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Probing the infiltrating character of brain tumors: inhibition of RhoA/ROK-mediated CD44 cell surface shedding from glioma cells by the green tea catechin EGCg

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Abstract

Glioma cell-surface binding to hyaluronan (HA), a major constituent of the brain extracellular matrix (ECM) environment, is regulated through a complex membrane type-1 matrix metalloproteinase (MT1-MMP)/CD44/caveolin interaction that takes place at the leading edges of invading cells. In the present study, intracellular transduction pathways required for the HA-mediated recognition by infiltrating glioma cells in brain was investigated. We show that the overexpression of the GTPase RhoA up-regulated MT1-MMP expression and triggered CD44 shedding from the U-87 glioma cell surface. This potential implication in cerebral metastatic processes was also observed in cells overexpressing the full-length recombinant MT1-MMP, while the overexpression of a cytoplasmic domain truncated from of MT1-MMP failed to do so. This suggests that the cytoplasmic domain of MT1-MMP transduces intracellular signaling leading to RhoA-mediated CD44 shedding. Treatment of glioma cells with the Rho-kinase (ROK) inhibitor Y27632, or with EGCg, a green tea catechin with anti-MMP and anti-angiogenesis activities, antagonized both RhoA- and MT1-MMP-induced CD44 shedding. Conversely, overexpression of recombinant ROK stimulated CD44 release. Taken together, our results suggest that RhoA/ROK intracellular signaling regulates MT1-MMP-mediated CD44 recognition of HA. These molecular processes may partly explain the diffuse brain-infiltrating character of glioma cells within the surrounding parenchyma and thus be a target for new approaches to anti-tumor therapy.

Keywords: CD44, green tea, human glioma metastasis, membrane type-1 matrix metalloproteinase, hyaluronan, RhoA.

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Although intrinsic brain tumors fail to metastasize, they do exhibit diffuse infiltration of the surrounding brain parenchyma (Bolteus *et al*. 2001). Local diffuse invasion is poorly understood, but is thought to be a multifaceted biological phenomenon of interactive mechanisms involving cell motility, adhesion and enzymatic remodeling of the extracellular matrix (ECM) components. These features of the infiltrating tumor cells presently preclude successful therapy, regardless of the histological type or grade of malignancy (Pilkington 1997). A better understanding of the molecular mechanisms controling tumor astrocyte detachment from initial brain tumor sites is thus needed to combat these tumors.

The principal ECM molecules that have been identified in the normal brain parenchyma are hyaluronic acid (HA) and chondroitin sulfate (Bignami *et al*. 1992). Of these two, small HA polymers are known to efficiently promote tumor cell migration (Sugahara *et al*. 2003). HA is in fact the principal, but by no means the only, ligand of CD44, a membrane glycoprotein belonging to the superfamily of

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Abbreviations used: ECM, extracellular matrix; EGCg, epigallocatechin-(3)-gallate; HA, hyaluronic acid, hyaluronate, hyaluronan; MEM, minimal essential medium; MMP, matrix metalloproteinase; MT1-MMP, membrane type-1 MMP; ROK, Rho-associated kinase; SDS–PAGE, sodium dodecyl sufate – polyacrylamide gel electrophoresis; Wt, wild-type.

immunoglobulin receptors. CD44 is also implicated in the promotion of tumor growth, invasiveness, and metastatic potential in experimental and human cancers (Gunthert *et al*. 1998). Recent findings suggest that CD44 provides a docking site for matrix metalloproteinase (MMP)-9 on the surface of melanoma and carcinoma cells and can thus indirectly contribute to pericellular proteolysis of types IV and V collagen (Yu and Stamenkovic 1999). Although several other cell surface receptors for HA have been reported, recent works have demonstrated that gliomas express significant levels of CD44 and that such expression could be relevant in determining their highly invasive behaviour (Akiyama *et al*. 2001; Ranuncolo *et al*. 2002).

Several studies have revealed different molecular and cellular mechanisms regulating CD44-mediated processes (Gal *et al*. 2003; Xu and Yu 2003). Among these, the role of intracellular Rho-mediated signaling, leading to cytokine production and breast tumor progression, was reported (Bourguignon *et al*. 2003). Interestingly, quantitative RT–PCR analysis in U-87 glioma cells showed that levels of the small RhoA GTPase, a potent modulator of actin polymerization/depolymerization dynamics triggering assembly of filopodia, lamellipodia and stress fibers, increased by 75% after the addition of galectin-1, an ECM glycoprotein that is synthesised by tumor astrocytes and which favours their migration (Camby *et al*. 2002). Important molecular processes regulating cell migration, tumor invasion and metastasis have also recently been highlighted by the common cell surface localization of CD44 with a membrane type (MT)-1 MMP at the leading lamellipodia edge of motile cells (Kajita *et al*. 2001; Mori *et al*. 2002). These studies demonstrated that CD44 directed MT1-MMP to lamellipodia by associating with its hemopexin-like domain, and that cell-surface MT1-MMP-mediated cleavage of CD44 subsequently played a critical role in promoting tumor cell migration. Lamellipodia formation is also known to be, at least partly, orchestrated by the small GTPase of the Rho family (Ridley *et al*. 2003). Noteworthy, one other common feature between RhoA, MT1-MMP and CD44 is their partial localization within Triton X-100-insoluble and cholesterol-enriched membrane domains termed caveolae (Perschl *et al*. 1995; Gingras *et al*. 1998; Annabi *et al*. 2001). Interestingly, the caveolar location of MT1-MMP was recently suggested to provide a regulatory mechanism in glioma (Annabi *et al*. 2004) and breast carcinoma cell invasion (Rozanov *et al*. 2004).

In light of the common caveolar localization of MT1-MMP, CD44, and RhoA at the leading edges of migrating glioma cells, we hypothesise that a crosstalk between these players may regulate the infiltrating phenotype of brain tumors. In the present study, we have investigated the mechanisms involved in the regulation of CD44 functions in cells derived from a highly infiltrating and vascularized brain tumor glioblastoma. Specifically, we addressed the intracellular signaling pathways that lead to CD44-mediated detachment of U-87 glioma cells from HA. Collectively, our results provide the first evidence for a cell-surface functional cross-talk between MT1-MMP/RhoA/ROK that impacts on the ability of gliomas to bind HA through CD44, and that may be efficiently targeted by the anti-cancer properties of the green tea polyphenol epigallocatechin-(3)-gallate EGCg (Annabi *et al*. 2002; Demeule *et al*. 2002).

