Introduction

Recently, tissue factor (TF) has attracted considerable attention as a receptor capable of mediating intracellular signals closely involved in metastasis, angiogenesis and tumor growth[1]. TF is expressed in a wide variety of malignancies[2] and can induce a variety of non-coagulant cellular responses related to angiogenesis, metastasis, tumor growth, and cell migration[3, 4]. It has been found that TF binding to factor VII (hereafter factor VII) or factor VIIa can trigger cell signal transduction via activation of protease-activated receptor 2 (PAR2)[5]. The activation of PAR2 induced by the TF/factor VIIa complex may contribute to the malignant behavior of certain cancer cells[6].

PAR2 is a G-protein-coupled receptor activated by proteolytic cleavage of its amino terminal domain. Experimentally, PARs can also be activated by synthetic peptides [referred to as an agonist peptide (AP)] that mimic the neo-amino terminus of the cleaved receptors. Specific PAR-APs are important tools for investigating the roles of PAR activation[7]. In a previous study, we observed that TF and PAR2 are highly expressed in the colon cancer cell line SW620[8]. Treatment with factor VIIa (10 nmol/L) induced SW620 cell proliferation and migration. The effects of factor VIIa on cells are similar to that of the PAR2 agonist peptide, PAR2-AP, and could be inhibited by anti-TF or anti-PAR2 antibodies. Furthermore, some intracellular signaling molecules were activated in these processes[9]. These results led us to hypothesize that there is a TF/factor VIIa/PAR2 axis in SW620 cells, and this axis might be a potential therapeutic target for colon cancers.

Epigallocatechin-3-gallate (EGCG), the major polyphenolic constituent in green tea, is well known to possess remarkable...
cancer chemo-preventive activity and therapeutic potential against various cancers due to its ability to inhibit cell growth, arrest cell cycle and induce apoptosis in some human carcinoma cells. One mechanism by which EGCG exerts its antitumor effects is through the inhibition of cell signaling associated with tumor cell proliferation, apoptosis, invasion and metastasis[10, 11].

Although it has been known for several years that EGCG has potent anticancer effects, whether or not it can affect TF/factor VIIa/PAR2 axis-mediated proliferation and migration of SW620 cells remains unclear. In the current study, we investigated whether EGCG is capable of blocking the stimulating effects of the TF/factor VIIa/PAR2 axis on SW620 cells and the possible mechanisms involved in this process.

Materials and methods

Materials

Leibovitz’s L-15 medium and fetal bovine serum (FBS) were obtained from Gibco BRL (Grand Island, NY, USA). EGCG (purity >98%) was purchased from Sigma (St Louis, MO, USA). The PAR2 agonist (SLIGKV-NH2, PAR2-AP) was synthesized by Proteintech Group Inc (Wuhan, China). Recombinant human factor VIIa was obtained from Novo Nordisk (Maaloev, Denmark). All reagents (EGCG, PAR2-AP, and factor VIIa) were dissolved in distilled water and then diluted (purity >98%) was purchased from Sigma (St Louis, MO, USA). The PAR2 agonist (SLIGKV-NH2, PAR2-AP) was synthesized by Proteintech Group Inc (Wuhan, China). Recombinant human factor VIIa was obtained from Novo Nordisk (Maaloev, Denmark). All reagents (EGCG, PAR2-AP, and factor VIIa) were dissolved in distilled water and then diluted in medium. Trizol and RT-PCR reagents were purchased from Invitrogen (Carlsbad, CA, USA). MMP-9 ELISA assay kits were purchased from R&D Systems, Inc (Minneapolis, MN, USA). TF activity assay kits (Actichrome™ TF) were purchased from American Diagnostic, Inc (Greenwich, CT, USA). Polyclonal anti-caspase-7, anti-ERK1/2 and anti-NF-κB (p65) antibodies were obtained from Cell Signaling Technology (Beverly, MA, USA). Polyclonal anti-histone H3 antibody was purchased from Biosynthesis Biotechnology Co, Ltd (Beijing, China). Nuclear and cytoplasmic extraction kits were purchased from KeyGen Biotech Co, Ltd (Nanjing, China). All other chemicals and reagents used in the experiments were commercially available.

Cell lines and cell culture

The human colon cancer cell line SW620 was obtained from Shanghai Institutes for Biological Sciences (Shanghai, China). The cells were cultured in Leibovitz’s L-15 medium supplemented with 10% FBS. The cells were maintained at 37 °C in a humidified atmosphere of 5% CO2 and were deprived of serum for 16 h before they were used in the experiments. All of the experimental data were obtained from cells passaged between 3 and 10 times.

