Herstatin, an Autoinhibitor of the Epidermal Growth Factor (EGF) Receptor Family, Blocks the Intracranial Growth of Glioblastoma

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INTRODUCTION

Activation of the epidermal growth factor (EGF) family of receptor tyrosine kinases by ligands results in dimerization, tyrosine autophosphorylation, and initiation of two major signaling cascades: the PI3K/Akt pathway and the Ras/Raf/mitogen-activated protein kinase (MAPK) pathway (1). Overexpression and/or autocrine activation of normal EGF receptor tyrosine kinases plays an important role in several human carcinomas (2). In addition, an N-terminally truncated mutant, ΔEGFR (EGFRvIII), which is constitutively active in the absence of ligand, is critical in a subgroup of human glioblastoma (3, 4).

The central role of two members of the EGF receptor tyrosine kinase family, EGFR and HER-2, in human cancers places them as attractive targets in anticancer drug development (2). Monoclonal antibodies directed at the receptor extracellular domains and small molecule kinase inhibitors have generated encouraging preclinical and clinical results (2, 5, 6). Nevertheless, there is a critical need for development of novel receptor inhibitors with alternative mechanisms of action.

Herstatin, distinct from kinase inhibitors and monoclonal antibodies, is a naturally occurring product of the HER-2 gene created by alternative mRNA splicing (7). Herstatin functions as a secreted inhibitor that binds to the extracellular domains of EGFR and HER-2 with nanomolar affinity, disabling multiple receptor combinations in response to a variety of ligands (7–10). In this study, we investigated the antitumor activity of Herstatin against glioblastoma because the targets of Herstatin, EGFR (3, 4) and HER-2 (11), play an important role and because current treatment options are toxic and ineffective (12). Herstatin dramatically blocked U87MG glioblastoma tumor formation corresponding to in vitro inhibition of EGFR signaling through Akt but not MAPK pathways, whereas constitutive signaling and tumorigenic growth driven by ΔEGFR were resistant to Herstatin. Because Herstatin is a secreted protein that can spread from the site of production and act outside the cell, it may be an effective therapeutic against intracerebral tumors.

METHODS

Cell Culture. U87MG and U87MG/ΔEGFR (henceforth U87 and U87/Δ) human glioblastoma cell lines were a generous gift from Dr. Webster Cavenee, Ludwig Institute for Cancer Research, Univ. of California at San Diego, CA (13). Stable transfections were conducted as described previously (9). Stable U87/Herstatin (Hst) cell lines were selected with 0.05 mg/mL hygromycin B and maintained in DMEM, 10% fetal bovine serum (FBS), and 0.05 mg/mL hygromycin B. Stable U87/Δ/Hst cells were selected and maintained in DMEM, 10% FBS, 0.2 mg/mL G418, and 0.1 mg/mL hygromycin B.

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Herstatin Blocks Brain Tumor Growth

**Tumor Implantation and Growth.** The care and use of the animals was approved by the Institutional Animal Care and Use Committee and was under the supervision of the Oregon Health & Science University Department of Animal Care. Female athymic nude rats (nu/nu) (200-220g, n = 16) were anesthetized with i.p. ketamine (60 mg/kg) and diazepam (97.5 mg/kg). Tumor cells (12 µl, ~10^6 cells, ≥90% viable by trypan blue exclusion) were inoculated at stereotactic coordinates for intracerebral localization in the right caudate putamen (vertical bregma: −6.5 mm, −3.1 mm lateral). Cells were inoculated over a 5-minute period to limit backflow along the injection tract. Rats were followed for survival and were sacrificed by barbiturate overdose when condition warranted or at 8 weeks after tumor implantation. Rat brains were fixed by immersion in 10% neutral buffered formalin for vibratome sectioning (100-µm coronal sections).

**Herstatin Purification.** S2 insect cells transfected with 6xHis tagged-Herstatin in the pM7/BP expression plasmid (Invitrogen, Carlsbad, CA) were maintained in insect serum-free medium with 1-glutamine (JRH Biosciences, Lenexa KS) supplemented with 300 µg/mL hygromycin. Cells were induced in fresh medium with cupric sulfate (100 µM) for ~16 hours. Herstatin was purified to 80% to 90% purity by Ni-NT (Qiagen, Valencia CA) affinity chromatography as previously described (10).

**In vitro Growth Assays.** The different glioblastoma cell lines were plated into 24-well plates overnight in medium with 5% FBS. The cells were then washed with PBS and medium with 0.2% FBS were added. For the Herstatin treatments, triplicate wells were treated with the indicated concentrations of purified Herstatin or with control vector (20 mM Tris, 0.3 mol/L NaCl, 0.15 mol/L histidine, pH 8.0) in medium with 0.2% FBS on days 1 and 3. Viable cells were measured in triplicate wells using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt (MTS) assay (Promega, Madison, WI) as described (10). The results of the assay were quantified by absorbance at 492 nm in a Fluorostar plate reader.

