

Combined Administration of Antibodies to Human Interleukin 8 and Epidermal Growth Factor Receptor Results in Increased Antimetastatic Effects on Human Breast Carcinoma Xenografts¹

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ABSTRACT

Purpose: Current antibody-based immunotherapeutic approaches under evaluation for breast carcinoma are limited in target scope. For example, administration of the human epidermal growth factor receptor (EGFR) antibody, alone or in combination with a chemotherapeutic drug, is thought to primarily inhibit tumor cell proliferation. The aim of this study was to assess the effects of a combined blockade designed to inhibit tumor growth by inhibition of proliferation rate and the proinflammatory effects of interleukin (IL) 8.

Experimental Design: A human breast carcinoma cell line that produces high levels of IL-8 was injected s.c. into severe combined immunodeficient mice. IL-8 has been reported to augment the progression of some human tumors; thus, we used a human IL-8 antibody, ABXIL8, in combination with anti-EGFR, ABXEGFR, to inhibit the metastasis of MDA231 tumors.

Results: Whereas anti-IL-8 alone had no appreciable antimetastatic effect, the combination of ABXIL8 significantly enhanced the antitumor effects of ABXEGFR, resulting in greater survival of SCID tumor-bearing mice. This effect on survival was correlated with decreased metastatic spread and decreased tumor size in mice receiving both antibodies. Intriguingly, *in vitro* studies indicate that this antibody combination markedly inhibited matrix metallo-

proteinase activity associated with MDA-231 cells to a greater degree than either antibody alone.

Conclusion: Combined administration of these two human antibodies using growth factor blockade in conjunction with chemokine blockade may thus provide a more effective approach for treatment of metastatic human breast carcinoma.

INTRODUCTION

Tumor cells arise in hostile environments and secrete factors that contribute to their survival. In addition, they express receptors that support proliferation and tumor growth. The EGFR³ and the chemokine IL-8 are reportedly survival factors for a variety of cell types including tumor cells (1–4) and are abundantly expressed by several breast carcinomas (5–8). Overexpression of EGFR by tumors has been associated with a poor clinical prognosis, and blocking EGFR inhibits the growth of several tumor cell types in mice (9–11). Consequently, therapeutic approaches that block EGFR in combination with chemotherapeutic agents such as paclitaxel, doxorubicin, and cisplatin are currently being tested in clinical trials on several tumor cell types including head and neck, non-small cell lung cancer, and breast carcinoma (6, 7, 12). In contrast to the proliferative effects of EGFR activation, the contribution of IL-8 to tumor growth is based on its chemotactic and angiogenic effects. However, IL-8 expression by ovarian carcinomas leads to reduce tumorigenicity, perhaps because of enhanced neutrophil infiltration into tumors sensitive to polymorphonuclear activities (13). Furthermore, the antitumor effect of Taxol has been attributed to an enhancement of tumor infiltration by neutrophils via up-regulation of tumor-associated IL-8 (14). There is also contrary evidence that blocking IL-8 prevents angiogenesis (15–17) and reduces tumorigenesis of human non-small cell lung cancer in SCID mice (18). Additionally, IL-8 production by breast carcinoma lines has been shown to correlate directly with their metastatic potential (19), perhaps by promoting their invasive capacity. In addition to IL-8, other chemokines such as MCP-1 and stromal cell-derived factor-1 α are reported to induce tumor growth by promoting angiogenesis and metastasis (20, 21).

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³ The abbreviations used are: EGFR, epidermal growth factor receptor; IL, interleukin; MCP, monocyte chemotactic protein; SCID, severe combined immune deficient; TGF, transforming growth factor; NCI-FCRDC, National Cancer Institute-Frederick Cancer Research and Development Center; VEGF, vascular endothelial growth factor; MI, metastatic index; MMP, matrix metalloproteinase; VEGF, vascular endothelial growth factor.

Because ~30% of human breast tumors exhibit up-regulated receptors for EGF (22) and a high percentage of metastatic breast carcinomas express IL-8 (19), we analyzed the diverse effects of blocking IL-8 and EGFR in combination using an experimental metastatic breast carcinoma-SCID mouse model. Furthermore, considering that anti-EGFR is known to target tumor growth by interfering with binding of EGF and TGF- α to EGFR (23), it follows that using the combination of these two antibodies might not only directly inhibit tumor growth but also interfere with the blood supply. The MDA-231 human breast carcinoma cell line was selected based on its coexpression of EGFR and IL-8 production. Because human antibodies have been shown to be less immunogenic and exert higher biological activities than rodent monoclonal antibodies (7, 23), we used human monoclonal antibodies to IL-8 and EGFR, termed ABXIL8 and ABXEGFR, respectively.

ABXEGFR can abolish EGF-dependent cell activation by completely blocking the binding of EGF and TGF- α to EGFR on human carcinoma cell lines and can also completely eradicate established human epidermoid carcinoma A431 xenografts in athymic mice without concomitant chemotherapy (23). ABXIL8 is currently being used in clinical trials for psoriasis and has been shown to effectively block *in vitro* IL-8-mediated neutrophil responses including neutrophil chemotaxis, activation, and *in vivo* IL-8-induced skin inflammation (24). Here we present evidence that therapeutic administration of anti-IL-8 potentiates the antitumor effects of anti-EGFR therapy in tumor-bearing SCID mice. Further analysis of the mechanistic effect of these antibodies led to the detection of inhibition of matrix metalloproteinase activity by IL-8 and EGFR blockage *in vitro*.

MATERIALS AND METHODS

Mice. Female C.B-17 scid/scid (SCID) mice were obtained from the Animal Production Area, NCI-FCRDC (Frederick, MD). Mice were used at 8–12 weeks of age. Animal housing and management were in accordance with the procedures outlined in the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 86-23, 1985; National Academy of Sciences, Institute of Laboratory Animal Resources, National Research Council, 1996). The experiments were conducted in accordance with the procedures outlined in the Guide for the Care and Use of Laboratory Animals, Institute of Animal Resources, National Research Council, Department of Health, Education and Welfare Publication 78-23 (National Institute of Health, Bethesda, MD).

Cells, Chemokines, and Antibodies. MDA231 cells were purchased from American Type Culture Collection (Rockville, MD). Cells were grown in RPMI 1640 containing 5% FCS, 2 mM glutamine, and 100 units/ml gentamicin. Human dermal microvascular endothelial cells were obtained from cell systems.com (Kirkland, WA). Endothelial cells were cultured on collagen type I-coated plastic wells (Biocoat; Becton Dickinson, Bedford, MA) in endothelial growth medium (Clonetics, Walkersville, MD) containing 5% FCS, 10 ng/ml VEGF, 10 ng/ml basic fibroblast growth factor, 2 mM glutamine, and 100 units/ml gentamicin. All experiments with endothelial cells were performed using subcultures between the second to four passages. Recombinant human IL-8, recombinant human EGF, and

recombinant murine EGF were purchased from Pepro Tech, Inc. (Rocky Hill, NJ). The human monoclonal IL-8 antibody (ABXIL8), human antibody to EGFR (ABX-EGFR), and human IgG were kindly provided by Abgenix, Inc. (Fremont, CA). Mouse antihuman EGFR was purchased from Upstate Biotechnology (Lake Placid, NY), and anti CXCR1 and CXCR2 were kindly provided by Genentech (South San Francisco, CA). Mouse IgG and human IgG were used as negative controls (Coulter, Miami, FL).

