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RECK-Mediated Inhibition of Glioma Migration and Invasion

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ABSTRACT

RECK is an anti-tumoral gene whose activity has been associated with its inhibitory effects regulating MMP-2, MMP-9, and MT1-MMP. RECK level decreases as gliobastoma progresses, varying from less invasive grade II gliomas to very invasive human glioblastoma multiforme (GBM). Since RECK expression and glioma invasiveness show an inverse correlation, the aim of the present study is to investigate whether RECK expression would inhibit glioma invasive behavior. We conducted this study to explore forced RECK expression in the highly invasive T98G human GBM cell line. Expression levels as well as protein levels of RECK, MMP-2, MMP-9, and MT1-MMP were assessed by qPCR and immunoblotting in T98G/RECK+ cells. The invasion and migration capacity of RECK+ cells was inhibited in transwell and wound assays. Dramatic cytoskeleton modifications were observed in the T98G/RECK+ cells, when compared to control cells, such as the abundance of stress fibers (contractile actin–myosin II bundles) and alteration of lamellipodia. T98G/RECK+ cells also displayed phosphorylated focal adhesion kinase (P-FAK) in mature focal adhesions associated with stress fibers; whereas P-FAK in control cells was mostly associated with immature focal complexes. Interestingly, the RECK protein was predominantly localized at the leading edge of migrating cells, associated with membrane ruffles. Unexpectedly, introduced expression of RECK effectively inhibited the invasive process through rearrangement of actin filaments, promoting a decrease in migratory ability. This work has associated RECK tumor-suppressing activity with the inhibition of motility and invasion in this GBM model, which are two glioma characteristics responsible for the inefficiency of current available treatments. J. Cell. Biochem. 110: 52–61, 2010.

KEY WORDS: GLIOBLASTOMA; RECK GENE; CYTOSKELETON; INVASION; MIGRATION; MMP

Glioma is the most common primary brain tumor in adults and among them, glioblastoma multiforme (GBM), the more aggressive type, shows the higher incidence while the prognosis remains dismal. Gliomas display high vascularization, aggressive growth, and invasion into surrounding brain tissue, often remaining refractory to therapy [Lassman and Holland, 2007]. Because gliomas rapidly grow and invade the surrounding brain parenchyma, it is often impossible to achieve complete surgical resection without causing severe neurologic damage [Iwamaru et al., 2007]. While individual glioma cells migrate into the surrounding brain parenchyma, other cancers metastatic to this site do not intermingle with host cells and grow as circumscribed destructive masses. Invasiveness is probably the greatest barrier to glioma treatment, since its diffuse infiltration is also the basis of surgical treatment failure.

The process of tumor cell invasion into the stromal tissue is closely related to interactions between the tumor cells and the extracellular matrix (ECM). Altered expression and modification of ECM proteins in tumor cells play a significant role in their invasion into surrounding normal tissue [Honma et al., 2007]. The matrix

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metalloproteinase (MMP) family has long been associated with cancer invasion and metastasis owing to its ability to degrade the ECM [Lopez-Otìn and Matrisian, 2007]. Among the MMPs, upregulation of gelatinases, MMP-2 and MMP-9, has shown a strong correlation with glioma progression [Levicar et al., 2003]. Hence, the development of novel invasion inhibitors along with anti-proliferative therapies may contribute to the control of local tumor growth as well as tumor spread.

Reversion-inducing cysteine-rich protein with Kazal motifs (*RECK*) was first identified as a cDNA clone inducing morphological reversion (''flat reversion'') in NIH3T3 cells transformed by the *v*-K-*ras* oncogene. RECK has been shown to inhibit MMP activity through several mechanisms, including direct inhibition of protease activity, regulation of cellular release as well as possible sequestration at the cell surface [Welm et al., 2002]. RECK inhibits the activity of at least three MMP members, including MMP-2, MMP-9, and MT1-MMP [Sasahara et al., 2002]. Recently, Takagi and co-authors (2009) described for the first time that RECK is also able to regulate MMP-9 not only post-transcriptionally, as broadly described before for many tumor types, but also at gene expression level. Due to its role in regulation of various ECM proteins, accumulating evidence has now suggested that the altered expression of RECK plays a role in various human disorders, such as rheumatoid arthritis [van Lent et al., 2005] and asthma [Paulissen et al., 2006]. Other functions have recently been attributed to differential expression of RECK in processes such as angiogenesis [Oh et al., 2001; Muraguchi et al., 2007], chondrogenesis [Kondo et al., 2007], and myogenesis [Echizenya et al., 2005].

Our previous data have shown higher *RECK* mRNA expression in non-invasive glioma cells, when compared to invasive T98G cells in samples obtained from cultures where the invasive process was fully established; while MMPs-2 and -9 displayed the opposite pattern: higher levels of expression and activity in T98G cells when compared to A172, the non-invasive cell line [Corrêa et al., 2006]. In glioma progression, from less invasive grade II gliomas to highly invasive GBMs, *RECK* mRNA level decreases [Gabriely et al., 2008]. This inverse correlation between *RECK* expression and glioma invasiveness conducted to this study, in which we explore *RECK* forced expression in T98G invasive human glioma cell line, hypothesizing that *RECK* would impair the invasive behavior. Interestingly, this effect is not associated with the modulation of MMP activity, as has previously been described referring to RECK function but rather we reveal a novel role of RECK in cytoskeleton rearrangement that impairs cell motility and hence partially prevents tumor invasion. Furthermore, we show here for the first time that *RECK* overexpression effectively decreases cell motility and invasion, through actin cytoskeleton rearrangements and stabilization of focal adhesions in tumor cell lines.

These results suggest that *RECK* may be a suitable target in the development of new strategies for glioma treatment.

