Catechins prevent vascular smooth muscle cell invasion by inhibiting MT1-MMP activity and MMP-2 expression

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Abstract

Objective: Regular consumption of green tea is associated with a reduced risk of mortality due to coronary diseases and cancer. The present study examined whether a green tea extract (GTE) inhibits activation of matrix metalloproteinase-2 (MMP-2), a major collagenase involved in vascular remodeling of atherosclerotic plaques, in vascular smooth muscle cells (VSMCs).

Methods and results: The expression of MMP-2 was assessed by Northern and Western blot analyses in human aortic VSMCs. MMP-2 activity was evaluated by zymography, membrane-type1-MMP (MT1-MMP, MMP-14) activity by an enzymatic assay, and cell invasion by a modified Boyden chamber assay. The thrombin-induced activation of secreted MMP-2 was abolished by GTE and the green tea polyphenols (−)-epigallocatechin-3-gallate (EGCG) and (−)-epicatechin-3-gallate (ECG). GTE reduced the expression of MMP-2 mRNA and protein. GTE, EGCG and ECG directly inhibited cell-associated MT1-MMP activity, the physiological activator of MMP-2, in a reversible manner. Thrombin-stimulated VSMCs invasion was abolished by EGCG and ECG, and reduced by GTE.

Conclusions: GTE inhibits thrombin-induced VSMCs invasion most likely by preventing MMP-2 expression and its activation by a direct inhibition of MT1-MMP. The ability of green tea to prevent cell invasion and matrix degradation might contribute to its protective effect on atherosclerosis and cancer.

Keywords: Matrix metalloproteinases; Atherosclerosis; Smooth muscle

1. Introduction

Regular consumption of tea has been associated with a reduced risk of coronary heart disease and cancer in some but not all epidemiological studies [1–4]. Clinical studies have indicated that intake of green tea or black tea reduced the risk of myocardial infarction [3,5] and increased survival after acute myocardial infarction [6]. Moreover, chronic administration of green tea reduced the development of atherosclerotic lesions without influencing established atherosclerosis in experimental models of atherosclerosis [7,8]. Although the mechanism underlying the beneficial effects of tea intake remains unclear, it has been attributed at least in part to the high content of flavonoids, especially of the catechin group of the flavanols [7]. Green tea extract (GTE) comprises approximately 30% of the dry weight of green tea leaves and includes predominantly (−)-epigallocatechin-3-gallate (EGCG), (−)-epicatechin (EC), (−)-epicatechin-3-gallate (ECG), (−)-epigallocatechin (EGC), and catechin [9]. Potential protective effects of tea catechins include their ability to prevent LDL oxidation [10,11], to inhibit platelet aggregation [12,13] and pro-thrombotic responses [13] and to decrease smooth muscle cell proliferation and migration [14–16]. In addition, green tea catechins can also increase

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the protective effect of endothelial cells on the arterial wall by stimulating the endothelial formation of nitric oxide [17,18] and prostacyclin [19].

The development of human atherosclerotic lesions to advanced plaques is characterized by extensive vascular remodeling with local accumulation of lipids, vascular smooth muscle cells and macrophages and the formation of microvessels [20]. Analysis of human atherosclerotic lesions and advanced plaques has indicated a strong expression of matrix metalloproteinases (MMPs), especially the gelatinases MMP-2 and MMP-9, predominantly by vascular smooth muscle cells, macrophages and possibly also endothelial cells [21–24]. MMP-2 is constitutively expressed and secreted as a latent zymogen, pro-MMP-2, and its main activation takes place on the cell surface and is mediated by membrane-type metalloproteinases (MT-MMPs) such as MT1-MMP [25,26]. Once activated, MMP-2 is able to digest components of the basement membrane such as type IV collagen and fibronectin [27]. Potential physiological inducers of MMP-2 activation include thrombin, plasmin, lysophosphatidylcholine, a major component of oxidized low density lipoprotein, oxidative stress, and mechanical forces [28–33]. Altogether, these findings suggest that gelatinases are likely to promote the development of atherosclerotic lesions and possibly also of clinical complications of atherosclerosis, such as plaque vulnerability by inducing a collagen poor environment, which facilitates cell migration, proliferation and neovascularization [34]. Therefore, the purpose of the present study was to determine whether GTE affects gelatinase expression and activation in VSMCs and the invasion capacity of these cells. Moreover, experiments were also performed to determine active catechins and to elucidate the underlying mechanism.

2. Methods

2.1. Reagents

GTE and the catechins were purchased from Alexis Corporation (Lausen, Switzerland). α-Thrombin was obtained from Etablissement Français du Sang (Strasbourg, France), GM6001 from Chemicon Europe (Hofheim/TS, Germany), and the chromogenic substrate S-2238 from Haemochrom Diagnostica GmbH (Essen, Germany).

