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Green tea polyphenol (−)-epigallocatechin 3-gallate inhibits MMP-2 secretion and MT1-MMP-driven migration in glioblastoma cells

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Abstract

We have recently shown that green tea polyphenols, and especially (−)-epigallocatechin 3-gallate (EGCg), acted as potent inhibitors of matrix metalloproteinase activities as well as of proMMP-2 activation (M. Demeule, M. Brossard, M. Page, D. Gingras, R. Beliveau, Biochim. Biophys. Acta 1478 (2000)). In the present work, we sought to examine the involvement of MT1-MMP in the EGCg-induced inhibition of proMMP-2 activation. The incubation of U-87 glioblastoma cells in the presence of concanavalin A or cytochalasin D, two potent activators of MT1-MMP, resulted in proMMP-2 activation that was correlated with the cell surface proteolytic processing of MT1-MMP to its inactive 43 kDa form. Addition of EGCg strongly inhibited the MT1-MMP-dependent proMMP-2 activation. The inhibitory effect of EGCg on MT1-MMP was also demonstrated by the down-regulation of MT1-MMP transcript levels and by the inhibition of MT1-MMP-driven cell migration of transfected COS-7 cells. These observations suggest that this catechin may act at both the MT1-MMP gene and protein expression levels. In addition, treatment of cells with non-cytotoxic doses of EGCg significantly reduced the amount of secreted proMMP-2, and led to a concomitant increase in intracellular levels of that protein. This effect was similar to that observed using well-characterized secretion inhibitors such as brefeldin A and manumycin, suggesting that EGCg could also potentially act on intracellular secretory pathways. Taken together, these results indicate that EGCg targets multiple MMP-mediated cellular events in cancer cells and provides a new mechanism for the anticancer properties of that molecule. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Angiogenesis; Cancer; Glioblastoma; Green tea; Membrane-type 1 matrix metalloproteinase

1. Introduction

Green tea consumption has been associated in epidemiologic studies with decreased risk of various proliferative diseases such as cancer and atherosclerosis in humans [1,2]. These studies are based on the observation that green tea possesses a number of inhibitory activities against pathological or cell-damaging processes such as carcinogenesis [3] and free radical formation [4]. Its low toxicity, low cost, and

Abbreviations: Con-A, concanavalin A; Cyto-D, cytochalasin D; MMP, matrix metalloproteinase; MT-MMP, membrane-type matrix metalloproteinase; RT-PCR, reverse transcriptase-polymerase chain reaction; TIMP, tissue inhibitor of matrix metalloproteinases

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natural abundance thus make it an attractive substance to investigate. Green tea consists mainly of polyphenols (catechins) which constitute about 40% of the dry weight of solids in brewed green tea, of which (−)-epigallocatechin gallate (EGCg) is the most abundant and the most extensively studied catechin [5–7]. Numerous studies have suggested that this catechin could influence tumor formation through an inhibition of various cellular processes involved in cell replication and DNA synthesis, by interfering with cell-to-cell adhesion [8], or by inhibiting some of the intracellular communication pathways required for cell division [9,10]. The mechanisms underlying the anticancer properties of EGCg and green tea as a whole remain, however, largely unknown.

The polyphenols present in green tea, particularly EGCg, thus appear to affect a number of molecular processes that include induction of tumor cell apoptosis and inhibition of tumor growth and invasion. Since matrix metalloproteinases (MMPs) play an important role in these processes and have been linked to tumor cell invasion [11,12], we thus examined whether green tea catechins may further inhibit other cellular MMP-mediated events in a highly metastatic U-87 glioblastoma cell line. MMPs represent a growing family of zinc-dependent proteases involved in matrix turnover occurring during normal and pathological processes [12]. MMPs are secreted by cells as proenzymes that must be cleaved in order to become functional. This latency of the MMP zymogens is due to the presence of the N-terminal pro-domain that shields the catalytic cleft through coordination of the catalytic zinc molecule [13]. Cleavage of the pro-domains of MMPs is mediated in most cases by soluble MMPs or by proteases of the serine families such as plasmin, plasma kallikrein and neutrophil elastase [12,14]. In contrast to most MMPs, proMMP-2 possesses a propeptide that is not susceptible to proteolytic cleavage by serine proteinases [15] and recent evidence has suggested that its activation is distinct and involves another member of the MMP family, the membrane-type 1 MMP (MT1-MMP) [16]. The intracellular molecular mechanisms involved in MT1-MMP-dependent activation of proMMP-2 at the cell surface is regulated by the balance between MT1-MMP complexed by tissue inhibitor of matrix metalloproteinases (TIMP)-2 and TIMP-2-free MT1-MMP [17] and results in MT1-MMP proteolytic processing to its 43 kDa inactive form [18–20]. The apparition of this inactive MT1-MMP form was strongly correlated with MMP-2 activation [21]. However, the biological function and regulation of this inactive MT1-MMP form remains debated since it retains both its hemopexin and cytoplasmic tail [19]. Such MT1-MMP proteolytic mechanism was interestingly found to be operative in specialized plasma membrane domains such as caveolae of U-87 glioma cells [22], and this localization may prove important in the acquisition of cancer cells' invasive properties [23]. Whether any of the MT1-MMP-mediated events is affected by green tea catechins is yet unknown.