MATERIALS AND METHODS

CELL CULTURE

Human glioma T98G cells, derived from a glioblastoma, were obtained from the American Type Culture Collection and donated by Dr. Mari Cleide Sogayar (Chemistry Institute, University of São Paulo, Brazil). Cells were grown in Dulbecco's modified Eagle's minimum essential (DMEM) medium supplemented with 10% fetal bovine serum (FBS), 50 U/ml penicillin, and 50 μg/ml streptomycin and maintained at 37°C in 5% CO_2 humidified atmosphere.

OVEREXPRESSION OF RECK IN T98G CELLS

The mammalian expression vector pCXN2-hRECK, used to overexpress the *RECK* gene [Niwa et al., 1991], was a kind donation from Dr. Makoto Noda's Laboratory. T98G cells were transfected with either pCXN2-hRECK or pCXN2 (control vector), using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Cells were selected in media containing 1 mg/ml of G418. G418-resistant colonies were cloned and expanded. Eleven G418-resistant cell colonies were screened for the expression of *RECK* by real-time PCR, immunoblotting, and immunohistochemistry (see Supplemental Data, Fig. 1). We present here, in all figures, the following samples: T98G cell, as a wild-type cell; MOCK cells, as a control, and RECK+, as a representative clone.

REAL-TIME PCR

Total RNA was isolated from cells using TRIzol Reagent (Invitrogen). One microgram of total RNA was used to assess relative levels of mRNA using Syber-Green quantitative real-time PCR (qRT-PCR) (Applied Biosystems) and normalized to tubulin mRNA. Relative expression was calculated using the DDCT method [Livak and Schmittgen, 2001]. All qRT-PCR reactions were performed in triplicates and the data are presented as the mean ± SD. The following primers used for qRT-PCR were designed using Primer3 Software:

- *h-Tubulin*
  F: 5′-TCAACACCTTCTTCAGTGAAACG;
  R: 5′-AGTGCCAGTGCGAACTTCATC.
- *h-RECK*
  F: 5′-TGCAAGCAGGCATCTTCAAA;
  R: 5′-ACCGAGCCCATTTCATTTCTG.
- *h-MMP-2*
  F: 5′-AGCTCCCGGAAAAGATTGATG-3′;
  R: 5′-CAGGGTGCTGGCTGAGTAGAT-3′.
- *h-MMP-9*
  F: 5′-GAGGTGGACCGGATGTTCC-3′;
  R: 5′-AACTCACGCGCCAGTAGAAG-3′.
- *h-MMP-14* (*MT1-MMP*)
  F: 5′-GCAGAAGTTTTACGGCTTGCA-3′;
  R: 5′-TCGAACATTGGCCTTGATCTC-3′.
- *h-TIMP-2*
  F: 5′-CGACATTTATGGCAACCCTATCA-3′;
  R: 5′-GGGCCGTGTAGATAAACTCTATATCC-3′.

SCRATCH ASSAY

Scratch assays were performed as described by Liang et al. [2007]. Briefly, cells were seeded in 24-well plates and, upon reaching complete confluence, a scratch was made through the culture dish using a P200 tip. Cells were washed twice with PBS before their

subsequent incubation with culture medium. After 24 h the scratches were analyzed for cell motility.

IN VITRO INVASION AND MOTILITY ASSAY

In vitro invasion assays were performed as previously described [Lochter et al., 1997]. A 24-well Boyden chamber (8 μm pore size, Becton Dickinson) was coated with 20 μl of Matrigel (BD, diluted 1:6 in serum-free medium), and 200 μl of a 10^5 cell suspension in serum-free DMEM was added to the inner compartment of the chamber. Three hundred microliters of medium supplemented with 10% serum was added to the outer cup. After 24 h, cells that had migrated through the Matrigel were fixed in 3.7% formaldehyde in PBS, stained with 2% toluidine blue, and counted under a light microscope. Each experiment was performed in triplicate. Cell motility assays were performed in a similar manner, except that the 8 μm pore membrane was not coated with Matrigel and the incubation time was reduced to 12 h. GM6001 (Ilomastat) (Chemicon International, Inc., Temecula, CA) was used as MMPs inhibitor at the final concentration of 10 μM, incubated in both sides of the transwell chamber.

GELATIN ZYMOGRAPHY

MMP-2 and MMP-9 enzymatic activities were assayed by gelatin zymography. Approximately 10^5 cells were seeded in 24-well plates and, upon reaching 80% confluency, cells were incubated with serum-free media for 48 h. Cultured supernatants were collected and protein concentrations determined by the Lowry method. Twenty-five micrograms of protein was resolved in 8% SDS acrylamide gels containing 1 mg/ml gelatin. Following electrophoresis, gels were incubated in 0.05 M Tris (pH 8.5) 5 mM $CaCl_2$, and 5 μM $ZnCl_2$ overnight at 37°C. Gels were then stained with Coomassie Brilliant Blue R-250 (0.5% in 10% methanol + 10% acetic acid in water) for 30 min, and destained in the same solution in the absence of dye.

IMMUNOBLOTTING

Adherent cells were collected by trypsinization and lysed by a standard procedure using RIPA buffer containing a protease inhibitor cocktail (Sigma). Protein concentrations of total cell lysates were measured using the Bradford method, and 50 μg of total cell lysate was resolved by electrophoresis on (sodium dodecyl sulfate) SDS–polyacrylamide gels. Proteins were transferred to nitrocellulose membranes (Hybond GE Healthcare) followed by detection and visualization with ECL Western blotting detection reagents (GE Healthcare). Immunoblotting was performed with the following primary antibodies: mouse anti-RECK (1:250; BD Biosciences Pharmingen), mouse anti-MMP-2, (1:1,500; Chemicon International, Inc.), mouse anti-MMP-9 (1:100; Chemicon International, Inc.), mouse anti-MMP-14 (1:400; Chemicon International, Inc.), and mouse anti-tubulin (1:5,000; Sigma). Secondary antibodies used were goat anti-rabbit IgG (Chemicon International, Inc.) and goat anti-mouse IgG (KPL Laboratories, Maryland) both at a dilution of 1:1,000.