2.2. Cell culture

Human aortic VSMCs were obtained by permission of the Declaration of Helsinki and supplied by BioWhittaker (Walkersville, USA). They were maintained in MCDB131 containing 10% fetal calf serum and antibiotics. All experiments were performed with VSMCs from passages 5 to 15, which were exposed to serum-free culture medium containing 0.1% bovine serum albumin for 24 h.

2.3. Gelatin zymography

MMP-2 activity in conditioned medium of cultured VSMCs was analyzed by substrate-gel electrophoresis (zymography) using SDS-PAGE (10%) containing 0.1% gelatin. Equal volumes of samples of conditioned cell culture medium were mixed with Laemmli-buffer under non-reducing conditions, loaded onto the gel and separated by electrophoresis. Thereafter, gels were washed 3 times for 30 min at room temperature in buffer (50 mM Tris–HCl, pH 8.0, 5 mM CaCl2, 0.02% NaN3, and 2.5% Triton X-100) and incubated for 18 h at 37 °C with same buffer except Triton X-100. Gels were stained with Coomassie Brilliant Blue R-2500 (0.1%) and destained in 5% methanol and 7% acetic acid. Gelatinolytic activity appeared as a clear band on a blue background.

2.4. Western blot and Northern blot analyses

The serine protease activity of thrombin was assessed using the chromogenic substrate S-2238. Optical densities were measured in a spectrometer at 405 nm. MT1-MMP activity was determined by using a commercial MT1-MMP activity assay kit (Amersham; Orsay, France).

2.5. Thrombin and MT1-MMP activity assays

2.6. Cell invasion assay

VSMCs invasion through the extracellular matrix was determined by using a commercial cell invasion assay kit (Chemicon Europe).
2.7. Statistical analysis

Results are shown as mean±SEM. Statistical analyses were performed using ANOVA followed by Fisher’s test to compare 2 treatments. A value of \(P<0.05\) was considered statistically significant.

3. Results

3.1. Green tea extract prevents thrombin-induced activation of MMP-2

Exposure of untreated VSMCs to serum-free culture medium was associated with the release of large amounts of the inactive zymogen pro-MMP-2 and small amounts of active MMP-2 into conditioned medium (Fig. 1A). Treatment of VSMCs with thrombin increased the conversion of secreted pro-MMP-2 into MMP-2 in a time- and concentration-dependent manner (Fig. 1A, [38]). The stimulatory effect of thrombin was reduced in a concentration-dependent manner by the treatment of VSMCs with GTE for 30 min before the addition of thrombin (Fig. 1A). A significant inhibitory effect was observed at concentrations as low as 3 \(\mu\)g/ml of GTE (Fig. 1A). GTE at concentrations below 10 \(\mu\)g/ml did not affect the basal secretion of pro-MMP-2 whereas a significant reduction was observed with higher concentrations (Fig. 1A). In contrast to GTE, the basal secretion of pro-MMP-2 and the thrombin-induced activation of MMP-2 were not affected by antioxidants such as vitamin C (200 \(\mu\)M) and the combination of the membrane permeant analogues of superoxide dismutase and catalase.

Fig. 1. GTE inhibits the thrombin-induced activation of MMP-2 in a concentration-dependent manner in VSMCs without affecting the catalytic activity of thrombin. (A) VSMCs were incubated with different concentrations of GTE for 30 min before the addition of thrombin for 24 h. Thereafter, the conditioned medium was analyzed by gelatin zymography. (B) GTE did not affect the catalytic activity of thrombin as assessed using the chromogenic substrate, S-2238. Thrombin was incubated with S-2238 (200 \(\mu\)M) in absence or presence of GTE for 2 min, and thereafter optical densities were measured in a spectrophotometer at 405 nm. Results are shown as mean±SEM for 4 different experiments. *\(P<0.05\) vs. control; #\(P<0.05\) vs. thrombin treatment.
(MnTMPyP, 100 μM and polyethylene glycol-catalase 500 U/ml, respectively, data not shown). Since the proteolytic activity of thrombin is essential for MMP-2 activation [28,38,39], the direct effect of GTE on the catalytic activity of thrombin was assessed using the chromogenic substrate S-2238. GTE did not affect the proteolytic activity of thrombin (Fig. 1B).

Similarly to GTE, exposure of VSMCs to the green tea polyphenols EGCG and ECG markedly reduced the thrombin-induced activation of MMP-2, whereas only a small inhibitory effect was obtained with EGC and no such effect was obtained with EC and catechin (Fig. 2A). The inhibitory effect of EGCG and ECG was concentration-dependent with a significant inhibition obtained at concentrations greater than 10 and 30 μM, respectively (Fig. 2B). At high concentration (50 μM), both catechins alone also significantly reduced the basal secretion of pro-MMP-2 by 74.8% and 62.2%, respectively (Fig 2B). EGCG (30 μM) and ECG (30 μM) did not affect the catalytic activity of thrombin (2 U/ml, the values were 97.5% and 98.7%, respectively, n = 4).

