Soluble endoglin antagonizes Met signaling in spindle carcinoma cells

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Abstract

Increased levels of soluble endoglin (Sol-Eng) correlate with poor outcome in human cancer. We have previously shown that shedding of membrane endoglin, and concomitant release of Sol-Eng is a late event in chemical mouse skin carcinogenesis associated with the development of undifferentiated spindle cell carcinomas (SpCCs). In this report, we show that mouse skin SpCCs exhibit a high expression of hepatocyte growth factor (HGF) and an elevated ratio of its active tyrosine kinase receptor Met versus total Met levels. We have evaluated the effect of Sol-Eng in spindle carcinoma cells by transfection of a cDNA encoding most of the endoglin ectodomain or by using purified recombinant Sol-Eng. We found that Sol-Eng inhibited both mitogen-activated protein kinase (MAPK) activity and cell growth in vitro and in vivo. Sol-Eng also blocked MAPK activation by transforming growth factor-β1 (TGF-β1) and impaired both basal and HGF-induced activation of Met and downstream MAPK. Moreover, Sol-Eng strongly reduced basal and HGF-stimulated spindle cell migration and invasion. Both Sol-Eng and full-length endoglin were shown to interact with Met by coimmunoprecipitation experiments. However, full-length endoglin expressed at the plasma membrane of spindle carcinoma cells had no effect on Met signaling activity, and was unable to inhibit HGF-induced cell migration/invasion. These results point to a paradoxical suppressor role for Sol-Eng in carcinogenesis.

Introduction

Many cell-surface membrane receptors are proteolitically cleaved at their juxtamembrane region in a process called 'shedding', which results in the release of the ectodomain into the extracellular space. Shedding may downregulate receptors at the cell surface and generate soluble forms that either antagonize or favor the formation of active signaling complexes with ligands (1). Endoglin (CD105) is a membrane glycoprotein that acts as a coreceptor for members of the transforming growth factor-β (TGF-β) superfamily, including TGF-β1, TGF-β3, activin-A, BMP2, BMP7, BMP9 and BMP10 (2). Endoglin plays a crucial role in cardiovascular development and angiogenesis (3,4). Mutations in the endoglin gene are responsible for the hereditary hemorrhagic telangiectasia type 1 (HHT1), a vascular disorder characterized by epistaxis, cutaneous telangiectases and arteriovenous malformations in brain, lung and liver (5). In cancer, endoglin has also emerged as an important modulator of malignant progression by influencing tumor cell proliferation, motility, invasion and metastasis (reviewed in refs. 2,6). Thus, endoglin behaves...
as a suppressor of malignancy in skin, esophageal, prostate and breast carcinomas (7–10), while promotes tumor growth and invasiveness in Ewing sarcoma and melanoma (11).

A soluble endoglin form (Sol-Eng) resulting from the cleavage of full-length endoglin by the membrane-anchored matrix metalloprotease MMP14 has been detected in the circulation associated with different pathologies, including preeclampsia and cancer (12–14). Preeclampsia is a specific syndrome of pregnancy characterized by hypertension and proteinuria, which is linked to elevated levels of Sol-Eng and placenta-derived soluble VEGF receptor 1 (also known as soluble fms-like tyrosine kinase-1, sFlt1 (15)). Sol-Eng inhibits angiogenesis (12,14) and increases blood pressure and vascular permeability (13,14). Whereas it has been proposed that these effects are mediated by Sol-Eng trapping circulating TGF-β1, thus impairing its binding to the functional receptors (14), other authors have found that Sol-Eng binds directly and with high affinity BMP9 and BMP10, but not TGF-β1 or TGF-β3 (15-17). Sol-Eng has also been detected in the plasma, serum and urine of cancer patients. Interestingly, elevated levels of circulating Sol-Eng is a bad prognostic factor in breast, colorectal and prostate cancer that correlates with metastasis (18–21). We have shown that cleavage of membrane endoglin, and concomitant generation of Sol-Eng occurs during progression of chemical mouse skin carcinogenesis, which is associated with the development of highly malignant undifferentiated spindle cell carcinomas (SpCCs (22)). Though Hawinkels et al. (12) suggested an anti-angiogenic role for Sol-Eng in the tumor microenvironment, the function of Sol-Eng in carcinogenesis remains to be determined.