IMMUNOCYTOCHEMICAL STAINING

For actin, tubulin, and RECK fluorescent staining, migrating cells of a scratch assay were fixed and permeabilized with 3.7% paraformaldehyde/0.2% Triton-X-100 at room temperature for 15 min, in PEM buffer (10 mM Pipes; 5 mM EGTA; 2 mM $MgCl_2$). For phosphorylated FAK staining, cells were fixed with 3.7% paraformaldehyde for 30 min at room temperature and permeabilized with 0.2% Triton-X-100 for 5 min, followed by several washes with PBS. Blocking of non-specific binding sites was carried out in 10% non-immune goat serum in PBS for 1 h at room temperature. Primary antibodies were diluted in 0.2% Triton-X-100 in PBS and incubated at 4°C overnight. Dilutions employed were 1:50 for RECK (BD Biosciences Pharmingen), 1:50 for phospho-FAK (Chemicon International, Inc.), and 1:100 for β-tubulin (Chemicon International, Inc.). After washing with PBS, Alexa 488-conjugated goat anti-mouse IgG or goat anti-rabbit IgG (1:200) were used for staining (Invitrogen). Rhodamine–phalloidin (Invitrogen) was employed for F-actin staining, according to the manufacturer's instructions. Samples were imaged with a Nikon LSM 2000 Laser Scanning Confocal microscope. No changes in brightness or contrast were applied to the pictures.

ELECTRON TRANSMISSION MICROSCOPY

For light microscopy, cultures maintained on collagen gels for 7 days were immediately fixed in Karnovsky fixative (0.1 M Sörensën phosphate buffer, pH 7.2, containing 5% paraformaldehyde and 2.5% glutaraldehyde). Fixed samples were dehydrated in a grade series of ethanol and embedded in paraffin (Histosec, Merck) or glycol methacrylate resin (Leica Historesin Embedding Kit). Sections (3 μm thick) were stained with hematoxylin–eosin for general studies. These sections were visualized under either a Zeiss Jenaval or an Olympus light microscope. Tissue fragments were fixed by immersion in 3% glutaraldehyde and 0.25% tannic acid solution in Millonig's buffer, pH 7.3, containing 0.54% glucose, for 24 h. After being washed with the same buffer, the tissue was post-fixed with 1% osmium tetroxide for 1 h, washed, dehydrated in graded acetone series, and embedded in Araldite. Ultra thin silver sections were cut using a diamond knife and contrasted with 2% alcoholic uranyl acetate followed by 2% lead citrate in 1 N sodium hydroxide for 10 min. The grids were examined under a Leo-906 transmission electron microscope operating at 80 kV.

STATISTICAL ANALYSIS

All experiments were conducted in, at least, biological triplicates. Data were expressed as the mean ± SD (standard deviation). Statistical analysis was carried out with the GraphPad InStat software (version 3.01 for Windows 95, GraphPad Software, San Diego, CA). One-way ANOVA with a multiple comparison test (Tukey test) was used for data analysis. Statistical significance was defined by a $P < 0.05$.

RESULTS

OVEREXPRESSION OF RECK IN T98G GLIOMA CELLS

Following transfection of T98G glioma cells with the vector pCXN2 with or without *h-RECK*, we confirmed RECK overexpression by Western blot analyses. *RECK* stable expression was also confirmed in whole cell lysates by both qPCR and Western blot analyses, while

RECK protein was not detectable in wild-type T98G and mock glioma cells (Fig. 1A,B). A band of correct size corresponding to the overexpressed RECK protein was observed in the positive clone, while it was absent in parental and mock cell lysates (Fig. 1B). No alteration of proliferation rates was observed in RECK+ cells compared to the controls (see the Supplementary Data, Fig. 2 for BrDu, senescence, and cell-cycle performed assays).

EFFECTS OF FORCED RECK OVEREXPRESSION ON MMPs

The forced increase in RECK expression did not alter MMP-2 and -14 gene expression but decreased MMP-9 mRNA levels ($P < 0.05$), as shown by qRT-PCR were observed (Fig. 1A). Furthermore, neither protein levels nor activity (Fig. 1B and C, respectively) were altered upon RECK overexpression. No effects were detected in the protein levels or in the activity evaluation, which evidences that the role of the studied MMPs in the positive clone is similar to that in the control cells (Fig. 1B,C).

ROLE OF RECK IN INHIBITION OF CELL INVASION AND MOTILITY

Considering invasion as an important hallmark of gliomas, assays were performed in order to investigate whether *RECK* expression alters the invasive properties of glioma cells. In vitro invasion model used Matrigel-coated membranes to assess the number of invading glioma cells. In fact, a considerable decrease in the number of invading RECK+ cells (60%) was observed when compared to T98G wild-type and mock cells (Fig. 2, left panel). In order to verify the involvement of MMPs in the invasive process, this assay was also performed in the presence of Ilomastat (GM 6001), a synthetic MMP inhibitor, and specific to MMPs including MMP-2 and -9 which are often associated with tumor progression [Galardy et al., 1994]. The results indicate that the presence of this MMP inhibitor produced a decrease in the invasion of control cells (parental and mock cells) but had little effect on the RECK+ cells (Fig. 2, left panel). These data show that invasion of RECK+ cells in the presence of MMP inhibitor GM 6001 had no