Establishment of Three-dimensional MDA-231 Tumors *in Vitro*. MDA-231 cells were resuspended at 1×10^6 cells/ml in RPMI 1640 containing 2 mM glutamine and 100 units/ml gentamicin in the presence or absence of FCS. Five hundred μ l of Matrigel/well were added to 24-well plates and left to solidify for 30 min at 37°C. Thereafter, 200 μ l of the cell suspension were added on top of the Matrigel and incubated at 37°C in a CO₂ atmosphere. The cells were fed every other day with 1 ml of medium. Initially, tumor cells formed tube-like structures; thereafter, they aggregated in small clumps, and three-dimensional tumors arose after 4–6 days.

ELISA. Determination for human cytokines was performed by ELISA (R&D, Minneapolis, MN).

Cell Permeabilization and Flow Cytometric Analysis. Indirect immunofluorescence was performed on MDA-231 cells by using saturating amounts of the corresponding antibodies. Fluorescein-conjugated F(ab)₂ fragments of goat antimouse (Sigma) was used as the secondary antibody. Cells were analyzed using a FACScan flow cytometer (Becton Dickinson, Mountain View, CA). For intracellular staining, cells were fixed in 2% paraformaldehyde for 5 min. Thereafter, cells were permeabilized with 0.8% octyl-glucopyranoside in buffer containing 0.5% gelatin for 30 min, washed twice with PBS, and subjected to staining with corresponding antibodies.

***In Vivo* Tumor Studies.** CB-17 SCID mice were used at 6–8 weeks of age and purchased from the animal production area (NCI-FCRDC, Frederick, MD). For survival experiments, SCID mice were injected i.v. with 20 μ l of anti-ASGM1 (Wako Chemicals, Richmond, VA) on day 0, and 3×10^5 MDA231 human breast carcinoma cells were injected i.v. on day 1. On day 2, antibody to IL-8, EGFR, and control human IgG (25 μ g/mouse, 1 mg/kg) were given i.p. Each antibody treatment was done twice a week for 10 weeks. The dosing for these antibodies was based on schedules from previous reports using antibodies to EGFR in gastric and squamous cell carcinomas (25, 26). Survival was monitored daily, and moribund mice were euthanized. For experimental metastasis experiments, mice from each group were sacrificed on the 35th day after i.v. injection of the tumor cells. Lungs, liver, and spleen were extracted and fixed in formalin. At this point, micrometastases were quantitated as described previously (20). Histological sections were stained with H&E, and tumor micrometastasis was quantitated using the Bioquant Program, counting the total tissue area/field $\times 40$ field (*D1*). The micrometastases present within the same field were gated, and the area within the gates was measured (*D2*). The MI was calculated by the ratio *D2:D1*. A minimum of 20 fields was analyzed per slide, and 10 mice were used per group in each experiment. The metastasis and survival experiments were repeated three times with 10 mice/group in each experiment.

MDA-231 Proliferation Assay. MDA-231 cells were resuspended at 1×10^6 cells/ml in RPMI 1640 containing 2 mM glutamine and 100 units/ml penicillin, 100 μ g/ml streptomycin, with or without FCS. One hundred μ l of the cell suspension/well were placed in 96-well plates. Cells were stimulated either with different concentrations of IL-8 (CXCL8) or EGF. Plates were incubated at 37°C in 5% CO₂ for 24 and 48 h. To determine cell proliferation, cells were incubated with [³H]thymidine (0.5–1 μ Ci/well) 4 h before uptake determination. After the incubation, plates were kept at –70°C overnight, and finally the plates were thawed and harvested, and [³H]thymidine incorporation was determined using a beta counter.

MDA-231 and Endothelial Cell Chemotaxis Assays. MDA-231 cell and endothelial cell chemotaxis was performed using micro-Boyden chambers. Polycarbonate filters of 10 μ m pore size (Nucleopore; NeuroProbe, Cabin John, MD) were coated with 10 μ g/ml collagen I (Sigma) overnight at 4°C. Binding medium containing 1.0% BSA in RPMI 1640 with or without various amounts of EGF were placed in the lower compartment of the chamber, and 50 μ l of cells resuspended at a concentration of 0.5×10^6 cells/ml in binding medium were then added to the upper compartment. The chambers were incubated for 3 h at 37°C. Membranes were scraped and stained with Leukostat (Fisher Scientific, Pittsburgh, PA) and analyzed using the BIOQUANT program (R & M Biometrics, Inc., Nashville, TN). The results were expressed as the mean number of migrated cells/10 fields at $\times 20$. For inhibitory assays, antibody to human EGFR was added together to the cells for 10 min before performing chemotaxis. Each sample was tested in triplicate, and each experiment was performed at least three times.

Immunoprecipitation, SDS-PAGE, and Immunoblot. For immunoblot, MDA-231 cells were lysed in lysis buffer [1% Triton X-100, 0.5% NP40, 150 mM NaCl, 10 mM Tris HCl (pH 7.4), 1 mM EDTA, 1 mM EGTA, 1 mM aprotinin, 1 mM leupeptin, and 0.2 mM phenylmethylsulfonyl fluoride] for 45 min at 4°C, followed by centrifugation (14,000 $\times g$, 15 min at 4°C). Protein extracts were precleared using 10 μ g of protein G-Sepharose (Sigma; 60 min at 4°C) and thereafter centrifuged as above. Cell lysates were incubated with anti-EGFR antibody (4 μ g/sample, 60 min, at 4°C) The immune complexes were precipitated by using protein G-Sepharose (60 min, 4°C). Immunoprecipitates were separated in 10% polyacrylamide gels. After SDS-PAGE, the proteins were transferred into nitrocellulose membranes. After blocking (5% skin milk, 2 h, 4°C), the membranes were exposed to anti-phosphotyrosine antibody (60 min, 4°C; Santa Cruz Biotechnology, Santa Cruz, CA) and developed using peroxidase-conjugated goat antimouse IgG (60 min, 4°C). Membranes were washed and developed by ECL (Amersham) according to the manufacturer's instructions.

