BRAK/CXCL14 Is a Potent Inhibitor of Angiogenesis and a Chemotactic Factor for Immature Dendritic Cells

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ABSTRACT

BRAK/CXCL14 is a CXC chemokine constitutively expressed at the mRNA level in certain normal tissues but absent from many established tumor cell lines and human cancers. Although multiple investigators cloned BRAK, little is known regarding the physiologic function of BRAK or the reason for decreased expression in cancer. To understand the possible significance associated with loss of BRAK mRNA in tumors, we examined the pattern of BRAK protein expression in normal and tumor specimens from patients with squamous cell carcinoma (SCC) of the head and neck and used recombinant BRAK (rBRAK) to investigate potential biological functions. Using a peptide-specific antiserum, abundant expression of BRAK protein was found in suprabasal layers of normal tongue mucosa but consistently was absent in tongue SCC. Consistent with previous in situ mRNA studies, BRAK protein also was expressed strongly by stromal cells adjacent to tumors. In the rat corneal micropocket assay, BRAK was a potent inhibitor of in vivo angiogenesis stimulated by multiple angiogenic factors, including interleukin 8, basic fibroblast growth factor, and vascular endothelial growth factor. In vitro, rBRAK blocked endothelial cell chemotaxis at concentrations as low as 1 nmol/L, suggesting this was a major mechanism for angiogenesis inhibition. Although only low affinity receptors for BRAK could be found on endothelial cells, human immature monocyte-derived dendritic cells (iDCs) bound rBRAK with high affinity (i.e., $K_d \sim 2$ nmol/L). Furthermore, rBRAK was chemotactic for iDCs at concentrations ranging from 1 to 10 nmol/L. Our findings support a hypothesis that loss of BRAK expression from tumors may facilitate neovascularization and possibly contributes to immunologic escape.

INTRODUCTION

To identify genes associated with the malignant phenotype of head and neck squamous cell carcinoma (SCC), we previously used differential display of matched tumor explants and normal oral squamous epithelial cells. One of the genes down-regulated in tumor specimens was BRAK/CXCL14, which encodes a novel chemokine (1). BRAK was independently cloned by Hromas et al. (2) and was found in normal kidney and breast tissues but was absent in the majority of established tumor cell lines. The work of multiple investigators (1–4) formed an early consensus that BRAK mRNA was constitutively expressed in normal tissues but was absent in a variety of transformed cells. The absence of BRAK from many tumor cell lines (1, 2) and head and neck SCC tumor specimens (1) is of currently unknown biological significance. Moreover, little is known regarding the physiologic functions of this gene.

To date, three groups of investigators have examined the chemotactic properties of BRAK for leukocytes and reported disparate results. Among cells proposed to respond to BRAK by various investigators are prostaglandin E2–treated monocytes (5), cell lines from B-cell and monocytic cell lineages (3), neutrophils, and dendritic cells (4). A plausible explanation for the lack of agreement could be the different sources of BRAK used by investigators, which included a synthetic polypeptide, a murine homologue, and unpurified conditioned supernatants from transfected mammalian cells. The recent availability of commercially purified recombinant human BRAK (rBRAK) should allow for better study of the physiologic targets and functions of the BRAK gene.

Although there are few published articles focusing on BRAK, one common finding appears to be the persistent absence of BRAK mRNA from established tumor cell lines despite constitutive expression in normal tissues. Consistent with these findings, we previously showed by in situ hybridization that BRAK mRNA is abundantly expressed in normal squamous mucosa but absent from a majority of head and neck SCC tumors (1). A role for chemokines in cancer is supported by evidence that these molecules can regulate fundamental biological processes, including tumor-associated angiogenesis, activation of host tumor-specific immunity, and autocrine stimulation of tumor growth (6–12).

Chemokines are classified into subfamilies based on variations in a structural motif of conserved aminoproximal cysteine residues and include the CXC, CC, CX3C, and the C families. BRAK belongs to the CXC family, which can be further subdivided by the presence or absence of a conserved “Glu-Leu-Arg” (ELR) motif at the NH2 terminus. ELR(+) CXC chemokines, such as GRO-α/CXCL1, IL-8/CXCL8, and ENA-78/CXCL5, are angiogenic, whereas ELR(−) CXC chemokines induced by interferon, such as PF-4/CXCL4, IP-10/CXCL10, and MIG/CXCL9, are angiostatic (10, 13–16). Although not induced by interferon (1), BRAK does lack an ELR motif similar to the angiostatic CXC chemokines. However, the role of BRAK in the regulation of angiogenesis remains to be established.

Chemokine action is mediated via members of the seven-transmembrane domain G protein–coupled receptors, which bind multiple ligands within chemokine subfamilies (17, 18). However, the receptors and mechanisms by which chemokines inhibit chemotaxis of endothelial cells currently are unknown. Although the angiostatic chemokines γ-interferon–inducible protein (IP-10), MIG, and I-TAC mediate chemotaxis of activated T cells through binding the high affinity chemokine receptor CXCR3 (19), there is little evidence that CXCR3 is involved in inhibition of endothelial cell chemotaxis. Although endothelial cells reportedly express low levels of CXCR3 (20, 21), a recent publication suggests that only the mRNA splice variant termed CXCR3B is present in these cells (22). This putative receptor was reported to mediate growth arrest of endothelial cells in response to PF-4 and IP-10. However, a role
for CXCR3B in inhibition of endothelial chemotaxis has not been investigated. The chemokine receptor that binds BRAK currently has not been identified.