**Western Blot Analysis.** Western blotting and Herstatin antibodies were as described (7, 8). Polyclonal Akt, phosphoAkt (phosphorylated at S473), MAPK, phospho-MAPK (phosphorylated at T202 and Y204) were from Cell Signaling (Beverly, MA). Polyclonal anti-EGFR was from Santa Cruz Biotechnology (Santa Cruz, CA) and antiphosphotyrosine monoclonal antibody was from Sigma (St. Louis, MO).

**Immunohistochemistry.** Primary antibodies used for immunostaining were the Herstatin polyclonal (7), Herstatin monoclonal (Upstate, Lake Placid, NY), and an EGFR monoclonal antibody (NeoMarkers, Fremont CA). Secondary antibodies were Alexa 488 goat anti-mouse and Alexa 594 goat anti-rabbit (Molecular Probes, Eugene, OR). Immunofluorescence was visualized with an Olympus confocal inverted laser-scanning microscope (Melville, NY).

**Data Analysis.** Films exposed to Western blots were scanned and quantitated by imaging densitometry (model GS-700, Bio-Rad, Hercules CA) and standardized to the maximum phosphorylation signal. For tumor volumetrics, sections were stained with hematoxylin and imaged with a Zeiss AxioCam digital camera attached to a Zeiss Axioplan Universal microscope (Thornwood, NY). Every sixth section (~10 per brain) was analyzed using Adobe Photoshop and NIH Image software tools. Tumor volume means and SDs were compared with Microsoft Excel software. Survival times (in days) were compared by paired Student t test using Microsoft Excel. In vitro cell growth inhibition was evaluated with Microsoft Excel software.

**RESULTS**

**Effect of Herstatin Expression on In vitro Growth and Tumor Formation.** We chose the U87 cell line from a human glioblastoma as a model of a highly lethal primary brain tumor for these studies. The U87 parental cells controlled by the full-length EGFR (14, 15) and the U87/Δ cell line driven by the N-terminally truncated mutant, ΔEGFR (13), were stably transfected with Herstatin to test effects on growth and tumorigenesis. Two clonal cell lines, U87/Hst and U87/Δ/Hst, which expressed comparable levels of Herstatin (Fig. 1A), were selected for further studies. The U87/Hst cells proliferated more slowly than the parental U87 cells (Fig. 1B) suggesting that Herstatin expression inhibited their growth. In contrast, Herstatin expression did not appear to affect the growth of the glioma cells that overexpressed ΔEGFR because the U87/Δ/Hst and U87/Δ cell lines grew at a similar rate (Fig. 1B, right).

We next examined the intracranial tumorigenic growth of the U87 and U87/Δ cell lines with and without Herstatin expression. The different cells were inoculated into the brain of female athymic nude rats and the animals were monitored for signs of toxicity and survival (Fig. 2A). Rats implanted with either U87/Δ or U87/Δ/Hst displayed adverse symptoms and died or were sacrificed at 15.0 ± 1.4 or 16.8 ± 1.0 days respectively.

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Fig. 1 Effect of Herstatin expression on in vitro growth. A, U87 or U87/Δ glioblastoma cells mock transfected or transfected with Herstatin were extracted and 20 µg of protein from each were subjected to Western blot (WB) analysis using antibodies against Herstatin. The blot was developed by chemiluminescence and exposed to X-ray film. B, U87, U87/Hst, U87/Δ, and U87/Δ/Hst were plated at 20,000 cells/well into 24-well plates overnight in medium with 5% FBS, washed, and serum was reduced to 0.2%. Viable cells were quantified by the MTS assay in triplicate wells after plating overnight to determine the zero time point and at 1, 2, and 3 days in culture. Points, mean of the A492 nm readout from the MTS assay; bars, SE.
Rats bearing the parental U87 cells survived 23.8 ± 3.2 days, which is a significantly greater survival time than those animals with tumors that expressed ΔEGFR (P < .01), in agreement with previous studies (16). The U87, U87Δ, and U87Δ/Hst all formed large tumors that filled the right hemisphere of the brain (Fig. 2B and data not shown) with tumor volumes at sacrifice of 170 ± 42 mm³. In marked contrast, rats implanted with the U87/Hst cells survived >8 weeks (Fig. 2A) and were sacrificed at day 57. Inspection of brain slices revealed no tumor formation at the caudate inoculation site (Fig. 2C, arrow). There was evidence of residual blood from the needle trauma, but no sign of toxicity in the adjacent normal region of the brain. There was histologic evidence of tumor cells on the cortical surface at the injection site (Fig. 2C, box, and Fig. 3B).