MMP Gelatinase Activity Assay. Twenty μ g/ml of antibodies to EGFR or IL-8 were added to cell monolayers or three-dimensional cultures of MDA231 tumors. After 4 or 36 h incubation, supernatants were collected and aliquoted and stored at –70°C. Fifty μ l of sample were added to 160 μ l of diluted biotinylated gelatin substrate according to the manufacturer's instructions (Chemicon International, Temecula, CA). Samples were incubated for 30 min at 37°C. Thereafter, samples were transferred to a biotin-binding plate and incubated for 30 min at 37°C. After five washes, 100 μ l/well of streptavidin-enzyme

conjugate was added, and plates were incubated for 30 min at 37°C. Thereafter, plates were washed five times, and 100 μ l/well of substrate were added and incubated for 10 min at room temperature. The absorbance was measure at 450 nm. MMP2 activated with α -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid was used as positive control.

Statistical Analysis. The statistical analyses for chemotaxis, cell proliferation, cytokine production, and MMP activity assays were done by the conventional Student's *t* test and the Welch modified *t* test. The survival analyses were done by means of the log rank test and also the Cox proportional hazards model. The latter technique was used to determine whether the effects of anti-IL-8 and anti-EGFR were synergistic or additive. The one-way ANOVA was used in the analysis of the metastatic index. Pairwise comparisons were done as follow-up tests to the one-way ANOVA. The Games-Howell procedure was used for these comparisons, because it is robust with respect to violation of the assumption of equal variances across groups.

RESULTS

MDA-231 Cells Produce IL-8 and Express Receptors for Both IL-8 and EGF. To test the hypothesis that MDA-231 breast tumor cells actively contribute to their survival by secreting IL-8 and/or EGF, we first tested the expression of IL-8 by MDA-231 cell culture monolayers grown *in vitro*. Although baseline levels of secreted IL-8 were generally <1 ng/ml per 10^7 cells, as shown in Fig. 1A, this production was enhanced >4-fold ($P < 0.05$) when cells were grown under serum starvation conditions (absence of FCS). However, no EGF production was detected under any of the culture conditions used.

To determine whether modifications of the cell culture conditions could induce EGF production by MDA-231 cells, three-dimensional cultures were established as described in "Materials and Methods." As shown in Fig. 1A, although IL-8 production was enhanced at least 12-fold on a per cell basis, in three-dimensional tumor cultures ($P < 0.001$), EGF could still not be detected in the culture supernatants under these conditions.

To test the hypothesis that MDA-231 cells are capable of being driven in an autocrine fashion by IL-8 and in a paracrine fashion by EGF, we next tested the cell surface expression of IL-8 receptors (CXCR1 and CXCR2) and of EGFR and of intracellular receptors in permeabilized MDA-231 cells. As shown in Fig. 1B, CXCR1 was expressed more abundantly than CXCR2, with a mean fluorescence intensity of 18.7 (± 4.7) and 5.0 (± 1.3), respectively ($P < 0.05$). More EGFR was expressed on a per cell basis on MDA-231 cells (222 ± 24 ; $P < 0.01$).

Because these cells constitutively produce IL-8, we tested the possibility that the IL-8 might be internalizing its receptors on MDA-231 cells. We therefore examined permeabilized MDA-231 cells as described in "Materials and Methods." As shown in Fig. 1, B and C, a significant amount of the CXCR1 (74 ± 35) and CXCR2 (20 ± 6.3) on MDA-231 cells were internalized. In contrast, the majority of the EGFR on MDA-231 is expressed on the cell surface (218 ± 33), which could be attributable to absence of internalization by endogenous EGF (Fig. 1A) and TGF- α (Table 1). Thus, MDA-231 cells express IL-8 and its cognate receptors. They also express EGFR but do

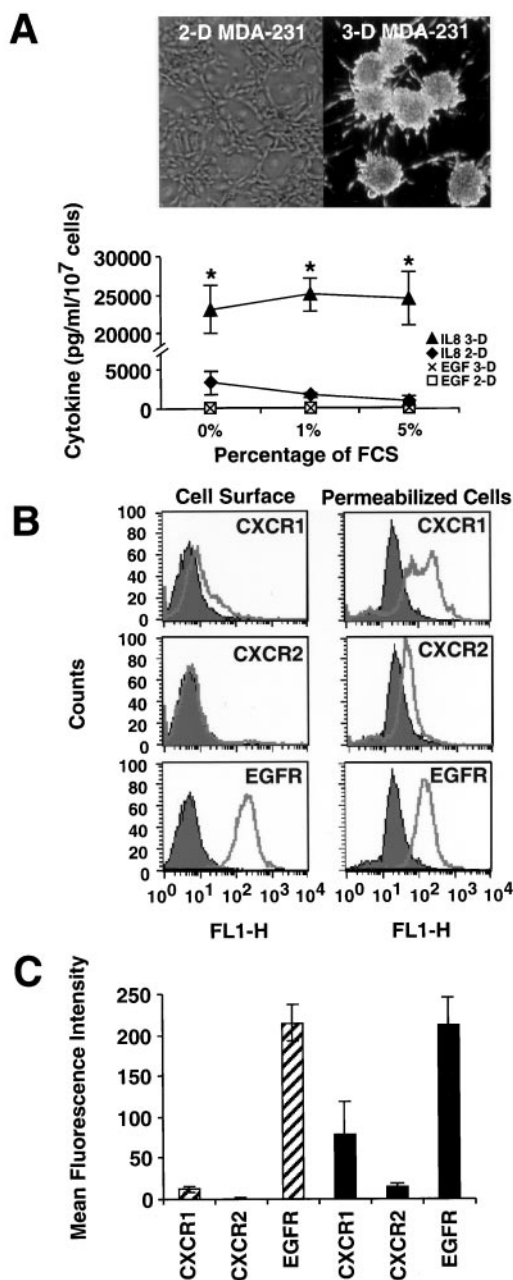


Table 1 Effect of culture conditions on cytokine profile produced by MDA-231 cells^a

^a Supernatants of MDA-231 cells grown under two-dimensional or three-dimensional conditions in RPMI 1640 containing 1% FCS were assessed for cytokine content by ELISA.

not produce EGF under any of the conditions assayed. It is of note that this tumor cell line expresses several other angiogenic factors in addition to IL-8, and that the cytokine profile was enhanced in the tumors grown under three-dimensional conditions.

Human Anti-EGFR and Anti-IL-8 Inhibited Chemotaxis of MDA-231 Cells in Response to EGF and IL-8, Respectively. To study the potential effects of anti-EGFR and anti-IL-8 on the migration of MDA-231 cells, we performed *in vitro* chemotaxis experiments. As shown in Fig. 2A, these cells responded chemotactically to human or murine EGF with similar dose-response curves, with a maximal chemotactic response in the range of 1–10 ng/ml of EGF (0.16–1.6 μ M). Additionally, MDA-231 cells also migrated in response to IL-8 with a maximal chemotactic dose at 1 ng/ml. The chemotactic response of MDA-231 cells toward IL-8 were at least 5-fold lower than the chemotactic response toward EGF ($P < 0.01$). These chemotactic responses to either human or murine EGF were completely blocked by addition of anti-EGFR (Fig. 2B), when used at a concentration of 10 μ g/ml (33 nM; $p < 0.01$). In the same manner, anti-IL-8 completely abolished MDA-231 responses to IL-8 at 10 μ g/ml ($P < 0.01$; Fig. 2C). Thus, MDA-231 cells express functional receptors for EGF and IL-8, and both antibodies effectively block functional interactions with the breast cancer cells.