On the basis of the loss of BRAK mRNA in head and neck SCC, as well as a structural relationship to other angiostatic CXC chemokines, we hypothesized that BRAK might inhibit angiogenesis. In this report, we examine the expression of BRAK protein in SCC of the tongue and confirm the antiangiogenic effect of this chemokine. We also show that rBRAK is a potent chemoattractant for human immature monocycto-derived dendritic cells (iDCs) through a specific high affinity receptor for BRAK.

MATERIALS AND METHODS

Reagents. Recombinant human cytokines including rBRAK, interleukin 8 (IL-8), vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), IP-10, stromal cell–derived factor 1 (SDF-1), BLC/BCA, macrophage inflammatory protein 1α (MIP-1α), monocyte chemoattractant protein 1 (MCP-1), RANTES, TARC, MIP-3α, and MIP-3β were obtained from Peprotech (Rocky Hill, NJ). Human BRAK was iodinated by Amersham Biosciences (Piscataway, NJ) to a specific activity of 1250 Ci/mmol using the lactoperoxidase method. The [125I]–IL-8 (2200 Ci/mmol) and [125I]–Bolton Hunter–labeled IP-10 (2200 Ci/mmol) were purchased from Perkin-Elmer Life Sciences (Piscataway, NJ) to a specific activity of 1250 Ci/mmol using the lactoperoxidase method. The [125I]–IL-2 (2200 Ci/mmol) and [125I]–Bolton Hunter–labeled IP-10 (2200 Ci/mmol) were purchased from Perkin-Elmer Life Sciences (Piscataway, NJ) to a specific activity of 1250 Ci/mmol using the lactoperoxidase method.

Immunohistochemistry. Rabbit antiserum to BRAK was raised against two synthetic peptides derived from the amino and COOH-terminal sequence of BRAK, which contained minimal homology to other chemokines. Whole antiserum was affinity purified using the synthetic peptides and confirmed to specifically react with BRAK and not other chemokines by Western blot analysis (data not shown). Immunohistochemistry was performed using reagents supplied in an alkaline phosphatase rabbit Vectastain ABC kit (Vector Labs, Burlingame, CA). Cryostat sections were fixed in cold acetone, washed, incubated in PBS containing 0.05% Triton X-100, and blocked overnight at 4°C with normal goat serum blocking solution. After a PBS/Triton wash, sections were incubated in avidin blocking solution (Vector Labs), washed again in PBS/Triton, and further incubated in biotin blocking solution (Vector Labs). Following additional washes, sections were incubated with rabbit anti-BRAK or normal rabbit IgG at a final concentration of 3 μg/mL in PBS/Triton at 4°C overnight. Sections then were rinsed in PBS/Triton, incubated with biotinylated goat antirabbit serum for 30 minutes, washed in PBS, and incubated with Avidin:Biocytinylated enzyme complex reagent. After rinsing with water, sections were incubated with 5-bromo-4-chloro-3-indolyl phosphate/ nitroblue tetrazolium substrate, counterstained with nuclear fast red, dehydrated, and mounted under coverslips. The specificity of the antibody in immunohistochemistry was validated by preincubating anti-BRAK antiserum with a 100-fold molar excess of immunizing BRAK peptides.

Cell Lines and Cell Cultures. Primary cultures of human umbilical vein endothelial cells (HUVECs) and human dermal microvascular endothelial cells (HMECs) were purchased from Cambrex Biosciences (Walkersville, MD), maintained in medium 131 plus microvascular growth supplement (Cascade Biologicals, Portland, OR), and cultured on gelatin-coated flasks. Murine lung microvascular endothelial cells (LEH), provided by Dr. Kari Alitalo (Helsinki, Finland), were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS). The iDCs were derived from peripheral blood monocytes as described previously (23). In brief, mononuclear cells from healthy volunteers were isolated over Histopaque, labeled with CD14 microbeads (Miltenyi Biotec, Auburn, CA), the CD14-positive population isolated with an MACS LS separation column (Miltenyi Biotec), and placed in a magnetic field. Purified CD14-positive cells (1 × 10^6/mL) were cultured in RPMI containing 10% FBS, 1000 units/mL IL-4 (R&D Systems, Minneapolis, MN), and 1050 units/mL granulocyte macrophage colony-stimulating factor (R&D Systems) for 6 to 7 days. By adding lipopolysaccharide (1 μg/mL) on day 4 of culture, mature dendritic cells were generated. Dendritic cells were phenotyped by staining with phycoerythrin-conjugated anti-CD83, allopurinol–conjugated anti-CD14, and FITC-conjugated anti-CD1a (all obtained from PharMingen, San Diego, CA). The iDCs were typically CD14 negative, CD83 negative, and 95% CD1a positive by flow cytometry, whereas mature dendritic cells were >80% CD83/CD1a positive and CD14 negative.