GTE and EGCG did also not affect cell viability as assessed by CellTiter 96 MDSU aqueous one solution cell proliferation assay (Promega; the values were 97.7% and 99% in GTE (30 μg/ml) and EGCG (30 μM)-treated cells after a 24-h incubation period, respectively, n = 4–6).

### 3.2 GTE and green tea polyphenols inhibit pro-MMP-2 expression

Next, the possibility that thrombin and GTE affect pro-MMP-2 expression in VSMCs was assessed by Western and Northern blot analyses. A significant expression of pro-MMP-2 mRNA was found in untreated VSMCs and this signal was not affected by thrombin (Fig. 3A). Exposure of VSMCs to GTE significantly reduced pro-MMP-2 mRNA

**Fig. 2.** The green tea polyphenols EGCG and ECG inhibit the thrombin-induced activation of MMP-2 in a concentration-dependent manner in VSMCs. (A) VSMCs were incubated with GTE or a green tea polyphenol (EGCG, catechin, EC, ECG, or EGC) for 30 min before the addition of thrombin for 24 h. Thereafter, the conditioned medium was analyzed by gelatin zymography. (B) VSMCs were incubated with different concentrations of either EGCG or ECG for 30 min before the addition of thrombin for 24 h. Results are shown as mean ± SEM for 3 different experiments. *P < 0.05 vs. control; #P < 0.05 vs. thrombin treatment.
levels in the absence or presence of thrombin (Fig. 3A). Consistent with the expression of pro-MMP-2 mRNA observed in VSMCs, pro-MMP-2 protein was detected by Western blot analysis in cell lysates and conditioned media from untreated VSMCs (Fig. 3B). Thrombin significantly increased cell-associated pro-MMP-2 protein levels but did not affect its secreted levels (Fig. 3B). GTE markedly reduced pro-MMP-2 protein levels in both cell lysates and conditioned media of control and thrombin-stimulated cells (Fig. 3B). EGCG (30 μM) and ECG (30 μM) also significantly reduced pro-MMP-2 levels in cell lysates of control cells by 51.6%±12.8% and 18.8%±4.7% and those induced by thrombin (2 U/ml) by 63.8%±10.7% and 51.5%±15.5%, respectively (n=4).

3.3. Green tea polyphenols directly inhibit MT1-MMP activity

Previous studies have indicated that the cell membrane-dependent activation of pro-MMP-2 by thrombin is mediated by MT-MMPs and, in particular by MT1-MMP in endothelial cells [28,39]. In addition, thrombin has also been shown to induce MT1-MMP expression in endothelial cells [39]. Experiments were therefore performed to test the effect of thrombin and GTE on MT1-MMP activity and expression in VSMCs. MT1-MMP mRNA and protein were observed in untreated cells and these signals were affected by neither thrombin nor GTE (Fig. 4). In contrast, the cell-associated MT1-MMP activity was strongly
increased in thrombin-treated cells compared to control cells (Fig. 5A). This thrombin-induced MT1-MMP activity was abolished by pretreatment of VSMCs with GTE that also repressed the basal MT1-MMP activity by about 63% (Fig. 5A). In addition, GTE, EGCG and ECG also repressed MT1-MMP activity extracted from basal and thrombin-stimulated cells when added directly to the enzymatic assay (Fig. 5B). The inhibitory effect of GTE was observed at concentrations as low as 1 µg/ml (Fig. 5C) and it was mimicked by the broad-spectrum MMP inhibitor GM6001 (thrombin-induced MT1-MMP activity was abolished at 1 µM, n = 4). Moreover, the inhibitory effect of GTE (30 µg/ml) on MT1-MMP activity was reversible since MT1-MMP activity recovered to 94.8 ± 7.5% (n = 3) after three sequential changes of the incubation medium to wash out polyphenols.

3.4. Green tea polyphenols inhibit thrombin-induced extracellular matrix invasion by VSMCs

The possibility that green tea polyphenols prevent thrombin-induced VSMCs invasion through extracellular matrix was tested using a cell invasion assay. VSMCs were stimulated with thrombin in presence or absence of GTE, EGCG, ECG, EC or GM6001 for 72 h. Thrombin-stimulated, the matrix invasion of VSMCs and this response was almost abolished by EGCG (Fig. 6). The stimulatory effect of thrombin was also significantly reduced by GTE, ECG and GM6001 but not affected by EC (Fig. 6).

