Mechanisms of the Inhibitory Effect of Epigallocatechin-3-Gallate on Cultured Human Vascular Smooth Muscle Cell Invasion

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Objective—Although we recently showed that the administration of catechins reduced the neointimal formation in a rat balloon-injury model, the precise molecular mechanisms are largely unknown. In the present study, we tried to determine these mechanisms using an in vitro SMC invasion system.

Methods and Results—Boyden chamber assay was used to examine the effect of catechins on the invasive behavior of SMCs. The invasive activity of SMCs through collagen gel was restrained by EGCG in a concentration-dependent manner. The data from gelatin and collagen zymography and Western blot revealed that EGCG blocks the activation of pro-matrix metalloproteinase (MMP)-2 during an invasion assay and in the conditioned medium of cultured SMCs as well as the activities of MMP-2 and membrane type 1-MMP (MT1-MMP) even at 0.1 to 0.3 μmol/L of EGCG. EGCG was found to restrain MT1-MMPcat–dependent pro–MMP-2 activation. EGCG upregulated the expression of tissue inhibitor of MMP-2 (TIMP-2) protein. Reverse zymography showed that the increased TIMP-2 to expression was validated by an increased activity. The data from decreased TIMP-2 activity using its siRNA suggested that upregulation of TIMP-2 expression may be one of the major mechanisms for inhibition of SMC invasion by EGCG.

Conclusions—These results indicate that EGCG targets multiple MMP-mediated SMC cellular events and provides a new major mechanism for the SMC invasion through upregulation of TIMP-2 expression to modulate MMP activity.

(Key Words: smooth muscle cell ■ migration ■ matrix metalloproteinase ■ catechins

Several epidemiologic and experimental observations have confirmed that there is a close relationship between green tea consumption and cardiovascular disease.1–3 These effects have been largely attributed to the most prevalent polyphenol contained in green tea, epigallocatechin gallate (EGCG). Additionally, recent experiments suggest that green tea catechins reduce atherosclerotic lesions in various animal models of hyperlipidemia.3–6 Although the precise mechanisms are not yet clear, it has been postulated that flavonoids such as EGCG may act as scavengers of reactive oxygen species, thereby preventing the pathogenesis of cardiovascular disease.3–6

Because vascular smooth muscle cells (SMCs) in the large vessels are usually surrounded by and embedded in extracellular matrix (ECM) proteins, the migration of SMCs and the remodeling of tissues during the intimal thickening require controlled degradation of the ECM.7 It is known that SMCs can produce proteolytic enzymes such as matrix metalloproteinases (MMPs), which are a family of zinc-dependent proteinases involved in the degradation and remodeling of the connective tissue.7–10 The data from our groups and others showed that, among MMPs, MMP-2 is most frequently overexpressed in the carotid artery after injury, and that MMP-2 deficiency impaired neointimal formation.7,9 The activation of MMP-2 is in fact one of the crucial steps of the enzymatic cascade leading to ECM degradation. Like other MMPs, MMP-2 is produced as pro–MMP-2 and requires activation to perform its activity,8 although MMP-2 has a peculiar pathway of pro–MMP-2 activation initiated by membrane type 1-MMPs (MT1-MMP) and urokinase (urokinase plasminogen activator [uPA]).11

It has been reported that uPA, one of the hydrolases implicated in the degradation of ECM and cell invasion, is directly inhibited by EGCG.12 However, the effective concentration of EGCG seems to be too high for it to be physiologically relevant in the tea drinker.13

We recently reported that the administration of green tea catechins suppressed the neointimal hyperplasia in a rat...
carotid arterial injury model. In the study we assumed that the ingestion of catechins inhibits the neointimal formation by shifting the proteolytic balance toward the anti-proteolytic state in the injured vascular wall, resulting in the inhibition of SMC migration from the media into the subendothelial space, although no direct evidence of this has been observed. However, the exact molecular mechanisms of the inhibitory effect of green tea catechins on SMC migration remain unknown. In present study, we tried to demonstrate the mechanisms by which EGCG inhibits SMC migration through a type I collagen barrier using an in vitro cultured system.

Materials and Methods

Cell Culture
Cell culture section is described in the online data supplement available at http://atvb.ahajournals.org.