We have recently reported that green tea polyphenols, and particularly EGCg, potently inhibited MMP activity both in vitro and in vivo [24], as well as the activation of proMMP-2 in glioblastoma cells and MT1-MMP-transfected COS-7 cells [22]. In this report, we extended these results by showing that green tea catechins, and especially EGCg, inhibited proMMP-2 secretion from U-87 glioblastoma cells and interfered with proMMP-2 activation through a transcriptional regulation of the MT1-MMP gene. Moreover, EGCg also specifically antagonized MT1-MMP-driven cell migration demonstrating a new potential cellular target for this catechin.

2. Materials and methods

2.1. Materials

The human glioblastoma cell line U-87 was purchased from the American Tissue Culture Collection and maintained in modified Eagle's medium (MEM) containing 10% fetal bovine serum (HyClone Laboratories), 100 units/ml penicillin, 100 μg/ml streptomycin. Concanavalin A (Con-A), cytochalasin D (Cyto-D), brefeldin A, EGCg, (−)-epigallocatechin (EGC), (−)-epicatechin (EC), (−)-epicatechin gallate (ECg), manumycin, and nocodazole were purchased from Sigma. The anti-MT1-MMP polyclonal antibody (raised against the hinge region) and monoclonal antibody against human MMP-2 were from Chemicon. The TriZOL reagent was from Gibco and the Titan one-tube RT-PCR kit was from Roche

Molecular Biochemicals. Reagents for electrophoresis were purchased from Bio-Rad.

2.2. Activation of proMMP-2 and isolation of cell lysates

U-87 cells were plated in Nunc 33 mm six-well plastic dishes at 10^5 cells/well and grown in a humidified atmosphere containing 5% CO_2 and 90% air at 37°C. The extent of proMMP-2 activity in the conditioned medium and in cell lysates was evaluated by the appearance of a 62 kDa gelatinolytic band on gelatin zymograms following stimulation of the cells, as described recently [21]. To induce activation, Con-A (10 μg/ml) or Cyto-D (1 μM) was added to the conditioned medium and the cells were further incubated in the presence or absence of the individual catechins for different periods of time. No difference on U-87 cell adhesion was observed when cells were either plated on plastic or gelatin-coated dishes in the presence of the catechins (not shown). After removal of the conditioned medium, cells were washed two times in phosphate-buffered saline and collected by scraping. Crude membranes were isolated from U-87 cells in 20 mM Tris–HCl pH 7.4 following centrifugation for 1 h at 200 000 × *g* at 4°C. The resulting particulate (membranes) and soluble (cytosol) fractions were then examined by immunoblotting. For the preparation of whole cell lysates, cells were resuspended in a buffer containing 1% Triton X-100, 0.5% Nonidet P-40, 150 mM NaCl and 20 mM Tris–HCl pH 7.4. The mixture was incubated on ice for 1 h and insoluble matter was removed by centrifugation.

2.3. MMP-2 fluorimetric assays

Fluorimetric assays of the gelatinolytic activities of MMP-2 were performed using the EnzChek Collagenase/Gelatinase kit as described previously [24]. Briefly, 0.005–0.02 units (25–100 ng) of MMP-2 were mixed with 0.2 μg/assay of quenched fluorescein-conjugated gelatin in a final volume of 100 μl of reaction buffer in 96-well microplates. The rate of proteolysis, in the presence or absence of EGC, EGCg or brefeldin A, was determined by measuring the increase in fluorescence using a Fluoroskan II Fluorimeter (Lab System), and was linear for at least 15 min under these conditions.

2.4. Immunoblotting procedures

Equal amounts of proteins from control and treated cells were resuspended in sample buffer and separated by SDS–PAGE, using 9% acrylamide gels. After electrophoresis, proteins were electrotransferred to a 0.45 μm pore size polyvinylidene difluoride (PVDF) membrane using a Milliblot graphite electroblotter (Millipore) in the presence of a transfer buffer containing 96 mM glycine, 10 mM Tris–HCl and 10% methanol. Hydrophobic or non-specific sites were blocked overnight at 4°C with 5% non-fat dry milk in Tris-buffered saline (150 mM Tris, 20 mM Tris–HCl, pH 7.5) containing 0.3% Tween 20 (TBST). Membranes were washed three times in TBST and incubated with the primary antibodies (1/1000 dilution) in TBST containing 3% bovine serum albumin and 0.02% sodium azide, followed by a 1 h incubation at room temperature with horseradish peroxidase-conjugated anti-rabbit IgG (1/10 000 dilution) or anti-mouse IgG (1/2500 dilution) in TBST containing 5% non-fat dry milk. PVDF membranes were washed three times in TBST and immunoreactive material was visualized by enhanced chemiluminescence (ECL, Amersham-Pharmacia Biotech).

2.5. RT-PCR analysis

U-87 cells were plated in Nunc 33 mm six-well plastic dishes at 10^5 cells/well and were treated the next day with Con-A (10 μg/ml), or Cyto-D (1 μM), in the presence or absence of 25 μM EGCg for 18 h in serum-deprived medium. Then cell monolayers were washed with PBS and total RNA was prepared using TriZOL reagent following the manufacturer's instructions. One microgram of RNA was used for first-strand cDNA synthesis followed by specific gene product amplification using the Titan One Tube RT-PCR Kit as described previously [22]. Primers were all derived from human sequences; PCR conditions were optimized so that the gene products were found to be within the linear range for PCR amplification [18]. PCR products were resolved on 2% (w/v) agarose gels containing 1 μg/ml ethidium bromide.