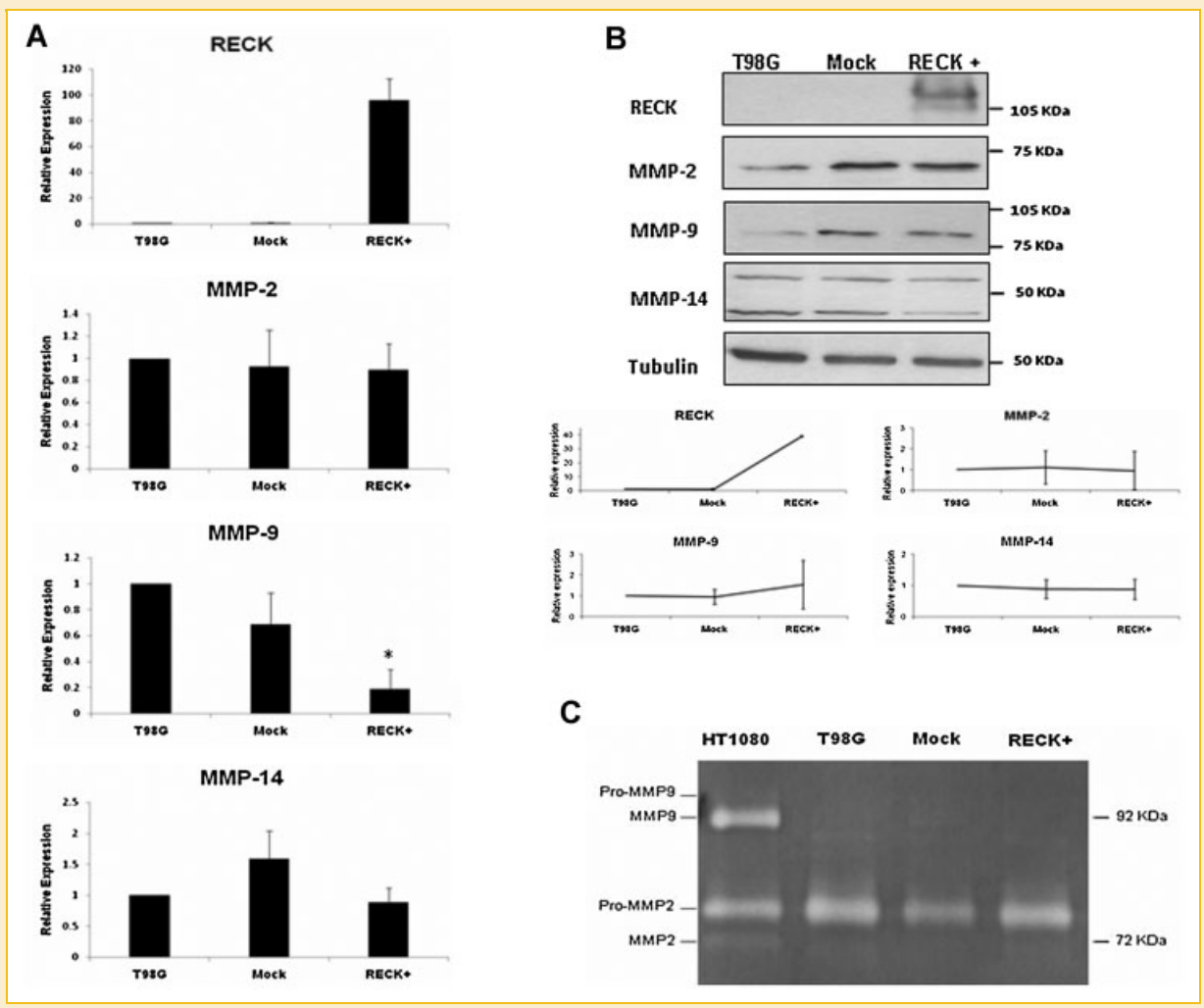

Fig. 1. RECK overexpression and its effect on MMPs. Evaluation of gene expressions (A, real-time qPCR); protein expression (B, immunoblotting) and activity (C, zymography) of RECK, MMP-2, -9, and -14 following RECK overexpression. HT1080 supernatant was used as a control for MMP-2 and -9 activities (C). $^{*}P < 0.05$.