Materials and methods

Materials

Agarose (–)-epigallocatechin 3-gallate (EGCg), sodium dodecyl sulfate (SDS), gelatin, and bovine serum albumin (BSA) were purchased from Sigma (Oakville, ON, Canada). TriZOL reagent was from Life Technologies (Gaithersburg, MD, USA). Fugene-6 transfection reagent was from Roche Diagnostics Canada (Laval, QC, Canada). The anti-CD44 R-phycoerythrin-conjugated mouse anti-human monoclonal antibody (G44-26) and mouse IgG2bκ (clone 27–35) were from BD Pharmingen (Franklin Lakes, NJ, USA). The anti-MT1-MMP polyclonal antibody AB-815, and the anti-Erk antibody were from Chemicon (Temecula, CA, USA). The anti-Myc and anti-RhoA antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cell culture and cDNA transfection method

The U-87 glioma cell line was purchased from American Type Culture Collection and maintained in Eagle's minimum essential medium (MEM) containing 10% (v/v) bovine calf serum (BCS) (HyClone Laboratories, Logan, UT, USA), 2 mM glutamine, 100 units/mL penicillin, 100 μg/mL streptomycin, and were cultured at 37°C under a humidified atmosphere containing 5% CO_2. The MT1-MMP cDNA constructs have been previously generated and validated by us (Annabi *et al*. 2001) as follows: wild-type (Wt) encodes the full-length MT1-MMP protein (Met1–Val582); Δ1 encodes a protein which lacks the entire C-terminal 20 amino acid cytoplasmic domain (Met1–Phe562). The cDNA encoding the Wt Myc-tagged RhoA and constitutively active Myc-tagged RhoA-associated kinase (ROK) were generously provided by Dr Allan Hall (University College London, London, UK) and Dr Salhia Bodour (The Hospital for Sick Children, Toronto, ON, Canada), respectively. U-87 cells were transiently transfected with cDNA constructs using the non-liposomal formulation Fugene-6 transfection reagent. Transfection efficiency was confirmed with a cDNA plasmid encoding green fluorescent protein (GFP) that was cloned in the same plasmid backbone (pcDNA3.1+). Fluorescent microscopy visualisation confirmed cell transfection by the presence of green fluorescent cells that were routinely found to represent 8–15% of total cells transfected (not shown). All experiments involving these cells were performed 36 h following transfection. Mock transfections of U-87 cultures with pcDNA (3.1+) expression vector alone were used as controls.

Total RNA isolation and reverse transcriptase–polymerase chain reaction (RT–PCR) analysis

Total RNA was extracted from monolayers of cultured U-87 cells using the Trizol reagent. One microgram of total RNA was used for first-strand cDNA synthesis followed by specific gene product

amplification with the One-Step RT–PCR Kit (Invitrogen, Burlington, ON, Canada). Primers for CD44s (forward: 5′-TTTGCCTCTTACAGTTGAGCCTG-3′, reverse: 5′-GGTGCCATCACGGTTGACAATAG-3′) (Annabi *et al*. 2004), MT1-MMP (forward: 5′-ATTGATGCTGCTCTCTTCTGG-3′, reverse: 5′-GTGAAGACTTCATCGCTGCC-3′) (Annabi *et al*. 2001), and for RhoA (forward: 5′-CTGGTGATTGTTGGTGATGG-3′, reverse: 5′-GCGATCATAATCTTCCTGCC-3′) (Turcotte *et al*. 2003) were derived from human sequences and PCR conditions were optimized so that the gene products were at the exponential phase of the amplification. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA amplification was used as an internal housekeeping gene control. PCR products were resolved on 1.5% agarose gels containing 1 μg/mL ethidium bromide.

Immunoblotting procedures

Proteins from control and treated cells were separated by SDS–polyacrylamide gel electrophoresis (PAGE). After electrophoresis, proteins were electrotransferred to polyvinylidene difluoride membranes which were then blocked overnight at 4°C with 5% non-fat dry milk in Tris-buffered saline (150 mM NaCl, 20 mM Tris–HCl, pH 7.5) containing 0.3% Tween-20 (TBST). Membranes were further washed in TBST and incubated with the primary antibodies (1/1000 dilution) in TBST containing 3% bovine serum albumin, followed by a 1-h incubation with horseradish peroxidase-conjugated anti-rabbit IgG (1/10 000 dilution for MT1-MMP detection) or anti-mouse IgG (1/5000 dilution for RhoA and CD44 detection) in TBST containing 5% non-fat dry milk. Immunoreactive material was visualized by enhanced chemiluminescence (Amersham Biosciences, Baie d'Urfée, QC, USA).

Flow cytometry analysis

For assessment of cell surface CD44 expression, cells were detached from plates, as previously described by us (Annabi *et al*. 2004), and re-suspended in 10% FBS/Dulbecco's modified Eagle's medium (DMEM) at a concentration of 10^6 cells/mL, washed two times and blocked for 15 min at 25°C in PBS containing 5% inactivated fetal calf serum (FCS/PBS). The cells were then incubated in 0.5% FCS/PBS with 0.5 μg/mL of the CD44 mAb or mouse IgG2bκ at room temperature for 30 min, washed once and re-suspended in 0.5% FCS/PBS. Flow cytometry data was analyzed on a FACS Calibur flow cytometer with CellQuestPro software (BD Biosciences, Mississauga, ON, Canada).

Cell migration assay

Cells were dislodged after brief trypsinization, washed extensively and re-suspended in MEM at a concentration of 10^6 cells/mL (Annabi *et al*. 2004). Cells (5×10^4) were then dispersed onto 1 mg/mL HA/PBS-coated chemotaxis filters (Costar; 8-μm pore size) within Boyden chamber inserts. Migration proceeded for 3 h at 37°C in 5% CO_2. Cells that had migrated to the lower surface of the filters were fixed with 10% formalin phosphate, coloured with 0.1% crystal violet/20% methanol and counted by microscopic examination. The average number of migrating cells per field was assessed by counting at least four random fields per filter using Northern Eclipse software (Empix Imaging Inc., Mississauga, ON, Canada). Data points indicate the mean obtained from three separate chambers within one representative experiment.