2.6. Cell migration assay

To assess whether the different green tea polyphenols affected U-87 glioma cells migratory potential and MT1-MMP-driven migration of COS-7 cells, transwells (Costar; 8 μm pore size) were precoated with 0.5% gelatin/PBS by adding 200 μl of the solution per transwell and allowing the membranes to air dry in a laminar flow hood at room temperature. The transwells were then assembled in a 24-well plate (Falcon 3097) and the lower chambers filled with 600 μl of MEM supplemented with 10% fetal bovine serum. Cells were incubated for 2 h at 37°C with the particular catechin, spun down to remove the inhibitor from the medium, resuspended in 200 μl fresh MEM medium at 7.5×10^4 cells/ml, and inoculated into the upper chamber of each transwell. The plate was then placed at 37°C in 5% CO_2/95% air for 2 h. Cells that had migrated to the lower surface of the filters were fixed and stained with 0.1% crystal violet/

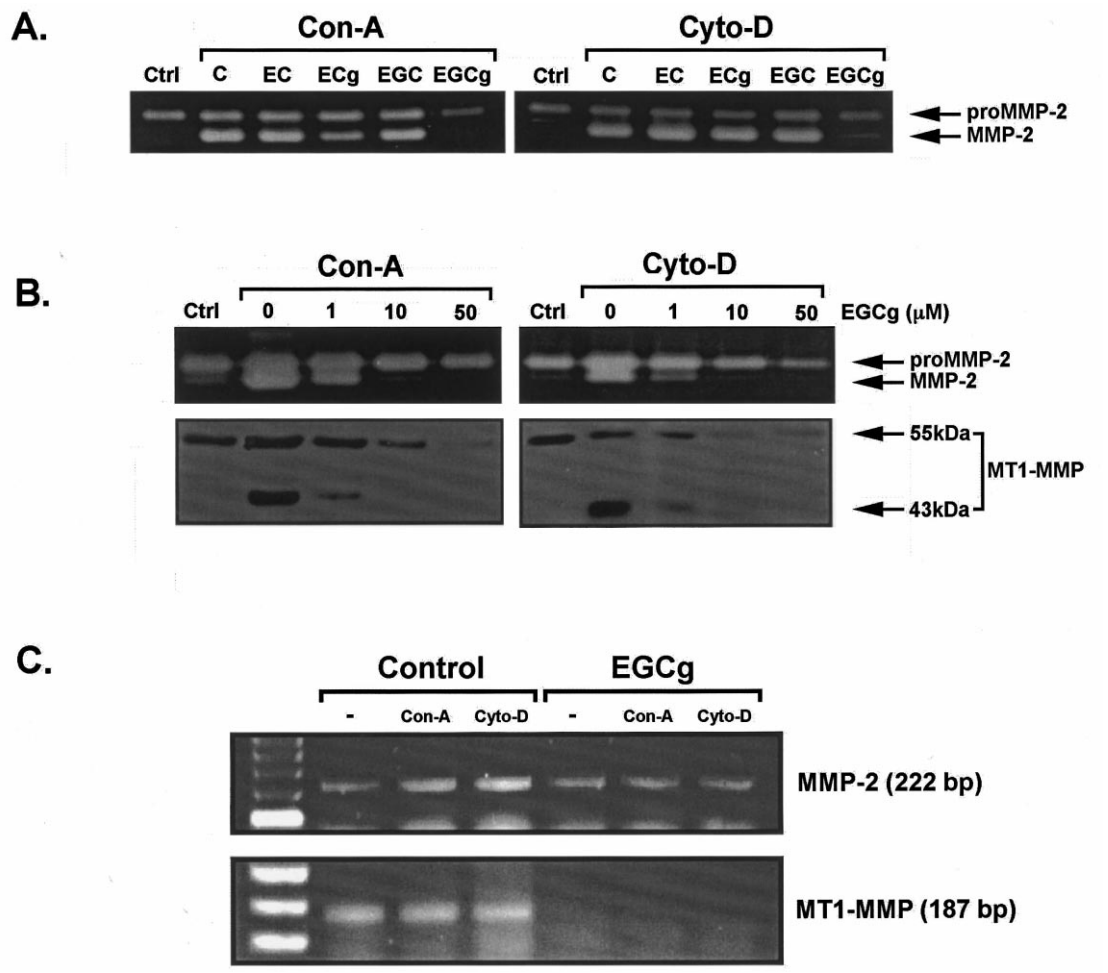

Fig. 1. Specific transcriptional regulation of MT1-MMP-dependent activation of proMMP-2 in U-87 glioblastoma cells by EGCg. (A) Control U-87 glioma cells were incubated in serum-deprived medium for 18 h to induce proMMP-2 secretion, and then treated with 25 μM of the indicated catechins for another 18 h. 20 μl of the conditioned medium were subjected to gelatin zymography. (B) U-87 cells were treated as described in A with increasing EGCg concentrations in the presence or absence of 5 μg/ml Con-A or 1 μM Cyto-D. Conditioned medium was saved to perform gelatin zymography, and cell lysates (20 μg/well) were loaded on a 9% SDS-gel to assess the level of the active 55 kDa and inactive 43 kDa MT1-MMP immunoreactive forms by Western blot. (C) Total RNA was extracted from U-87 glioblastoma cells treated or not with 25 μM EGCg, 5 μg/ml Con-A, or 1 μM Cyto-D for 18 h in serum-deprived medium. 1 μg was subjected to RT-PCR analysis of MMP-2. The resulting amplified products of 222 bp for MMP-2, and 187 bp for MT1-MMP were resolved on 2% agarose gels containing 1 μg/ml ethidium bromide.

20% MeOH, and counted. Data are presented as the average number of migrated cells per five fields ($\times 100$).