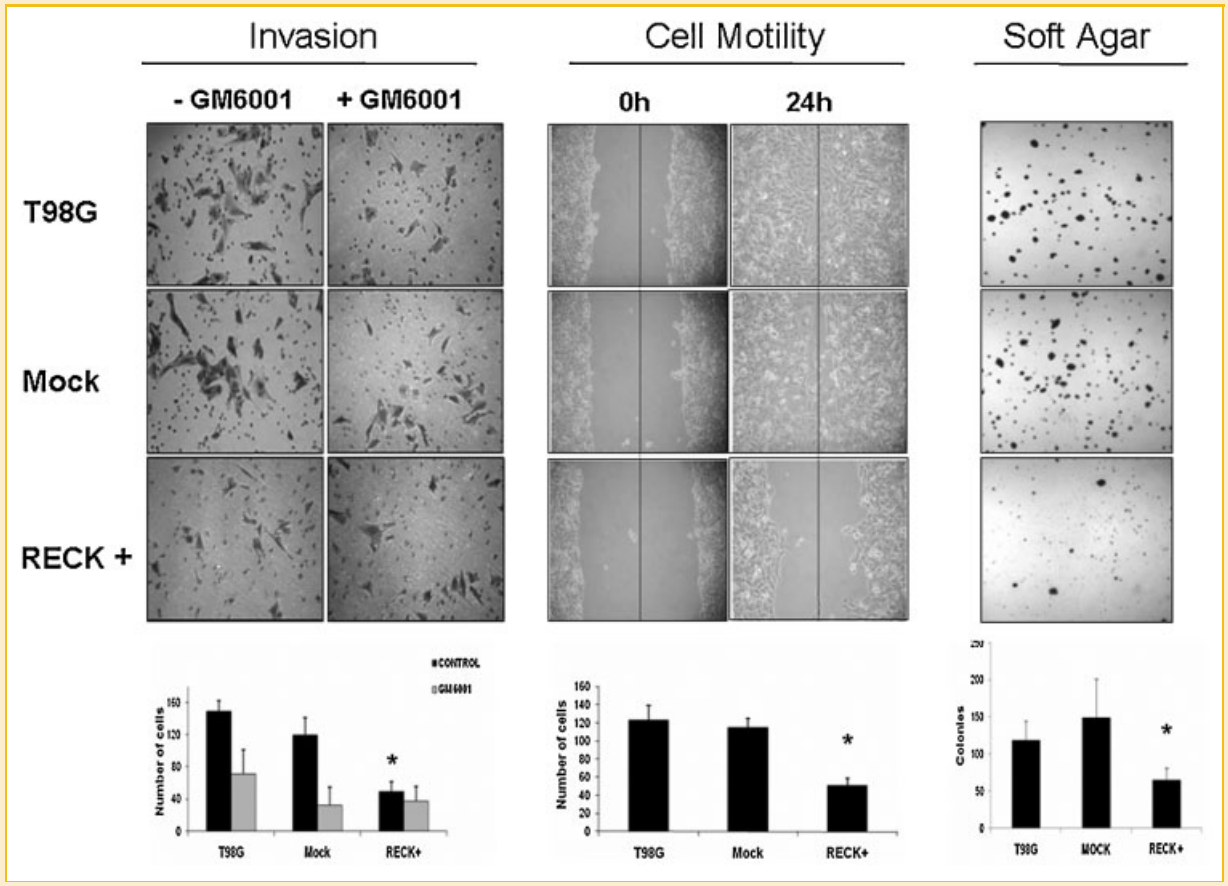

Fig. 2. RECK effects on invasion (left), motility (middle), and colony formation (right) in human glioma cell line by RECK overexpression. Left panel: RECK+ and control cells were added to a transwell chamber covered with Matrigel, and the invasion capacity was evaluated in the presence and absence of a synthetic MMP inhibitor (GM 6001). Invaded cells were fixed, stained and visualized by microscopy. Top: Microphotographs of representative cell fields for each treatment (20× magnification). Bottom: Data are represented as mean ± SD obtained by counting six different fields for each treatment. Quantification of invading cell numbers. Note that GM6001 inhibited invasion of control cells (T98G and mock) only, evidence that invasion inhibition caused by RECK overexpression is independent of MMP activity. $^{*}P < 0.05$. Middle panel: RECK overexpression affects glioma cell motility. Top: Visualization of cell migration following a scratch through confluent monolayer of T98G, Mock, or RECK+ glioma cells. Images were taken at 0 and 24 h postscratch. Bottom: Numerical representation of transwell assay, performed without ECM coating, to assess the motility of these cells. Data represented as mean ± SD, obtained by counting six different fields for each treatment. The experiment was performed in duplicate and repeated four times. $^{*}P < 0.05$. Right panel: Soft agar assay. Top: Colonies formed in soft agar assay, after 30 days in culture, for the control cell lines and RECK+ cells. Bottom: Quantification of colonies, showing a significant decrease both in size and in number of colonies formed in RECK+ cells. $^{*}P < 0.05$.

further effects on invading RECK+ cells, which was already minimal.

These results led us to assess other events that might influence tumor invasion, such as cellular motility. This was assessed by both a scratch assay as well as a transwell motility assay. The scratch-wound assay measures the in vitro migration of individual cells at the leading edge of an inflicted scratch through a culture monolayer. As shown in Figure 2 middle panel, control cells were able to cover the open space left by the scratch in 24 h, whereas migration of RECK+ cells was significantly impaired, leaving an exposed area through the cellular monolayer. Effects of RECK overexpression on glioma cell migration were then quantified using a transwell assay; again RECK forced expression resulted in a remarkable inhibition of glioma cell migration (Fig. 2, middle panel). These data support the concept that RECK expression in glioma cells affects not only the invasive capacity of these tumor cells but tumor cell motility as well.

SOFT AGAR COLONY FORMATION

Since the tumor stem cells are considered the most relevant cell type in the development of metastases and recurrences due to their ability to divide and to form colonies in a semisolid matrix [agar or methylcellulose; Fiebig et al., 2004], we can assume that the capacity to form colonies is an indicative of tumoral grade. From transplantable tumors in rat and mice, harvested cells can be cultured directly and quantitatively in vitro to produce clones and provide information on the effectiveness of tumor development in vivo [Franken et al., 2006]. After 30 days in soft agar culture, RECK+ cells formed remarkably less colonies than control cells which display smaller diameter (Fig. 2, right panel).

RECK EFFECTS ON THE CYTOSKELETON AND PHOSPHO-FAK DISTRIBUTION

Histochemical staining of polymerized actin with rhodamine–phalloidin and the immunocytochemical staining of tubulin to

visualize microtubules showed profound differences between control (T98G and Mock, Fig. 3A–C and D–F, respectively) and RECK+ migrating cells (Fig. 3G–I). Insets represent the negative control (Fig. 3J–L). Control cells were evidently polarized in the direction of the movement, and large motility-associated processes such as lamellipodia were observed, containing both actin and microtubules. The cells also presented stress fibers, which are contractile bundles of actin–myosin II, important for the movement of the cell body forward. RECK+ cells, on the other hand, showed few lamellipodia and a presence of stress fibers traversing the cell body. This image suggests that those cells were closely packed together, and microtubules were present mostly in the cell body, probably close to the centrosome.

The distribution of phosphorylated FAK was also affected by RECK forced expression. While control cells showed the presence of phospho-FAK mostly in immature focal complexes (Fig. 4, arrowhead) located in lamellipodia and membrane ruffles, RECK+ cells showed an accumulation of phospho-FAK in mature focal contacts, where stress fibers were inserted (Fig. 4, arrows). Insets represent the negative control (Fig. 4J–L).

RECK DISTRIBUTION IN MIGRATING CELLS

RECK immunocytochemical localization showed the presence of the protein almost exclusively in lamellipodia, at the leading edge, mostly in areas of membrane ruffling in RECK+ cells (Fig. 5H,I). We imaged a few RECK+ cells presenting lamellipodia, just to show that those cells had higher RECK protein levels than control cells. The distribution pattern in the control cells (Fig. 5A–F), however, was not altered by RECK overexpression. Insets represent the negative control (Fig. 5J–L).