Statistical data analysis

Data are representative of three or more independent experiments. Statistical significance was assessed using Student's unpaired *t*-test and was used to compare the relative RhoA- or MT1-MMP-induced effects on HA cell adherence, CD44 cell surface expression, or migration on HA with untreated (Mock or control) U-87 cells. Probability values of less than 0.05 were considered significant, and an asterisk (*) identifies such significance in each figure.

Results

Overexpression of recombinant RhoA or MT1-MMP decreases U-87 glioma cells adhesion to HA

RhoA is thought to regulate several processes involved in cancer invasion and it was recently found to regulate CD44 binding to HA (Ito *et al*. 2004). We have shown that the function of the HA receptor CD44 was also regulated through a complex interplay involving MT1-MMP (Annabi *et al*. 2004), which is of particular importance in brain tumor development. We decided to analyze the roles of RhoA and MT1-MMP in cell–HA interaction. Cells were cultured on plastic and transiently transfected with cDNA plasmids encoding the full-length (Wt) MT1-MMP and RhoA recombinant proteins, as well as plasmid encoding a truncated cytoplasmic form (Δ1) of MT1-MMP that anchors to the plasma membrane but fails to transduce any intracellular signaling (Gingras *et al*. 2001). Transfected cells were then trypsinized, seeded on HA-coated dishes, and adhesion left to proceed for 2 h. We found that overexpression of RhoA or Wt-MT1-MMP triggered a loss of approximately 60–65% in cell-HA adhesion, while Mock or Δ1-MT1-MMP-transfected cells had their respective adhesion to HA unaffected (Figs 1a and b). Generation and selection of stably transfected U-87 cells with the respective cDNA plasmids is currently underway in order to confirm and further characterize the new RhoA- and MT1-MMP-mediated phenotype observed. Interestingly, a bidirectional crosstalk was seen between RhoA and MT1-MMP expression. Indeed, overexpression of recombinant RhoA triggered an increase in endogenous MT1-MMP expression, while Wt-MT1-MMP overexpression induced RhoA expression in U-87 cells (Fig. 1c). Moreover, the cytoplasmic domain of MT1-MMP appears to be essential for that induction as Δ1-MT1-MMP did not trigger RhoA expression in U-87 cells. This suggests that the cytoplasmic domain of MT1-MMP regulates crucial intracellular signaling leading to the induction of RhoA.

RhoA and MT1-MMP overexpression trigger CD44 cell surface shedding from U-87 glioma cells

We have previously reported that MT1-MMP overexpression antagonized functional recognition and binding of HA by reducing CD44 cell surface expression (Annabi *et al*. 2004). Whether CD44 was shed from the cell surface of glioma cells

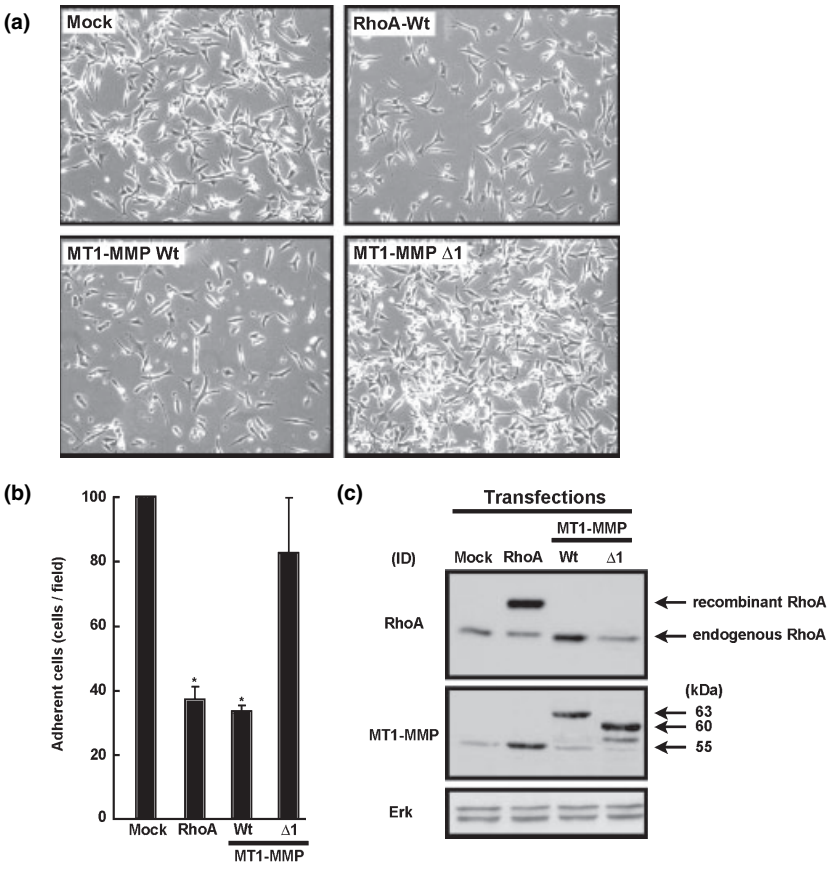

Fig. 1 Recombinant RhoA and MT1-MMP overexpression induces loss of cell–HA adhesion in U-87 glioma cells. U-87 glioma cells were cultured on plastic dishes until they reached approximately 60% confluency. Cells were then transfected with cDNA plasmids as follows: Mock (pcDNA3.1$^+$), Wt-RhoA, Wt-MT1-MMP or Δ1-MT1-MMP, as described in Materials and methods. Thirty-six hours post-transfection, cells were trypsinised and seeded on HA-coated dishes (1 mg/mL). (a) Pictures of the adherent cells were taken 2 h after seeding and quantified (b). Western blotting of the cell lysates generated from the respective cell transfections (20 μg/well) was performed on 12% SDS–PAGE for RhoA and ERK, and 9% SDS–PAGE for MT1-MMP. Immunodetection was performed as described in Materials and methods (c). The 63-, 60-, and 55-kDa immunoreactive bands observed for MT1-MMP represent, respectively, the full-length-proMT1-MMP form, the cytoplasmic (Δ1)-truncated form, and the mature 55 kDa processed form of the endogenous MT1-MMP. Data are representative of three independent experiments.

remained to be evaluated. We transfected U-87 cells grown on plastic dishes with Wt-MT1-MMP, Δ1-MT1-MMP and RhoA cDNAs and assessed the presence of CD44 in the conditioned media by western blotting. We observed a 75-kDa CD44-immunoreactive protein that appeared in the conditioned media isolated from RhoA and Wt-MT1-MMP-expressing cells (Fig. 2a). Interestingly, no CD44 shedding was triggered by the overexpression of a cytoplasmic-deleted recombinant form of MT1-MMP. Furthermore, immunophenotyping of CD44 cell surface expression of the transfected cells was performed using flow cytometry. Accordingly, a significant shift to lower fluorescence levels was observed in RhoA- and MT1-MMP-transfected cells (Fig. 2b), suggesting that less CD44 remained at the cell surface (Fig. 2c) and supporting the assumption that CD44 is released in the culture media.