3. Results

3.1. EGCg inhibits MT1-MMP-dependent proMMP-2 activation through transcriptional events in U-87 glioblastoma cells

We have previously shown that U-87 human glioblastoma cells constitutively secreted large amounts of proMMP-2 into the conditioned medium and thus represented a convenient model to study proMMP-2 activation [21]. We also showed that treatment of these cells with Con-A or Cyto-D promoted a massive activation of proMMP-2 and that, among green tea polyphenols, EGCg selectively inhibited this activation [24]. At that time, we hypothesized that this catechin could inhibit proMMP-2 activation through its interference with MT1-MMP activity.

In order to further assess the involvement of MT1-MMP, we now examined the effects of EGCg on proMMP-2 activation induced by Con-A and Cyto-D in highly invasive U-87 cells. As shown by gelatin zymography in Fig. 1A, incubation of U-87 cells with either Con-A or Cyto-D resulted in a marked activation of proMMP-2 to its active form, and this was specifically antagonized by co-incubation of the cells with EGCg but not with the other catechins tested. Moreover, a dose-response incubation with EGCg shows that MT1-MMP-dependent activation of proMMP-2 by Con-A and Cyto-D was indeed antagonized (Fig. 1B). This reduction in the activation of proMMP-2 correlated with a decrease in both the active 55 kDa and inactive 43 kDa protein levels of MT1-MMP suggesting that EGCg may act on both the intracellular protein turnover of the protein and at a transcriptional level. IC_{50} values for the inhibition of MT1-MMP protein turnover by EGCg were calculated and derived from semi-log plots of densitometric measurements of the 55 kDa form of MT1-MMP (Fig. 1B), and were 3.2 ± 0.9 and 1.1 ± 0.8 μM respectively for the Con-A- and Cyto-D-treated cells.

Total RNA was then isolated from control U-87 glioma cells or treated in the presence of EGCg. We found that, while MMP-2 transcript levels were increased in Con-A- and Cyto-D-treated cells (Fig.

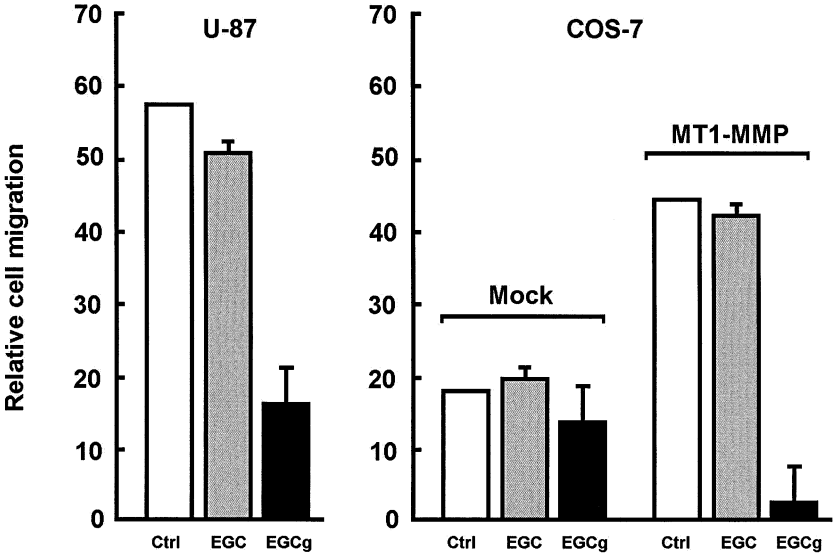

Fig. 2. EGCg antagonizes MT1-MMP-induced cellular migration. Migration was assayed in a modified Boyden chamber as described in Section 2. U-87 glioma or COS-7 cells were treated as described in the legend of Fig. 1 for 2 h. The cells were then trypsinized and resuspended in MEM supplemented with serum. Cells (7.5×10^4 cells/ml) were inoculated into the upper chamber of each transwell. Migration was performed for 2 h at 37°C, the number of cells that had migrated was determined by visually counting the cells on the lower compartment of the filter. The white bars represent no treatment, gray bars represent EGC treatment, and black bars represent EGCg treatment. Data represent the mean of cells per microscope field of duplicate experiments.

1C), they remained unaffected in EGCg-treated U-87 cells. However, EGCg antagonized the increase of either Con-A- or Cyto-D-induced MMP-2 gene expression. On the other hand, MT1-MMP transcript levels were strikingly decreased in all EGCg-treated cells (Fig. 1C). Since MT1-MMP is responsible for the activation of proMMP-2 at the cell surface, these results strongly suggest that EGCg may interfere with the activity of this enzyme by regulating intracellular MT1-MMP gene expression.

3.2. *Specific inhibitory effect of EGCg on MT1-MMP-induced cell migration*

We have previously shown that recombinant MT1-MMP expression induced COS-7 cell migration [22]. To further establish the involvement of MT1-MMP in the inhibitory effect of EGCg, we thus examined the effect of this catechin on the migratory potential of these cells. A 2 h preincubation in the presence or absence of the catechins EGC or EGCg was performed, and migration of U-87 cells was compared to COS-7 cells transfected or not with MT1-MMP cDNA. As shown in Fig. 2, migration of U-87 cells was specifically inhibited by EGCg but not by EGC. COS-7 cells, which lack endogenous MT1-MMP [21,22], had their cell migration increased only when they overexpressed MT1-MMP protein, and this migration was also specifically antagonized by EGCg. This observation implies that EGCg may directly inhibit MT1-MMP activity that is crucial for cell migration as it did not affect the MT1-MMP transcript levels in transiently MT1-MMP-transfected COS-7 cells [22]. Direct demonstration for such interaction could potentially be provided by showing precipitation of EGCg using appropriate MT1-MMP antibodies. MT1-MMP immunoprecipitation was, however, unsuccessful in our conditions. Finally, the rapid kinetic (2 h) of EGCg inhibition of cell migration prompted us to further examine