Chemotactic Assays. In vitro endothelial chemotaxis assays were performed in a 48-well chemotaxis chamber (Neuro Probe, Gaithersburg, MD) using an 8-μm pore-sized filter precoated with type IV collagen. Cytokines known to stimulate migration of endothelial cells (IL-8, bFGF, or VEGF) were added at 10 ng/mL to the bottom wells of chemotaxis chambers containing assay medium, rBRAK, or IP-10. Following starvation in DMEM containing 0.1% FBS for 2 hours, HUVECs or HMECs were trypsinized, seeded at 12,500 cells per well in the upper chamber, and allowed to migrate for 4 hours at 37°C. Unmigrated cells were scraped from the tops of filters, which were fixed, stained with Dil Quik (Baxter Scientific, Deerfield, IL.), and mounted under oil immersion. Migratory cells were counted from nine random high-power fields from each well. The mean counts of cells from multiple wells were averaged and plotted graphically along with the SE of the means. Chemotaxis assays were repeated at least three times. Results were analyzed for significance using the honest statistical difference test of unequal Ns.

Chemotaxis of dendritic cells also was measured in a microchemotaxis chamber using an 8-μm pore-sized polyvinyl pyrrolidone-free filter (uncoated). Assay media alone or in combination with rBRAK or MIP-1α was placed in the bottom wells. Dendritic cells were seeded at 50,000 cells per well in the upper chambers and allowed to migrate for 90 minutes at 37°C. To determine the effects of abolishing the chemotactic gradient, rBRAK was added to the upper and lower wells of the chemotactic chamber. In other experiments, dendritic cells were pretreated overnight with 100 ng/mL pertussis toxin. Fixation, mounting, cell counting, and statistical analysis were performed as described for endothelial cells.

In vivo Angiogenesis. In vivo angiogenesis was examined using a modification of the rat corneal micropocket assay as described previously (24). Essentially, 5 μL hydrocarbon pellets were prepared with cytokines (maintaining a polymer to cytokine ratio of 4:1) and polymerized overnight in the presence of UV light. Male Long Evans rats (2 months old) were anesthetized via intraperitoneal injection of mixture containing ketamine (63 mg/ mL), atropine (0.7 mg/mL), and xylazine (3.6 mg/mL) in a volume to deliver a ketamine dose of 150 mg/kg body weight. An ophthalmic solution of 0.5% proparacaine hydrochloride was applied to corneas for local anesthesia, and corneal micropockets were created using microsurgical technique. Six days following implantation of rehydrated pellets, rats were reanesthetized with ketamine/atropine/xylazine mixture, given 0.2 mL of heparin (5000 units/mL) intraperitoneally, perfused with 10 mL of a 1:1 solution of colloidal carbon (Sanford Design Higgins Waterproof Drawing Ink, black India 4415; Sanford, Bellwood, IL) and normal saline via direct intracardiac injection, and sacrificed. Globes were harvested and fixed in 4% paraformaldehyde overnight. Dissected corneas were mounted and imaged with a microscope equipped with a digital camera.

Image analysis was carried out using Image Pro Plus software (Media Cybernetics, Silver Spring, MD) to measure the area of neovascularization and calculate the total vascularity by summing the pixel intensities over the area of neovascularization. Data were analyzed using a one-way ANOVA test. All of the animals were handled in accordance with the University of Texas/M.D. Anderson Department of Veterinary Medicine, and an Institutional Animal Care Use Committee approved the procedures.

The optimal dose of angiogenic cytokine per pellet was determined to be 100 ng for IL-8, 50 ng for bFGF, and 200 ng for VEGF. Pellets containing angiogenic cytokines alone were implanted into the right eyes of rats, whereas pellets containing the combination of angiogenic cytokines plus rBRAK were placed into the opposite eye (i.e., left eye) of the same animals.

Competitive Binding Assays. HUVECs and HMECs were plated at 75,000 cells per well in 24-well plates and left in growth medium overnight at 37°C. Before experiments, cells were washed twice with PBS and once with wash buffer containing 50 mmol/L HEPES, 1 mmol/L calcium chloride, 5 mmol/L magnesium chloride, 500 mmol/L sodium chloride, and 1% bovine serum albumin (BSA) adjusted to pH 7.4. Cells then were incubated with 0.1 mmol/L [125I]–BRAK, [125I]–IP-10, or [125I]–FGF and increasing concentrations of unlabeled rBRAK, IP-10, bFGF, or heparin sodium in binding buffer (wash buffer containing no sodium chloride) for 2 hours at 4°C. Cells then were washed three times, and bound radioactivity extracted with 1 N sodium hydroxide was measured in a gamma counter. In some experiments, the concentration of NaCl in washes was changed to either 0.15 mol/L (to detect
low affinity receptors) or 2 mol/L NaCl (to detect high affinity receptors). Total binding was determined in the absence of unlabeled ligand. Nonspecific binding was determined in an excess (i.e., 1 µmol/L) of unlabeled ligand and was usually <20% total binding. Percent specific binding then was calculated using the formula:

\[
\% \text{Specific binding} = \frac{\text{Sample counts} - \text{NSB}}{\text{Total binding} - \text{NSB}} \times 100\%
\]

Values for the \(K_d\) and maximum number of receptors per cell (\(B_{max}\)) were calculated using GraphPad Prism V4.0 software (GraphPad Software, Inc., San Diego, CA). Binding of \([^{125}\text{I}]-\text{FGF}\) to LEII cells was essentially as described for HUVECs, except assays were performed in six-well plates containing 500,000 cells/plate, and wash buffer routinely contained 2 mol/L NaCl to remove ligand from low affinity sites.

