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Regulation of urokinase plasminogen activator by epigallocatechin-3-gallate in human fibrosarcoma cells

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Abstract

(−)-Epigallocatechin-3-gallate (EGCG), a main flavanol of green tea, potently suppressed the urokinase-type plasminogen activator (uPA) expression in human fibrosarcoma HT 1080 cells. EGCG induced not only the suppression of the uPA promoter activity but also the destabilization of uPA mRNA. EGCG inhibited the phosphorylation of extracellular signal-regulated kinases 1 and 2 (Erk-1/2) and P38 mitogen-activated protein kinase (MAPK), but not the phosphorylation of c-*jun* N-terminal kinase (JNK) and Akt. Specific inhibitors of Erk-1/2 (2′ -amino-3′ -methoxyflavone, PD98059) and P38 MAPK (pyridinylimidazole, SB203580) were found to suppress the uPA expression and the uPA promoter activity. However, the specific inhibitors did not affect the uPA mRNA stability. These results suggest that EGCG could regulate the uPA expression by at least two different mechanisms: EGCG may inhibit the Erk-1/2 and P38 MAPK, leading to suppression of the uPA promoter activity, and EGCG may destabilize the uPA mRNA in an Erk-1/2- and p38 MAPK-independent way.

Keywords: EGCG ((-)-epigallocatechin-3-gallate); uPA (urokinase-type plasminogen activator); Erk-1/2; P38 MAPK; Transcription; mRNA stability

1. Introduction

Epidemiological studies have suggested that ingestion of green tea may decrease the cancer risk (Yu et al., 1995; Ji et al., 1997). In addition, many preclinical studies have provided the convincing evidence that some substances of green tea may afford protection against cancer (Jung et al., 2001; Rogers et al., 1998). The anticarcinogenic effects of green tea have been attributed to the biological activities of its polyphenol components. Green tea extract contains catechins such as (−)-epigallocatechin gallate (EGCG), (−)-epigallocatechin (EGC), (−)-epicatechin gallate (ECG), and (−)-epicatechin (EC; Stoner and Mukhtar, 1995). EGCG, the most abundant polyphenol in green tea, has been shown to inhibit proliferation and induce apoptosis of tumor cells (Jung et al., 2001).

In addition to having a cancer chemopreventive activity, polyphenols have been shown to inhibit tumor invasion which is a crucial step for the growth and metastasis of all solid tumors. Cancer invasion and metastasis are multifactorial processes and require the coordinated action of cell-secreted proteolytic enzymes and their inhibitors (Liotta, 1986). Urokinase-type plasminogen activator (uPA) and uPA receptor form a complex proteolytic system which has been implicated in cancer invasion and metastasis. Inhibition of uPA activity leads to inhibition of tumor invasion in several experimental systems, and uPA is selectively expressed at invasive foci in some experimental and human cancers (Ossowski et al., 1991).

Recently, it was proposed that the anti-invasive activity of EGCG is associated with inhibition of the activity of uPA (Jankun et al., 1997). EGCG can directly impair the activity of urokinase and its role in degradation of extracellular matrix. Jankun et al. (1997) demonstrated, through the use of computer-based molecular modeling, that EGGC binds to urokinase, blocking the histidine 57 and serine 195 residues of the urokinase catalytic triad and extending towards arginine 35 from a positively charged loop of urokinase. Such localization of EGCG would interfere with the ability of uPA to recognize its substrates and inhibit its enzymatic activity.

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We have found that EGCG can potently suppress the uPA expression in tumor cells. In this study, to investigate the mechanism for the EGCG-mediated regulation of uPA expression, we examined the effects of EGCG on the uPA promoter activity, uPA mRNA stability, and relevant signalings in human fibrosarcoma HT 1080 cells. Understanding the basic principles of the inhibitory effects of EGCG on tumor invasion may lead to development of new therapeutic strategies, in addition to supporting the role of green tea as a cancer chemopreventive agent.