MDA-231 Cell Proliferation Is EGF Dependent and IL-8 Independent. Because EGF is a classical mitogen for epithelial cells, we tested the effect of EGF on MDA-231 cells. We observed that human EGF (Fig. 3) and murine EGF (data not shown) induced cell proliferation. The proliferative effects of EGF were obtained at doses as low as 1 ng/ml with a plateau >100 ng/ml (data not shown). This mitogenic effect was completely abolished by the addition of anti-EGFR when used at a concentration of 20 μ g/ml (66 nM). Thus, anti-EGFR completely blocks proliferation of MDA-231 cells in response to EGF.

In a similar manner to EGF, IL-8 can also act as a mitogenic factor for some cell types and because MDA-231 cells express IL-8 and its receptors, we tested whether these tumor cells could potentially use IL-8 in an autocrine manner. As

Fig. 1 MDA-231 cells produce IL-8 but not EGF and express CXCR1, CXCR2, and EGFR. A, the production of IL-8 and EGF by MDA-231 cells was tested in two-dimensional (2D) and three-dimensional (3D) tumors in the presence of different concentrations of FCS. The data represent the means of three different experiments; bars, SE. *, $P < 0.001$ as assessed by Student's *t* test. B, flow cytometric analysis of the cell surface expression of CXCR1, CXCR2, and EGFR on the cell surface and in permeabilized MDA-231 cells. The background level of fluorescence in the presence of mouse IgG control is depicted by the filled histograms. CXCR1, CXCR2, and EGFR (open histograms) were detected using monoclonal antibodies as described in the methods. A representative experiment of three is shown. C, MDA-231 cells express more receptors for EGF than for IL-8. The intensity of CXCR1, CXCR2, and EGFR expressed by MDA-231 cells was determined, and the results are expressed as mean fluorescence intensity for each receptor with the Ab control subtracted. The means of three experiments are shown; bars, SE.

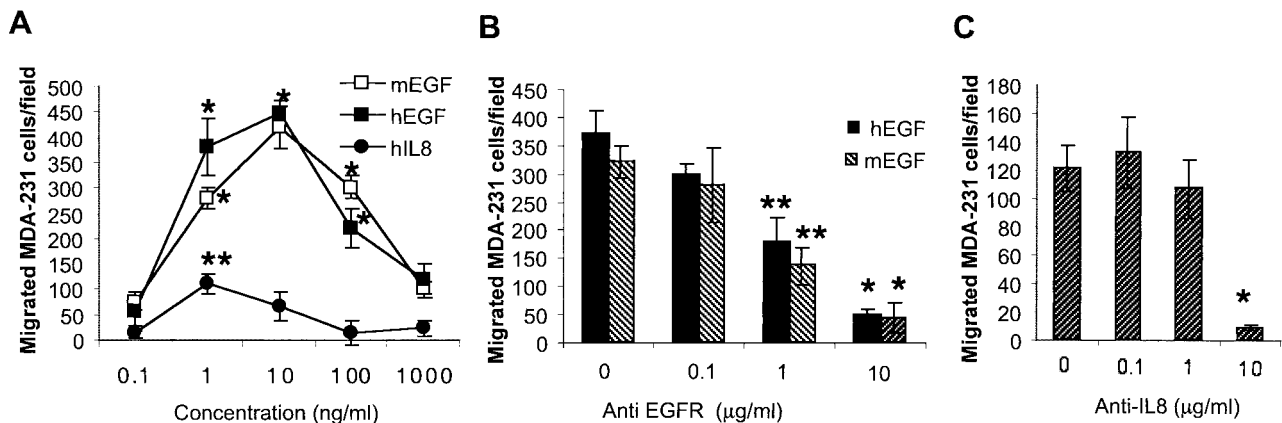


Fig. 2 Human antibodies to EGFR and IL-8 inhibited chemotaxis of MDA-231 cells toward EGF and IL-8, respectively. *A*, MDA-231 cells migrate toward human (*hEGF*), murine EGF (*mEGF*), and human IL-8 (*hIL8*). *, $P < 0.01$ and **, $P < 0.05$ in relation to the spontaneous basal migration as assessed by Student's *t* test. *B*, the chemotaxis of MDA-231 cells in response to the optimal dose of hEGF and mEGF (10 ng/ml; 1.6 μM) were inhibited by anti-EGFR (10 μg/ml). *, $P < 0.01$ and **, $P < 0.05$ as assessed by Student's *t* test. *C*, human antibody to IL-8 (10 μg/ml) inhibited chemotaxis of MDA-231 cells to IL-8. The means of three experiments are shown; bars, SE. *, $P < 0.01$ as assessed by Student's *t* test. The chemotaxis data are expressed as number of migrated cells/×20 field.

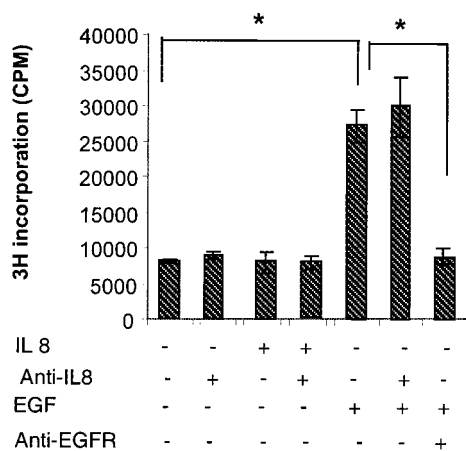


Fig. 3 Anti-EGFR inhibited MDA-231 proliferation in response to EGF. MDA-231 cell proliferation in response to the optimal dose of hEGF (1 ng/ml; data not shown) was compared with the effect of exogenous hIL (10 ng/ml). For inhibitory experiments, antibodies to EGFR and IL-8 were used at 20 μg/ml. Proliferation was measured by [³H]thymidine incorporation. The means of five experiments are shown; bars, SE. *, $P < 0.001$ as assessed by Student's *t* test.

shown in Fig. 3, blocking endogenous IL-8 using anti-IL-8 did not have any effect on the rate of MDA-231 proliferation. Moreover, addition of exogenous IL-8 to MDA-231 cultures did not enhance cell proliferation, nor did anti-IL-8 have any detectable effect on EGF-mediated proliferation. Thus, MDA-231 cells apparently do not use IL-8 as a mitogenic factor.