Hepatocyte growth factor (HGF) signaling through its membrane tyrosine kinase receptor Met plays an important role during embryonic development and tissue remodeling. Yet, HGF/Met aberrant signaling is also associated with cancer, since Met hyperactivation promotes different processes related to malignant progression, including tumor cell proliferation, migration, invasion, metastasis and angiogenesis (23,24).

In this work, we show that mouse spindle cell carcinomas generated by chemical carcinogenesis express elevated levels of HGF. Sol-Eng, but not membrane-bound full-length endoglin, inhibited baseline and HGF stimulated Met signaling, impairing proliferation, migration and invasion of spindle carcinoma cells. These results suggest an anti-oncogenic role for Sol-Eng in carcinogenesis.

### Real-time quantitative RT-PCR analysis

RNA from tissues and cell lines was obtained using the RNAeasy kit (Qiagen). Quantitative reverse transcription-PCR analysis was performed using the iScript Reverse Transcription Supermix kit (BioRad) in a 7900HT Fast (Life Technologies) instrument. The ribosomal protein RPLPO was used as an internal control of RNA quality and amplification. Taqman probes for HGF (Mm01135193-m1) and Met (Mm01156972-m1) were from Life Technologies.

### Western blot and coimmunoprecipitation experiments

For detection of proteins in Western blots, cells were lysed at 4°C in modified RIPA buffer and a cocktail of protease (2 μg/ml aprotinin; 2 g / ml leupeptin; 1mM phenylmethylsulfonyl fluoride) and phosphatase (1 mM sodium orthovanadate; 2 mM β-glycerophosphate; 0.2 mM sodium fluoride) inhibitors (Sigma–Aldrich). Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred to Immobilon-P membranes (Millipore). For immunodetection of proteins, the following antibodies were used: 12CA5 mAb (Roche Diagnostics) for HA-Sol-Eng; P4A4 mAb (Santa Cruz Biotechnology) for human full-length.
endoglin (7); the mAb 1D4 recognizing glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was purchased from Enzo Life Sciences AG; the Abs specific for p44/42 MAPK (ERK1/2) (#9102), phospho-p44/42 MAPK (Thr202/Tyr204) (#9110S), as well as a mAb specific for phospho-Met (Tyr1234/1235) (D2E) (#3077), all were from Cell Signaling; a polyclonal Ab recognizing Met (sc-162) was from Santa Cruz Biotechnology Inc. Appropriate secondary Abs coupled to horseradish peroxidase were used. Peroxidase activity was developed using an enhanced chemiluminescence kit as indicated by the manufacturer (Pierce).

For communoprecipitation experiments, cells were lysed in IP buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 5 mM EDTA, 0.5% NP-40) with a cocktail of protein inhibitors. Tagged Sol-Eng and Met were communoprecipitated using anti-HA mAb (Roche Diagnostics) and anti-Met Ab sc-162 (Santa Cruz Biotechnology Inc.), respectively.

Cell growth, migration and invasion assays

For growth assays, cells were plated in triplicate in a 24-wells/plate at a density of 30,000 cells/well and grown in the absence of serum (with or without TGF-β1) for the indicated times. The relative number of cells was determined by staining with crystal violet and spectrophotometric reading (560 nm) of the solubilized dye. For cell proliferation assays, cells were seeded in 96 wells/plate at a density of 10,000 cells/well and grown in the presence or absence of serum for the indicated times. Incorporation of 5-bromo-2-deoxyuridine (BrdU) was determined by using the Cell Proliferation ELISA BrdU Kit (Roche Diagnostic).