Migration and Invasion Assay
SMC migration and invasion were assessed with a modified Boyden chambers as previously described.

MT1-MMP–Dependent Pro–MMP-2 Activation Assay
To study the effect of EGCG on MT1-MMP–dependent pro–MMP-2 activation, human recombinant pro–MMP-2 (0.5 μg/mL; Oncogene) was incubated with human recombinant MT1-MMP catalytic domain (MT1-MMPcat, 15 μg/mL; Chemicon International, Inc) in reaction buffer (50 mmol/L Tris-HCl, 150 mmol/L NaCl, 20 mmol/L CaCl2, pH 7.5) with or without catechins at the indicated concentrations for 4 hours at 37°C. The results were analyzed by gelatin zymography (n=5).

Quantitative Real-Time RT-PCR
Quantitative real-time RT-PCR was performed using Assay-on-Demand and ABI PRISM 7700 Sequence Detection System on human targeted genes, MMPs (MMP-1, -2, -9, -13, and MT1-MMP), TIMP-2, and lamin A/C (assay IDs Hs00233958_m1, Hs00234422_m1, Hs00234579_m1, Hs00233992_m1, Hs00237119_m1, Hs00234278_m1, and Hs00153462_m1; Bio-systems) as previously described.

Attachment Assay
After pretreatment with an increasing concentration of EGCG (0 to 10 μmol/L) for 30 minutes, SMC attachment assay was performed as previously described.

Zymography
Gelatinase activity was detected in the SMC-conditioned media and lysates as previously described. Reverse zymography was used to detect activity of TIMP-2 in conditioned media as previously described. Equal amount of each sample was loaded for gelatin zymographic (25 μg/line) and reverse zymographic assays (60 μg/line).

Western Blotting
The levels of TIMPs and MMPs proteins as above were determined by Western blot in the SMC-conditioned media and lysates as previously described. Antibodies against for MT1-MMP, TIMP-1, TIMP-2 (3 from Sigma-Aldrich), MMP-2 (Fuji Chemical Co), MMP-9 (Santa Cruz Biotechnology, Inc), and MMP-13 (Neo Markers) were used. Each time this procedure was done, we prepared 2 gels, 1 for Western blot analysis and 1 for coomassie blue staining, to check the loading of protein as the same amounts.

Statistical Analysis
All data included in the present text were considered to be normally distributed and are presented as mean±SD unless otherwise indicated. Significant differences were analyzed by using Student t test or ANOVA followed by Scheffe multiple comparison post-hoc test. A value of P<0.05 was considered to be statistically significant.

Results

Effects of Catechins on SMC Adhesion, Migration, and Invasion
Green tea catechins that contain the galloyl group in the 3-position in the catechin structure, namely EGCG and ECG, significantly suppressed SMC invasion through a thick layer of collagen lattice, whereas little or no effect was observed with other polyphenols such as EC, C, or EGC (Figure 1A). GA alone also produced no effect (Figure 1A). Moreover, neither green tea catechins nor GA suppressed SMC migration across a filter lightly coated with type I collagen (Figure 1B).
1A). As shown in Figure 1B, the invasive activity of SMCs through the type I collagen lattice toward a chemoattractant was restrained by EGCG in a concentration-dependent manner with a minimum detected effect even at 0.1 μmol/L.

To establish the involvement of adhesion in the effect of EGCG on SMC invasion, SMCs pretreated with an increasing concentration of EGCG (0 to 10 μmol/L) were adhered to native or denatured type I collagen. No difference in the morphology and the number of attached cells was observed between native and denatured type I collagen. No difference in the morphology and the number of attached cells was observed between native and denatured type I collagen (Figure 1C and 1D). As shown in Figure 1C and 1D, SMC attachment and spreading were significantly inhibited on the denatured collagen at the high dose of EGCG (3 and 10 μmol/L). However, no significant effect of EGCG was detected on SMC attachment and spreading on native type I collagen (Figure 1C and 1D).