Cell migration on hyaluronic acid is inhibited in RhoA- and MT1-MMP-transfected U-87 glioma cells

Overexpression of recombinant RhoA and MT1-MMP was achieved by transfecting U-87 cells grown on plastic. Cells were then briefly trypsinized and seeded on HA- or gelatin-coated filters inserted into Boyden Chambers. We found that overexpression of either RhoA or MT1-MMP inhibited U-87 cell migration on HA by 72 and 67%, respectively (Fig. 3, grey bars). This was an expected result as the binding of the cells to HA was already shown to be reduced as a result of lower CD44 cell surface expression, as demonstrated by the increased CD44 shedding induced by both RhoA and MT1-MMP in transfected cells. In contrast, cell migration on gelatin was only found significantly increased in Wt-MT1-MMP transfected cells (Fig. 3, black bars). This suggests that the cell surface receptors that are involved in HA recognition are specifically decreased.

The actions of green tea catechin EGCg and of ROK inhibition reverse RhoA- and MT1-MMP-induced shedding of CD44. We have demonstrated that treatment of U-87 glioma cells with EGCg, a naturally occurring green tea catechin for which we have reported anti-MMP and anti-angiogenic activity (Demeule *et al*. 2002), also inhibited HA binding to CD44 in Type-I collagen-treated U-87 glioma cells (Annabi *et al*. 2004), while it had no influence on basal cell migration on HA (not shown). Whether EGCg antagonized RhoA-mediated CD44 cell surface shedding was assessed in parallel with functional inhibition of RhoA functions by the Rho-associated kinase (ROK) inhibitor Y27632. Interestingly, ROK was recently suggested to regulate CD44 expression in osteoclasts (Chellaiah *et al*. 2003), while its inhibition led to a decreased interaction of CD44 with a Na+–H+ exchanger, suggesting a potential role

for RhoA in transducing CD44 signaling (Bourguignon *et al.* 2004). Inhibition of ROK, as well as treatment of U-87 cells with EGCg, significantly inhibited both RhoA- and MT1-MMP-mediated CD44 shedding into the conditioned media (Fig. 4a). CD44 cell-surface expression was also measured by flow cytometry and this was quantified in Fig. 4(b). Moreover, the observed EGCg inhibition of RhoA- and MT1-MMP-mediated shedding was primarily because of a

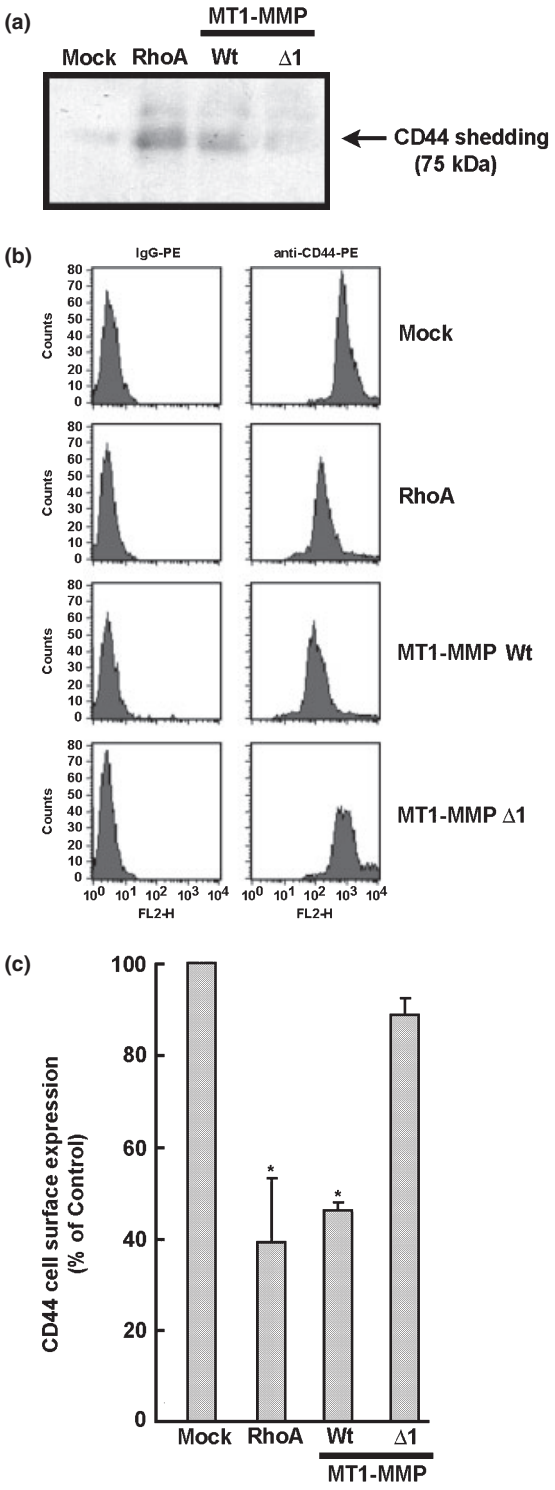

Fig. 2 RhoA and MT1-MMP overexpression trigger CD44 cell surface shedding from U-87 glioma cells and inhibit cell migration on hyaluronic acid. (a) U-87 glioma cells were cultured on plastic dishes and subsequently transfected with empty vector (Mock) or with cDNA plasmids encoding RhoA, Wt-MT1-MMP, or Δ1-MT1-MMP. Thirty-six hours post-transfection, cells were starved in serum-free media for 18 h. Conditioned media was then collected and centrifuged to eliminate any floating cells. Equal volumes (600 μL) of the conditioned media were TCA-precipitated and subjected to western blotting and immunodetection of CD44. (b) Flow cytometry was used to monitor CD44 cell surface protein expression as described in Materials and methods. (c) Flow cytometric results were quantified and expressed as the ratio of relative geometric mean values from the transfected cells to their Mock (controls) and are representative of three independent experiments.