2. Materials and methods

2.1. Cell culture and reagents

Human fibrosarcoma HT 1080 cells were obtained from the American Type Culture Collection (Manassas, VA). The cells were cultured at 37 °C in a 5% CO_2 atmosphere in RPMI1640 supplemented with 10% fetal bovine serum and 1% penicillin–streptomycin. Purified EGCG, EC, and ECG were obtained from Sigma (St. Louis, MO) and PD98059, SB203580, wortmannin, and actinomycin D were from Calbiochem (San Diego, CA). The rabbit polyclonal antiphosphospecific p44/42 mitogen-activated protein kinase (MAPK; extracellular signal-regulated kinases 1 and 2, Erk-1/2), P38 MAPK, Akt, and c-*jun* N-terminal kinase (JNK) antibodies were purchased from New England Biolabs (Beverly, MA). Goat polyclonal antihuman uPA immunoglobin G (IgG) was purchased from American Diagnostica (Greenwich, CT). cDNAs for uPA and GAPDH were obtained from the American Type Culture Collection.

2.2. Zymography

This was performed as previously described (Yoo et al., 2002). Culture supernatants collected from equal number of cells were denatured and electrophoresed in a 10% polyacrylamide gel containing 0.2% (w/v) casein with 5 μg/ml plasminogen. The gel was incubated at room temperature for 2 h in the presence of 2.5% Triton X-100 and subsequently at 37 °C overnight in a buffer containing 10 mM $CaCl_2$, 0.15 M NaCl, and 50 mM Tris (pH 7.5). The gel was then stained for protein with 0.25% Coomassie Blue and photographed on a light box. Proteolysis was detected as a white zone in a dark field. As a control, the same samples were run in a gel without plasminogen.

2.3. Northern blot analysis

Total RNA extraction and Northern blot hybridization were performed as previously described (Jung et al., 2001). To examine the role of Erk-1/2 and P38 MAPK in uPA expression, cells were pretreated with PD98059 (a specific inhibitor of MEK-1, an upstream effector of Erk-1/2), and SB203580 (a specific P38 MAPK inhibitor) for 24 h, and total RNA was then isolated using Trizol reagent (Life Technologies, Grand Island, NY). Each cDNA probe was radiolabeled with [α-^{32}P]deoxyribonucleoside triphosphate by the random-priming technique using the Rediprime labeling system (Amersham, Arlington Heights, IL). The probed nylon membranes were exposed to radiographic films (Life Technologies). To study the uPA mRNA stability, 1 μg/ml actinomycin D was added to control or EGCG (50 μM, 1 h)-treated HT 1080 cells. Total RNA was isolated from these cells at varying times (0–24 h), and Northern blot analysis was then performed as described above.

2.4. Western blot hybridization

Protein extraction and Western blot analysis were performed as previously described (Jung et al., 2001). The activated forms of Erk-1/2, JNK, P38 MAPK, and Akt were detected using polyclonal antiphosphospecific antibodies. Protein bands were visualized using a commercially available chemiluminescence kit (Amersham). Total protein levels were assayed by washing the membrane with stripping solution [100 mM 2-mercaptoethanol, 2% sodium dodecyl sulfate, and 62.5 mM Tris–HCl (pH 6.7)] for 30 min at 50 °C and then reprobing the membrane with polyclonal anti-Erk-1/2, JNK, P38 MAPK, and Akt antibodies (all at a 1:1000 dilution).

2.5. Chloramphenicol acetyl transferase (CAT) assay

The transcriptional regulation of uPA was studied using transient transfection with a uPA promotor–CAT reporter construct (Lengyel et al., 1996). HT 1080 cells (5×10^5) were seeded on a 6-well plate and grown to 60–70% confluence, and 1 μg of an expression vector bearing the uPA promotor–CAT reporter construct was transfected into cells using FuGENE^6 (Boehringer Mannheim, Indianapolis, IN) according to the manufacturer's protocol. The CAT activity was subsequently measured by incubating cell lysate at 37 °C for 8 h with 4 μM [^{14}C]chloramphenicol and 1 mg/ml acetyl coenzyme A. After 4 h incubation, the acetyl coenzyme A was replenished. The acetylated products were extracted with ethyl acetate and were subjected to thin layer chromatography using chloroform/methanol (95:5) as the mobile phase.

