mCXCR2^{-/-} mice. CXCR2 is the major mediator of neutrophil migration to sites of inflammation in addition to its angiogenic effect (17, 18). Mice that lacked an intact *mIL-8R α* gene had a loss of neutrophil chemotactic response and intracellular calcium flux to mMIP2 and hIL-8/CXCL-8 (11, 19), suggesting that mCXCR2 is the primary (or only) chemokine receptor for these ligands on mouse neutrophils. In the present study, we have compared neutrophil infiltration in tumors from wild-type and mCXCR2^{+/-} with mCXCR2^{-/-} nude mice. Our results show that mCXCR2^{-/-} mice had minimal neutrophil infiltration in tumors. The decrease in neutrophil infiltration in tumors from mCXCR2^{-/-} mice and their inability to respond to tumor-derived CXCL-8, in turn, decreases melanoma neovascularization, tumor growth, and metastasis in a paracrine manner. Neutrophils have been shown to play a role in promoting the metastatic phenotype of tumors releasing CXCL-8 (20).

In summary, our results suggest that host CXCR2 plays a critical role in melanoma growth, angiogenesis, and experimental lung metastasis. We have observed that loss of host CXCR2 (endothelial

cells and inflammatory cells) has a significant effect on melanoma growth, angiogenesis, and experimental lung metastasis. Hence, targeting the expression of host CXCR2 may represent a potential target for therapeutic intervention in CXCR2 ligand-producing malignant tumors.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

Received 9/3/2008; revised 10/27/2008; accepted 11/21/2008.

Grant support: CA72781 (R.K. Singh), Cancer Center Support grant (P30CA036727) from the National Cancer Institute, NIH, and Nebraska Research Initiative Molecular Therapeutics Program (R.K. Singh).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank Dr. Ajay P. Singh, University of Nebraska Medical Center, Nebraska for careful reading of this manuscript.

References

- Jemal A, Siegel R, Ward E, et al. Cancer statistics, 2008. *CA Cancer J Clin* 2008;58:71–96.
- Singh RK, Gutman M, Radinsky R, Bucana CD, Fidler IJ. Expression of interleukin 8 correlates with the metastatic potential of human melanoma cells in nude mice. *Cancer Res* 1994;54:3242–7.
- Ugurel S, Rappi G, Tilgen W, Reinhold U. Increased serum concentration of angiogenic factors in malignant melanoma patients correlates with tumor progression and survival. *J Clin Oncol* 2001;19:577–83.
- Varney ML, Johansson SL, Singh RK. Distinct expression of CXCL8 and its receptors CXCR1 and CXCR2 and their association with vessel density and aggressiveness in malignant melanoma. *Am J Clin Pathol* 2006;125:209–16.
- Addison CL, Daniel TO, Burdick MD, et al. The CXC chemokine receptor 2, CXCR2, is the putative receptor for ELR+ CXC chemokine-induced angiogenic activity. *J Immunol* 2000;165:5269–77.
- Clark-Lewis I, Dewald B, Geiser T, Moser B, Baggiolini M. Platelet factor 4 binds to interleukin 8 receptors and activates neutrophils when its N terminus is modified with Glu-Leu-Arg. *Proc Natl Acad Sci U S A* 1993;90:3574–7.
- Salcedo R, Resau JH, Halverson D, et al. Differential expression and responsiveness of chemokine receptors (CXCR1-3) by human microvascular endothelial cells and umbilical vein endothelial cells. *FASEB J* 2000;14:2055–64.
- Li A, Dubey S, Varney ML, Dave BJ, Singh RK. IL-8 directly enhanced endothelial cell survival, proliferation, and matrix metalloproteinases production and regulated angiogenesis. *J Immunol* 2003;170:3369–76.
- Li A, Varney ML, Valasek J, Godfrey M, Dave BJ, Singh RK. Autocrine role of interleukin-8 in induction of endothelial cell proliferation, survival, migration and MMP-2 production and angiogenesis. *Angiogenesis* 2005;8:63–71.
- Heidemann J, Ogawa H, Dwinell MB, et al. Angiogenic effects of interleukin 8 (CXCL8) in human intestinal microvascular endothelial cells are mediated by CXCR2. *J Biol Chem* 2003;278:8508–15.
- Calalano G, Lee J, Kikly K, et al. Neutrophil and B cell expansion in mice that lack the murine IL-8 receptor homolog. *Science* 1994;265:682–4.
- Sadanandam A, Varney ML, Kinarsky L, Ali H, Mosley RL, Singh RK. Identification of functional cell adhesion molecules with a potential role in metastasis by a combination of *in vivo* phage display and *in silico* analysis. *OMICS* 2007;11:41–57.
- Johnston RA, Mizgerd JP, Shore SA. CXCR2 is essential for maximal neutrophil recruitment and methacholine responsiveness after ozone exposure. *Am J Physiol Lung Cell Mol Physiol* 2005;288:L61–67.
- Gutman M, Singh RK, Xie K, Bucana CD, Fidler IJ. Regulation of IL-8 expression in human melanoma cells by the organ environment. *Cancer Res* 1995;55:2470–5.
- Mestas J, Burdick MD, Reckamp K, Pantuck A, Figlin RA, Strieter RM. The role of CXCR2/CXCR2 ligand biological axis in renal cell carcinoma. *J Immunol* 2005;175:5351–7.
- Keane MP, Belperio JA, Xue YY, Burdick MD, Strieter RM. Depletion of CXCR2 inhibits tumor growth and angiogenesis in a murine model of lung cancer. *J Immunol* 2004;172:2853–60.
- Terkeltaub R, Baird S, Sears P, Santiago R, Boisvert W. The murine homolog of the interleukin-8 receptor CXCR-2 is essential for the occurrence of neutrophilic inflammation in the air pouch model of acute urate crystal-induced gouty synovitis. *Arthritis Rheum* 1998;41:900–9.
- Benelli R, Albini A, Noonan D. Neutrophils and angiogenesis: potential initiators of the angiogenic cascade. *Chem Immunol Allergy* 2003;83:167–81.
- Lee J, Calalano G, Camerato T, Toy K, Moore MW, Wood WI. Chemokine binding and activities mediated by the mouse IL-8 receptor. *J Immunol* 1995;155:2158–64.
- De Larco JE, Wuertz BR, Furcht LT. The potential role of neutrophils in promoting the metastatic phenotype of tumors releasing interleukin-8. *Clin Cancer Res* 2004;10:4895–900.