Inhibition of angiogenesis by growth factor receptor bound protein 2-Src homology 2 domain binding antagonists

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Abstract

Growth factor receptor bound protein 2 (Grb2) is an intracellular adaptor protein that participates in the signal transduction cascades of several angiogenic factors, including hepatocyte growth factor, basic fibroblast growth factor, and vascular endothelial growth factor. We described previously the potent blockade of hepatocyte growth factor–stimulated cell motility, matrix invasion, and epithelial tubulogenesis by synthetic Grb2-Src homology 2 (SH2) domain binding antagonists. Here, we show that these binding antagonists block basic morphogenetic events required for angiogenesis, including hepatocyte growth factor–, vascular endothelial growth factor–, and basic fibroblast growth factor–stimulated endothelial cell proliferation and migration, as well as phorbol 12-myristate 13-acetate–stimulated endothelial cell migration and matrix invasion. The Grb2-SH2 domain binding antagonists also impair angiogenesis in vitro, as shown by the inhibition of cord formation by macromolecular endothelial cells on Matrigel. We further show that a representative compound inhibits angiogenesis in vivo as measured using a chick chorioallantoic membrane assay. These results suggest that Grb2 is an important mediator of key proangiogenic events, with potential application to pathologic conditions where neovascularization contributes to disease progression. In particular, the well-characterized role of Grb2 in signaling cell cycle progression together with our present findings suggests that Grb2-SH2 domain binding antagonists have the potential to act as anticancer drugs that target both tumor and vascular cell compartments. [Mol Cancer Ther 2004;3(10):1289–99]

Introduction

Angiogenesis, the sprouting of new capillaries from existing blood vessels, consists of a complex cascade of events including endothelial cell–mediated degradation and invasion of the extracellular matrix, endothelial cell migration, proliferation, differentiation, basement membrane deposition, and the organization of endothelial cords into capillary structures. This sequence of events is regulated by soluble angiogenic growth factors such as fibroblast growth factor (FGF)-2, vascular endothelial growth factor (VEGF), and hepatocyte growth factor (HGF) operating through an intricate network of tyrosine kinase receptor–mediated signaling pathways (1–5). The intracellular signal transduction pathways activated by various receptor tyrosine kinases share a subset of common events and mediators. On ligand binding, receptor kinases undergo autophosphorylation on multiple cytoplasmic tyrosine residues. Phosphorylated tyrosine residues on receptors or receptor substrates then become binding sites for Src homology 2 (SH2) domain containing effector proteins. Growth factor receptor bound protein 2 (Grb2; refs. 6–8) is an adaptor protein that binds phosphotyrosyl proteins through its SH2 domain and interacts through its two SH3 domains with proteins containing proline-rich motifs (9–11).

Grb2 was first found to link growth factor receptor tyrosine kinases to the Ras signal transduction pathway (6, 12) as well as to other intracellular signaling proteins including Gab family members and phosphatidylinositol 3 (P1-3)-kinase, leading ultimately to cell cycle progression. In the epidermal growth factor (EGF) signaling pathway, receptor autophosphorylation allows Grb2 binding via its SH2 domain, bringing SH3 domain–bound SOS-1 into the EGF receptor (EGFR) signaling complex. SOS-1 then facilitates guanine nucleotide exchange and activation of Ras and subsequently a cascade of kinase activation steps involving Raf and mitogen-activated protein kinases (MAPKs), which in turn stimulate the transcription of key cell cycle regulators (13). SH2 domain–mediated recognition by Grb2 is specific for the phosphopeptide motif pYXNX (where pY is phosphotyrosine, N is asparagine, and X is any residue), which is found in several tyrosine kinases and their substrates. Thus, ligand-independent EGFR activation, such as growth hormone–induced EGFR tyrosine phosphorylation by JAK2, also...
leads to Grb2-mediated MAPK pathway activation and c-fos expression (14, 15). Similarly, mechanical stress leading to increased angiotensin II production and transactivation of EGFR and other intracellular kinases implicates Grb2 recruitment in cardiac hypertrophy and myocardial remodeling (16, 17).