2.6. Matrigel invasion assay

Cell invasion assay was performed using BioCoat Matrigel invasion chambers (Becton-Dickinson, Bedford, MA) according to the manufacturer's protocol. Briefly, 10^5 cells in 300 μl were added to each chamber and allowed to invade Matrigel for 24 h. The noninvading cells on the upper surface of the membrane were removed from the

chamber, and the invading cells on the lower surface of the membrane were stained with Quick-Diff stain kit (Becton-Dickinson). After two washes with water, the chambers were allowed to air-dry. The number of invading cells were counted using a phase-contrast microscope. To determine the effect of EGCG on HT 1080 cell invasion, cells were pretreated with 0–100 μM EGCG for 12 h and the Matrigel invasion assay was performed in the presence of 0–100 μM EGCG.

3. Results

3.1. Effects of EGCG on uPA expression

To examine whether EGCG could inhibit the uPA expression in HT 1080 cells, cells were treated with 0–100 μM EGCG and changes in the uPA mRNA level and uPA activity were determined. EGCG at 30 μM remarkably inhibited the uPA mRNA expression. However, other tea catechins such as EC and ECG had no significant effect on the uPA mRNA expression at the same concentration (Fig. 1A). EGCG inhibited the uPA mRNA expression in a dose-dependent manner (Fig. 1B). In addition, the EGCG treatment abrogated the uPA activity, as determined by plasminogen zymography in conditioned media collected from cultured HT 1080 cells (Fig. 1C). EGCG used at the concentration did not affect the cell viability. The above results indicated that EGCG could inhibit the uPA expression.

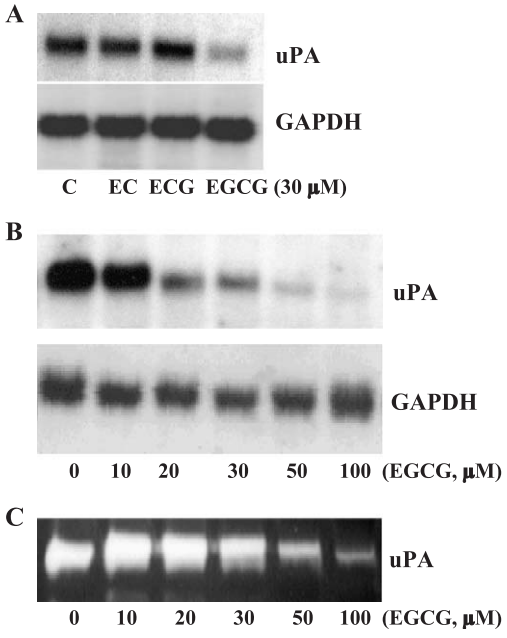

Fig. 1. Effects of tea catechins on uPA expression in HT 1080 cells. (A) After HT 1080 cells were treated with 30 μM EC, ECG, and EGCG for 8 h, Northern blot analysis was performed for the uPA and GAPDH expression. (B) After HT 1080 cells were treated with 0–100 μM EGCG for 8 h, Northern blot analysis was performed. (C) After HT 1080 cells were treated with 0–100 μM EGCG for 24 h, plasminogen zymography was performed for the uPA activity in conditioned media.