Human IL-8 Antibody (ABXIL8) Abolished *in Vitro* Chemotaxis of Endothelial Cells in Response to IL-8. IL-8-mediated angiogenic responses directly correlate with the ability of IL-8 to induce endothelial cell chemotaxis; therefore, we tested the effect of anti-IL-8 on IL-8-induced chemotaxis of endothelial cells. As shown in Fig. 4, endothelial cells re-

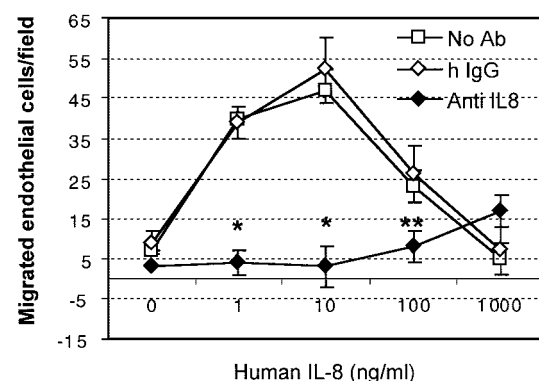


Fig. 4 Human antibody to IL-8 inhibited chemotaxis of endothelial cells to IL-8. The migration of endothelial cells to IL-8 was inhibited by anti-IL-8 (10 μg/ml). The chemotaxis data are expressed as number of migrated cells/×20 field. The means of three experiments are shown; bars, SE. *, $P < 0.01$ and **, $P < 0.05$ as assessed by Student's *t* test.

sponded chemotactically to IL-8 with a maximal chemotactic response over a range of 1–10 ng/ml of IL-8. Anti-IL-8 at a dose of 10 μg/ml completely abolished this chemotactic response ($P < 0.01$). Thus, the human anti-IL-8 functionally blocks IL-8-induced responses by other cell types *in vitro*.

Administration of Anti-IL-8 Enhances the Antitumor Effect of Anti-EGFR in SCID Mice Bearing MDA-231 Human Breast Carcinoma Cells. Our *in vitro* results on the characterization of the effects of anti-EGFR and anti-IL-8 on MDA-231 tumor model indicated that EGF-mediated chemotaxis and proliferation of MDA-231 cells and IL-8-mediated endothelial cell chemotaxis were each inhibited completely by the respective antibodies. On the basis of these data, we hypothesized that using these antibodies in combination would have a greater antitumor effect compared with each antibody administered alone.

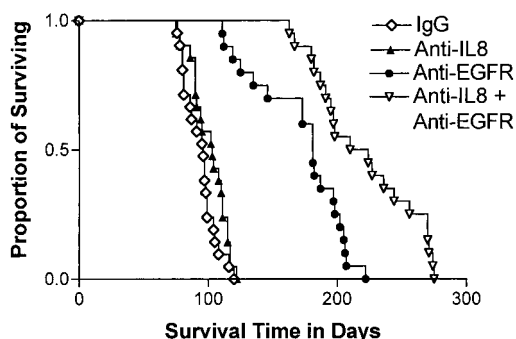


Fig. 5 Anti-IL-8 enhances anti-EGFR antitumor effects in SCID mice bearing MDA-231 human tumor breast carcinoma cells. MDA-231 human breast cancer cells (3×10^5) were injected i.v. on day 1. Antibodies including human IgG, ABXIL-8, and ABXEGFR (25 μ g/mouse, 1 mg/kg) were given i.p. Each antibody treatment was administered biweekly for 10 weeks. Survival was monitored daily, and moribund mice were euthanized. The log rank test statistical analysis indicates a $P < 0.001$ for the anti-EGFR-treated group in comparison with control antibody and a $P = 0.002$ for the combined anti-EGFR + anti-IL-8 treatment in comparison with anti-EGFR treatment alone. According to the Cox proportional hazards analysis, anti-IL-8 + anti-EGFR effects were additive. The experiment was repeated three times with at least 10 mice/group in each experiment. The figure represents the data obtained in two independent experiments.

Numerous reports demonstrate that EGF is abundantly produced by lung tissue (27–33). Thus, because MDA-231 cells appear to be capable of responding to an exogenous source of EGF, they would presumably thrive in the lung. The expression of receptors for IL-8 and EGF on MDA-231 cells is consistent with the possibility that these factors might play a contributory role in lung metastasis. To test our hypothesis, we injected MDA-231 cells i.v. into SCID mice and administered anti-IL-8, anti-EGFR, or a combination of these antibodies twice a week for 10 weeks. As shown in Fig. 5, anti-IL-8 therapy did not result in any significant increase in survival when compared with the control, human IgG-treated group. In contrast, anti-EGFR therapy significantly increased the survival of SCID mice bearing MDA-231 tumors ($P < 0.001$). It is notable that the combination anti-EGFR and anti-IL-8 administration resulted in a significant enhancement of survival when compared with anti-EGFR therapy used alone ($P = 0.002$). Thus, the combination of EGFR and IL-8 antibody therapy results in improved antitumor response *in vivo*.

Greater Antimetastatic Effects Were Observed after Combined Administration of Anti-IL-8 and Anti-EGFR Relative to Anti-EGFR Alone. To further evaluate the observed therapeutic effects, we dissected the lungs, liver, and spleen of tumor-bearing mice treated with each antibody individually or combination on day 35 after i.v. injection of MDA-231 tumor cells. Histological sections revealed metastatic lesions only in the lungs. These experimental metastases were much larger in mice treated with control human IgG or anti-IL-8 (Fig. 6A) alone. In contrast, mice treated with anti-EGFR or the combination of both antibodies showed fewer and much smaller metastatic lesions (Fig. 6A).

To quantify the metastatic tumor burden, we performed a grading analysis by calculating the total area invaded by tumor

cells as a percentage of the total tissue, as explained in “Materials and Methods.” A significant decrease in the MI in mice treated with anti-EGFR (MI, 0.195; SE, ± 0.027) was observed as compared with human IgG alone (MI, 0.336; SE, ± 0.037) with $P < 0.005$ (Fig. 6B). The anti-IL-8-treated group exhibited a MI of 0.364 (SE ± 0.04), which was not significantly different from the control mice. In contrast, the combination of anti-IL-8 and anti-EGFR exhibited a MI of 0.130 (SE ± 0.20), which is statistically greater than the effect of anti-EGFR therapy alone ($P < 0.025$) and in agreement with *in vivo* survival data (Fig. 5). Thus, it appeared that blockage of both IL-8 and EGF cooperates to inhibit MDA-231 tumor growth and metastatic spread to a greater degree.

EGF-mediated Responses Are Not Affected by IL-8 *in Vitro*. Because our *in vivo* data showing that combination of IL-8 and EGFR antibodies have greater antitumor effect than EGFR antibody alone and because in some ovarian carcinomas IL-8 is reported to enhance EGF-mediated signal transduction events including phosphorylation (34), we tested the possibility that in our model IL-8 might be interacting with EGFR by augmenting EGFR phosphorylation in response to EGF. As shown in Fig. 7, EGF induced EGFR phosphorylation in MDA-231 cells, and this phosphorylation was completely inhibited by addition of EGFR antibody. In contrast, IL-8 did not have any detectable effect on EGFR phosphorylation. Moreover, anti-IL-8 did not inhibit EGF-mediated phosphorylation of EGFR. Therefore, interference with EGF-mediated phosphorylation of its receptor does not appear to account for the enhanced *in vivo* therapeutic effect of anti-IL-8 when used in combination with anti-EGFR.