Many of the signaling pathways in which Grb2 functions are critical for vasculogenesis, angiogenesis, and lymphangiogenesis. The VEGF receptors VEGFR2 and VEGFR3 bind Grb2 directly, and Grb2 is also recruited to receptors through its interaction with Shc (18–21). Similarly, Grb2 participates in the signaling pathways of FGF-2, angiopoietin-1, and HGF-stimulated vasculogenesis and angiogenesis via direct association with activated receptors and/or through intermediaries such as FGF receptor substrate 2, Gab1, and Shc (22–25). As a mediator of signals downstream of potent epithelial and endothelial growth and motility factors, it is not surprising that Grb2 has been implicated in tumor progression and metastasis. For example, Grb2 is critical in metastatic signaling downstream of ErbB-2/Neu in a mouse model of mammary tumorigenesis (26), and the critical role of Grb2 in linking HGF-stimulated c-Met activation with Ras and Rac-regulated cell migration in normal development seems to extend to c-Met-driven cell transformation and tumor metastasis as well (27–30).

While all of these properties make Grb2 an excellent target for the design of therapeutic antitumor agents, evaluating Grb2-SH2 domain binding antagonist phosphopeptides using preclinical models has been hampered by their relative inability to cross the cell membrane, resulting in poor bioavailability (31, 32). The binding antagonists used in this study (33, 34) are potent tripeptide mimetic inhibitors that seem to have significantly improved potential for cell membrane penetration (33); their chemical structures are shown in Fig. 1. Replacement of phosphotyrosine at position X (Fig. 1) with phosphonomethyl phenylalanine in compound C126 or with two carboxylic acid moieties in C90, as shown below the backbone structure. Both moieties confer resistance to cellular phosphatases.

![Figure 1. Chemical structures of Grb2-SH2 domain binding antagonists. X: position of phosphotyrosine (pY) in the basic peptidomimetic structure recognized by the Grb2-SH2 domain, which is replaced with phosphonomethyl phenylalanine in compound C126 or with two carboxylic acid moieties in C90, as shown below the backbone structure. Both moieties confer resistance to cellular phosphatases.](https://example.com/figure1.png)

**Materials and Methods**

**Reagents**

Full-length human HGF protein was a gift from Dr. George Vande Woude (Van Andel Research Institute, Grand Rapids, MI). The HGF isoform HGF/NK1, which has the same biological properties as full-length HGF, was produced in a bacterial expression system and then purified and refolded as described previously (38). The Grb2-SH2 domain binding antagonists designated C126 and C90 were synthesized and purified as described (33, 34). Human recombinant basic FGF (bFGF) and human recombinant VEGF were from R&D Systems (Minneapolis, MN). Phorbol 12-myristate 13-acetate (PMA) was from Sigma Chemical Co. (St. Louis, MO).

**Cultured Cell Lines**

Human dermal microvascular (HMEC-1) endothelial cells (39) were grown in RPMI 1640 (Biofluids, Rockville, MD) containing 10% fetal bovine serum (Biofluids) and 2 mmol/L glutamine (Sigma Chemical). Human microvascular endothelial cells (HMVEC) from neonatal dermis were purchased from Cascade Biologicals (Portland, OR) and cultured in medium 131 containing MVGS (media supplement) and 1% glutamine as indicated by the manufacturer. Human umbilical vein endothelial cells (HUVEC) were isolated from freshly delivered cords as reported previously (40) and grown on Nunclon dishes (Nunc, Roskilde, Denmark) in RPMI 1640 supplemented with 20% bovine calf serum (HyClone Laboratories, Logan, UT), 50 μg/mL gentamycin, 2.5 μg/mL amphotericin B (fungizone, Life Technologies, Gaithersburg, MD), 5 units/mL sodium heparin (Fisher Scientific, Pittsburgh, PA), and 200 μg/mL endothelial cell growth supplement (Collaborative Research, Bedford, MA) and were used between passages 3 and 6. HMEC-1 and HUVEC were gifts from Dr. Hynda Kleinman (NIDCR, NIH, Bethesda, MD).
Cell Migration Assays