Binding assays for dendritic cells were performed as described previously for nonadherent cells (25). In brief, cells were washed in PBS and resuspended to obtain 750,000 cells/100 µL of binding buffer containing 75 mmol/L HEPES, 1 mmol/L calcium chloride, 5 mmol/L magnesium chloride, 150 mmol/L sodium chloride, and 1% BSA at a pH of 7.4. The cell suspension was incubated with 0.5 mmol/L \([^{125}\text{I}]-\text{BRAK}\) and either increasing concentrations of unlabeled BRAK or 25 mmol/L unlabeled chemokines for 90 minutes at 4°C. Following incubation, the binding reaction was centrifuged through a binding column containing a 300 µL mixture of phathate and bisphtate oil (4:1) to separate cells from unbound radiolabeled ligand. Binding columns were snap frozen in a dry ice/ EtOH bath, and the bottoms containing the cell pellets were cut off for counting in a gamma counter.

**Binding to Immobilized IL-8 and bFGF.** Binding assays were performed on immobilized cytokines as described previously (26). Ninety-six-well HB isolates (Perkin-Elmer) were coated with 15 ng of IL-8 or bFGF in 100 µL of 0.1 mol/L carbonate buffer (pH 8.5) overnight at 4°C. Plates then were washed three times with PBS containing 0.05% Tween-20 and once with PBS alone. Blocking was performed with 1% BSA in PBS for 2 hours at room temperature, followed by three washes with PBS/Tween-20 and an additional PBS wash. Fifty microliters of a 0.1% BSA solution in PBS with or without unlabeled competitor ligands at various concentrations then were added to the plate. Subsequently, 50 µL of PBS/0.1% BSA containing 4 nmol/L \([^{125}\text{I}]-\text{BRAK}\) was added to each well. After 1 hour at 37°C, the plate was washed four times with 0.05% Tween-20 in PBS; scintillation fluid was added to the wells; and the radioactivity was counted in a MicroBeta TriLux scintillation counter (Perkin-Elmer).

**RESULTS**

**BRAK Protein Is Abundantly Expressed in Normal Mucosa and Absent from SCC Tumors of the Tongue.** Our previous demonstration that BRAK mRNA is abundant in normal squamous mucosa but absent from many head and neck SCC tumors (1) led to our analysis of BRAK protein expression in tumor specimens. Immunohistochemistry with antiserum raised against BRAK peptide was performed on frozen section specimens of tumor and adjacent normal tissue derived from patients with SCC of the tongue. Intense staining for BRAK protein was observed in the suprabasal layers of histologically normal squamous epithelium of tongue (Fig. 1A) but found to be virtually absent in an adjacent SCC of the tongue from the same patient (Fig. 1C). Control normal rabbit immunoglobulin in the absence of BRAK antiserum showed negative staining (Fig. 1B and D). Similar results showing intense suprabasal expression of BRAK in normal squamous epithelium of tongue were observed in an additional three patients, whereas BRAK staining was weak or absent in six of eight tongue SCC samples examined (data not shown). The specificity of the BRAK antiserum was confirmed by preincubating specimens of normal mucosa with antiserum in the presence of a 500-fold molar excess of immunizing peptides, which led to a substantial reduction in staining intensity (See Supplementary Fig. 1). Strong expression of BRAK protein also was observed in stromal fibroblasts adjacent to nests of tongue SCC that were clearly negative for BRAK (See Supplementary Fig. 2).

**rBRAK Protein Inhibits Chemotaxis of Human Endothelial Cells Stimulated by IL-8, bFGF, and VEGF.** We investigated the effects or rBRAK on migration of HUVECs and HMECs in vitro. Initial findings indicated that rBRAK did not stimulate the chemotaxis of either of these human endothelial cells. Therefore, we examined whether rBRAK would block chemotaxis of endothelial cells stimulated by IL-8, bFGF, and VEGF. In HUVECs, profound inhibition of the chemotactic response stimulated by IL-8 (Fig. 2A) or bFGF (Fig. 2B) occurred at rBRAK concentrations of 10 ng/mL (\(P < 0.008\)), 50 ng/mL (\(P < 0.0002\)), or 100 ng/mL (\(P < 0.0002\)). In HMECs, a similar profound inhibition of the chemotactic response stimulated by either cytokine (Fig. 2C and D) occurred at all three concentrations of rBRAK (\(P < 0.0002\)). The potency of rBRAK inhibition is shown alongside that of IP-10, a member of the CXC subfamily that is known to block endothelial cell migration (ref. 27; Fig. 2). Complete inhibition of chemotaxis was shown at 100 ng/mL rBRAK by the reduction of migrating cells to the level of control conditions without cytokine. The inhibitory response in HUVECs was concentration dependent and reached maximal inhibition at 100 ng/mL. However, in HMECs a nearly complete inhibitory response was reached at the lowest concentration of 10 ng/mL (i.e., 1 mmol/L), and inhibition persisted at higher concentrations. Nearly identical results were found for the inhibition of chemotaxis stimulated by VEGF at the same rBRAK concentrations (data not shown). Chemotactic assays were repeated a minimum of three times, with similar results obtained.

**rBRAK Protein Inhibits Angiogenesis in the Rat Corneal Micropocket Assay.** Our findings in endothelial cell migration led us to investigate the effects of rBRAK on angiogenesis in vivo using a rat corneal micropocket assay. Because rBRAK did not stimulate angiogenesis in the rat cornea, we studied the effects of rBRAK in the presence of cytokines known to stimulate angiogenesis, including IL-8 (27), bFGF (28), and VEGF (29). In the corneal micropocket assay, rBRAK profoundly inhibited the angiogenic response stimulated by IL-8, bFGF, or VEGF.