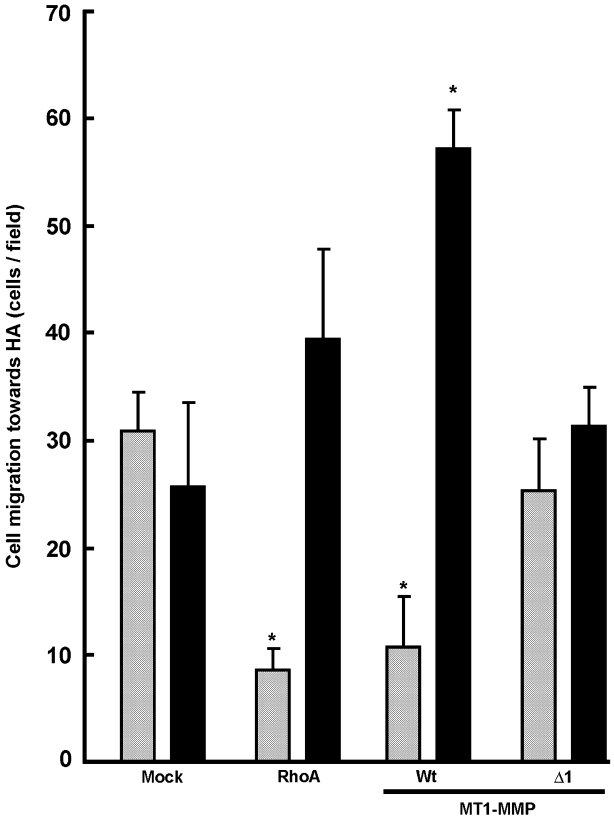

Fig. 3 Cell migration on hyaluronic acid is inhibited in RhoA- and MT1-MMP-transfected U-87 glioma cells. U-87 glioma cells were cultured on plastic dishes and subsequently transfected with empty vector (Mock) or cDNA plasmids encoding RhoA, Wt-MT1-MMP, or Δ1-MT1-MMP. Thirty-six hours post-transfection, cells (5×10^4) were harvested by brief trypsinization and seeded on hyaluronic acid- (grey boxes) or gelatin- (black boxes) coated filters. Migration was allowed to proceed as described in Materials and methods.

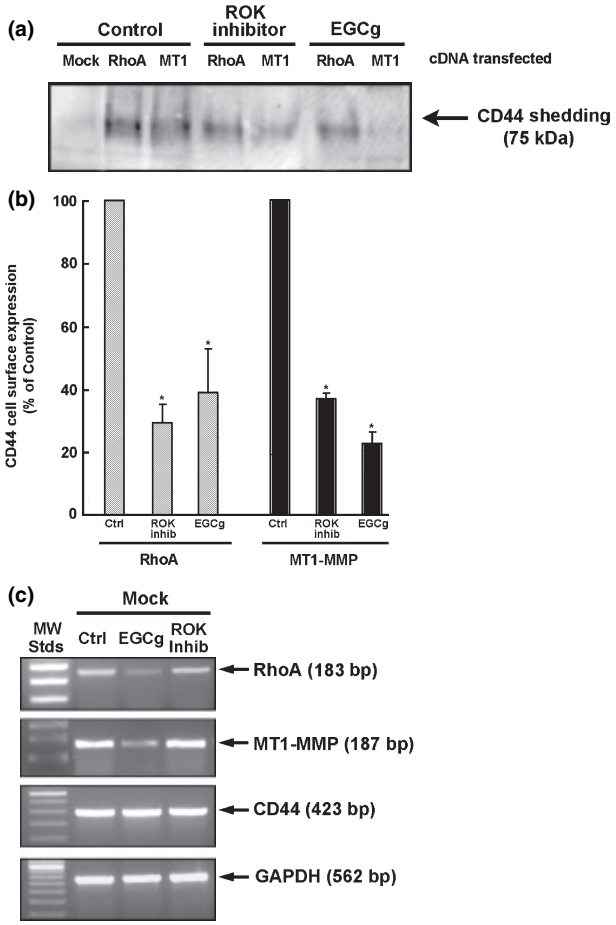

Fig. 4 Functional inhibition of ROK and the green tea catechin EGCg reverse RhoA- and MT1-MMP-induced shedding of CD44. (a) U-87 glioma cells were cultured on plastic dishes and subsequently transfected with empty vector (Mock) or cDNA plasmids encoding RhoA or Wt-MT1-MMP. Thirty-six hours post-transfection, cells (5×10^5) were starved in serum-free media supplemented (or not) with 20 μM EGCg or 5 μM Y27632 (a Rho-kinase inhibitor) for 18 h. The conditioned media were then assessed for CD44 as described in the legend to Fig. 2. (b) Quantification of the CD44 cell-surface expression was performed by flow cytometry as described in the legend of Fig. 2(c) for the RhoA- and MT1-MMP-transfected cells. (c) The effects of EGCg and of the ROK inhibitor were also assessed on RhoA, MT1-MMP, and CD44 gene expression by RT-PCR as described in Materials and methods.