The migration of HUVEC or HMVEC-1 was measured in modified Boyden chambers adapted from procedures described previously (41, 42). In brief, Biocoat Cell Environment control inserts (8 μm pore size, Becton Dickinson, Bedford, MA) were coated with 0.1% gelatin (Sigma Chemical) for at least 1 hour at 37°C and air dried. Lower chambers contained 0.7 mL RPMI plus 0.1% bovine serum albumin, to which 20 ng/mL HGF and/or 300 nmol/L Grb2-SH2 domain binding antagonists were added. Cells were pretreated with the indicated concentrations of Grb2 binding antagonists for 18 to 24 hours, trypsinized, washed twice in RPMI plus 0.1% bovine serum albumin, added to upper chambers (5 x 10⁴ cells/well) with or without binding antagonists in a final volume of 0.5 mL, and incubated for 4 hours at 37°C. Cells on the upper surface of each filter were removed with a cotton swab, whereas cells that had traversed to the bottom surface of the filter were fixed and stained using Diff-Quik (Dade Diagnostics, Deerfield, IL) and counted by bright-field microscopy using a 10× objective. Mean values from four randomly selected fields (1 × 1.4 mm) were calculated for each of triplicate wells per experimental condition. Values are expressed either as the ratio of growth factor–treated cells to control proliferating cells designated as “Migration (Fold Increase)” or as the mean number of cells per optical field.

Cell Proliferation Assays

HUVECs or HMVECs were seeded in triplicate into type I collagen-coated 48-well plates (3,000 cells/well, Biocoat) in 500 μL complete ECM-Bullet Kit medium (BioWhittaker, Walkersville, MD), allowed to attach for 4 hours, and incubated for 16 hours with or without the indicated concentrations of binding antagonists. The cultures were rinsed twice in serum-free medium and incubated with endothelial cell basal medium (BioWhittaker) supplemented with 50 μg/mL heparin, 50 μg/mL ascorbic acid, 10% FCS, and the indicated concentrations of binding antagonists. After either 4 days (HUVECs) or 5 days (HMVECs), cells were harvested with trypsin-EDTA and counted with a hemocytometer. The mean number of cells per milliliter was calculated from three independent measurements for each of triplicate wells per experimental condition. Values are expressed as the ratio of growth factor–treated cells or in the form of cell cords as described (44). Values were compared using Student’s unpaired t test and a significant value was taken as P < 0.001. Results were expressed as mean total length (μm) per field.

HUVEC Cord Formation Assay on Matrigel

The HUVEC tube formation assay was performed as described previously (41, 43). Briefly, 96-well plates were coated with 90 μL Matrigel (10 mg/mL, Collaborative Research) and incubated at 37°C for 30 minutes to promote gelling. HUVECs (10,000 cells/sample) were resuspended in reduced growth medium (serum concentration 1% and 5 units/mL heparin) and added to each well with the indicated reagents in a final volume of 100 μL. After 18 hours, the plates were fixed with Diff-Quik and at least four randomly selected fields per experimental condition were digitally recorded under bright-field illumination using a 10× objective. The mean additive length of all of the cords present in each optical field was measured using IPLab software and compared with controls using the Student’s unpaired t test.

Chick Chorioallantoic Membrane Assay

To assess in vivo angiogenesis, a chick chorioallantoic membrane (CAM) assay was performed using a modification of a procedure described previously (46, 47). To this end, apical holes were drilled in 10-day-old chicken eggs to expose the CAMs, and filter paper discs (0.5 cm in diameter) containing 20 μL of a 1 or 3 μmol/L C90 in PBS were placed on the surface of each CAM (day 0). The holes were covered with parafilm and the eggs were incubated in a humidified atmosphere at 37°C for 24 hours after which 100 μL of 1 or 3 μmol/L C90 was given i.v. using a 30-gauge needle. Three days later (day 4), the discs and associated CAM were excised and immediately fixed in 3.7% formaldehyde. For computer-assisted image analysis, the discs from 10 eggs per experimental condition in each of two separate experiments were divided into quadrants using fine forceps, the blood vessels in each quarter were digitally photographed, and the mean vessel area was normalized to the total quadrant area and was quantitated using (5% FCS and 150 μg/mL endothelial cell growth supplement). At confluence, cells were pretreated with or without Grb2 binding antagonists for 24 hours after which time medium was replaced with fresh medium supplemented with or without the indicated concentration of binding antagonists and/or 20 ng/mL HGF. Medium and compounds were changed every day. After 5 days, the cultures were fixed in situ in 2.5% glutaraldehyde in 0.1 mol/L sodium cacodylate buffer (pH 7.4) and five randomly selected optical fields (measuring 1 × 1.4 mm) per experimental condition in each of three separate experiments were digitally recorded under phase-contrast microscopy using a 20× objective by focusing 20 μm beneath the surface of the gel. Invasion was quantitated using IPLab software (Scanalytics, Fairfax, VA) by measuring the total length of all cellular structures that had penetrated beneath the surface monolayer either as apparently single cells or in the form of cell cords as described (44). Values were compared using Student’s unpaired t test and a significant value was taken as P < 0.001. Results were expressed as mean total length (μm) per field.
Grb2 Binding Antagonist C90 Inhibits Endothelial Cell Migration Induced by PMA