Combination of Anti-EGFR and Anti-IL-8 Results in Greater Inhibition of MMP Activity and MMP9 Expression by MDA-231 Cells. It is well established that MMP generation plays an important role in tumor metastasis (35). Both IL-8 and EGF have been shown to trigger the production of MMPs by several cell types (36–41). We therefore hypothesized that inhibition of MMP induction could be underlying the *in vivo* antitumor effects seen using combined administration of IL-8 and EGFR antibodies.

We first studied the expression of MMPs by MDA-231 cells, and we detected abundant expression of MMP9 and very low levels of expression MMP2 by immunoprecipitation followed by Western blot (data not shown). We then assessed the functional activity of these the proteases by using a functional gelatinase assay for MMP2 and MMP9 as described in “Materials and Methods.” As shown in Fig. 8A, MDA-231 cells display higher gelatinase activity when grown under three-dimensional conditions than monolayers, and this correlated with higher levels of IL-8 found in the culture supernatant under the three-dimensional conditions. It is noteworthy that in our three-dimensional cell cultures, Matrigel (0.5–1.3 ng/ml) and FCS (0.1–0.4 ng/ml) are sources of EGF. We next tested the combined effects of anti-EGFR and anti-IL-8 on MDA-231-associated MMP activity. As shown in Fig. 8B, treatment of three-dimensional MDA-231 cell tumors with either anti-IL-8 or anti-EGFR significantly inhibited MMP activity by about 26 and 33%, respectively, in relation to the treatment with control antibody alone ($P < 0.05$). When anti-IL-8 and anti-EGFR were used in combination, the inhibitory effect was enhanced to

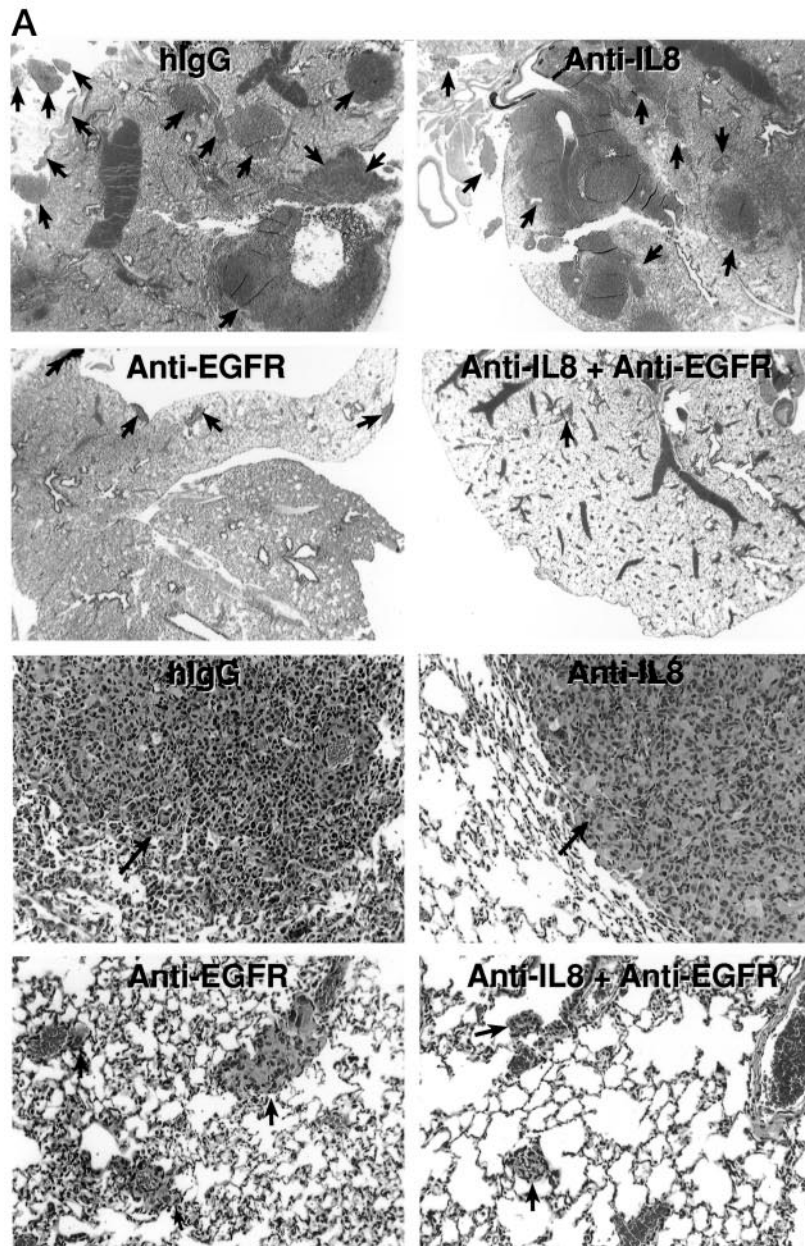
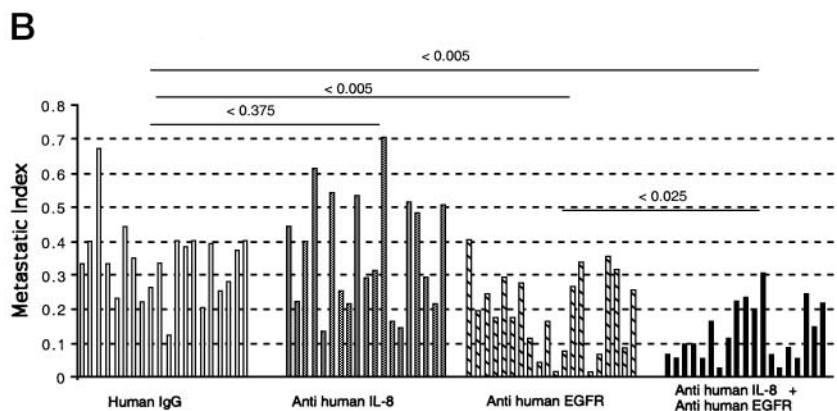


Fig. 6 Anti-IL-8 enhances the antimetastatic effect of anti-EGFR treatment in SCID mice bearing MDA-231 human tumor breast carcinoma cells. SCID mice were injected i.v. with 20 μ l of antiserum to ASGM1; 3×10^5 MDA-231 human breast cancer cells were injected i.v. on day 1. Antibodies including human IgG, anti-IL-8, and anti-EGFR (25 μ g/mouse, 1 mg/kg) were given i.p. For experimental metastasis, each antibody treatment was given twice a week for 5 weeks, and mice were sacrificed on the 35th day after i.v. injection of the tumor cells. Lungs, liver, and spleen were extracted and fixed in formalin. **A**, histological sections were stained with H&E to evaluate tumor metastases. Photographs were taken at $\times 20$ (upper photographs) and $\times 200$ (lower photographs). Arrows, metastatic lesions. One representative field from two experiments is shown. **B**, micrometastases were quantitated as described in "Materials and Methods." The experiment was repeated three times. Statistical analysis between the different groups was performed using one-way ANOVA with the Games Howell procedure for pairwise comparison; the *P*s are depicted in the figure.