Transwell migration assays were performed using Transwell chambers with 8-μm-pore polycarbonate filters (Corning) coated or not with 30 μg of Matrigel (Becton Dickinson). Cells were seeded in the upper compartment (4×10^5 cells per well) in medium without serum and allowed to transmigrate for 15 h at 37°C using 10% FBS, in the presence or absence of 100 ng/ml HGF, as chemottractant. Cells on the upper side of the Transwell were then removed and those on the underside were fixed with methanol and stained with 1 μg/ml solution of 4',6-diamino-2-phenylindol (DAPI). Cell migration was quantified by counting the number of cells that migrated through the inserts. Four-five different fields were counted using an Axioshot fluorescence microscope (Carl Zeiss).

Chemical carcinogenesis and tumorigenicity assays

All animal experiments were approved by the Animal Care and Use Committees of the CSIC and UAM. Mice were cared for following institutional guidelines for animal care and in accordance with the standards established in the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Induction of mouse skin tumors by initiation of CSE1 and CSE3 is presently unknown since both clones express similar levels of Sol-Eng in vitro (Figure 2A), whereas no variation was observed in the total ERK1/2 expression levels. In vitro, this reduction was more pronounced in CSE3 (Figure 2C), which may account for the slightly weaker tumorigenic potential of CSE3 with respect to CSE1 (Figure 2A).

Sol-Eng blocks TGF-β1-induced cell growth

In contrast to normal keratinocytes and SCC cells, spindle carcinoma cells are not only insensitive to the TGF-β1 growth inhibitory response, but their growth is stimulated by the cytokine (32). Accordingly, proliferation of mock cells was slightly increased by TGF-β1 in a dose-dependent manner (Figure 3A). In contrast, this TGF-β1-dependent growth stimulation did not occur in CSE3 cells. Moreover, TGF-β1 at 1 ng/ml was able to activate the ERK1/2 pathway in mock but not in CSE3 cells (Figure 3B). A similar inhibitory effect on the TGF-β1-dependent activation of ERK1/2 was seen in parental CarC cells pre-incubated with rsSol-Eng (Figure 3C). These results indicate that Sol-Eng inhibits TGF-β1 stimulation of MAPK signaling activity in CarC spindle cells.

Enhanced expression of HGF is a late event in mouse skin chemical carcinogenesis

Next, we asked whether Sol-Eng could inhibit cell growth and MAPK signaling activity triggered by activation of tyrosine kinase receptors. In a search for growth factors expressed/secreted by transformed keratinocytes, we found that CarC cells express remarkably high levels of HGF mRNA compared to other keratinocyte cell lines such as premalignant keratinocytes (MCA3D and PB), SCC cells (PDV and HaCa4) and SpCC cell lines (CarB and MSC11A5) (Figure 4A). As a matter of fact,
increased levels of HGF transcripts are mainly associated with the spindle phenotype during in vivo mouse skin carcinogenesis (Figure 4B). The transcript levels of the HGF receptor Met remain low in transformed keratinocyte cell lines, with the exception of MCA3D and MSC11A5 that express relatively high levels (Figure 4C). In vivo, Met expression is increased in SCCs with respect to premalignant stages of carcinogenesis, but Met transcript levels appear to diminish in SpCCs (Figure 4D). Interestingly, when the levels of total and phosphorylated Met proteins were analyzed in the cell lines, spindle carcinoma cells (CarB, CarC and MSC11A5) showed enhanced levels of activated versus total Met protein expression compared with the other cell lines, and CarC displayed the highest phospho-Met/Met ratio (Figure 4E). However, Met polypeptides (both the 170-kDa single chain precursor and the 145-kDa product corresponding to the β-chain of the mature receptor) were clearly downregulated in CarC compared to the other cell lines (Figure 4E). These results might indicate that an autocrine loop exists in CarC by which...
secreted HGF continuously activates Met on the cell surface inducing its degradation, and are consistent with reports showing Met internalization and degradation induced by HGF (33,34).