To determine whether the effect of Grb2 binding antagonists is restricted to the inhibition of natural angiogenic factors or might also block the effect of artificial proangiogenic molecules, we assessed the effect of C90 on the migratory properties of HUVECs in the presence of PMA, a potent tumor and angiogenesis promoter. Upon exposure to PMA in vitro, both microvascular and macrovascular endothelial cells undergo a vascular morphogenetic program by invading the surrounding extracellular matrix and subsequently forming extensive network of capillary-like tubular structures (43). Addition of PMA (40 ng/mL) to cultures of HUVECs in modified Boyden chambers resulted in a 3-fold increase in cell migration. This proangiogenic activity, which cannot be mimicked by the maximal effective concentration (5 ng/mL) of bFGF, was reduced to near-basal levels in the presence of 300 nmol/L C90 (Table 1).

Blockade of Growth Factor–Induced Endothelial Cell Proliferation by Grb2 Binding Antagonist C90

To investigate the activity of compound C90 during growth factor–induced endothelial cell proliferation, we cultivated HUVECs and HMVECs on type I collagen-coated wells in partially supplemented endothelial culture medium as described in Materials and Methods. Under these stringent culture conditions, HGF (25 ng/mL), VEGF (10 ng/mL), and bFGF (5 ng/mL) induced significant (P < 0.0001) increases of 2.3-, 2-, and 4.1-fold, respectively, in the mean number of macrovascular HUVECs per milliliter (Fig. 4A, open columns). Similar significant (P < 0.001) increases in cell numbers were observed in HMVECs (Fig. 4B, open columns). Addition of Grb2 inhibitor C90 (30 and 300 nmol/L) resulted in significant, but markedly different, levels of inhibition of proliferation in HUVECs.

Results

Grb2-SH2 Domain Binding Antagonists Inhibit Endothelial Cell Motility

We initially assessed the effect of C126 on the chemokinetic response of immortalized HMEC-1 and primary HUVECs to well-characterized angiogenic factors. Compound C126 (300 nmol/L) did not significantly alter the basal levels of endothelial cell motility (Fig. 2A and B, open columns). However, it reduced to near-basal levels by 5.5- and 3.5-fold increases in cell motility induced by 20 ng/mL HGF on HMEC-1 and HUVECs, respectively. Interestingly, this inhibitory activity was not limited to HGF stimulation as shown by the complete blockade of bFGF-induced HUVEC migration by 300 nmol/L C126 (Fig. 2A and B, solid columns). To further characterize the potential antiangiogenic effect of the Grb2 binding antagonists, we assessed neonatal HMVEC migration in the presence or absence of increasing concentrations of either HGF (0–50 ng/mL) or bFGF (0.2–50 ng/mL) and compound C90 (300 nmol/L). Compound C90 abolished (P < 0.001) cell migration induced by HGF (Fig. 2C, left) and significantly (P < 0.001) inhibited the biphasic stimulatory activity of bFGF (Fig. 2C, right). Notably, the effect of the optimal concentration of bFGF (5 ng/mL) was reduced by 72% (Fig. 2C, right). We next assessed the effect of C90 on the activity of VEGF, the most potent angiogenic molecule known to date. When incubated in modified Boyden chambers in the presence of VEGF, HMEC-1, HMVEC, and HUVEC underwent different, albeit significant, degrees of migration (Fig. 3). However, the addition of compound C90 significantly (P < 0.001) reverted the effect of VEGF in all three cell lines, although the degree of inhibition of VEGF activity differed among the endothelial cell types. Similar results were observed with binding antagonist C126 (Fig. 3 and data not shown).