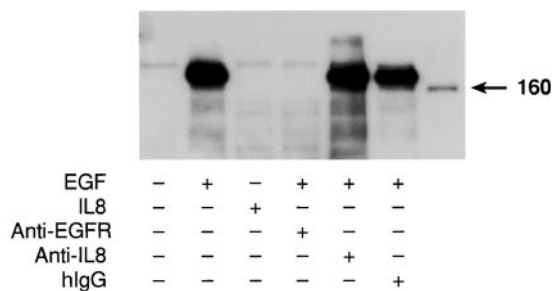


Fig. 7 EGF-induced phosphorylation of EGFR on MDA-231 cells is inhibited by anti-EGFR but not by IL-8 antibody. Phosphorylation experiments were performed as described in "Materials and Methods." MDA-231 cells were stimulated for 20 min with EGF (10 ng/ml) or IL-8 (50 ng/ml). Antibodies to EGFR and IL-8 or human IgG were used at 20 μ g/ml. The figure shows one representative experiment of at least five experiments.

~50% in relation to the treatment with control antibody alone ($P < 0.01$; Fig. 8B), which was statistically different in comparison with each antibody alone ($P < 0.05$).

Analysis of the effect of anti-IL-8 and anti-EGFR on the expression of MMP2 and MMP9 by MDA-231 cells indicated that both antibodies partially inhibited the expression of MMP9 by MDA-231 cells (Fig. 8C). When both antibodies were used in combination, the cell surface expression of MMP9 by MDA-231 cells was decreased significantly in comparison with each antibody alone ($P < 0.05$; Table 2). Thus, the combination of antibodies to IL-8 and EGFR can inhibit MDA-231-associated MMP activity. A downstream consequence of the combined administration of anti-IL-8 and anti-EGFR antibodies would therefore be the inhibition of the capacity of MDA-231 breast tumor cells to invade normal lung tissue.

DISCUSSION

Tumors persist and thrive in immunocompetent hosts via autocrine/paracrine mechanisms that maintain proliferation using escape mechanisms, resulting in the defeat of immune system-dependent elimination, and by producing proteases such as MMPs that facilitate invasion of normal tissue and metastasis. In this study, we used an experimental model of metastatic spread of breast cancer cells to the lung and sought to evaluate the contribution of IL-8 and EGF to tumor growth, based on the high incidence of IL-8 and EGFR expression by human breast tumors (19, 22).

IL-8 has been shown to act as a mitogenic factor via CXCR1 and CXCR2 for certain tumor cell types (34) by a mechanism that involves Erk and EGFR phosphorylation. In contrast to previously published results, we did not detect evidence of cross-talk between the IL-8 and EGF receptors. In our model, IL-8 was not a proliferative factor for MDA-231 cells, and IL-8 did not induce EGFR phosphorylation. Moreover, in contrast to what was observed for gastric carcinomas in which IL-8 increased the expression of the EGFR as well as its own levels (42), in our tumor model, stimulation of MDA231 cells with exogenous IL-8 or blocking by anti-IL-8 of the endogenously produced IL-8 has no effect on the expression of EGF or EGFR (data not shown). Neither did stimulation of MDA-231

cells with exogenous EGF have any effect on the expression of IL-8, CXCR1, or CXCR2 (data not shown). Thus, the mechanism of anti-IL-8 enhancing the antimetastatic effect of EGFR antibody is not attributable to a mutual up-regulation of IL-8 and EGF or their cognate receptors.

IL-8 production by tumors such as melanomas directly correlates with metastatic spread (43), and IL-8 levels are higher in metastatic malignant melanoma than in primary melanomas (44). Moreover, UV light-induced increases in the aggressiveness and metastasis of cutaneous melanoma are mediated by IL-8 (45). In line with these observations, IL-8 enhances MMP production in a variety of tumor types including melanoma (37), gastric (42), renal (46), and prostate (47) carcinomas. Here we show that blocking IL-8 only partially inhibited the MMP9 activity produced by MDA-231 cells *in vitro*. Indeed, administration of anti-IL-8 alone did not enhance the survival or inhibit the number of experimental metastases in SCID mice. It is important to note that mice do not produce IL-8; therefore in this model anti-IL-8 is expected to block exclusively tumor-produced IL-8.

The fact that IL-8 therapy alone was ineffective may be based on production of redundant compensatory cytokines such as MCP-1, GRO α , VEGF, and others (Table 1). IL-8 is also not an exclusive inducer of MMP activities because other mediators such as EGF can also indirectly activate MMPs. In addition to matrix degradation, other activities such as chemotaxis must occur concomitantly to account for metastasis (21). Therefore, in our study, anti-IL-8 may have potentiated the effect of anti-EGFR by a common mechanism that involved inhibition of MMPs and also by divergent mechanisms, because anti-EGFR can inhibit MDA-231 growth and motility, whereas anti-IL-8 potentially targeted angiogenesis. Although anti-IL-8 is an effective inhibitor of human endothelial cell chemotaxis toward IL-8 *in vitro*, this antibody only marginally blocked endothelial cell migration toward MDA-231 supernatants (data not shown). The low efficiency of this antibody in blocking angiogenic responses *in vitro* may be attributable to the redundant repertoire of angiogenic mediators in the MDA-231 supernatant including MCP-1 and VEGF (Table 1). Furthermore, EGF is also an angiogenic factor (48), and our *in vitro* data indicated that anti-EGFR (ABXEGFR) inhibited human endothelial cell chemotaxis in response to EGF (data not shown). Nevertheless, in our *in vivo* murine model, anti-EGFR would not be expected to have any angiostatic effect, because this antibody targets exclusively human EGFR expressed only by MDA-231 cells and not by murine endothelial cells or other cell types such as epithelial cells. On the other hand, in human tissues, this antibody can target endothelial EGFR; therefore, anti-EGFR may have beneficial angiostatic effects in patients. Thus, although EGF and IL-8 have overlapping angiogenic effects in humans because they use distinct signal transduction pathways, the antiangiogenic effect of antibodies to these cytokines may be synergistic in the human.