The typical dense angiogenic response to 100 ng IL-8 alone is shown in Fig. 3 (top), in which IL-8 alone was implanted in the right eye of the animal, whereas inhibition of the response is shown in the animal's left eye, in which 100 ng of rBRAK was combined with IL-8. Similar results were found with inhibition of angiogenesis...
stimulated by 50 ng of bFGF (Fig. 3, middle) and 200 ng of VEGF (Fig. 3, bottom) by the introduction of 100 ng of rBRAK.

Computer-assisted image analysis was used to objectively quantify the area of neovascularization and total vascularity, thus allowing comparisons to be made between the right eye (angiogenic substance alone) and the left eye (angiogenic substance plus 100 ng rBRAK) for each group of 12 animals. For corneas implanted with IL-8 plus rBRAK, the reduction in area of neovascularization ranged from 34.9% to 88.1% with a mean reduction of 67.6% (P < 0.001). The reduction in total vascularity ranged from 44.0% to 91.6% with a mean reduction of 67.6% (P < 0.001). For assays in which bFGF was introduced as the angiogenic cytokine, rBRAK caused a 58.4 ± 14.0% mean reduction in area of neovascularization (P < 0.01) and a 58.9 ± 16.1% mean reduction in total vascularity (P < 0.05). For assays in which VEGF was introduced as the angiogenic cytokine, rBRAK caused a similar reduction in both parameters (P < 0.03). The ability of rBRAK to inhibit corneal neovascularization induced by IL-8, bFGF, and VEGF has been confirmed in two independent experiments. Neutralizing BRAK antibody was found to attenuate the inhibition of angiogenesis when added to the combination of IL-8 and rBRAK in pellets, indicating that the angiostatic properties were attributable to rBRAK itself and not a contaminant of the commercial chemokine (See Supplementary Fig. 3).

**rBRAK Ligand Binds Low Affinity Sites that AreCompeted by Heparin Sodium.** The time-dependent binding of [125I]-rBRAK to HUVECs was examined by incubating cells with 0.1 nmol/L labeled rBRAK for increasing time points at 4°C. Binding required 2 hours to reach equilibrium (Fig. 4A). To characterize the receptor for rBRAK on HUVECs, a homologous competitive binding assay was performed by incubating cells with 0.1 nmol/L of [125I]-BRAK in the presence of increasing concentrations of competing, unlabeled rBRAK. An IC50 of 300 nmol/L was measured for unlabeled BRAK (Fig. 4B), which amounts to a KD of ~300 nmol/L when the Cheng and Prusoff equation is applied. The number of receptors per cell or Bmax calculated was on the order of several million, consistent with low affinity binding sites on HUVECs. High affinity receptors were undetectable even by pretreatment with acid wash or by varying the salt concentrations of the binding and wash conditions. Similar results were found with HMECs (data not shown). Thus, only low affinity binding sites for rBRAK could be shown on either HUVECs or HMECs.

The binding of chemokine and growth factor ligands to endothelial cells has been shown through low affinity binding sites on cell surface glycosaminoglycans, such as heparin moieties (30–33), and can be overcome in the presence of excess, free glycosaminoglycan. To determine the presence of such interaction between rBRAK and glycosaminoglycans, a competitive binding assay was performed in HUVECs or HMECs by incubating cells with 0.1 nmol/L [125I]-rBRAK in the presence of increasing concentrations of heparin sodium. Heparin sodium effectively blocked [125I]-rBRAK binding to both types of endothelial cells (Fig. 4C).

The CXC chemokine IP-10, which like rBRAK inhibits endothelial cell chemotaxis and angiogenesis, has been reported to bind high affinity receptors on endothelial cells (34). The inability to detect such high affinity receptors for BRAK on endothelial cells led us to investigate HUVECs for the binding of IP-10. A competitive binding assay was performed by incubating HUVECs with 0.1 nmol/L [125I]-IP-10 in the presence of increasing concentrations of unlabeled IP-10 or rBRAK. An IC50 and KD for unlabeled IP-10 at ~2 nmol/L was found (Fig. 4D). Unlabeled rBRAK did not compete for the binding of [125I]-IP-10 in HUVECs, suggesting that rBRAK and IP-10 act via disparate receptors.
Recombinant BRAK Protein Binds Immobilized IL-8 and bFGF and Inhibits Binding of bFGF to High Affinity Receptors. Several groups have hypothesized that chemokines inhibit angiogenesis through direct interaction with angiogenic ligands (26, 35, 36). To determine the presence of such interactions between BRAK and angiogenic ligands, we performed binding assays using IL-8 or bFGF immobilized on 96-well plates. Preliminary results showed that [125I]-rBRAK bound to immobilized IL-8 or bFGF with high affinity as compared with binding of control wells coated with immobilized BSA alone. A competitive binding assay was performed by incubating immobilized IL-8 with 0.2 nmol/L [125I]-rBRAK in the presence of increasing concentrations of unlabeled BRAK or IL-8. As shown in Fig. 5A, [125I]-rBRAK binds immobilized IL-8 with high affinity and an IC50 of 2 nmol/L, whereas unlabeled, soluble IL-8 did not block [125I]-rBRAK binding even at high concentration. Similar results are shown in Fig. 5B for the binding [125I]-rBRAK to immobilized bFGF, in which labeled rBRAK also binds bFGF with high affinity and an IC50 of 2 nmol/L. In contrast to IL-8, unlabeled soluble bFGF did inhibit [125I]-rBRAK binding to immobilized bFGF.