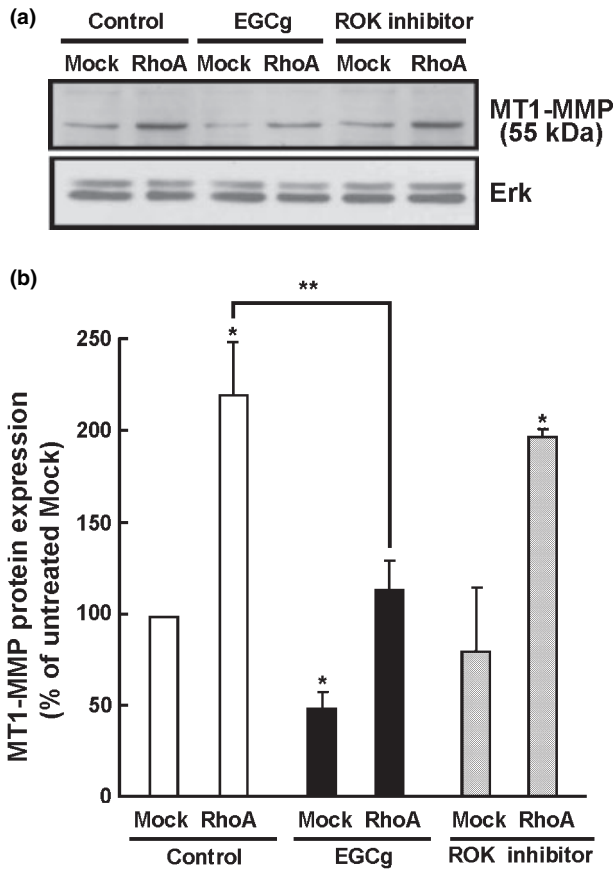

Fig. 5 The green tea catechin EGCg and ROK inhibition reverse RhoA-induced expression of MT1-MMP. (a) U-87 glioma cells were cultured on plastic and subsequently transfected with empty vector (Mock) or RhoA cDNA. Thirty-six hours post-transfection, cells were treated (or not) for 18 h with either 20 μM EGCg or 5 μM Y27603 (ROK inhibitor). Cells were then lysed and subjected to 9% SDS–PAGE, followed by an immunodetection of MT1-MMP or ERK. (b) Densitometric quantification was performed on three independent experiments. Probability values of less than 0.05 were considered significant, and an asterisk (*) identifies such significance against control (Mock-transfected cells), while (**) identifies significant difference between RhoA-transfected cells and RhoA-transfected cells followed by EGCg treatment.

down-regulation in RhoA and MT1-MMP mRNA levels (Fig. 4c) 21, while the functional inhibition of downstream signaling from RhoA with the ROK inhibitor did not affect significantly the expression of either gene.

The green tea catechin EGCg, but not functional inhibition of ROK, reverses RhoA-induced expression of MT1-MMP

Whether RhoA-induced MT1-MMP protein expression could be regulated by EGCg or functional inhibition of ROK was next assessed. U-87 glioma cells were cultured on plastic and subsequently transfected with empty vector (Mock) or RhoA cDNA. Thirty-six hours post-transfection cells were treated or not for 18 h with either 20 μM EGCg or 5 μM Y27632. Western blotting followed by MT1-MMP immunodetection revealed that the only significant inhibition of MT1-MMP protein expression was observed in EGCg-treated U-87 cells (Fig. 5a). Protein expression of basal and of RhoA-induced MT1-MMP was unaffected in U-87 cells treated with the ROK inhibitor Y27632 (Fig. 5b). This suggests that functional ROK inhibition cannot overcome the MT1-MMP induction by constitutive expression of recombinant Wt-RhoA, or that MT1-MMP induction was mediated by

another RhoA effector. Alternate yet unidentified targets of EGCg affecting RhoA functions may also be assumed. Importantly, although EGCg reduced basal MT1-MMP protein levels, it also significantly diminished the RhoA-induced increase in MT1-MMP protein levels.

Rho-associated kinase-induced CD44 shedding is inhibited by green tea catechin EGCg

Several different enzymes have been identified as possible downstream targets for RhoA signaling. One such enzyme is Rho-associated kinase (ROK), which is a serine–threonine kinase known to interact with Rho in a GTP-dependent manner (Manser *et al*. 1998). U-87 glioma cells were transfected with a cDNA encoding a constitutively active ROK, and CD44 shedding was assessed in the conditioned media. The overexpression of a recombinant constitutively active form of ROK was found to induce CD44 shedding from the cell surface (Fig. 6a) in agreement with the effect of the ROK inhibitor (Fig. 4a), while CD44 shedding was undetectable in Mock-transfected cells (Fig. 6a). Interestingly, the green tea catechin EGCg reduced ROK-induced CD44 shedding, suggesting that a crucial RhoA/ROK-mediated intracellular signaling pathway is involved in the shedding of CD44 in glioma cells. This effect of EGCg was correlated to the expression of CD44 at the cell surface (Fig. 6b) as assessed by flow cytometry. CD44 levels (shaded plots on right side) decreased in RhoA- and ROK-transfected cells as seen by the shift of signal intensity to the left, while EGCg treatment (dotted lines) antagonized both of the RhoA- and ROK-mediated effects on CD44.

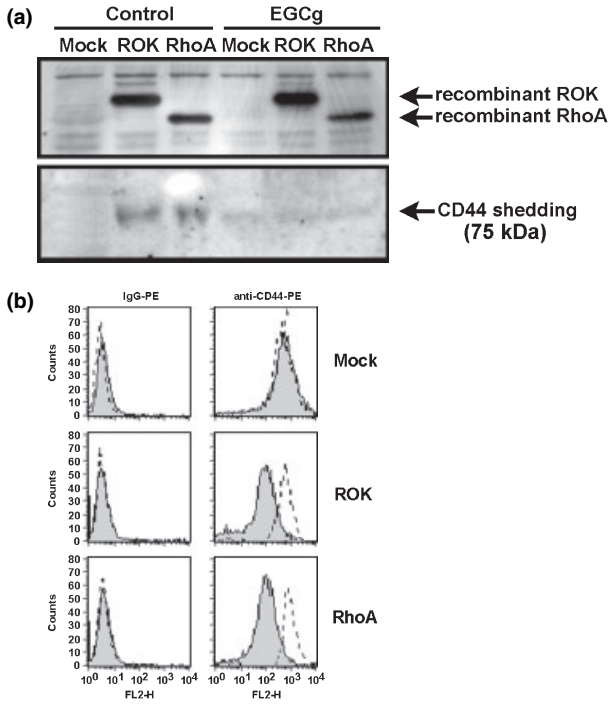

Fig. 6 ROK-induced CD44 shedding is inhibited by the green tea catechin EGCg. U-87 glioma cells were cultured on plastic and subsequently transfected with either an empty vector (Mock), a cDNA encoding a constitutively active ROK or a cDNA encoding Wt RhoA. Thirty-six hours post-transfection, cells were treated (or not) for 18 h with 20 μM EGCg. (a) Cells were then lysed and subjected to 9% SDS–PAGE, followed by immunodetection of Myc in order to detect the recombinant myc-tagged ROK and myc-tagged RhoA. The conditioned media from Mock-, ROK-, and RhoA-transfected cells was also assessed for CD44 content as described in the legend to Fig. 2. (b) Flow cytometry was used to monitor CD44 cell-surface protein expression as described in Materials and methods. Shaded plots represent the untreated cells, while the dotted plots represent the EGCg-treated cells. A representative experiment out of two independent treatments is shown.