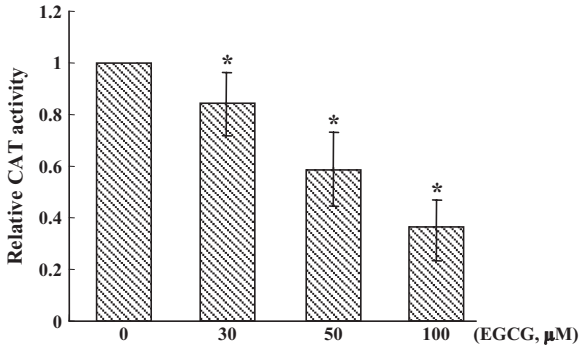

Fig. 2. Effect of EGCG on uPA transcription activity. HT1080 cells were transiently transfected with uPA promoter–CAT reporter constructs. After the cells were treated with 0–100 μM EGCG for 24 h, the uPA promoter activity was determined by thin-layer chromatography. The acetylated products, then, were quantitated using a densitometer. Data represent the means ± S.D. from triplicate measurements. *Significant difference from control $P < 0.05$.

3.2. Effects of EGCG on uPA transcription activity

To study the transcriptional regulation of uPA, transient transfection was performed with uPA promoter–reporter (CAT) constructs and the promoter-mediated CAT activities were determined in HT1080 cells. As shown in Fig. 2, cells treated with EGCG lost the uPA promoter activity as a function of EGCG concentration.

3.3. Effects of EGCG on signaling pathways involved in regulation of the uPA mRNA expression

After HT 1080 cells were exposed to 0–100 μM EGCG for 8 h, cells were lysed and determined for phosphorylated and total Erk-1/2 (p44/42), JNK, P38 MAPK, and Akt by Western blot analyses. As shown in Fig. 3, phosphorylation of Erk-1/2 and P38 MAPK decreased with EGCG treatment,

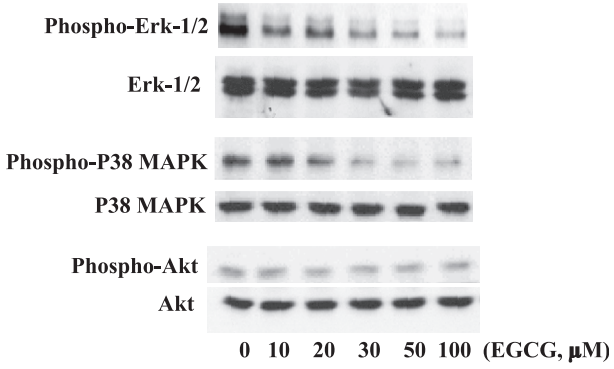

Fig. 3. Effects of EGCG on phosphorylation of Erk-1/2, P38 MAPK, and Akt in HT 1080 cells. HT 1080 cells were treated with 0–100 μM EGCG for 8 h and Western blot analyses were performed for the phosphorylated and total Erk-1/2, P38 MAPK, and Akt.

dose-dependently. However, EGCG did not reduce the phosphorylation of Akt. The phosphorylation of JNK was nearly not detected (data not shown). The levels of total Erk-1/2 and P38 MAPK were not significantly altered by EGCG treatment. To study the role of Erk-1/2, P38 MAPK, and PI-3 kinase in uPA expression, the effects of PD98059 (50 μM, a specific inhibitor of MEK-1, an upstream effector of Erk-1/2), SB203580 (25 μM, a specific P38 MAPK inhibitor), and wortmannin (300 nM, a PI-3 kinase inhibitor) on uPA mRNA expression were examined. As shown in Fig. 4A, PD98059 and SB203580 significantly reduced the uPA mRNA expression, whereas wortmannin showed only a negligible effect.

3.4. Effects of Erk-1/2 and P38 MAPK inhibitors on uPA transcription activity

To further study the mechanism by which Erk-1/2 and P38 MAPK regulate the uPA mRNA expression, the effects of Erk-1/2 and P38 MAPK inhibitors on the uPA promoter activity were examined. Transient transfection study showed that PD98059 and SB203580 inhibited the activity of uPA promoter in dose-dependent fashions (Fig. 4B).