CELL ELECTRON MICROSCOPY

Our previous data show that cells onto collagen gel showed a well-established invasive process after 7 days of culture [Corrêa et al., 2006]. In order to evaluate *RECK* impact on cell structure during the invasive process, we performed electron microscopy analyses. Figure 6 depicts T98G and mock cells showing membrane blebbing around collagen, engulfing part of the fibrils, which indicates a more invasive capacity (Fig. 6A,B). The cell membrane surface that has contact with the collagen is notably different in RECK+ cells (compare the arrows in Fig. 6). Conversely, RECK+ cells exhibit a smooth contact surface between the cell membrane and the collagen gel, with no evidence of degradation or invasion through the collagen (Fig. 6C). Control cells show higher migrating and invading capacities when compared to RECK+ cells.

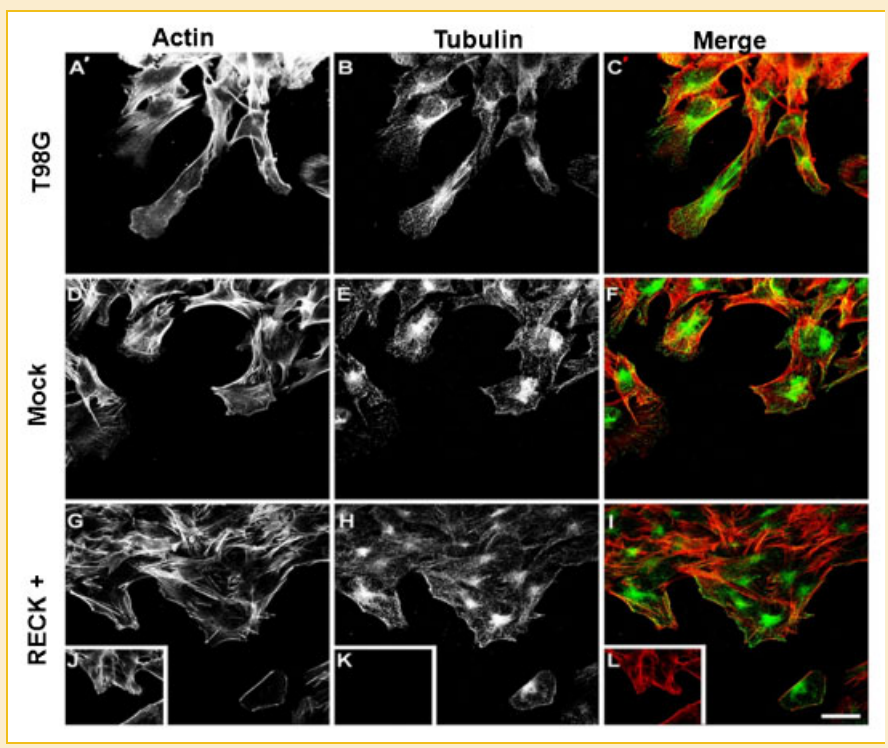

Fig. 3. Histochemical and immunohistochemical staining for cytoskeleton proteins of T98G, Mock, and RECK+ glioma cells (A–C; D–F; G–I, respectively): actin (left column) and tubulin (middle column) staining provides evidence that RECK overexpression causes cytoskeletal modifications. Control migrating cells present evident lamellipodia, which are dependent upon actin polymerization; their morphology is polarized and adhesions to the substrate tend to be immature (A,D). Microtubules are present throughout the cells, including in lamellipodia (B,E). RECK+ cells (G) display a greater amount of actin bundles, in contractile structures that include myosin II named stress fibers. Microtubules (H) are less dispersed throughout the cytoplasm and concentrated in a region close to the cell nucleus, probably the centrosome. These cells form strong adhesions to the substrate and are less motile. Insets represent the negative control (J–L). Bar = 30 and 60 μm for the inset.

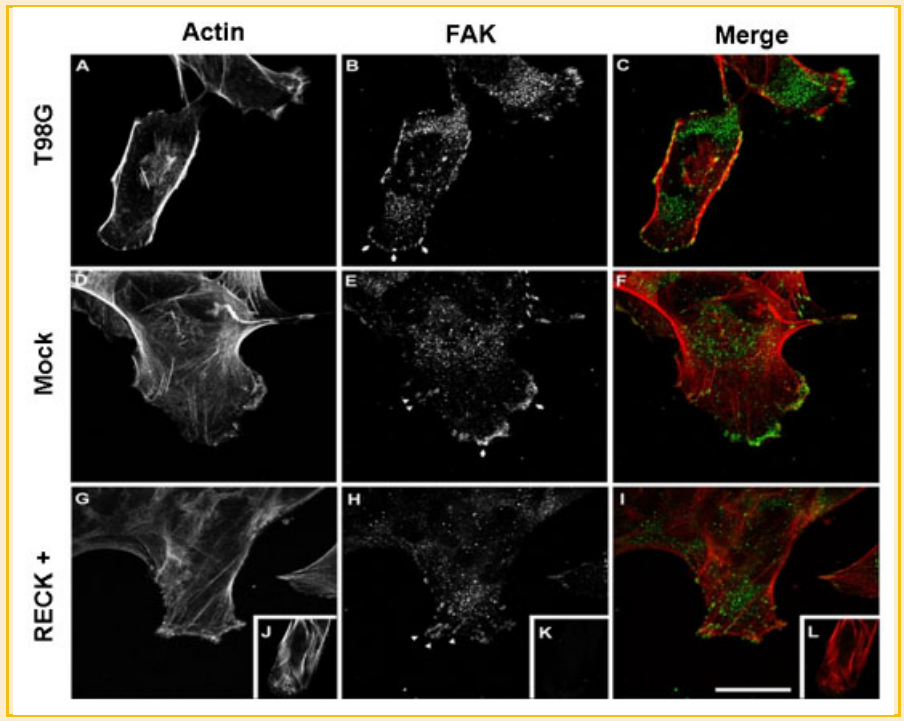

Fig. 4. Histochemical staining for F-actin (left column) and immunohistochemical staining for phospho-FAK (middle column) of T98G, Mock, or RECK+ glioma cells (A–C, D–F, G–I, respectively). Besides a cytoplasmic pool, phospho-FAK is mostly observed in immature focal complexes in control cells (arrows) and mature focal adhesions (arrowheads) in RECK+ cells (H). Observe the association of actin bundles with focal adhesions. Insets represent the negative control (J–L). Bar = 30 and 60 μm for the inset.