Discussion

Tumor astrocytes migrate into the normal brain parenchyma along preferred routes such as blood vessel walls situated in the grey matter (Giese *et al*. 1994) or myelin tract in the white matter (Giese *et al*. 1996). The fact that transformed astrocytes can detach themselves from initial tumor sites is the major reason why any type of therapy remains ineffective today against astrocytic tumors (Sehgal 1998). We have previously shown that the interaction between CD44 and hyaluronan in glioma cells is a complex process that involves specialized plasma membrane microdomains and that can mediate both cell–cell and cell–ECM interactions (Annabi *et al*. 2004). In the present study, we provide evidence regarding the mechanism by which shedding of CD44 is thought to stimulate brain tumor cell motility. Elucidation of such mechanism may impact on a variety of physiological and pathophysiological processes including tumor metastasis, wound healing and leukocyte extravasation at sites of inflammation.

Moreover, we illuminate the potential to reduce that infiltrating character of brain tumors by the use of EGCg, a green tea catechin that has anti-cancer properties (Demeule *et al*. 2002). Previous studies have shown that EGCg possessed the property to inhibit MT1-MMP-mediated ECM degradation and migration of glioma cells (Annabi *et al*. 2002) and to inhibit MT1-MMP-mediated angiogenesis (Oku *et al*. 2003). We now exhibit a new effect of EGCg inhibiting the MT1-MMP-induced signaling leading to CD44 cell surface shedding. This CD44 down-regulation from the cell surface via receptor shedding may be required for cells to detach from the brain ECM and facilitate movement. This infiltrating/metastatic character of glioma cells likely involves a RhoA/ROK-regulated release of a soluble extracellular fragment of CD44, which also involves intracellular signaling through MT1-MMP (see Fig. 7 for summarized scheme). In support to our observations, intravenous administration of EGCg was recently reported to target MT1-MMP-mediated *in vivo* tumor angiogenesis (Yamakawa *et al*.

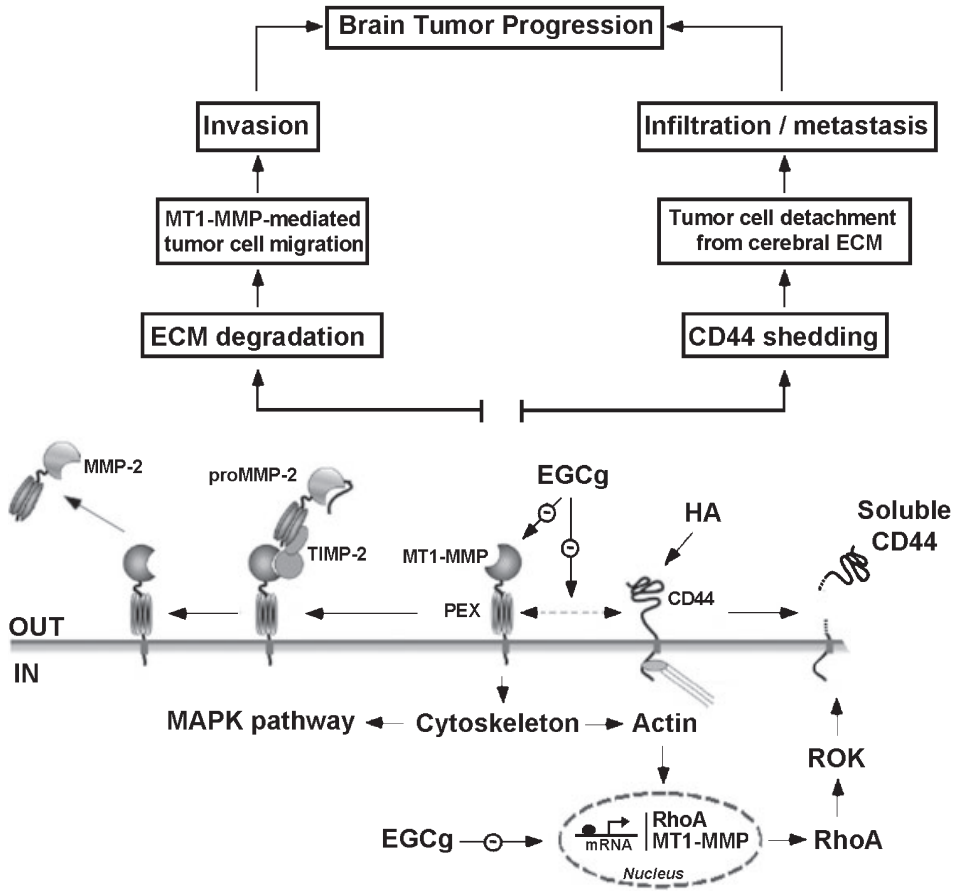

Fig. 7 Scheme of the proposed regulatory mechanism of RhoA/ROK-mediated CD44 cell surface shedding leading to glioma infiltration in brain parenchyma. Brain tumor progression is characterized by the invasive and infiltrating character of the invading cells. These properties are, in part, mediated by MT1-MMP, which regulates the degradation of the ECM through the formation of a trimolecular complex with TIMP-2 and the latent proMMP-2 form. This complex eventually leads to the release of an active MMP-2 form which regulates tumor invasion. In contrast, MT1-MMP is also thought to be a multifunctional protein which regulates several pericellular processes at the cell surface of glioma cells that may reflect the infiltrating character of the brain tumor. In the basal state, MT1-MMP regulates CD44 cell surface expression and hyaluronic acid (HA) binding through a MAPK-dependent pathway (Annabi *et al.* 2004), and this is antagonized by inhibition of MT1-MMP functions by EGCg, a green tea catechin with anti-cancer and anti-angiogenic properties. The profound cytoskeletal reorganisation induced by MT1-MMP's intracellular domain may also regulate CD44 cell surface functional expression through the up-regulation of both RhoA and MT1-MMP gene expression. EGCg can also antagonize this event by down-regulating their gene expression levels. Overall, RhoA/ROK intracellular signaling is an important step that regulates the mechanisms leading to infiltrating/metastatic processes involved in the interaction of glioma cells with their brain ECM environment and that could be efficiently targeted by the green tea catechin EGCg.