Grb2 Binding Antagonist C90 Inhibits Endothelial Cell Migration Induced by PMA

A

B

C

Figure 2. Grb2 binding antagonists inhibit HGF- and bFGF-induced endothelial cell migration. HUVECs were pretreated with or without 300 nmol/L C126 for 18 hours; after which time, cell migration in the presence of inhibitor and/or HGF (20 ng/mL) or bFGF (50 ng/mL) was quantified using modified Boyden chambers. A and B, open and solid columns, migration of cells in the absence or presence of HGF, respectively. Columns, ratio of migrating cells in growth factor–treated wells to control wells (CTRL). B, gray columns, migration of cells in the presence of bFGF. Results from one representative experiment. C, effect of Grb2 binding antagonist C90 on HGF- and bFGF-induced HMVEC migration. Cells were pretreated for 18 hours with or without 300 nmol/L C90; after which time, the experiments were done in the presence or absence of inhibitor and the indicated concentrations of growth factors. Columns, mean number of cells per optical field from triplicate wells per experimental condition; bars, SE. Where no bars are visible, the error is too small to be shown.
and HMVECs. Whereas C90 inhibited serum-dependent (P < 0.001) and bFGF-dependent (P < 0.0001) HUVEC proliferation at concentrations of 30 nmol/L, it failed to significantly inhibit HGF- and VEGF-induced proliferation at this concentration (Fig. 4A, gray columns). Only higher concentrations of compound (300 nmol/L) were able to induce significant (P < 0.001) reductions in cell counts under these conditions (Fig. 4A, solid columns). In contrast, no significant inhibition of HMVEC proliferation was observed with 30 nmol/L compound C90 in either the presence or the absence of growth factors, and generally less but significant (P < 0.001) inhibition of HMVEC growth was observed with 300 nmol/L compound (Fig. 4B).

Grb2 Binding Antagonists Block HGF-Induced Matrix Invasion by Microvascular Endothelial Cells

We investigated the effect of Grb2 binding inhibitors on extracellular matrix invasion using HMEC-1 grown to confluence on the surface of collagen I gels as described in Materials and Methods. When monolayers of HMEC-1 were grown under control conditions for 5 days, they discretely invaded the underlying collagen matrix as single cells (Fig. 5A, top). Addition of 20 ng/mL HGF to the cultures induced a 6-fold increase in collagen invasion by either single cells or short cell cords devoid of lumen (Fig. 5A, middle, and B). Coaddition of C126 (30-300 nmol/L) and HGF to the cultures suppressed collagen invasion induced by HGF (Fig. 5, bottom). Invasion was quantitated by measuring the total length of all cellular structures that had penetrated 20 μm beneath the surface monolayer either as apparently single cells or in the form of cell cords as described previously (43). Significant (P < 0.001) decreases in the total length of the cords penetrating the surface of the gel matrix were observed with concentrations of C126 as low as 30 nmol/L, and while no effect was observed on control levels of invasion, HGF-induced HMEC-1 matrix invasion was generally reversed in the presence of the Grb2 binding antagonist (Fig. 5B).

Table 1. Grb2 binding antagonist C90 inhibits PMA-induced HUVEC migration

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Migrating Cells per Field</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>43 ± 9</td>
</tr>
<tr>
<td>PMA</td>
<td>148 ± 15</td>
</tr>
<tr>
<td>PMA + C90</td>
<td>58 ± 8</td>
</tr>
<tr>
<td>bFGF</td>
<td>86 ± 14</td>
</tr>
</tbody>
</table>

NOTE: HUVECs pretreated with or without C90 (300 nmol/L) for 18 hours were seeded in modified Boyden chambers in the presence or absence of either 40 ng/mL PMA or 5 ng/mL bFGF as described in Materials and Methods. Values are the mean ± SE number of cells per optical field; four randomly selected fields were quantitated for each of triplicate wells per experimental condition.