4. Discussion

The present findings reveal that GTE is a strong inhibitor of the expression of pro-MMP-2 mRNA and protein, and, more importantly, of the activation of the secreted MMP-2 in response to thrombin in VSMCs. Two major tea catechins, EGCG and ECG are able to mimic the inhibitory effect of GTE on MMP-2 activation whereas catechin and EC were inactive and EGC caused only a modest inhibition. The inhibitory effect of GTE and EGCG is detected at
concentrations as low as low 3 µg/ml and 10 µM, respectively. Previous studies have indicated that consumption of green tea extract (1.5–4.5 g, 1–3 cups) by healthy humans is associated with the appearance of EGCG, ECG, and EC in the plasma, and that the plasma concentration of EGCG is about 4 µM [40–42]. Thus, the inhibitory effect of green tea catechins on MMP-2 activation observed in the present study occurs at concentrations that are likely to be achieved in the plasma of moderate green tea drinkers.

Previous studies have indicated that the thrombin-induced activation of MMP-2 is strictly dependent on its catalytic activity by a mechanism distinct from the proteolytic activation of protease-activated receptor-1, a major thrombin receptor expressed in VSMCs [28,38,39]. Therefore, the possibility that the inhibitory effect of green tea catechins is due to a direct inhibition thrombin was examined. However, since the present findings indicate that green tea catechins did not affect the proteolytic activity of thrombin, such an explanation is unlikely. An alternative explanation might be related to the antioxidant properties of green tea catechins [43]. Indeed, reactive oxygen species have been shown to activate MMP-2 and thrombin is a potent stimulator of the NADPH oxidase-dependent formation of reactive oxygen species in VSMCs [32,33,44–46]. However, in our experimental model of VSMCs, the stimulatory effect of thrombin remained unaffected by antioxidants such as N-acetylcysteine and vitamin C [38, present findings]. Moreover, MMP-2 activation was not induced by other potent activators of NADPH oxidase, including angiotensin II and platelet-derived growth factor AB, or by hydrogen peroxide [38, present findings]. Thus, the inhibitory effect of green tea catechins on MMP-2 activation appears to be independent of their antioxidant properties since the thrombin-induced MMP-2 activation is a redox-insensitive event in VSMCs.

The ability of thrombin to activate MMP-2 is markedly accelerated in the presence of cells and this effect has been attributable to the binding of pro-MMP-2 to cell-surface MT1-MMP and its activation through limited proteolysis by MT1-MMP [25]. In addition, immunohistochemical studies have shown that sites of intense vascular remodeling are associated with a pronounced expression of MT1-MMP such as in human atherosclerotic plaques predominantly in VSMCs and macrophages [47], and also in neointimal VSMCs following balloon injury [48,49]. VSMCs express MT1-MMP at low levels and this signal can be increased by pro-inflammatory cytokines such as interleukin-1β, tumor necrosis factor-α and oxidized LDL [47,49]. An increased expression of MT1-MMP both at the mRNA and protein level has also been observed in response to thrombin tested at 10 U/ml in endothelial cells [39]. In contrast, the present findings indicate that a lower concentration of thrombin (2 U/ml) did not affect the expression of MT1-MMP in VSMCs but it markedly increased cell-associated MT1-MMP activity. They also indicate that GTE directly and effectively inhibited the activity of MT1-MMP at concentrations similar to those preventing activation of MMP-2. The inhibitory effect of GTE was mimicked by EGCG and ECG. These findings are consistent with a previous one indicating a direct inhibition of the catalytic domain of MT1-MMP by EGCG [50]. In addition, neither GTE nor thrombin affected the expression of TIMP-2, the major inhibitor of MMP-2, in VSMCs (data not shown). Altogether, these findings indicate that the prevention of MMP-2 activation by green tea catechins is likely to be mediated by the direct inhibition of MT1-MMP activity. Because these effects are reversible, the continuous presence of green tea catechins is required to prevent efficiently the degradation of extracellular matrix components. Finally, and more importantly, the active green tea catechins prevented thrombin-induced VSMCs invasion at least as efficiently as a broad-spectrum MMP inhibitor. Since tea catechins did not affect VSMCs migration [14], this striking effect further supports the potential role of green tea catechins in the control of matrix degradation. Green tea catechins also prevented effectively matrix degradation and cell invasion in experiments with several cancer cell lines [51,52]. Besides matrix degradation and cell invasion, tea catechins might also prevent vascular remodeling and angiogenesis, by inhibiting cell proliferation [53] and the expression of vascular endothelial growth factor, a major pro-angiogenic factor [54,55] and of its receptor VEGF receptor-2 [56,57].

In conclusion, the present findings indicate that a GTE and green tea catechins are potent inhibitors of VSMCs invasion and that this effect involves most likely their ability to prevent MMP-2 expression and its activation via the direct inhibition of the physiological activator of MMP-2, MT1-MMP. The prevention of extracellular matrix degradation might contribute to the ability of green tea to retard the development of atherosclerotic lesions by preventing plaque vascularization and remodeling.
References