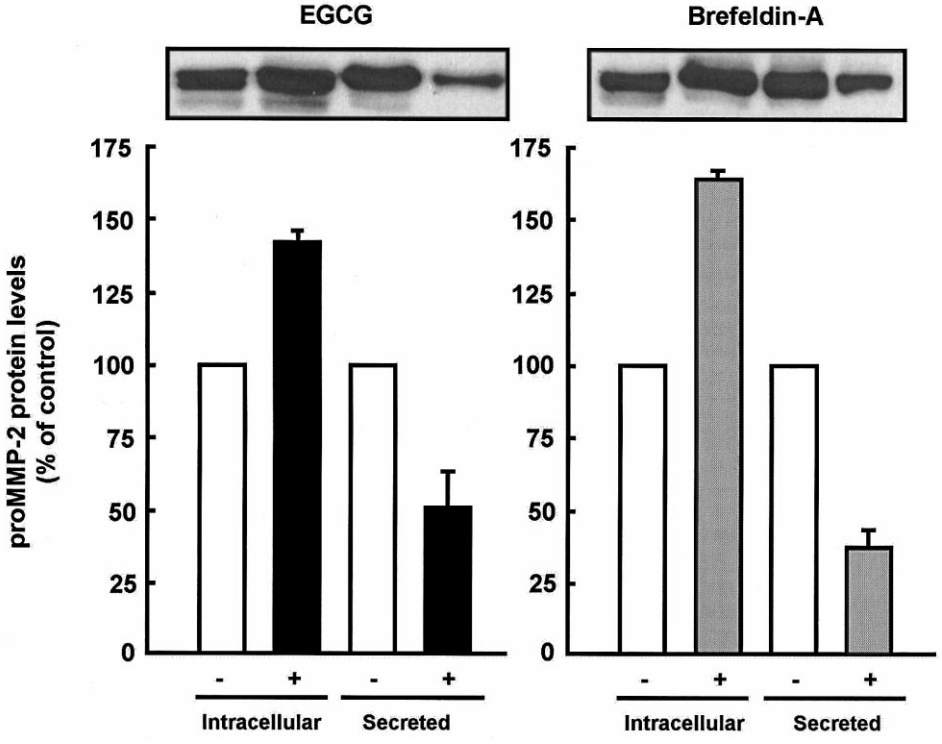

Fig. 3. EGCg and brefeldin A inhibit proMMP-2 protein secretion in U-87 glioblastoma cells. Control U-87 glioblastoma cells were cultured in serum-free medium and treated with 25 μM EGCg or 3 μM brefeldin A for 18 h. The conditioned medium was saved and the cells subsequently washed in PBS 1× and lysed. The intracellular proMMP-2 protein pool was compared to the extracellular secreted proMMP-2 protein using Western blot analysis. Densitometric measurement was used to quantify the extent of proMMP-2 protein levels. White bars represent no treatment, black bars represent EGCg treatment, and gray bars represent brefeldin A treatment. Data are the mean of three independent treatments.

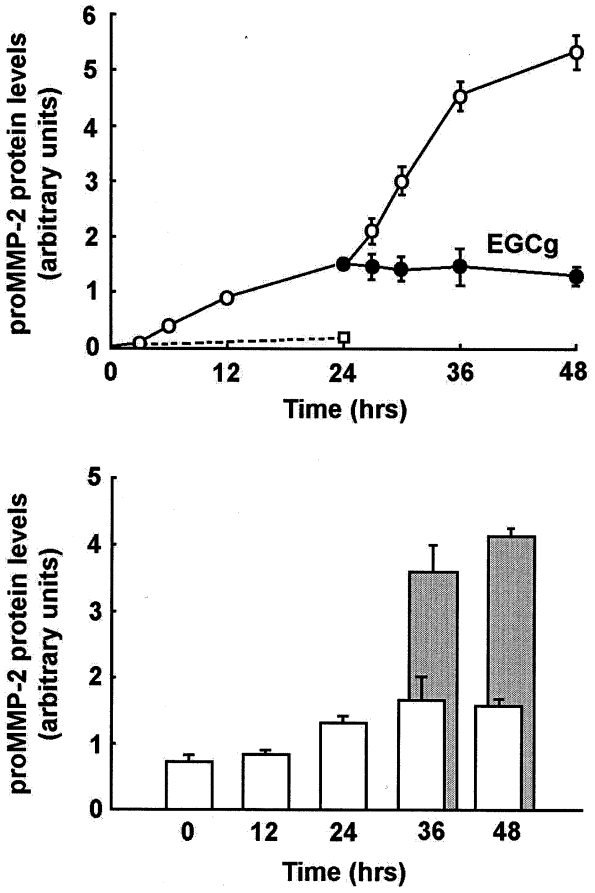

Fig. 4. Time-dependent inhibition of proMMP-2 secretion by EGCg correlates with intracellular proMMP-2 sequestration. U-87 glioblastoma cells were serum-starved for 48 h, and proMMP-2 protein secretion and intracellular levels were monitored by Western blot analysis and densitometric measurement. (A) 20 μl of the conditioned medium were saved at different times and monitored for proMMP-2 protein secretion (○). 25 μM EGCg was added either at the beginning (□) or after 24 h of serum deprivation (●). (B) 20 μg of the corresponding control (open bars) or EGCg-treated (closed bar) U-87 cell lysates were also isolated and blotted for proMMP-2 protein.

whether other intracellular MMP-mediated events were also affected.