Considering that the interaction of ligands with low affinity binding sites on cell surface glycosaminoglycans may facilitate the action of chemokines and growth factors on endothelial cells, we investigated the effects of rBRAK and heparin sodium on the binding of bFGF to low affinity receptors on HUVECs. A competitive binding assay was performed by incubating cells with 0.1 nmol/L [125I]-bFGF in the presence of increasing concentration of unlabeled rBRAK or heparin sodium under low salt wash conditions. Unlabeled rBRAK did not block binding of [125I]-bFGF to HUVECs (Fig. 5C) even at 1000 nmol/L. This concentration is 100 times higher than that required for inhibition in the chemotactic assays. Conversely, incubation with heparin sodium at 10 μg/mL resulted in near complete competition of binding by [125I]-bFGF (Fig. 5C).

We next examined whether BRAK could interfere with binding of bFGF to high affinity receptors on endothelial cells by increasing the salt concentration in washes (i.e., 2 mol/L NaCl). In preliminary experiments, binding of [125I]-bFGF to HUVECs and HMECs was barely measurable following high salt washes, suggesting that receptor numbers were low. Therefore, the murine microvascular endothelial...
Fig. 5. Interaction between BRAK and angiogenic factors. Fifteen nanograms of IL-8 (A) or bFGF (B) were preabsorbed onto the surface of a 96-well ELISA plate and subsequently incubated with 2 mmol/L [125I]-BRAK and increasing concentrations of the indicated unlabeled cytokine for 1 hour at 37°C. Nonspecific binding was determined in parallel wells precoated with BSA and was <20% of what bound to either immobilized IL-8 or bFGF. The ability of excess BRAK (1 mmol/L) to block binding of [125I]-bFGF to low affinity receptors on HUVECs was examined in C. The percent specific binding of [125I]-bFGF to high affinity receptors on murine LEII cells was examined in the presence of unlabeled bFGF or rBRAK (D). Binding was significantly different in the presence of unlabeled bFGF (P < 0.001) or rBRAK (P < 0.05) compared with no competitor (none).

Results were found in three independent experiments using iDCs derived from unrelated donors.

To determine whether abolishing the chemotactic gradient of rBRAK effects the migration of iDCs, experiments were performed by adding various concentrations of rBRAK to the upper and lower wells of the microchemotaxis chamber. The experiment depicted in Fig. 6B shows that chemotaxis of iDCs in response to rBRAK is abrogated by adding rBRAK to upper and lower wells. Abolishing the gradient of rBRAK resulted in only random migration of cells similar to the conditions of media alone. Consistent with what has been described for many other chemokines, supraoptimal concentrations of BAK (i.e., > 1.0 μg/mL) were inhibitory for chemotaxis, and pretreatment of iDCs with pertussis toxin completely blocked chemotaxis (data not shown).

rBRAK Ligand Binds a High Affinity Receptor on iDCs. Our finding that rBRAK stimulates chemotaxis of iDCs led us to investigate these cells for the presence of a high affinity chemokine receptor capable of binding rBRAK. Homologous competitive binding assays were performed by incubating iDCs with 0.5 mmol/L [125I]-BRAK in the presence of increasing concentrations of unlabeled rBRAK or 25 mmol/L unlabeled CXC chemokine. Fig. 6C shows the high affinity binding of rBRAK to iDCs with an IC50 of 2 nmol/L. Calculations revealed a Kd of 2.2 nmol/L and an estimated 20,000 receptors sites per cell, consistent with high affinity binding. IL-8, IP-10, SDF-1, and BCA did not compete with [125I]-rBRAK for this high affinity receptor (Fig. 6D). Binding experiments were repeated twice using iDCs from two unrelated donors. The CC chemokines MIP-1α, MCP-1, RANTES, TARC, MIP-3α, and MIP-3β also failed to compete with [125I]-BRAK binding to high affinity receptors on iDCs (data not shown).

DISCUSSION

In this study, we validated our previous findings for BRAK mRNA by confirming the abundant expression of BRAK protein in normal squamous mucosa and the absence of protein in tumors derived from the same tissue of origin. These findings give rise to the hypothesis that loss of BRAK may allow tumor cells to gain a selective advantage...
Fig. 6. BRAK is chemotactic for iDCs and binds to high affinity receptors. A. Six-day-old cultures of iDCs were seeded in upper wells of a 48-well chemotaxis chamber containing various concentrations of either rBRAK or MIP-1α in the lower chambers. B. checkerboard analysis in which increasing concentrations of rBRAK were added to the lower wells of the chemotaxis chamber and resulted in cell migration ( ). Adding increasing, equivalent concentrations of rBRAK to upper and lower wells abolished the chemotactic gradient and abrogated the migration of iDCs ( ). Chemotaxis assays were performed for 90 minutes at 37°C, and migratory cells were counted. The mean of the averages from replicate wells is depicted along with error bars corresponding to the SE. C. homologous competitive binding assay in which 75,000 iDCs per tube were incubated with 0.2 nmol/L [125I]-BRAK and increasing concentrations of unlabeled rBRAK for 1.5 hours at 4°C. A Kd of 2.2 nmol/L was calculated in the experiment shown. D. heterologous competitive binding assay in which 750,000 iDCs per tube were incubated with 0.5 nmol/L [125I]-BRAK and no competitor or unlabeled competitor at 25 nmol/L for 1.5 hours at 4°C. Nonspecific binding was determined in the presence of (1 µmol/L) unlabeled rBRAK. All of the reactions were performed in triplicate, and error bars represent the SD of triplicate measurements.

in vivo. Central to explaining the implications for the loss of BRAK in tumors is an understanding of the normal biological function of BRAK. However, little progress has been made in understanding BRAK function since the gene was cloned >5 years ago (2).