Figure 4. Effects of Grb2 binding antagonist C90 on endothelial cell proliferation. HUVECs (A) or HMVECs (B) were grown in 48-well plates, pretreated 16 hours without (open columns) or with 30 (gray columns) or 300 (solid columns) nmol/L compound C90, grown for either 4 days (HUVECs) or 5 days (HMVECs), harvested with trypsin/EDTA, and counted. The mean number of cells per milliliter was calculated from three independent measures for each of triplicate wells per experimental condition and compared with controls using the Student’s unpaired t test. Columns, ratio of growth factor–treated cells to control proliferating cells (Fold Increase).
Grb2 Binding Antagonist C126 Impairs Endothelial Cell Tubulogenesis In vitro

In an in vitro model of angiogenesis (41, 44, 45), we seeded HUVECs onto Matrigel gel layers and immediately treated the cells with the indicated agents (Fig. 6). Under control conditions, the cells migrated on the surface of the gel, and only a few cells have invaded the underlying matrix (top). Cells incubated with HGF invade the gel extensively either as whole cells or as cellular projections (middle). Coaddition of HGF and the Grb2 binding antagonist C126 results in a significantly reduced number of invading cells invading the matrix (bottom).

Figure 5. Grb2 binding antagonist C126 inhibits HGF-induced matrix invasion by HMEC-1. A, cells seeded on collagen gels were grown to confluence prior to incubation with or without inhibitors and/or HGF (20 ng/mL) for 24 hours as described in Materials and Methods. In the three panels, invasion was assessed by focusing 20 μm beneath the surface of the gel. HMEC-1 incubated under control conditions remain primarily on the surface of the collagen gel, which appears blurred in the background; bars, SE. Results are from at least three independent experiments.

Figure 6. Grb2 binding antagonist C126 inhibits HGF-stimulated angiogenesis in vitro. HUVECs were plated on Matrigel-coated 96-well plates and incubated with or without 300 ng/mL HGF/NK1 and or 1 μmol/L C126 for 18 hours as described in Materials and Methods. Under control conditions (top), cells form irregular aggregates on the surface of the Matrigel and short, discontinuous cell cords were visualized by dark-field microscopy (×40). Cells treated with HGF/NK1 form an extensive network of anastomosing, thick endothelial cords (middle). Cells treated with HGF/NK1 and C126 have formed only a few thin and discontinuous cords, similar to controls (bottom).
gel, established contact with each other, and after 12 to 18 hours formed irregular ridges or cords, a process reminiscent of the early steps of angiogenesis (Fig. 6, top). Addition of 20 to 50 ng/mL HGF (data not shown) or 300 ng/mL HGF/NK1 to the cultures resulted in the formation of a continuous, extensive network of interconnecting cords on the surface of the Matrigel visualized by phase-contrast microscopy (×40; top left). Simultaneous addition of C90 inhibits VEGF-driven cord formation, resulting in the formation of short, discontinuous cell cords and cell aggregates on the surface of the Matrigel (top right). In the presence of 5 ng/mL bFGF, HUVECs develop a network of anastomosing endothelial cords similar to that induced by VEGF (bottom left); coaddition of C90 to bFGF-treated cultures results in partial inhibition of cord formation (bottom right).

Grb2 Binding Antagonist C90 Inhibits Angiogenesis in the Chick CAM

Chick CAM assays performed on 10-day-old eggs demonstrated that compound C90 inhibits basal in vivo angiogenesis (Fig. 8). When paper discs treated with PBS alone were placed onto the CAM of 10-day-old eggs, which were subsequently given a single i.v. injection of PBS, a large number of vessels occupied the CAM area covered by the paper discs (Fig. 8A, left). In striking contrast, when the discs were treated with compound C90 (3 mmol/L), the number of vessels underlying the paper discs was significantly reduced (Fig. 8A, right). Morphometric analysis showed a significant (P < 0.05) and dose-dependent decrease in mean vessel area in CAM treated with C90 relative to control-treated CAM samples (Fig. 8B). Similar results were obtained when measuring mean vessel length (data not shown). We also observed that chick embryos treated with compound C90 were alive and showed the same extent of motility as embryos from PBS-treated eggs at the end of the experiment.