2004). Unfortunately, the levels of circulating CD44 were not assessed in that report. However, it is tempting to suggest that the circulating soluble CD44 increases could be considered as markers for tumor dissemination and could potentially also interfere competitively with the ability of membrane-bound CD44 to interact with HA (Ahrens *et al.* 2001). Whether alternate receptors for HA (such as RHAMM) may also be regulated at the cell surface remains to be investigated. Finally, and although not yet clearly established, ADAM10 was recently reported to shed CD44 from the cell surface through a similar mechanism to that of MT1-MMP (Nakamura *et al.* 2004). Whether RhoA, which is known to regulate the functions of ADAM12 (Thodeti *et al.* 2003), also regulates ADAM10 has yet to be determined. Altogether, these numerous effects of RhoA indeed highlight potential alternate molecular and cellular processes besides those involving MT1-MMP.

Of particular interest is the fact that several of the documented CD44 releasing processes from cells involve phorbol esters, a calcium ionophore ionomycin or cytokines (DeGrendele *et al.* 1997; Ristamaki *et al.* 1997), which are also known as MT1-MMP inducers in invading cells

(Yu *et al*. 1997; Park *et al*. 2000). More recently, structure–function analysis has shown the hemopexin-like domain of MT1-MMP to be responsible for the binding and subsequent shedding of the standard hematopoietic form of CD44 (Suenaga *et al*. 2005). Concomitant with CD44 shedding, cytoskeletal reorganization occurs. Pharmacological disruption of actin assembly reduced CD44 shedding, whereas activation of Rho family GTPases, which regulate actin filament assembly, enhanced CD44 cleavage (Shi *et al*. 2001). Shedding of CD44 has also been reported to be induced by Ras, an oncoprotein involved in cell motility and migration. The effect of Ras on CD44 processing appears to be mediated by members of the Rho family of GTPases (Kawano *et al*. 2000). Taken together, these data suggest that shedding of CD44 is controlled by Ras and Rho GTPases (Cdc42 and Rac1), possibly via regulation of the actin cytoskeleton. We now provide additional intracellular crosstalk linking RhoA to the cell surface proteolytic activity and expression of MT1-MMP. The specific contribution of MT1-MMP to cytoskeleton changes remains to be determined. Whether the intracellular domain of MT1-MMP transduces any RhoA-mediated cell morphology changes is also under investigation. Finally, in agreement with our data in glioma cells, transcriptional regulation of the MT1-MMP gene was also demonstrated in a study which showed that RhoA and functional inhibition of ROK restored MT1-MMP mRNA that was inhibited by LPA in human osteosarcoma cells (Matsumoto *et al*. 2001).

Rho family GTPases play an important role in a number of processes related to metastasis. It is thus not surprising that the overexpression of certain Rho GTPases in human tumors often correlates with poor prognosis (Fritz *et al*. 1999). In particular, survival prediction in human gliomas based on proteome analysis has recently identified the increased RhoA levels as potential biomarkers for anti-glioma therapy (Iwadate *et al*. 2004). Indeed, the high level of intracellular expression of RhoA facilitates its translocation to the membrane where it is activated, resulting in stimulation of the RhoA-ROK-actomyosin system, and leading to migration (Itoh *et al*. 1999). ROK has been shown to phosphorylate the cytoplasmic domain of the $CD44v_{3,\ 8-10}$ isoform and to up-regulate the interaction between the $CD44v_{3,\ 8-10}$ isoform and the cytoskeletal protein ankyrin during HA/CD44-regulated tumor cell migration (Bourguignon *et al*. 1999). Thus, ROK is clearly one of the important signaling molecules required for membrane–cytoskeleton interaction, Ca^{2+} regulation, and HA/CD44-mediated cell function. Moreover, several cellular proteins, including the cytoplasmic domain of CD44 and IP_3 receptors, have been identified as ROK-specific cellular substrates during HA-CD44 signaling (Singleton and Bourguignon 2002). Whether MT1-MMP's intracellular domain may also be a substrate for ROK-mediated phosphorylation remains to be established. Co-ordinated mechanisms involving RhoA/MT1-MMP crosstalk in cell motility and cell surface proteolysis that could also possibly influence glioma cells invasiveness have been reported. For instance, glioblastoma cell growth and proliferation *in vitro* was recently shown to be regulated by the chemokine stromal cell-derived factor (SDF-1α) that is expressed in several human glioblastoma multiform tumor tissues (Barbero *et al*. 2003). Interestingly, SDF-1α was also reported to promote invasion and to trigger the activation of the GTPase RhoA-dependent signaling in the highly metastatic BLM melanoma cell line, leading to the control of MT1-MMP expression (Bartolome *et al*. 2004). Altogether, these data indicate that SDF-1 through RhoA regulation could play important roles during glioma cell invasion and directional migration for basement membrane and brain parenchyma infiltration.

In the present study, we propose a RhoA/ROK-mediated CD44 cell surface shedding mechanism that may regulate glioma infiltration in brain parenchyma. We also provide *in vitro* evidence for a new cellular action of green tea catechin EGCg that could help optimize current therapeutic approaches for brain tumor treatments. We have already demonstrated that EGCg can be efficiently used in conjunction with radiotherapeutic modalities to efficiently target those tumor-derived endothelial cells that escaped ionizing radiation-induced apoptosis (Annabi *et al*. 2003). Even more exciting is the fact that a receptor for EGCg has recently been identified as the 67-kDa laminin receptor (Tachibana *et al*. 2004). Such laminin receptors have been shown to regulate the invasion of malignant glioma cells potentially through Rho GTPases intracellular signaling (Fukushima *et al*. 1998). Whether EGCg can directly interact and inhibit intracellular RhoA functions remains to be investigated. We now propose that the infiltrating character of brain tumors may also be efficiently targeted by the anti-cancerous properties of green tea catechin EGCg, which could then be used in synergy with currently employed therapeutic modalities.

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