**Sol-Eng inhibits basal and HGF-induced Met signaling activity and coimmunoprecipitates with Met**

Subsequently, we studied the effect of Sol-Eng on basal and HGF-mediated Met activation of CarC cells. As shown in Figure 5A, CSE1 and specially CSE3 cells displayed reduced levels of baseline active phospho-Met compared to mock cells. This result is consistent with the decreased baseline phospho-ERK1,2 levels and weaker tumorigenicity observed in CSE1 and CSE3 cells (Figure 2A and C). In addition, HGF-dependent activation of Met and its downstream effector ERK1/2 were blocked in both CSE1 and CSE3 cells that express/secrete Sol-Eng (Figure 5B and C). In mock cells, HGF induced the downregulation of Met shortly after stimulation for 5–10 min (Figure 5B and C). Interestingly, baseline Met levels in CSE1 and CSE3 were also reduced with respect to mock cells (Figure 5A–C), suggesting that expression of Sol-Eng induces the downregulation of Met. Likewise, preincubation of CarC cells with increasing concentrations of rhSol-Eng inhibited stimulation of Met signaling activity by HGF and downregulated Met levels in a dose-dependent manner (Figure 5D). We next studied whether full-length human endoglin (Eng) was able to inhibit HGF stimulation of Met signaling. As shown in Figure 5E, Eng stably expressed at the plasma membrane of CarC cells (22) was unable to inhibit HGF-induced phosphorylation of both Met and ERK1/2. Also, Met protein levels were not affected by expression of full-length endoglin, but were down-regulated upon stimulation with HGF, as expected (Figure 5E).

In order to ascertain whether Sol-Eng interacts with Met, we performed coimmunoprecipitation analysis. To this end, HEK-293T cells were cotransfected with constructs encoding Sol-Eng tagged with HA and untagged Met. Immunoprecipitation with anti-Met antibody clearly coprecipitated HA-Sol-Eng (Figure 5F, upper panel), whereas a weaker coprecipitation signal of Met with anti-HA-Sol-Eng was detected (Figure 5F, lower panel). We also cotransfected constructs encoding Met and either Sol-Eng or full-length Eng, both tagged with HA, in HEK-293T cells. Interestingly, Met immunoprecipitation coprecipitated both full-length and truncated soluble endoglin proteins (Figure 5G).

**Sol-Eng impairs migration/invasion of spindle carcinoma cells**

CarC cells are highly migratory and invasive in vitro and in vivo (35,36). Using Transwell migration assays, in which the filter was coated (invasion) or not (migration) with Matrigel, HGF was able to stimulate further CarC cell migration and invasion by ~1.5 and ~4 folds, respectively (Figure 6A and B). In order to test whether Sol-Eng and full-length Eng had some effect on this behavior, we compared the migratory/invasive abilities of CSE3 and CarC-Eng with that of the parental and control cells before and after HGF stimulation. Compared to mock cells, CSE3 showed strongly
decreased basal migratory and invasive abilities and were refractory to HGF stimulation (Figure 6C and D). In contrast, CarC-Eng cells exhibited a substantial reduction in their migratory capacities under basal conditions, and showed only a slight, but non-significant, decreased invasiveness (Figure 6A and B). Interestingly, HGF clearly stimulated cell migration and invasion in CarC-Eng and parental/control cells (~2 and ~4 folds, respectively; Figure 6A and B), indicating that whereas Sol-Eng does interfere with Met-mediated CarC pro-migratory and pro-invasive properties, full-length Eng does not. These results are in agreement with the respective abilities of Sol-Eng and full-length Eng to impair or not HGF-induced Met signaling activity (Figure 5).