Effects of EGCG on MT1-MMP–Dependent Pro-MMP2 Activation, MT1-MMP Activation, and TIMP-1 and -2 Expression

Our previous study suggested that the primary MMP derived from SMCs in an invasion assay is MMP-2 but not MMP-9.17 As in that previous work, we observed that both pro- and active forms of MMP-2 were detected in the conditioned media and lysate from cultured SMCs by gelatin zymographic and Western blotting assays (data not shown). Gelatin zymographic analysis of the conditioned medium from invasion assays revealed that EGCG attenuated pro–MMP-2 conversion into a 64-kDa active form of MMP-2 in a concentration-dependent manner (0.3 to 3 μmol/L; Figure 2A). As shown in Figure 2A, we also observed that EGCG suppressed total gelatinolytic activity in a concentration-dependent manner. We further analyzed the effect of EGCG on the production of MMP-2 by SMCs. As shown Figure 2B and 2C, EGCG did not affect total MMP-2 production even at 10 μmol/L, but it blocked the conversion of pro–MMP-2 into the active form in cultured SMC-conditioned medium. Similarly, the data from Western blotting analysis of the lysate showed that 10 μmol/L of EGCG did not affect MMP-2 and MT1-MMP production (data not shown).

Recent evidence has suggested that MT1-MMP activation itself is required to perform pro–MMP-2 activation.17 We next examined whether EGCG affects Con-A–induced MT1-MMP activation in SMCs. As shown in Figure 2D, EGCG had no effect on the Con-A–induced MT1-MMP activation of SMCs compared with the control SMC. To investigate whether EGCG directly inhibits MT1-MMP–dependent pro–MMP-2 activation, recombinant pro–MMP-2 was incubated with recombinant MT1-MMPcat and then incubated with catechins. Gelatin zymographic analysis revealed that the incubation of pro–MMP-2 with MT1-MMPcat yielded bands at 68 and 64 kDa, which seem to correspond to the intermediate and mature form of MMP-2 (Figure 3A). Again EGCG attenuated total gelatinolytic activity in a concentration-dependent man-

Figure 2. Effects of EGCG on the expression and activation of MMP-2 and MT1-MMP. A, The ratio of the activated form of MMP-2 to total MMP-2 was assessed by gelatin zymography of the conditioned medium derived from the invasion assay (n=5). The values were obtained by densitometric evaluation of gelatin zymography and are presented as relative gelatinolytic activity. B and C, The total MMP-2 (B) and the ratio of the activated form of MMP-2 to total MMP-2 (C) were assessed by a Western blot (n=5) of the conditioned medium derived from the cultured SMCs. D, The ratio of the activated form of MT1-MMP to total MT1-MMP was assessed by a Western blot (n=5) of the SMC lysates stimulated with Con-A (20 μg/mL). The values were obtained by densitometric evaluation of the Western blot. *P<0.05, **P<0.01, ***P<0.001 vs control.

Figure 3. Effects of EGCG on MT1-MMPcat–dependent pro–MMP-2 activation. A, Pro–MMP-2 (0.5 μg/mL) was incubated with MT1-MMPcat (15 μg/mL) in reaction buffer in the presence of an increasing concentration of EGCG for 4 hours. B, Pro–MMP-2 was incubated with MT1-MMPcat in the presence of EC, C, EGC, ECG, or EGCG at 1 μmol/L for 4 hours. Samples were analyzed by gelatin zymography (n=5), and MMP-2 activation is expressed as a percentage of both the intermediate and the active form band densities to pro–MMP-2 band density. In our zymograms, mature MMP-2 was created by the activation of pro–MMP-2 by APMA as a positive control. The values were expressed as relative gelatinolytic activity. *P<0.05, **P<0.01, ***P<0.001 vs control.
ner (Figure 3A). In addition, EGCG significantly inhibited MT1-MMP cat–dependent pro–MMP-2 activation in a concentration-dependent manner with a minimum detected even at 0.3 μmol/L (Figure 3A). ECG was also able to suppress MT1-MMP cat–dependent pro–MMP-2 activation, although other catechins had no effect (Figure 3B).

Analysis of the levels of TIMP-1 and TIMP-2 proteins in the conditioned media from an invasion assay revealed that EGCG significantly increased the TIMP-2 level in a concentration-dependent manner with a minimum detected at 0.1 μmol/L (Figure 4A) but had no effect on TIMP-1 (data not shown). Consistently, reverse zymography revealed TIMP-2 activity also increased in conditioned media from an invasion assay in a concentration-dependent manner (Figure 4B). When SMCs were cultured in serum-free medium with or without EGCG for 24 hours, the level of TIMP-2 protein was upregulated by EGCG not only in cultured medium but also in the lysates from cultured SMCs in a concentration-dependent manner (data not shown).