To further assess the effect of Herstatin on tumor growth, a series of rats received intracerebral inoculation of a second clonal line of U87 cells expressing about 10-fold lower levels of Herstatin (U87/Hst2) (data not shown). Four rats were sacrificed at 6 days after cell implantation to evaluate tumor viability and growth. At the caudate inoculation site, all animals showed a small (3.6 ± 1.5 mm³) diffuse tumor with signs of necrosis, unlike the solid tumor formed by parental U87 cells (data not shown). Two animals were followed up for survival: one died from brain tumor burden at 35 days after tumor implantation, whereas the second was sacrificed at 8 weeks with no evidence of disease. These results provide further support that Herstatin expression inhibited the tumorigenic growth of U87 cells.

Herstatin Expression in Implanted Cells. Because the size of the tumors and survival time of animals injected with U87/Δ versus U87/Δ/Hst were not significantly different, we examined by immunocytochemistry whether Herstatin expression was retained after intracerebral implantation. Herstatin was readily detected at the cell surface in tumors formed from the U87/Δ/Hst cells (Fig. 3A) but not from the U87/Δ cells (data not shown), showing that differential Herstatin expression was maintained. Abundant amounts of EGFR, localized to the cell surface, were detected in both the U87/Δ/Hst (Fig. 3A) and the U87/Δ (data not shown). Secretion of Herstatin from the U87/Δ/Hst tumor cells...
did not appear to cause toxicity in the normal brain around the tumor margin.

We examined whether Herstatin expression could also be detected in rat brains inoculated with the U87/Hst cells. Whereas there was no apparent staining around the needle track in the caudate nucleus, the cortex at the injection site showed Herstatin staining that coincided with the histologically identified residual tumor cells (Fig. 3B). This indicated that U87/Hst cells survived and expressed Herstatin but were unable to proliferate. EGFR staining is not shown because levels are low in the parental U87 cells.

**Effects of Purified Recombinant Herstatin on In vitro Growth.** Herstatin, expressed as a secreted protein in U87 cells, blocked their in vitro as well as their intracranial growth, suggesting that addition of purified Herstatin may also suppress their proliferation. On the other hand, the U87/Δ cells that were not inhibited by Herstatin expression were predicted to be resistant to exogenous Herstatin. To test this, the effect of Herstatin on in vitro growth was evaluated in cultured U87 cells and, in parallel, in U87/Δ cells. Figure 4 demonstrates that exogenous Herstatin caused a dose-responsive inhibition of viable U87 cells (\( P = 0.0002 \)), whereas the U87/Δ cells were unaffected (\( P = 0.535 \)). These results showed the efficacy of purified Herstatin as an antiproliferative agent against U87 cells. Moreover, these results supplied further evidence that the ΔEGFR conferred resistance to Herstatin.

**Effects of Herstatin on Receptor Phosphorylation and Intracellular Signaling.** Herstatin blocked in vivo and in vitro growth of parental but not U87 cells driven by ΔEGFR. Therefore, we hypothesized that the full-length EGFR in the parental cells may be inhibited, whereas the mutant ΔEGFR may resist inhibition by Herstatin. To test this we compared the in vitro effects of purified Herstatin on receptor activation and signaling in the paired glioblastoma cell lines. To measure intracellular signaling, Herstatin was added before EGF because treating with EGF causes receptor down-regulation (17, 18). Herstatin added to U87 cells inhibited EGF-induced EGFR tyrosine phosphorylation in a dose-dependent fashion (Fig. 5A). Inhibition was detected at 25 nmol/L. Herstatin and receptor tyrosine phosphorylation was reduced to basal levels at about 250 nmol/L. In contrast, the constitutive tyrosine phosphorylation of ΔEGFR was unaffected by Herstatin (Fig. 5B) even at high concentrations that were sufficient to cause maximum inhibition of EGFR in the U87 cells.

Signals initiated by EGFR receptor tyrosine kinases generally proceed simultaneously through both the MAPK and the PI3K/Akt signaling cascades (1). To investigate effects of exogenous Herstatin on these signaling pathways, the phosphorylation levels of MAPK and Akt were followed up over time. Based on the dose-response data, signaling studies were done using 200 nmol/L Herstatin, a concentration that prevented EGF-mediated activation of the receptor. Herstatin blocked EGF activation of Akt in U87 cells, whereas MAPK activation by EGF was unaffected (Fig. 5C). This finding is in agreement with previous studies demonstrating that Herstatin preferentially inhibits EGF activation of Akt (9). The cells that overexpress ΔEGFR exhibited constitutive activation of Akt, whereas MAPK was activated in response to EGF (Fig. 5D) presumably through the low levels of endogenous EGFR. This result is explained by previous studies demonstrating that ΔEGFR causes constitutive activation of Akt but not the MAPK pathway (13). In contrast to the results observed with the parental U87 cells, phospho-Akt levels in U87/Δ cells were unaffected by Herstatin treatment (Fig. 5D). MAPK activation was unaffected by Herstatin regardless of which form of the EGFR was expressed (Fig. 5C and D). In summary, Herstatin blocked EGF activation of the full-length receptor and Akt but not MAPK activation in the parental U87 cells, whereas the constitutive activation of ΔEGFR and Akt in the U87/Δ cells were unaffected by Herstatin.