3.3. EGCg rapidly inhibits proMMP-2 secretion from U-87 glioblastoma cells

During the course of this study, we observed that incubation of U-87 cells with EGCg significantly reduced the amount of proMMP-2 in the conditioned medium despite no apparent effects on cell viability and MMP-2 transcript levels (Fig. 1C). We thus investigated whether that specific catechin could affect the secretion of proMMP-2 from glioblastoma cells. As shown in Fig. 3 (left panel), the addition of 25 μM EGCg to U-87 cells indeed resulted in a marked reduction of proMMP-2 protein levels in the conditioned medium (~50%), whereas intracellular proMMP-2 levels concomitantly increased in the corresponding treated cell lysates. We next compared the effect of EGCg with that of brefeldin A, a well-characterized compound that specifically blocks translocation of proteins from the endoplasmic reticulum to the Golgi apparatus of the cell [25]. As shown in Fig. 3 (right panel), incubation of the cells in the presence of brefeldin A resulted in an accumulation of proMMP-2 in the cell lysates and in a marked reduction in the corresponding conditioned medium. Moreover, whether proMMP-2 secretion from U-87 cells was measured in the presence of EGCg added at the beginning or after 24 h of serum starvation, the catechin inhibitory effect was seen extremely rapidly (less than 2 h after the addition of EGCg) and resulted in an immediate buildup of an intracellular proMMP-2 protein pool (Fig. 4). This suggests that EGCg could reduce proMMP-2 secretion very efficiently and rapidly through a mechanism affecting some vesicular trafficking pathway from the intracellular MMP-2 pool to the plasma membrane of U-87 cells. Moreover, such effect could not be attributed to a potential binding of MMP-2 at the cell surface as intracellular MMP-2 buildup, induced

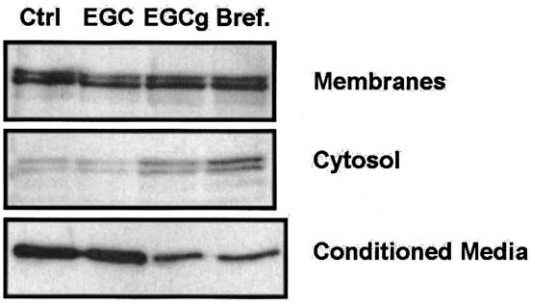

Fig. 5. EGCg-induced proMMP-2 intracellular accumulation is not due to binding at the cell surface membrane of U-87 glioblastoma cells. U-87 glioma cells were serum-deprived and incubated for 24 h in the presence of 25 μM EGC, 25 μM EGCg, or 3 μM brefeldin A. Conditioned medium was saved and subcellular fractionation was performed as described in Section 2 to isolate both the cytosol and a crude total membrane preparation. ProMMP-2 protein levels were examined by Western blot in the respective fractions.

through either brefeldin A or EGCg treatment of the cells, was specifically observed within the cytosolic fraction and was not associated with cell-derived membranes (Fig. 5). Other compounds known to antagonize intracellular vesicular trafficking such as nocodazole [26], or to suppress MMP secretion such as manumycin A [27] and U0126 [28] also decreased the proMMP-2 protein levels secreted in the extracellular media (not shown). This further defines the potential mechanistic effect that EGCg may exert on intracellular vesicular trafficking in U-87 glioma cells.

3.4. EGCg has a dual inhibitory enzymatic and secretory effect on MMP-2 in U-87 glioblastoma cells

EGCg was demonstrated to inhibit MMP-2 gelatinolytic activity and to a greater extent MMP-12 activity [24]. The question as to whether the extracel-