**Discussion**

Membrane endoglin is cleaved in the juxtamembrane region by MMP14 to release Sol-Eng into the circulation, and increased levels of Sol-Eng are linked to poor prognosis in human breast, colorectal and prostate cancers (reviewed in refs. 2, 6). In addition, we found that shedding of membrane endoglin leading to release of Sol-Eng is a hallmark of progression to highly aggressive SpCCs during chemical mouse skin carcinogenesis (22). In this work, we have studied the effect of Sol-Eng on the behavior of CarC, a highly tumorigenic and metastatic spindle carcinoma cell line (35) that has hyperactivated the MAPK signaling pathway due to the presence of two mutated H-Ras alleles (31,37). We report that Sol-Eng clearly inhibits MAPK activity and CarC cell growth in vitro. Although Sol-Eng was able to inhibit MAPK signaling activity also in vivo, its effect on the growth of tumors induced by CarC in nude mice was less pronounced (Figure 2A). This could be due to the undetermined actions of Sol-Eng on stromal cells that might compensate the direct growth inhibitory effect exerted by this molecule on tumor cells. In this regard, it is also worth emphasizing the active crosstalk between stromal and tumor cells by which stromal-dependent epithelial endoglin shedding constitutes a mechanism that may exert an environmental control of cell malignancy (38). We also show that Sol-Eng counteracts the mild stimulation of CarC cell proliferation as well as the further activation of MAPK signaling induced by TGF-β1. The mechanism for this blockade is uncertain since although it has been reported that Sol-Eng acts as a scavenger for TGF-β1 preventing its binding to the cell surface receptors (14), this has been questioned by other laboratories (16,17). Notwithstanding, the most striking observation of the present report is the inhibitory effect exerted by Sol-Eng on the activation of the tyrosine kinase receptor Met by its ligand HGF. Moreover, Sol-Eng strongly inhibited basal and HGF-induced spindle carcinoma cell migration and invasion (Fig. 6). Interestingly, this effect is specific for Sol-Eng as membrane-bound full-length Eng was unable to inhibit the activation of Met and its downstream mediator ERK1,2 as well as the stimulation of cell migration/invasion by HGF (Figures 5E and 6 A and B). Although full-length Eng significantly reduced basal spindle carcinoma cell migration, this effect could be due to the inhibition of TGF-β1/Smad3 pro-migratory signaling activity (22). It is well established that HGF induces the internalization of Met and its subsequent degradation in the lysosomal compartment.
The fact that both transfected and recombinant Sol-Eng were able to downregulate Met in the absence of HGF (Figure 5B–D), suggests the possibility that Sol-Eng can bind Met on the surface of CarC cells and promote its internalization. A schematic model depicting the role of HGF-induced Met internalization on Met signaling and the hypothetical inhibitory effect exerted by Sol-Eng is presented in Supplementary Figure 1, available at Carcinogenesis Online. As a matter of fact, we show that Sol-Eng interacts with Met by coimmunoprecipitation experiments (Figure 5F and G). Because Met can associate with different membrane proteins, including integrins, the hyaluronic receptor CD44 andplexins (23), the question whether Sol-Eng binds Met directly or indirectly remains to be investigated. Intriguingly, full-length endoglin while coimmunoprecipitating with Met (Figure 5G) was unable to downmodulate it and prevent its signaling activity.