**DISCUSSION**

Several inhibitors of EGFR including monoclonal antibodies and kinase inhibitors have been intensively investigated as potential cancer therapeutics (5, 6). Studies presented here suggest that Herstatin may be a potent inhibitor of malignant glioblastoma that is driven by the EGFR. Herstatin represents a novel addition to the repertoire of antireceptor agents, distinguished by its novel structure and human origin. It is the only naturally occurring (7) inhibitor of the EGFR receptor family that exerts its action on the initial events in receptor activation: dimerization and autophosphorylation. Furthermore, Herstatin blocks activation of both HER-2 and the EGFR (8, 9) that are implicated most often in the progression of numerous human cancers.
Herstatin effectively inhibited EGFR tyrosine phosphorylation and the PI3K/Akt pathway, but MAPK activation was unaffected in the U87 cells. This is in agreement with a previous report demonstrating that Herstatin uncouples EGF activation of these two major signaling pathways in murine fibroblasts (9). In both studies, inhibition of Akt correlated with suppression of proliferation indicated by blocked in vitro and in vivo tumorigenic growth of glioblastoma (this study) and inhibition of EGF– mediated proliferation of murine fibroblasts (9). Recent studies have showed that EGF activation of Akt in U87MG cells is important in the regulation of vascular endothelial growth factor production, which mediates tumor vascularity (15). Although both the Akt and MAPK pathway have showed involvement in both proliferative and survival signaling, our findings suggest that obstruction of Akt may be sufficient to achieve growth blockade. Herstatin expression may block tumorigenic growth by preferential inhibition of Akt even though MAPK activity is unabated.

Although Herstatin has been found to prevent activation of the full-length EGFR, our results show that ΔEGFR was resistant. Herstatin inhibits full-length EGFR by binding to its extracellular domain and blocking dimerization (8, 9). Herstatin contains a dimerization arm in its subdomain II (19) and may therefore block the dimerization arm of EGFR, preventing formation of productive receptor dimers. Because ΔEGFR is missing subdomains I and II (3, 4), Herstatin binding could be inhibited, or ΔEGFR, which is missing its dimerization arm, may resist Herstatin-mediated inhibition (19, 20). Preliminary observations indicate that Herstatin binds to ΔEGFR, suggesting that receptor subdomains I and II do not contain the binding site but rather are required for Herstatin-mediated inhibition.

Understanding the molecular profile of cancer cells, which predicts responsiveness to targeted inhibitors, is crucial to maximizing clinical efficacy. Results presented here indicate that Herstatin dramatically inhibits tumor growth of cells driven by the full-length EGFR, whereas cells overexpressing the truncated ΔEGFR are resistant to Herstatin. In a significant proportion of glioblastoma, amplification of the wild-type EGFR occurs and may precede the generation of ΔEGFR (2). The wild-type EGFR is overexpressed in a subset of glioma as well as in many carcinomas to a much greater extent than...
observed in the U87 cells used in this study. It is expected that Herstatin will also inhibit glioma with amplified wild-type EGFR because our previous studies have shown that Herstatin binds with nanomolar affinity and inhibits EGF signaling and proliferation in cells that overexpress EGFR (8, 9, 21). These studies further point to constitutively active Akt as a predictor of Herstatin insensitivity.

Inhibition of intracranial growth of U87 cells by Herstatin and the growth-inhibitory effects of purified recombinant Herstatin suggests potential as a therapeutic against human glioblastoma that are driven by the EGFR. Current treatments of this disease are largely limited to chemotherapy and radiotherapy, both of which have toxic side effects and minimal efficacy. Receptor-targeted therapeutics are less toxic than most conventional treatments (2, 5, 6). However, the blood-brain barrier presents an obstacle in the treatment of glioblastoma and other brain neoplasms by limiting agent access to the tumor mass and to infiltrating cells far from the main tumor mass (22). Osmotic opening of the blood-brain barrier provides a method to optimize delivery of peptides, such as Herstatin, and even viral vectors to brain tumors (22). Because Herstatin is a secreted protein that can spread to and inhibit adjacent tumor cells, it may be an effective therapeutic transgene for gene therapy of intracerebral tumors. Another delivery option is convection or clysis to enhance the distribution of injected therapeutic in brain parenchyma (22). Future experiments will test the antitumor efficacy of Herstatin protein as well as Herstatin gene expression in rat intracerebral tumor models after intratumor injection with convection in comparison to osmotic blood-brain barrier disruption delivery.

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