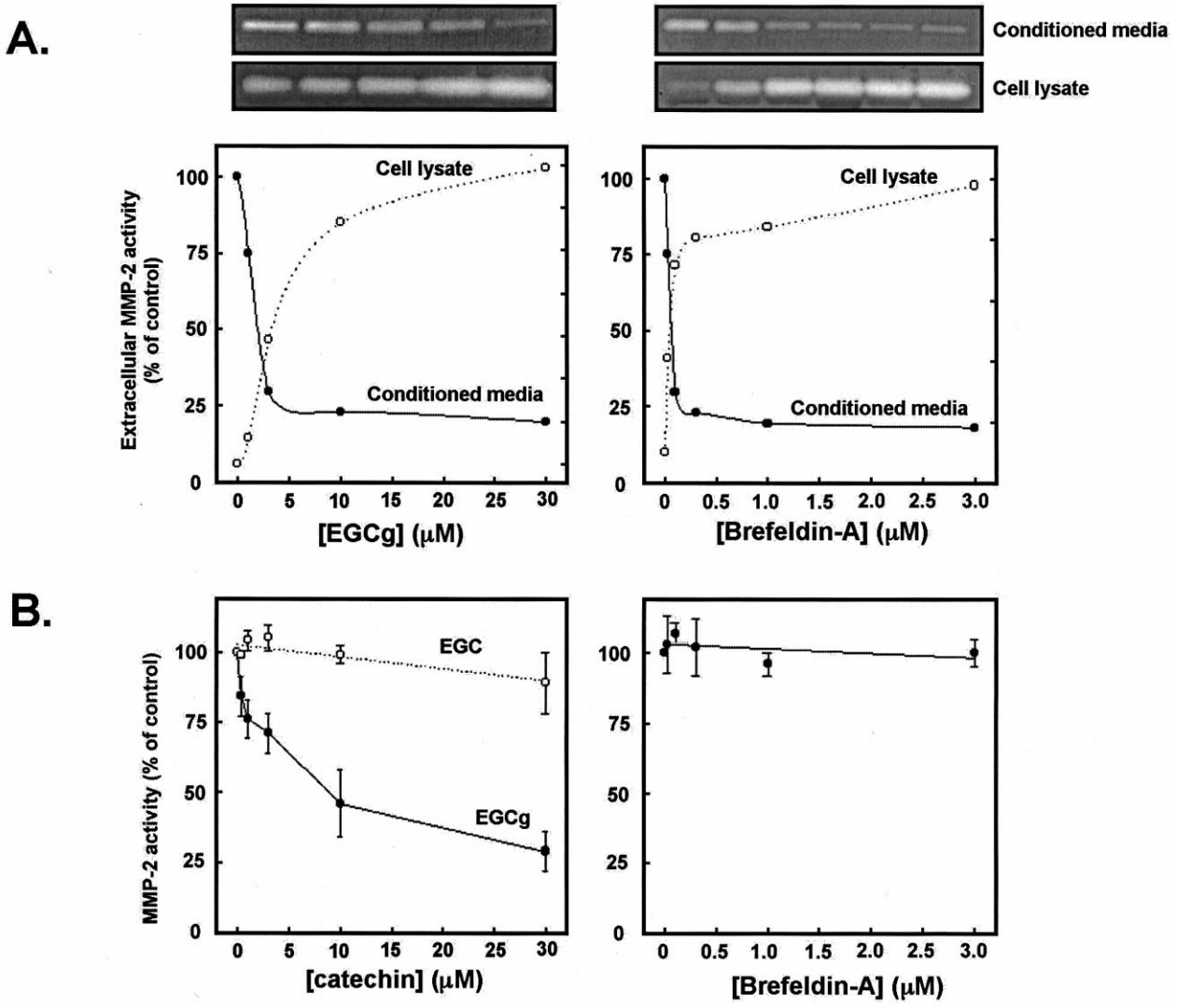

Fig. 6. EGCg, but not brefeldin A, has a dual effect on proMMP-2 gelatinolytic activity and protein secretion. U-87 glioblastoma cells were serum-starved and treated with increasing concentrations of brefeldin A or EGCg. (A) The proMMP-2 gelatinolytic activity was assessed by gelatin zymography using the corresponding conditioned medium (20 μl) and cell lysates (20 μg) after 18 h of treatment. The extent of gelatin hydrolysis was quantified by densitometry and expressed as the percentage of the respective control. (B) Recombinant MMP-2 gelatinolytic activity was assayed using a fluorescent gelatin substrate in the presence of increasing concentrations of brefeldin A, EGC, or EGCg ($n = 3$).

lular and intracellular proMMP-2 protein levels retained any enzymatic activity was thus assessed by gelatin zymography. Here we show that EGCg and brefeldin A antagonized proMMP-2 secretion in a dose-responsive manner as assayed by gelatin zymography (Fig. 6A), and that this resulted in a corresponding increase in intracellular proMMP-2 gelatinolytic activity in the cell lysate. Interestingly, while EGCg, but not EGC or brefeldin A, inhibited MMP-2 in vitro activity (Fig. 6B), it did not seem to affect significantly the activity of the intracellular proMMP-2 protein pool that had accumulated (Fig. 6A). This further demonstrates the dual action that EGCg exerts with regard to modulating both the intracellular MMP-2 secretory pathway and the catalytic activity of the MMP-2 enzyme.

4. Discussion

Activation of proMMP-2 by MT1-MMP located at the tumor cell surface is thought to represent a crucial step in tumor invasion and metastasis [11]. In gliomas, particularly, both MT1-MMP and the activation of proMMP-2 have been strongly correlated with glioma invasiveness [29,30]. These considerations led us to investigate the molecular events leading to proMMP-2 activation in glioma cells as well as the involvement of the MT1-MMP protein in this process. In addition, we report potential new intracellular mechanistic targets of EGCg, a green tea catechin, in MMP-mediated events that may prove crucial with regard to its reported antimetastatic effect.

The first new target of EGCg that we report in this study involves MT1-MMP per se and MT1-MMP-mediated events in glioblastoma cells. It is now well established that MT1-MMP enables and regulates cell invasion activity [23,31,32]. Accordingly, overexpression of MT1-, MT2-, and MT3-MMP in epithelial cells promoted invasion of basement membrane whereas a wide range of soluble MMPs did not [23]. In agreement with these observations, we are now showing that overexpression of recombinant MT1-MMP in COS-7 cells indeed increased cell migration through a gelatin matrix ([22]; this study). This MT1-MMP-driven migration is also antagonized by EGCg, which suggests a potential direct interaction of this catechin with the overexpressed MT1-MMP protein. Such hypothesis was indeed recently raised by Garbisa et al. [33], who, however, did not characterize the nature of the MT1-MMP (55 kDa active vs. 43 kDa inactive) form affected by EGCg. This inhibition of cellular migration was similarly observed in U-87 glioma cells and seemed specific to EGCg, since other catechins tested had little or no effect. Whether this effect was cell specific remains to be investigated as ECg, in addition to EGCg, was reported to suppress invasion of human HT-1080 fibrosarcoma cells [33,34] and of mouse lung carcinoma LL2 cells [35]. The inhibitory effect of EGCg observed on cellular migration thus suggests that, in addition to its ability to form complexes with metal ions such as zinc [36], this catechin may also bind and interact with specific intramolecular sites of proteins and, in the present study, specifically target MT1-MMP. Moreover, supporting this notion is the low IC_{50} value obtained for the EGCg effect on MT1-MMP that is comparable to the EGCg plasma concentration (0.3 μM) measured in human after consumption of tea [37]. Whether EGCg affects other membrane-type MMPs is currently under investigation.