3.5. Effects of EGCG and MAPK inhibitors on uPA mRNA stability

Next, we investigated whether the EGCG treatment altered the stability of uPA mRNA. To do this, HT 1080 cells were cultured with 1 mg/ml actinomycin D for 0–24 h in the presence or absence of 50 μM EGCG, and the uPA mRNA levels were determined by Northern blotting. In control HT 1080 cells, the time required for 50% loss of uPA mRNA was >8 h. However, the EGCG treatment reduced the half-life of uPA mRNA to approximately 4 h, indicating that EGCG altered the stability of uPA mRNA (Fig. 5A and B). However, as shown in Fig. 5B, PD98059 and SB203580 did not significantly affect the uPA mRNA stability.

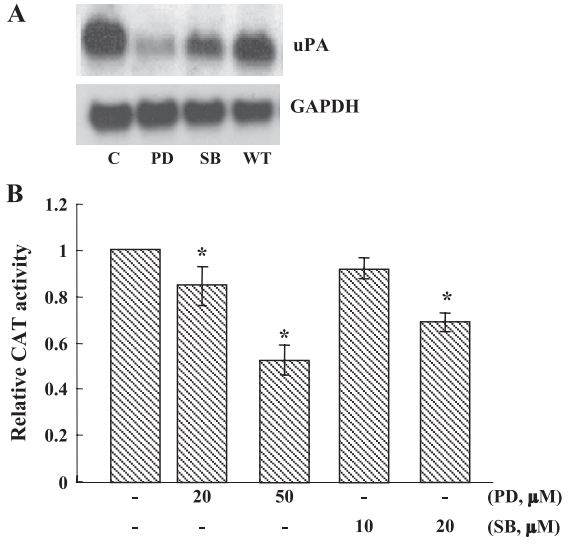

Fig. 4. Effects of PD98059 (PD), SB203580 (SB), and wortmannin (WT) on uPA mRNA expression. (A) After HT 1080 cells were exposed to 50 μM PD98059, 20 μM SB203580, and 300 nM wortmannin for 12 h, total RNA was extracted and Northern blot analysis was performed. (B) HT1080 cells were transiently transfected with uPA promoter–CAT reporter constructs. After the cells were treated with 0–50 μM PD98059 and 0–20 μM SB203580, the uPA promoter activity was determined by thin layer chromatography, and the acetylated products were quantitated using a densitometer. Data represent the means ± S.D. from triplicate measurements. *Significant difference from control $P < 0.05$.

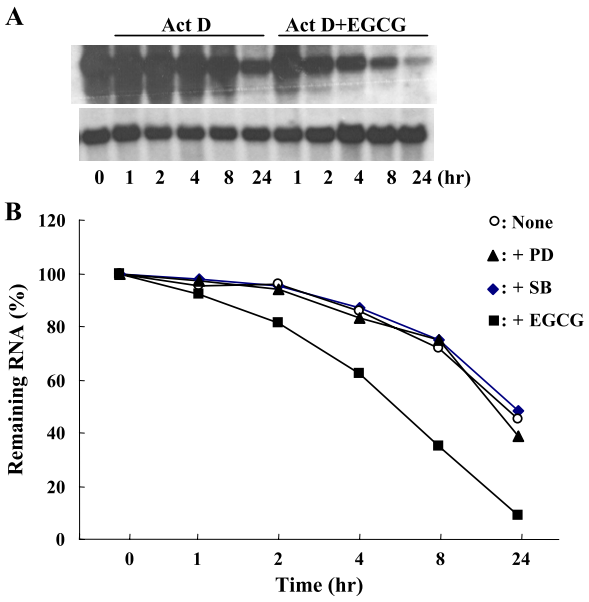

Fig. 5. Effect of EGCG, PD98059 (PD), and SB203580 (SB) on uPA mRNA stability. (A) After HT 1080 cells were cultured with or without 50 μM EGCG for 1 h, the cell cultures were added with 1 mg/ml of actinomycin D. Total RNA was extracted from the cells at the indicated times after the addition of actinomycin D, and the uPA mRNA level was determined by Northern blotting. (B) After HT 1080 cells were cultured with 50 μM EGCG, 50 μM PD98059, and 20 μM SB203580 for 1 h, the cell cultures were added with 1 mg/ml of actinomycin D. Total RNA was extracted from the cells at the indicated times after the addition of actinomycin D, and the uPA mRNA level was determined by Northern blotting. The uPA mRNA levels were quantitated densitometrically and expressed as percentages of the zero-time control.