Our data establish BRAK as a potent inhibitor of in vivo angiogenesis in the rat corneal micropocket assay. Neovascularization induced by multiple angiogenic factors was inhibited to a high degree at a biologically relevant dose of rBRAK. Although qualitative assessment of corneal images was sufficient to show a profound inhibition of neovascularization, we also developed a quantification method that provided objective evidence. Concurrence between separately defined parameters for the area of neovascularization and the total vascularity was found in each experimental group of 12 animals for angiogenesis in response to IL-8, bFGF, or VEGF. Finally, the specificity of inhibition was shown with a neutralizing antibody to BRAK.

Our findings of nearly complete inhibition of human endothelial cell migration in response to multiple chemotactic stimuli with 10 ng/mL rBRAK place BRAK alongside IP-10 and MIG (27) as ELR(−) CXC chemokines that are potent inhibitors of endothelial cell chemotaxis. Because angiogenesis occurs by a stepwise process of events that includes the migration and proliferation of endothelial cells accompanied by the formation of three-dimensional tubelike structures, inhibition of any one of these events is sufficient to interrupt in vivo angiogenesis. We found only a slight effect of BRAK on proliferation of endothelial cells (data not shown). Similar to the reported effects of IP-10 and PF-4 on proliferation (16, 26, 40), inhibition of proliferation required BRAK concentrations >1 µmol/L. Concentrations of chemokine up to 1000-fold greater than those required to inhibit chemotaxis suggest a limited role for BRAK in regulating endothelial cell proliferation. Thus, inhibition of endothelial migration appears to be a major mechanism by which BRAK interrupts in vivo angiogenesis.

A high affinity binding receptor for BRAK could not be shown on either HUVEC or HMEC lines. Several mechanisms can interfere with the detection of a high affinity binding site for ligands and may be overcome by varying conditions in receptor binding assays. Labeled ligand can bind to an abundance of low affinity cell surface receptors (e.g., surface glycosaminoglycans), which can obscure high affinity receptors and can be overcome by increasing the salt concentration in washes. We found that a 2 mol/L NaCl wash solution sufficient to disrupt the binding of [125I]-BRAK to immobilized heparin agarose beads (data not shown) did not reveal the presence of a high affinity receptor on HUVECs. Second, occupation of receptors by endogenous ligand before incubation can block binding of labeled ligand and can be overcome by acid stripping the cells to remove endogenous ligand. Pretreatment of HUVECs with an acid wash did not unmask a high affinity receptor. However, we cannot exclude the possibility that such receptors exist at levels beneath the capability of detection in binding assays.

A high affinity receptor for IP-10 is present on HUVECs as shown previously by Soejima et al. (34), and BRAK does not compete with IP-10 for this receptor. It currently is unknown whether this high affinity IP-10 receptor is the CXCR3B variant reported by Lasagni et al. (22). Nevertheless, our evidence suggests that BRAK does not bind CXCR3B because [125I]-BRAK failed to bind with high affinity to ACHN cells (data not shown), a human kidney adenocarcinoma line reported to express abundant levels of this receptor (22). Rather, our findings of low affinity receptor sites for BRAK with Kd of ~300 nmol/L are consistent with cell surface glycosaminoglycan heparin moieties and are supported by the demonstration of competition of [125I]-BRAK binding by soluble heparin in HUVECs and HMECs.

Interaction of ligands with cell surface glycosaminoglycans facilitates the specific receptor binding and signal transduction of angiogenic cytokines, such as IL-8, VEGF165, and bFGF (30–33). The demonstration that PF-4 inhibits the binding of bFGF and VEGF165 to endothelial cells mediated through glycosaminoglycans (26, 35) supports a proposed mechanism that chemokines may inhibit angiogenesis by competing for cell surface glycosaminoglycan binding sites (40). Our observation that BRAK binds immobilized heparin and the finding of low affinity receptor sites on endothelial cells support such a mechanism. However, we found that unlabeled BRAK at concentrations up to 1 µmol/L could not block the binding of [125I]-FGF to low affinity receptors on HUVECs. Therefore, competition with angiogenic cytokines for glycosaminoglycan binding sites does not appear responsible for the inhibition of endothelial cell chemotaxis mediated by BRAK.

Several published reports (26, 35, 36) support the hypothesis that angiostatic chemokines can inhibit angiogenic ligands by direct interaction. PF-4 has been shown to bind immobilized bFGF or VEGF165 in vitro and to interfere with bFGF dimerization. Our data suggest that
BRAK may act through a similar mechanism because rBRAK bound with high affinity to immobilized bFGF (i.e., IC_{50} < 10 nmol/L). The concentration of soluble bFGF required to block [125I]-BRAK binding to immobilized bFGF also was consistent with concentrations found by Perollet et al. (26) required to block PF-4 binding to immobilized bFGF. Our findings that unlabeled rBRAK could inhibit binding of bFGF to high affinity receptors further support a mechanism of direct interaction between BRAK and bFGF. Although [125I]-BRAK bound to immobilized IL-8 with a similar affinity, competition for [125I]-BRAK binding by excess soluble IL-8 did not occur at concentrations up to 2 μmol/L, suggesting that oligomerization of IL-8 may be necessary for interaction with BRAK.