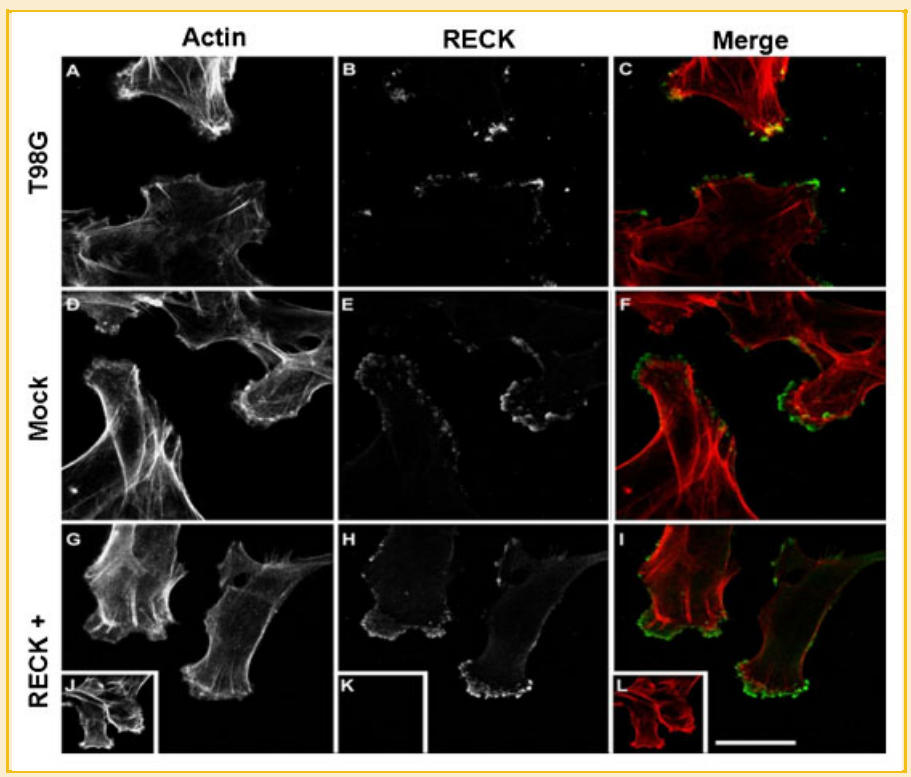

Fig. 5. Histochemical staining for F-actin (left column) and immunohistochemical staining for RECK (middle column) of T98G, Mock, or RECK+ glioma cells (A–C, D–F, G–I, respectively). Reck was present exclusively at the leading edge, mostly associated with membrane ruffles in lamellipodia. A higher amount of RECK in the overexpressing RECK+ cells was observed, as expected (in order to show this result, atypical RECK+ cells presenting large lamellipodia were picked). RECK distribution was similar between control and RECK+ cells. Insets represent the negative control (J–L). Bar = 30 and 60 μm for the inset.

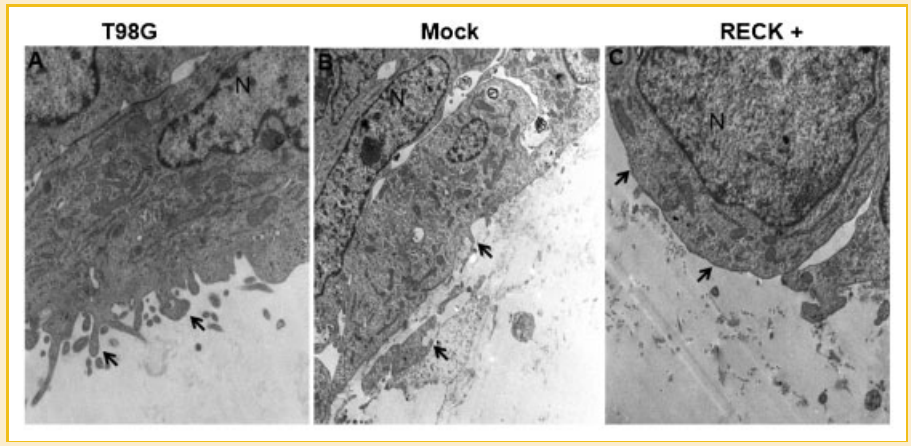

Fig. 6. Transmission electron microscopy showing the surface of T98G (A), Mock (B), and RECK+ (C) cells when cultured over collagen type I gel for 7 days. RECK+ cells clearly show less membrane activity, with no motility-associated processes. Arrows are indicating cell membrane protrusions and N indicates the nucleus (6000× magnification).

DISCUSSION

RECK AND MMPS

Brain tumor invasiveness involving tumor cell–ECM interactions, as well as tumor cell–normal astrocyte interactions [Louis, 2006; Aubert et al., 2008] will probably impair novel glioma therapeutic approaches. The ability of this malignant glioma to spread very fast has been associated with MMP activity [Giese et al., 2003; Aubert et al., 2008]. A number of proteases have been implicated, but most studies have focused on MMP-2 and -9. Expression of these proteases has been shown to increase with glioma grade, and interference with the function of these proteases in vitro results in decreased invasive and/or migratory properties [Louis, 2006].

RECK was initially identified as a transformation suppressor gene, and its function was correlated with inhibition of MMP-2, MMP-9, and MT1-MMP [Takahashi et al., 1998; Noda and Takahashi, 2007; Omura et al., 2008].