It is noteworthy that three-dimensional culture of tumors might provide a more representative model of *in vivo* tumors, because we noted a significant enhancement in cytokine and chemokine levels and MMP activity relative to the same tumor cell number grown as monolayers (Table 1). Thus, three-dimen-

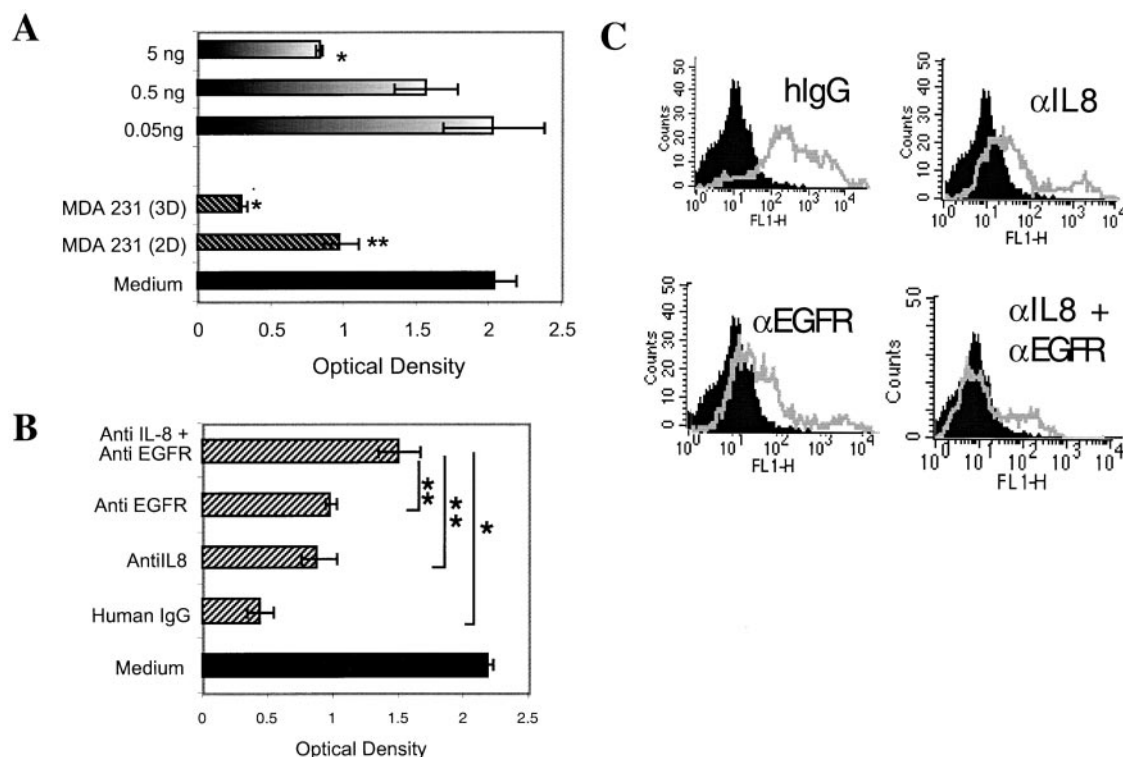


Fig. 8 Antibodies to IL-8 and EGFR inhibited MMP activity and cell surface expression of MMP-9 by MDA-231 cells. **A**, the MMP activity of two-dimensional (2D) and three-dimensional (3D) MDA-231 cultures was compared with different concentrations of activated MMP2 or medium alone. A major activity is reflected by a decrease in the absorbance. *, $P < 0.01$ and **, $P < 0.05$ as assessed by Student's *t* test. **B**, anti-EGFR and anti-IL-8 inhibited MMP activity of MDA-231 three-dimensional cultures. Human IgG, anti-EGFR, and anti-IL-8 were used at 20 $\mu\text{g}/\text{ml}$. The data represent the means of three experiments; bars, SE. *, $P < 0.01$ and **, $P < 0.05$ as assessed by Student's *t* test. **C**, antibodies to IL-8 and EGFR inhibited the expression of MMP-9 on the cell surface of MDA-231 cells. The expression of MMP-9 on the cell surface of three-dimensional MDA-231 cultures stimulated for 18 h with 20 $\mu\text{g}/\text{ml}$ of hlgG, anti-IL-8, anti-EGFR, or the combination is indicated. Rabbit IgG was used as negative control (black histograms). Rabbit anti human MMP-9 is shown by the gray histograms. The data show one representative experiment of three.

Table 2 Antibodies to IL-8 and EGFR decrease cell surface expression of MMP-9 by MDA 231 cells

Antibody treatment	Mean fluorescence	
	MMP-9	MMP-2
Human IgG	367 \pm 42	82 \pm 22
Anti-IL-8	176 \pm 23	76 \pm 36
Anti-EGFR	113 \pm 32	90 \pm 19
Anti-IL-8 + Anti-EGFR	45 \pm 10	73 \pm 31

Three-dimensional MDA-231 tumors were stimulated with human IgG, anti-IL-8, or anti-EGFR at 50 $\mu\text{g}/\text{ml}$ for 18 h. Thereafter, cells were assessed for the expression of MMP-9 and MMP2 by using polyclonal antibodies as described in "Materials and Methods".

sional tumor cells would appear more likely to persist and thrive than isolated tumor cells.

Several reports indicate a potential contribution of EGF to the production of MMPs in ovarian and head and neck carcinomas (36, 40, 41). In a similar manner, IL-8 production by tumors has also been shown to alter MMP production and activities in a variety of tumor cell types including melanoma, gastric, renal, and prostate carcinomas (37, 42, 47, 49). Here we present, to our knowledge, the first *in vivo* evidence in which simultaneous

inhibition of these two cytokines was associated with reduction in tumor metastasis, thus supporting that the use of this combined antibody administration could be potentially be useful for other IL-8-producing and EGFR-positive carcinoma cells types.

Many if not most clinical trials for cancer involve the administration of single agents, such as VEGF antibodies, Combrestatin, or TNP-40, which reportedly improve survival and inhibit tumor growth (50), but it seems more likely that combinations of agents will be more effective. In a complex tumor environment, targeting one of the many factors alone seems highly unlikely to result in durable suppression of tumor growth. Currently, one of the few new treatments for breast carcinoma involves the administration of Herceptin, a monoclonal antibody directed against the human EGFR. However, when presented with more complex tumor types that produce an array of growth and inflammatory factors, it seems unlikely that Herceptin alone will be sufficiently inhibitory in the majority of cases. We have shown previously that blocking MCP-1 also enhanced the survival and reduced the number of MDA-231 lung metastasis in SCID mice (20). We would expect that combined administration of MCP-1, IL-8, and EGFR antibodies would be even more effective in inhibiting tumor growth. On the other hand, a more universal treatment for breast carcinoma might involve the

administration of inhibitors such as IFN- β , which would be expected to inactivate the effects of many of the cytokines listed in Table 1. Histologically indistinguishable breast tumors can vary widely regarding their gene expression patterns (22); therefore, better tools for the characterization of tumors should help direct the formulation of efficacious combinations of antitumor agents. Thus, a multiprolonged therapeutic strategy using biological agents might ultimately be developed to produce more desirable and thorough tumor eradication.

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Clinical Cancer Research

Combined Administration of Antibodies to Human Interleukin 8 and Epidermal Growth Factor Receptor Results in Increased Antimetastatic Effects on Human Breast Carcinoma Xenografts

Rosalba Salcedo, Manuela Martins-Green, Barry Gertz, et al.

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