A clear consensus is lacking regarding the cellular target spectrum of chemotaxis in response to BRAK. An explanation could be the disparity in sources of BRAK protein used by various investigators before the recent commercial availability of rBRAK. One study used a COOH-terminal histidine-tagged protein corresponding to the murine homologue BMAC (97% identical to human) and found chemotaxis of human B-cell and mononcytic cell lines but not resting or activated T cells (3). Another reported that micromolar concentrations of chemically synthesized BRAK peptide were chemotactic for prostaglandin E_{2}-stimulated human monocytes but not for dendritic cells or other leukocyte subsets (5). A third group used unpurified BRAK (e.g., MIP-2-y) from supernatants of transfected mammalian cells to show chemotaxis for human neutrophils and dendritic cells but not other leukocyte subsets (4). Consistent in these reports is the finding that BRAK does not appear chemotactic for resting or activated T cells. Although each group reported chemotactic activity for a monoocyte-derived cell type, the activation or differentiation requirements varied among reports.

Our data unequivocally show that human BRAK is chemotactic for iDCs at 10 ng/ml of purified rBRAK. These findings were validated with the abolition of a chemotactic response by abolishing the concentration gradient. Consistent with the action of chemokines through G protein–coupled receptors, BRAK-mediated chemotaxis of iDCs was sensitive for pertussis toxin. Moreover, BRAK bound to high affinity receptors on iDCs with a K_{D} of 2.2 nmol/L. The binding of [125I]-BRAK to dendritic cells in our assays occurred at concentrations comparable with those of the chemotaxis assay and consistent with the demonstration of a receptor for BRAK on dendritic cells.

In summary, we found that BRAK protein is constitutively expressed in normal squamous mucosa of the tongue but absent in SCC tumors arising from this site. We showed that BRAK is a potent inhibitor of endothelial cell chemotaxis and angiogenesis in response to multiple angiogenic factors. Supporting that the loss of BRAK expression might dysregulate host immune mechanisms, we found that BRAK is chemotactic for iDCs. Therefore, the potential biological implications of rBRAK in SCC of the head and neck, either systemically introduced or pharmacologically induced, require further investigation.

Note Added in Proof

During preparation of this manuscript, Allinen et al. (Cancer Cell 2004;6:17–32) reported that CXCL14 (BRAK) is up-regulated in the myoepithelial stromal cells adjacent to invasive breast carcinomas and that breast carcinoma tumor lines bind BRAK and respond with chemotaxis and increased invasion. These observations are in agreement with our current findings that BRAK/CXCL14 protein is highly expressed in stromal fibroblasts immediately adjacent to nests of tongue SCC and suggest that breast carcinomas may express the same high affinity receptor for BRAK as iDCs.

REFERENCES

Corrections

p53 and BCNU Resistance in Astrocytes

In the article on p53 and BCNU Resistance in Astrocytes in the June 15, 1996 issue of Cancer Research (1), the title was incorrect. The title should have read “Wild-Type p53 Renders Mouse Astrocytes Resistant to 1,3-Bis(2-chloroethyl)-1-nitrosourea Despite the Absence of a p53-dependent Cell Cycle Arrest.”


AChE in Apoptosis

In the article on AChE in Apoptosis in the April 15, 2004, issue of Cancer Research (1), there is an error on page 2652, in the section under “Materials and Methods” on “siRNA Transfection”. The AChE target sequence should have read 5’-AAGAGUGUCGUAC-CAAUAU-3’.


Depletion of Methionine Aminopeptidase 2

In the article on Depletion of Methionine Aminopeptidase 2 in the May 1, 2004, issue of Cancer Research (1), there is an error on page 2984, in the section under “Materials and Methods” on “Cell and Enzyme Assays”. The text near the end of the section should have read the following: “The targeting sequence was AAUGCCGGUGA-CACAACAGUA (Dharmacon Research). The control mismatch sequence was AAUGCCGGUGA-CACAACAGUA.”


NIS Gene Therapy of Hepatocarcinoma

In the article on NIS Gene Therapy of Hepatocarcinoma in the November 1, 2004, issue of Cancer Research (1), a note should have been included indicating that J. Faivre and J. Clerc contributed equally to the study.


Novel Functions of BRAK

In the article on Novel Functions of BRAK in the November 15, 2004, issue of Cancer Research (1), the following grant support information should have appeared:

This work was supported in part by the University of Texas M.D. Anderson Cancer Center SPORE in Head and Neck Cancer NIH-NCI P50 CA097007 (G. Clayman and M. Frederick), NIH R01 DE013954 (G. Clayman), Cancer Center Support Grant NIH P30 CA016672, Alando J. Ballantyne Distinguished Chair in Head and Neck Surgery Award (G. Clayman), Michael A. O’Bannon Endowment for Cancer Research (G. Clayman), Betty Berry Cancer Research Fund (G. Clayman), and NIH INRS Award T32 CA060374 (G. Clayman).

BRAK/CXCL14 Is a Potent Inhibitor of Angiogenesis and a Chemotactic Factor for Immature Dendritic Cells

Thomas D. Shellenberger, Mary Wang, Manu Gujrati, et al.

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