3.6. Effects of EGCG and anti-uPA antibody on HT 1080 cell invasiveness

The effect of EGCG on the invasiveness of HT 1080 cells was determined by measuring the ability of cells to pass through the Matrigel chemoinvasion chambers. As shown in Fig. 6A, the EGCG treatment diminished the matrix invasiveness of HT 1080 cells, dose-dependently. EGCG at 100 μM inhibited the HT1080 cell invasion by over 50%. To evaluate the biologic role of uPA in the HT1080 cell invasion, cells were incubated with specific antibodies to uPA in chemoinvasion chambers prior to determination of cell invasiveness. The result showed that cells treated with uPA-neutralizing antibodies, but not the

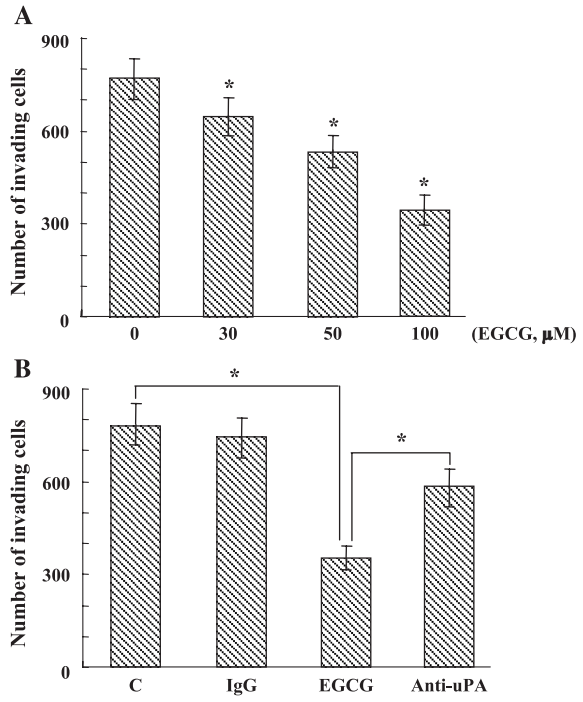

Fig. 6. Effects of EGCG and anti-uPA antibody on HT 1080 cell invasiveness. (A) After HT 1080 cells were cultured in BioCoat Matrigel invasion chambers containing 0–100 μM EGCG for 24 h, the invading cells on the undersurface of the chambers were counted using a phase contrast light microscope after staining with Diff-Quick Stain Kit. Data represent the means ± S.D. for triplicate measurements. (B) HT 1080 cells were cultured in BioCoat Matrigel invasion chambers containing 50 μM EGCG, 50 μg/ml anti-PA antibodies (anti-PA), and 50 μg/ml nonspecific IgG (control, C) for 24 h. The subsequent procedures were the same as in panel (A). *Significant difference from control $P < 0.05$.

control cells treated with nonspecific IgG, partially lost the Matrigel invasiveness (Fig. 6B).

4. Discussion

EGCG is the most abundant of the green tea polyphenols, accounting for more than 40% of the total polyphenolic mixture (Stoner and Mukhtar, 1995). Several molecular mechanisms have been suggested for EGCG's observed anticancer effect, including suppression of ligand binding to the EGF receptor (Liang et al., 1997), inhibition of protein kinase C (Kitano et al., 1997), lipoxygenase, and cyclooxygenase activities (Stoner and Mukhtar, 1995), and induction of apoptotic cell death and arrest of the cell cycle in tumor cells (Fujiki et al., 1998; Han, 2003). Recently, it was proposed that the anticancer activity of EGCG is also associated with suppression of cell invasion by inhibiting the activity of urokinase (Jankun et al., 1997) and matrix metalloproteases (Maeda-Yamamoto et al., 1999).