Effect of Transfection Using TIMP-2 siRNA on SMC Invasion

The 83% reduction of TIMP-2 mRNA levels was observed in the TIMP-2 siRNA oligo-transfected human SMCs (Figure 4C). As shown in Figure 4D, the impaired invasion ability of SMCs treated with 3 μmol/L of EGCG was significantly recovered by the reduction of TIMP-2 mRNA using TIMP-2 siRNA, although no significant difference of SMC invasive activity was detected without EGCG between control and TIMP-2 siRNA-treated SMCs. It should be noted that only low level of MMP-9 mRNA was detected in cultured SMCs.

Effects of EGCG on MMP-13 Expression and Activation in SMCs

It has recently been reported that SMCs are able to produce MMP-13 and that MMP-13 possesses a potential property for degrading collagen.7,18 The Western blotting data of the conditioned medium from the cultured SMCs showed that the high concentration of EGCG (10 μmol/L) significantly inhibited the production and activation of MMP-13, whereas no change was detected at the low concentration of EGCG (0.3 to 3 μmol/L) (Figure 5A and 5B). It is likely that the inhibitions of MMP-13 activation and expression would be insufficient to bring about SMC invasion suppression by EGCG at the physiological plasma concentration.13 We also observed that EGCG at low concentration (1 to 3 μmol/L) had no effect on the expression of MMP-1, -2, -13, and MT1-MMP mRNA (data not shown). It is consistent with the results of Western blotting analysis of their proteins in the SMC-conditioned media and lysates that EGCG at low concentration did not affect production of MPPs proteins (data not shown). It should be noted that only low level of MMP-9 mRNA was detected in cultured SMCs.

**Figure 4.** Effects of EGCG on TIMP-2 expression and activity. A and B, The content of TIMP-2 protein and the activity of increased TIMP-2 in the conditioned media from an invasion assay were examined by Western blot analysis and reverse zymography (n=5). The values were obtained by densitometric evaluation of the Western blot and reverse zymography. C, After transfection with TIMP-2 siRNA or siCONTROL Non-targeting siRNA, respectively, the reduction of TIMP-2 mRNA was analyzed by real-time RT-PCR. After being transfected with TIMP-2 siRNA or siCONTROL Non-targeting siRNA for 24 hours, respectively, the cells were used to perform invasion assays in presence or absence of EGCG. The values are expressed as a percentage of the control values. *P<0.05, **P<0.01, ***P<0.001 vs control. #P<0.05, ##P<0.01 vs those controls for transfection with Non-targeting siRNA.

**Figure 5.** Effects of EGCG on MMP-13 expression and activation and MT1-MMP collagenolytic activity. A and B, After being cultured in serum-free SmBM, SMCs were treated with an increasing concentration of EGCG for 24 hours in DMEM. The total MMP-13 (A) and the ratio of the active form to total MMP-13 (B) in the conditioned medium were analyzed by Western blot (n=5). C, The effect of EGCG on the collagenolytic activity of MT1-MMP cat was examined by collagen zymography. The values were obtained by densitometric evaluation of Western blot and collagen zymography and are presented as relative collagenolytic activity. *P<0.05, **P<0.01, ***P<0.001 vs control.
Discussion

As revealed by the invasion assay, green tea catechins that have the 3-galloyl group, EGCG and ECG, suppress SMC invasion at a physiological concentration as low as 0.1 μmol/L EGCG, whereas little or no effect is observed with EC, C, EGC, or GA. These findings, which suggest that the galloyl group in the 3-position in catechin structure is necessary for the inhibitory effect on SMC invasion, are in agreement with previous observations of tumor cell invasion in the same range of concentrations.15 The present work revealed that EGCG had no effects on SMC adhesion to the native and denatured type I collagen at the low concentration of EGCG. Taken together with the observation that neither green tea catechins nor GA suppressed SMC migration, it is suggested that the inhibitory effect of EGCG on SMC invasion is not attributable to the cell adhesion and motility impairment but rather to the inhibition of proteinases derived from SMCs, at least at the physiological concentrations of EGCG that we used.