Discussion

The ability of Grb2-SH2 domain binding antagonists to block HGF-stimulated epithelial cell migration and invasion has been described (48, 49). Our prior studies using an in vitro model of mammary epithelial morphogenesis revealed that these binding antagonists could block HGF-induced formation of branching duct-like structures (49) and prompted us to determine whether they might also...
modulate the morphogenetic response of endothelial cells to various angiogenic stimuli, including HGF. Here, we show that these Grb2-SH2 domain binding antagonists also block HGF-stimulated matrix invasion and branching morphogenesis by vascular endothelial cells as well as HGF-, VEGF-, and bFGF-stimulated vascular endothelial cell proliferation, migration, and the formation of capillary-like structures on reconstituted extracellular matrices.

Grb2-SH2 domain binding blockade was associated with inhibition of growth stimulated by HGF, bFGF, and VEGF more significantly in HUVECs than in HMVECs. These results contrast with our previous findings where little inhibition of HGF-stimulated epithelial cell proliferation was observed (49) and suggest that the roles of Grb2 in facilitating cell cycle progression and other activities are cell type dependent and may vary among different vascular beds. The results presented here are also consistent with the phenotypic heterogeneity generally observed among endothelial cells from different tissues and different levels of the vasculature. Most significant in the present study, and consistent with our prior report, was potent Grb2-binding antagonist-induced inhibition of motility observed in every cell line tested. In addition, and perhaps as a consequence of motility inhibition, matrix invasion and cord formation were also potently blocked.

While the molecular mechanisms by which SH2 binding antagonists inhibit motility, invasion, and cord formation are not yet fully defined, published evidence indicates that Grb2 may regulate cell motility at two levels: as a receptor proximal adaptor and as a direct participant in actin filament based motility. On ligand stimulation, Grb2 is recruited via its SH2 domain to growth factor receptors or to tyrosine-phosphorylated receptor proximal substrates and/or adaptors (9, 25, 48–52). VEGF and bFGF are among the most potent regulators of angiogenesis but share intracellular signaling mediators with a variety of angiogenesis signaling pathways (4). In porcine aortic endothelial cells, for example, VEGF-A-induced phosphorylation of VEGFR2 induces the recruitment of Shc, Grb2, and Nck and formation of the Shc-Grb2 complex (21). The FLT4L kinase, an alternatively spliced form of the VEGFR3/FLT4 receptor, binds Grb2 directly through its cytoplasmic region and recruits a second pool of Grb2 by phosphorylating tyrosine residues within appropriate sequence motifs in Shc (18, 20). Grb2 acting at this level is believed to link receptor activation to cell cycle progression and/or cell motility via the Sos/Ras and Rac1/Rho pathways, respectively (1, 18, 22, 26, 27, 29, 48, 52–55). We and others have reported direct evidence of the ability of Grb2-SH2 domain binding antagonists to block HGF-stimulated Grb2 signaling at this level, with primary impact on cell motility and motility-dependent processes (48, 49).

In addition to its role as a receptor-proximal adaptor protein, Grb2 participates directly in the regulation of actin filament formation and actin-based cell motility. Grb2 is important in T-cell receptor signaling (56) and is a critical link between Wiskott-Aldrich Syndrome protein (WASp) and the actin cytoskeleton; WAS patients show defects in T-cell polarization and migration in response to physiologic stimuli, resulting in thrombocytopenia, eczema, and immunodeficiency (57–59). Studies of WASp function and the intracellular motility of invasive microbial pathogens such as Listeria monocytogenes and Vaccinia virus have elucidated an important role for Grb2 in directly promoting actin-based motility (58, 60, 61). These microbes invade eukaryotic cells and use a limited number of microbial surface