EGCG has been shown to directly impair the activity of urokinase and its role in degradation of extracellular matrix (Jankun et al., 1997). The inhibitory effect of EGCG on uPA activity was verified with a spectrophotometric amidolytic assay. Of note, however, was that the effective EGCG concentration (4 mM) has been considered to far exceed the levels found in vivo after tea consumption (Cao and Cao, 1999). In this study, we found that EGCG at micromolar concentrations inhibited the uPA expression in human fibrosarcoma HT 1080 cells, as revealed by Northern blot and plasminogen zymographic analyses (Fig. 1).

The uPA expression may be regulated at either transcriptional or mRNA stability level or at both levels. First, through the transient transfection study with uPA promoter–reporter constructs, we found that EGCG inhibited the uPA promoter activity (Fig. 2A and B). Recently, several studies have documented that MAPKs have important roles in regulation of the uPA gene, although the profiles of MAPK activation appear to vary in a cell type-dependent fashion (Ward et al., 2001; Adeyinka et al., 2002). In mammals, three subgroups of MAPK have been detected: the Erk, JNK, and P38 MAPK (Cobb and Goldsmith, 1995). In this study, we found that EGCG inhibited the activation of Erk-1/2 and P38 MAPK, and the specific inhibitors of Erk-1/2 and P38 MAPK were capable of downregulating the uPA promoter activity. These results suggest that the inhibition of Erk-1/2 and P38 MAPK by EGCG may be an early cellular event that is partly responsible for the downregulation of uPA gene transcript in HT 1080 cells. The exact mechanisms by which ECGC inhibits the activation of Erk-1/2 and P38 MAPK are not known. One possible explanation is that EGCG may inhibit the kinases that are involved in MAPK activation (Jung et al., 1999). EGCG is known to be a strong metal ion chelator (Yang and Wang, 1993). Because some receptor kinases depend on divalent cations for their activity, EGCG may inhibit the activity of receptor kinases by chelating the divalent cations (Mahadevan et al., 1995).

The half-life of uPA mRNA in HT 1080 cells was estimated to be over 8 h (Fig. 5A and B). However, EGCG treatment reduced the half-life of uPA mRNA to approximately 4 h. Previous studies have suggested that uPA expression could be regulated at the level of mRNA stability through the AU-rich sequences in 3′ -UTR of uPA mRNA. P38 MAPK was reported to impact the stability of uPA mRNA through the AU-rich element-targeted mechanism in human breast BT 549 cells (Huang et al., 2000). In this study, to investigate if Erk-1/2 or P38 MAPK is involved in destabilizaion of uPA mRNA by EGCG, PD98059 and SB203580, which are specific inhibitors of Erk-1/2 and P38 MAPK, respectively, were employed. The results showed that neither the PD98059 nor SB203580 affected the uPA mRNA stability, suggesting that the EGCG-mediated destabilization of uPA mRNA is an Erk-1/2- or P38 MAPK-independent process. The discrepancy between the above results may be explained by difference in the cell system used, and further studies are required to clarify the mechanisms involved in the EGCG-mediated destabilization of uPA mRNA.

It has recently been reported that EGCG inhibits tumor gelatinases in HT 1080 cells. Maeda-Yamamoto et al. (1999) reported that suppression of Erk activation by EGCG was involved in the inhibition of expression of metalloproteinase (MMP)-2 and MMP-9 mRNAs, leading to the reduction of tumor cell invasion. Garbisa et al. (2001) demonstrated that EGCG directly inhibited the MMP-2 and MMP-9 activities, and that the inhibition was not due to metal chelating properties of EGCG but correlated with direct binding of EGCG to gelatinases. Our results suggest that the inhibition of uPA expression by EGCG is also important for the anti-invasive function of EGCG. This suggestion can be further supported by the observation that treatment with anti-uPA antibodies partially inhibited the invasiveness of HT 1080 cells (Fig. 6B).

Acknowledgements

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