A number of studies have shown that among MMPs, MMP-9 is expressed in injured arterial walls only in the early phase after injury, that MMP-2 is expressed constitutively in a latent form in the arterial wall and overexpressed in an activated form during the period from 5 to 14 days after injury, and that MMP-2 deficiency significantly reduced neointimal hyperplasia.9,14,20 In the present work we observed that both pro- and active-form MMP-2 but not MMP-9 were produced in the conditioned medium and lysates on the basis not only of an SMC invasion assay but also of cultured SMCs, a finding that is consistent with our previous findings.8 However, the data from our and other groups’ studies indicated that MMP-9 may be namely secreted by infiltrated inflammatory cells at early period after the injury and plays an important role for atherosclerotic lesion and neointimal formation.9,14,20,21 We previously demonstrated that the inclusion of MMP inhibitors blocked SMC invasion in accompany with the inhibition of pro–MMP-2 activation.8 These findings indicate that the activated MMP-2 plays a fundamental role in SMC invasion. Based on these observations, we conclude that MMP-2 is one of the most essential MMPs involved in the invasive behavior of SMCs through type I collagen barrier.

We and others previously demonstrated that EGCG inhibits MMP-2 activity even at physiological concentrations of EGCG.13,15,19 We also observed that EGCG binds to pro- as well as the mature form of MMP-2, leading to reversible conformational changes in MMP-2 molecules or to masking the catalytic region of MMP-2, either of which may be essential for proteolytic activity.15 The EGCC–MMP-2 complex is not dissociated during the zymographic analysis, resulting in the inhibition of the gelatinolytic activity of MMP-2 pretreated with EGCG in gelatin zymography. In the present study we confirmed that the treatment of MMP-2 with EGCG attenuated its gelatinolytic bands in a concentration-dependent manner. On the other hand, the activation of pro–MMP-2 by MT1-MMP is thought to represent a crucial step in tumor invasion and metastasis.10,22 In gliomas, particularly, MT1-MMP–dependent pro–MMP-2 activation has been strongly correlated with glioma invasiveness.23 These findings led us to investigate the effect of green tea catechins on MT1-MMP–dependent pro–MMP-2 activation in SMCs during their invasive process. In the present study, we clearly demonstrated that EGCG blocks the activation of SMC-derived pro–MMP-2 during SMC invasion and the culture of SMCs. It should be noted that EGCG was found to have no effect on Con-A–induced MT1-MMP activation in SMCs. A large number of studies have shown that EGCG inhibits MMPs expression including MMP-1, -2, -9, and MT1-MMP in various cell types.24,25 However, the effective concentrations of EGCG seem to be too high for it to be physiologically relevant for tea drinkers. In fact, EGCG at low concentration (1 to 3 μmol/L) had no effect on the expression of MMPs (MMP-1, -2, -13, and MT1-MMP). Overall, these results suggest that modulation of MMP expression is not a like mechanism for the inhibitory effect of EGCG on SMC invasion at the concentration of EGCG similar to those in the plasma of moderate green tea drinkers (≥0.1 to 0.3 μmol/L).13