Figure 8. Grb2 binding antagonist C90 inhibits angiogenesis in the chick CAM. Chick CAM assays were used to assess the impact of Grb2-SH2 domain antagonism on angiogenesis in vivo. Apical holes were drilled in 10-day-old chicken eggs to expose the CAM, and filter paper discs (0.5 cm diameter) containing 20 μL of a 1 or 3 μmol/L solution of C90 were placed on the CAM surface. After 24 hours, 100 μL of 1 or 3 μmol/L C90 was given again i.v. Three days later, the discs and associated CAM were excised, fixed, and photographed by bright-field microscopy. Representative filters from control (left) and C90-treated (right) CAM are in A. Discs from 10 eggs per experimental condition in each of two separate experiments were digitally photographed and mean vessel area was normalized to total area using Optima 5 software. B, columns, expressed as a mean area in pixels; bars, SD. *, P < 0.001, statistical significance from control, Student’s t test.
proteins to harness host cell actin and associated regulatory molecules for propulsion through the host cytoplasm. Unraveling these pathogenic events helped to define the roles of several eukaryotic proteins in normal actin-based motility. In most mammalian cells, the WASp family member neural WASp interacts with the ARP2/3 complex and G-actin to stimulate actin polymerization. Neural WASp activity is enhanced by other effectors such as Nck, Cdc42, and Grb2; disruption of Grb2-SH3 or -SH2 domains diminishes actin polymerization and thus actin-based motility (58, 60). The activity of the Grb2-SH2 domain binding antagonists described here at this level of Grb2 function is currently under investigation.

Grb2 also acts as a critical downstream intermediary in several angiogenic signaling pathways not tested here, including platelet-derived growth factor (PDGF), ELK, and Eph, suggesting that these pathways may also be susceptible to blockade by Grb2-SH2 domain binding antagonists. PDGF is implicated in different biological processes such as vascular remodeling, wound healing, and cancer (54, 62). Consistent with the spectrum of pathways disrupted by Grb2, we have also observed that the Grb2 binding antagonist C90 inhibits PDGF-BB–induced NIH3T3 cell migration. In microvascular endothelial cells, membrane-bound LERK-2 stimulates ELK tyrosine phosphorylation and recruitment of Grb2 and Grb10 via their SH2 domains, signaling the assembly of microvascular endothelial cells into capillary-like cords (52). Interestingly, natively expressed Eph-related receptors, including ELK, Mek4, and Eck, do not signal proliferative responses on ligand binding (52, 63), reinforcing the hypothesis that motility and morphogenesis are primary cellular responses downstream of Grb2. We also show that the Grb2-SH2 domain binding antagonists inhibit PMA-induced cell migration. PMA is an artificial activator of PKC; PKC-mediated migration plays an important role not only during angiogenesis but also during tumor development, tumor invasion, and metastasis. Signaling through the VEGF receptors FLT-1 and FLK-1 is thought to occur via PKC activation and the MAPK and PI-3-kinase pathways, ultimately leading to capillary formation in vitro and to vasculogenesis and angiogenesis in vivo (64, 65).

The widespread role of Grb2 in most, if not all, angiogenesis signaling pathways together with our observations that Grb2-SH2 domain binding antagonists blocked endothelial cell migration, matrix invasion, proliferation, and cord formation in vitro suggested that they might also inhibit angiogenesis in vivo. Results obtained using the chick CAM assay support this contention: SH2 domain binding antagonists displayed potent local inhibition of CAM vasculogenesis. We noted that these treatments did not seem to disrupt vasculogenesis generally and that chick embryos displayed grossly normal development and motility throughout the time course of the experiments. In preliminary pharmacokinetic studies, we have not observed toxicity in normal adult mice after 21 days of i.p. injections of millimolar doses of binding antagonist. Further studies of these compounds in other animal models are underway.

Despite the modest performance of angiogenesis inhibitors as single agents in early human clinical trials (66), more recent trials showing better efficacy have resulted in approval of the first rationally designed antiangiogenic drug (67). In parallel, the finding that angiogenesis inhibitors can enhance the potency of conventional chemotherapy and radiotherapy has brought widespread support to multidrug clinical trials where conventional and antiangiogenic therapies are combined (68). Combination trials, however, are inherently more complex with regard to their design, execution, and interpretation, in addition to potential obstacles related to the approval and/or commercialization of drug combinations (69). These circumstances make single agents that target both tumor and vascular compartments very attractive from the earliest phases of rational drug design and development. Together with our previous studies, the results presented here suggest that potent simultaneous disruption of Grb2-SH2 domain–mediated signaling downstream of HGF, VEGF, and bFGF and potentially other angiogenic regulators in the tumor microenvironment by a single agent may be a clinically viable strategy to inhibit tumor cell invasion into the surrounding tissue, tumor metastasis, and the recruitment of new blood vessels needed to sustain these pathologic processes.

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