RECK is normally expressed in adult human tissues and it is downregulated in many solid tumors, such as lung cancer, human breast carcinoma, pancreatic cancer, non-small cell lung cancer, and colorectal cancer [Liu et al., 2002; Masui et al., 2003; Span et al., 2003; Takeuchi et al., 2004; Takenaka et al., 2005; Takagi et al., 2007]. Restored expression of this gene in tumor cells suppresses tumor angiogenesis, invasion, and metastasis in animal models [Takahashi et al., 1998; Oh et al., 2001], and its residual expression level in tumor tissues often correlates with better prognosis [Noda and Takahashi, 2007; Omura et al., 2008]. However, knowledge of underlying molecular mechanisms mediated by RECK in tumor suppression is limited, and little is known about their functions at the single cell level. Therefore, better understanding of RECK gene function would provide valuable insight into the reason why tumors with lower expression tend to be more aggressive [Morioka et al., 2009].

The aim of this study was to investigate the effects of RECK overexpression during the migration and invasion processes in a glioma model. We have observed cells with highly impaired migration and invasion ability even though activities of various MMPs were not altered. However, a decreased MMP-9 in mRNA levels was observed. Migration and invasion assays were performed in the presence of GM 6001, an MMP synthetic inhibitor, in order to investigate the role of MMPs. Invasion of glioma control cells (low RECK expression) was significantly inhibited by the presence of GM 6001, indicating that MMP activity is essential for the invasive process through Matrigel®, as expected. Interestingly, invasion remained unaltered in the GM 6001-treated RECK+ cells, suggesting that RECK overexpression has effects on the invasion process per se. Besides that, RECK+ cells were able to form less colonies than control cells resembling their inability to form metastasis.

Recently, Gabriely et al. [2008] observed that RECK expression, as a target of miR-21, was accompanied by a decrease in migratory ability and invasion due to a decrease in MMP-2 activity in vitro in glioma model. It has been shown that microRNA-21 is upregulated in gliomas, and that its inhibition leads to elevated levels of RECK, suggesting a possible explanation as to why there is low RECK expression in many gliomas. On the other hand, our results suggest that migratory ability invasion can be inhibited by RECK expression through another effect different from MMPs regulation, such as cytoskeleton modifications showed here. We, however, cannot discard the possibility that RECK can regulate ECM metalloproteinases distinct from those examined in this study, as described for ADAM10 [Muraguchi et al., 2007] and CD13 aminopeptidase N [Miki et al., 2007].

RECK AND CYTOSKELETON REARRANGEMENT

Cell migration requires polarization and actin polymerization for the formation of motility-associated processes, such as lamellipodia. While cell polarization involves the centrosome reorientation in the direction of the movement, lamellipodia are actin-based protrusions that establish new contacts with the ECM substrate. These immature contacts are very dynamic and may disappear or mature into focal contacts, which are sites of insertion of stress fibers. Excessive membrane activity at the border of lamellipodia generates membrane ruffles. In tumor cells and transformed cells, metalloproteases associated with the lamellipodium mediate proteolysis of

ECM constituents, including fibronectin, laminins, and collagens [Small et al., 2008]. To further investigate the modifications on motile capacity of RECK overexpressing cells, the actin and tubulin cytoskeleton was analyzed. Interestingly, we observed that RECK+ clones revealed greater amounts of actin stress fibers, which are known to form strong adhesions to substrates, and lesser structures of lamellipodia and membrane ruffles, when compared to control cells. This phenotype is usually associated with low motility and great stability.

FAK overexpression in gliomas has been reported extensively [Golubovskaya et al., 2009]. In different tumors, including glioma, increased FAK expression is observed when compared to non-neoplastic tissues. FAK can promote tumor growth, and a role for FAK in glioma pathogenesis is suggested by its expression and localization [Shi et al., 2007]. Compared to phospho-FAK distribution in focal complexes (as small dots, more present in Fig. 4B,E than in Fig. 4H), phospho-FAK in focal contacts formed elongated structures clearly associated with the insertion of stress fibers, suggesting an accumulation at these junctions.

Our results illustrate that in a human glioma model, forced RECK expression was responsible for cytoskeletal rearrangement and differences in phospho-FAK distribution, which were certainly underlying the defective cell motility. This is an important finding in glioma, since its accelerated invasion is often a main feature contributing to treatment failure.

These findings are in accordance with those published by Morioka et al. [2009], regarding RECK-mediated effects on the cytoskeleton of fibroblasts and fibrosarcoma cells. They reported that cells lacking RECK lose their anterior–posterior polarity, decrease spreading with increased speed, and decrease directional persistence in migration. Our data suggest that in a glioma model, forced expression of RECK correlates with a significant decrease in invasion and migration, associated with a highly contractile and stable phenotype. Morioka et al. [2009] also showed that focal adhesions and stress fibers were affected in normal cells that lacked RECK expression, since these cells were rimmed by dense accumulation of amorphous focal adhesions and thin, short actin fibers.

T98G glioma cells are able to invade as well as migrate, and introduced RECK expression in these cells inhibits this ability. Regarding the inversion correlation of glioma grade and RECK expression described by Gabriely et al. [2008], the present results could contribute to reveal a role of RECK in the invasiveness of glioma. Cytoskeleton arrangement in human tissue samples and its correlation with RECK gene must be further explored to elect RECK as a real molecular therapy target.

CONCLUSION

In contrast to previous data indicating that tumor suppressor functions of RECK can be mediated via inhibition of MMP activity, we have now shown that overexpression of RECK has little to no effect on MMP-2 and -9 activity in a glioma model. Rather, our data have shown that RECK effectively inhibits the invasive process, most likely through cytoskeleton rearrangement of actin filaments, preventing the migratory ability of these cells. These data support the concept that low level RECK found in gliomas provides migration and invasion advantages to these highly motile and aggressive tumor cells, characteristics responsible for the poor efficacy of current available treatments.

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