In addition, Western blot and reverse zymography data revealed that EGCG significantly stimulated the production of TIMP-2 protein in SMCs during their invasion process and cultured SMC but had no effect on TIMP-1, a finding that is consistent with our previous finding that the administration of green tea catechin upregulates TIMP-2 levels in the balloon injured carotid arteries.14 As sufficient amounts of TIMP-2 specifically inhibit both MMP-2 gelatinolytic activity and pro–MMP-2 activation by MT1-MMP,8,23 it is possible that EGCG inhibits not only MMP-2 gelatinolytic activity but also its transformation into an activated form of MMP-2 through TIMP-2 upregulation. Additionally, we clearly showed that EGCG directly blocks MT1-MMP cat–dependent pro–MMP-2 activation, suggesting a possible mechanism whereby the influence of EGCG on SMC invasion is mediated by the inhibition of MT1-MMP–dependent pro–MMP-2 activation via its direct effect on MT1-MMP activity. This hypothesis is supported by the present finding that, in collagen zymographic analysis, EGCG inhibited MT1-MMP activity. Given that EGCG inhibits the gelatinolytic activity of mature MMP-2, there are 3 possible mechanisms by which EGCG inhibits MMP-dependent SMC invasion through the fibrillar collagen barrier (Figure I, available online at http://atvb.ahajournals.org). First, it is possible that EGCG directly inhibits the gelatinolytic activity of MMP-2 and the collagenolytic activity of MT1-MMP; second, EGCG may directly block MT1-MMP–dependent pro–MMP-2 transformation into an active form of MMP-2 without the influence of MT1-MMP activation but via the inhibition of MT1-MMP activity; and third, EGCG may inhibit MMP-2 gelatinolytic activity and pro–MMP-2 activation through TIMP-2 upregulation in SMCs. In fact, we clearly showed that the reduction of TIMP-2 mRNA using TIMP-2 siRNA recovered the inhibitory effect of EGCG on SMC invasive behavior, and especially TIMP-2 siRNA treatment completely recovered the effect of low concentration of EGCG (0.3 μmol/L). These results suggest that the third one may be one of the major effects of EGCG, at least at the physiological concentration of EGCG.
In this report, we demonstrated that EGCG inhibited the invasion of SMC through a type I collagen barrier via the modulation of MT1-MMP–dependent pro–MMP-2 activation and upregulation of TIMP-2 expression and directly inhibition of MMP-2 and MT1-MMP. These findings 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 atherosclerosis- and restenosis-preventive agents in humans. Drinking green tea daily would contribute to maintaining atherosclerosis- and restenosis-preventive agents in humans.

Increasing evidence suggests that clinical trials of catechins for arterial remodeling such as neointimal formation of MMP-2 and MT1-MMP. These findings 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 atherosclerosis- and restenosis-preventive agents in humans. Drinking green tea daily would contribute to maintaining atherosclerosis- and restenosis-preventive agents in humans.

References

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Figure I

- Pro-MMP-2 to Activated MMP-2
- Activated MT1-MMP
- Pro-MT1-MMP to Smooth muscle cells
- TIMP-2

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Online Figure Legend

**Figure I.** Proposed mechanism of the inhibitory effects of EGCG on multiple MMP-mediated SMC cellular events. Three possible mechanisms (1: EGCG directly inhibits the gelatinolytic activity of MMP-2 and the collagenolytic activity of MT1-MMP; 2: EGCG directly block MT1-MMP-dependent pro-MMP-2 transformation into an active form of MMP-2 without the influence of MT1-MMP activation but via the inhibition of MT1-MMP activity; 3: EGCG inhibit MMP-2 gelatinolytic activity and pro-MMP-2 activation through TIMP-2 up-regulation in SMCs) may be responsible for the inhibitory effect of EGCG on SMC invasion.
Online Data Supplement

Materials and Methods

Cell Culture

Human aortic SMCs (HSMCs, Bio Whittaker) were subcultured at passages 4-10 for the following experiments in SmBM supplemented with SmGM-2 Single Quots (both from Cambrex Bio Science Walkersville, Inc). The catechins used in this study were EGCG, epigallocatechin (EGC), epicatechin gallate (ECG), epicatechin (EC), catechin (C), and gallic acid (GA) (Sigma-Aldrich, Louis, MO).

To examine the effects of EGCG on MMP expression and activation, SMCs at a density of $10^5$ cells/well were cultured in serum-free SmBM for 24 hours, and then treated with an increasing concentration of EGCG (0 to 10 µmol/L) for 24 hours. At the end of the incubation, the lysates and conditioned media were collected and concentrated to examine the levels of pro-MMP-2, MMP-9, and MMP-13, as well as their active forms, using zymography and Western blot. We also examined the levels of TIMP-1 and –2 in the lysates. For quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR) analysis, total RNA was extracted using the Rneasy Mini Kit according to the manufacture’s protocol (Qiagen Inc., Valencia, CA). Following treatment with or without concanavalin A (Con-A, 20 µg/mL; Sigma-Aldrich, Louis, MO) in the presence of an increasing concentration of EGCG, the SMCs were harvested and the levels of the pro- and activated-MT1-MMP were examined by Western blot.