Recent studies have provided evidence that the mechanisms of the inhibitory actions of EGCg may involve, among other pathways, the inhibition of phospho-ERK and phospho-c-jun formation. These intermediate pathways are involved in transformation, differentiation, and cell proliferation but, more importantly, EGCg also inhibited activator protein 1 (AP-1) function which has been associated with invasive and metastatic characteristics of cancer cells [38]. Interestingly, one class of genes that AP-1 regulates is MMPs, and increased MMP secretion has been associated with AP-1 activity in the MCF-7 breast cancer cell line [39]. This suggests that EGCg may have access to and interact with extracellular and intracellular proteins potentially involved in signal transduction pathways. Recent studies have indeed shown that [^{3}H]EGCg was able to incorporate into the cytosol, as well as in the nuclei [40]. Here, we show that EGCg specifically down-regulated MT1-MMP gene expression in glioma cells, but not that of MMP-2, indicating that specific regulatory mechanisms underlying transcriptional regulation of MT1-MMP may impair the initiation

of the invasive phenotype of these cells. Intriguingly, although most MMP genes are known to be strongly regulated by AP-1 and AP-2 [38,41], the consensus binding sites for these two transcription factors are absent within the MT1-MMP promoter region [42] and only AP-2 binding sites were found in the MMP-2 promoter region [43]. This implies that some unique MT1-MMP transcriptional control elements, which remain to be identified, exist and are involved in the EGCg inhibitory effect. Interestingly and although still debated, recent evidence has linked Egr-1, a key transcription factor, to the initiation of a migratory and invasive phenotype in tumor cells [44,45]. It is thus tempting to suggest this candidate transcription factor, whose consensus sites were absent from the TIMP-2 [46] and MMP-2 [47] promoter regions, as possibly involved in the EGCg inhibitory effect of both the MT1-MMP-dependent activation of proMMP-2 and MT1-MMP-driven migration in glioma cells. Supporting this hypothesis is the recently reported observation that, in EGCg-treated vascular smooth muscle cells, Egr-1 mRNA expression was strikingly reduced [10].

The second new and important intracellular effect that we observed in EGCg-treated glioma cells is the inhibition of proMMP-2 protein secretion. This observation proved to be crucial as this effect was not observed in non-metastatic cell lines. EGCg seemed to perturb the general intracellular vesicular trafficking and, thus, the secretion of other soluble proteins such as TIMP-2 (not shown). As the activation of proMMP-2 at the cell surface is regulated by the balance between MT1-MMP complexed by TIMP-2 and TIMP-2-free MT1-MMP, very low concentrations of TIMP-2 should eventually prevent the formation of the tri-molecular complex between proMMP-2/TIMP-2/MT1-MMP that would lead to MMP-2 activation and MT1-MMP autocatalytic processing to its inactive form. Such regulation of MT1-MMP proteolytic processing was shown to be specifically inhibited by TIMP-2 but not by TIMP-1, and was also recently investigated by us [50–52]. Whether EGCg acts by limiting the extracellular levels of TIMP-2 or surface binding to MT1-MMP is presently not known, but certainly deserves further investigation. We also demonstrated that EGCg acted through a brefeldin A-like mechanism and thus potentially blocked protein translocation from endoplasmic reticulum to the Golgi apparatus [25], as a concomitant increase in intracellular proMMP-2 protein levels was also observed. Interestingly, EGCg's effect on proMMP-2 secretion was comparable to that of manumycin A, a potent inhibitor of Ras farnesyltransferase. Ras signaling was indeed recently shown to play a critical role in the activation of MMP-2 secretion [27,28]. Whether some of the EGCg actions are mediated through the Ras pathway remains speculative. Taken together, all these observations thus emphasize the pleiotropic activity of EGCg on intracellular MMP-mediated events and may provide new insights into the mechanisms of action of EGCg and green tea extract as cancer-preventive agents in humans.

During the last decade, green tea made from the leaves of the plant *Camellia sinensis* has received considerable attention as a preventing agent against cancer [7,48,49]. However, although the inhibition of soluble extracellular MMPs by tea catechins and related polyphenols has been reported [24,33,35], few studies have investigated the intracellular molecular events involved in MMP secretion into the extracellular media and those involved in activation processes of the MMP zymogens. In this report, we present evidence that green tea catechins, and particularly EGCg, target multiple new intracellular functions involved in the activation and secretion of proMMP-2 from a highly invasive tumor cell line. Whether this mechanism can be generalized to other protease systems deserves further investigation. Moreover, we also provide support to a central role of MT1-MMP as being responsible in the acquisition of the invasive properties of cancer cells and that may also represent a direct target of green tea in chemoprevention of cancer. Taken together, with numerous recent reports on the inhibitory effects of these molecules on various aspects of cancer cell biology, our results emphasize the pleiotropic anticancer properties of green tea constituents. The clinical usefulness of these molecules either as chemopreventive agents or for the design of structural analogues thus represents an interesting area for future research.

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