Spindle carcinoma cell lines exhibit a high ratio of active phospho-Met respect to total Met protein levels compared with...
Figure 5. Sol-Eng coimmunoprecipitates with Met and attenuates basal and HGF-induced Met signaling activity. (A) Western blot analysis of basal Met phosphorylation relative to the total Met expression levels in mock, CSE1 and CSE3 cells. (B, C) Western blot analysis of phospho-Met, Met, phospho-ERK1/2 and ERK1/2 in mock and CSE1 (B) or CSE3 (C) cells after stimulation with 20 ng/ml HGF for the indicated times. (D) Western blot analysis of phospho-Met, Met, phospho-ERK1/2, ERK1/2 in CarC cells untreated or treated with 20 ng/ml HGF for 5 min, pre-incubated with the indicated concentrations of purified rhSol-Eng for 1 h. The discontinuous line indicates that lanes fifth and sixth are not contiguous but belong to the same gel (see Supplementary Figure 6A, available at Carcinogenesis Online). (E) Western blot analysis of phospho-Met, Met, phospho-ERK1/2, ERK1/2 and endoglin (Eng) in CarC cells stably transfected with a cDNA encoding full-length endoglin (CarC-Eng) or the empty vector (CarC-neo) after stimulation with 20 ng/ml HGF for the indicated times. GAPDH was used as a loading control. (F) HEK293T cells were transiently cotransfected with Met and HA-tagged Sol-Eng. After 24 h of transfection, the indicated amounts of total lysates were immunoprecipitated with anti-Met Ab (upper panel), anti-HA mAb (lower panel) and rat IgG as a control. Immunoprecipitates were subjected to SDS-PAGE electrophoresis and immunoblotted with anti-HA (upper panel) and anti-Met (lower panel) antibodies. The presence of Met and HA-Sol-Eng in the precipitates were determined with the corresponding antibodies. (G) HEK-293T cells were transiently cotransfected with untagged full-length, wild type c-Met and either full-length Eng (Eng) or Sol-Eng, both tagged with HA. After 24 h of transfection, total lysates were immunoprecipitated with anti-Met Ab or rabbit IgG as a control. Immunoprecipitates were subjected to SDS-PAGE electrophoresis and immunoblotted with anti-HA mAb. The presence of Met in the precipitate was determined with anti-Met Ab.
Figure 6. Sol-Eng but not full-length Eng inhibits stimulation of CarC cell migration and invasion by HGF. (A, B) Transwell migration assays through uncoated (A) and Matrigel-coated (B) filters of parental CarC, CarC-neo and CarC-Eng cells. (C, D) Transwell migration assays through uncoated (C) and Matrigel-coated (D) filters of mock and CSE3 cells. HGF (100 ng/ml) was used as a chemoattractant. Migrated cells on the underside of the filter were fixed, stained with 4′, 6-diamino-2-phenylindol and counted. Values are represented as percentages with respect to unstimulated CarC cells. P values were obtained using the Student’s t test. *P < 0.05; **P ≤ 0.01; ***P ≤ 0.001; ns, non-significant. Panels below the graphics show representative examples of migrated/invaded cells stained with 4′, 6-diamino-2-phenylindol.
cell lines corresponding to earlier stages of tumor progression (Figure 4F). This is due to the fact that spindle carcinoma cells tend to express higher levels of HGF. Indeed, elevated expression of HGF appears to be a hallmark of malignant progression during mouse skin carcinogenesis in vivo, since HGF levels increase during progression from papillomas to SCCs and are further enhanced in SpCCs (Figure 4B). High levels of HGF in the serum of breast cancer patients correlate with shorter disease-free survival and increased metastasis in lymph nodes (29,40). In this respect, skin spindle cell carcinomas (represented by the CarC cell line) resemble claudin-low, basal-type breast carcinomas (41), where Met activation is associated with high aggressiveness and poor patient outcome (42,43).

In summary, our results suggest that Sol-Eng antagonizes Met signaling activity at advanced stages of carcinogenesis. These data together with the anti-angiogenic action ascribed to Sol-Eng (12,14,17) point to a malignancy suppressor role for this circulating form of endoglin. Paradoxically, the levels of Sol-Eng increase at later stages of carcinogenesis and correlate with bad prognosis in human epithelial cancer (2,4). It could be argued that Sol-Eng cannot counteract the full malignancy displayed in the tumor microenvironment at late stages of carcinogenesis. In addition, Sol-Eng might act by preventing the development of cancer in premalignant stages. Supporting this view there is epidemiological evidence indicating that preeclamptic women expressing elevated levels of placental Sol-Eng are protected against the development of different types of cancer, including breast carcinomas, in later life. Nonetheless, there are dissenting reports on this matter (reviewed in ref. 44). Further studies are necessary to understand the role of soluble endoglin on tumor initiation and progression.

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Supplementary material
Supplementary Table 1 and Figures 1–7 can be found at http://carcin.oxfordjournals.org/

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