Cell proliferation assay

The SW620 cells were seeded in 96-well plates at 5×10^4 cells per well and grown to 70% confluence in culture medium. The cells were pretreated with various concentrations of EGCG (0, 25, 50, 75, and 100 µg/mL) for 15 min and then stimulated with PAR2-AP (100 µmol/L) or factor VIIa (10 nmol/L) in the presence of EGCG for 24 h. Then, 20 µL of MTT solution (5 mg/mL in PBS) was added to each well, and the cells were incubated for another 4 h at 37°C. Finally, the culture media were removed and the cells were solubilized with 100 µL dimethylsulfoxide (DMSO), and the absorbance of the cells was measured at 490 nm using a microtiter plate photometer (BioTek, USA).

Cell migration assay

Cell migration assays were performed using modified Boyden chambers with a 65 µm diameter and a porous (8 µm) polycarbonate membrane separating the two chambers (Transwell, Corning Inc, USA). The cells were harvested by brief exposure to trypsin/EDTA and neutralized with Leibovitz’s L-15 medium. The cells were washed and resuspended in medium with 0.1% bovine serum albumin (BSA) in an adequate concentration. At the indicated times, cells (1.0×10^5 in 100 µL) were incubated with EGCG (100 µg/mL) for 15 min at 37°C, then placed in the upper compartments in the continued presence of EGCG. Cells were placed in the upper compartment of the migration chamber with PAR2-AP (100 µmol/L) or factor VIIa (10 nmol/L) in both the upper and lower compartments. The cells without any agonists or inhibitors in the assay were used as a control. After 8 h of incubation, the cells that had migrated through the pores to the lower side of membrane were fixed with methanol and stained with Giemsa. The number of cells was counted in five randomly chosen fields using light microscopy (×200). For each triplicate, the number of cells in ten high power fields was determined, and the counts were averaged.

Cellular cytoskeleton detection

The SW620 cells were placed in 24-well culture dishes at a density of 3×10^4 cells per well in culture medium containing 10% FBS at 37 °C in 5% CO2. After 12 h, the cells were serum-starved for 16 h prior to stimulation with PAR2-AP (100 µmol/L) or factor VIIa (10 nmol/L) for 1 h. The cells in some wells were pretreated with EGCG (100 µg/mL) for 15 min as described above. Then, the cells were fixed on glass coverslips with 4% paraformaldehyde and washed with PBS three times. The DNA was stained with Hoechst 33342 (Sigma), and the actin cytoskeleton was stained with rhodamine-conjugated phalloidin (Sigma). The samples were observed under a laser scanning confocal fluorescence microscope (Carl Zeiss, Germany).

Quantitative real-time PCR analysis

The SW620 cells were seeded at 1×10^4 cells/mL into 6-well plates and serum-starved for 16 h prior to stimulation with PAR2-AP (100 µmol/L) or factor VIIa (10 nmol/L) for 2 h. The cells in some wells were pretreated with EGCG (100 µg/mL) for 15 min. Then, the total cellular RNA was isolated, and first-strand cDNA was synthesized using SuperScript II reverse transcriptase (Life Technologies). Finally, Q-PCR was performed using a Rotor-Gene 2000 (Corbett Research, Australia). The caspase-7 forward primer was 5’-TGACCTATCATGGCTGCCCTCA-3’, and the reverse primer was 5’-TCTCCTGC-
The SW620 cells were seeded at 1×10⁶ cells/mL into 6-well plates and serum-starved for 16 h prior to stimulation with PAR2-AP (100 μmol/L) or factor VIIa (10 nmol/L) for 24 h. The cells in some wells were pretreated with EGCG (100 μg/mL) for 15 min. The MMP-9 secreted into the cell supernatant was measured using MMP-9 ELISA assay kits following the manufacturer’s instructions. The MMP-9 protein level was expressed as ng/mL.

Detection of MMP-9 secretion

The SW620 cells were seeded at 1×10⁶ cells/mL into 6-well plates and serum-starved for 16 h prior to stimulation with PAR2-AP (100 μmol/L) or factor VIIa (10 nmol/L) for 24 h. The cells in some wells were pretreated with EGCG (100 μg/mL) for 15 min. After each Q-PCR run, a melting curve was performed to ensure that only a single amplicon was generated. Caspase-7 mRNA levels were normalized to control values of β-actin (%).

Western blotting analysis

For detection of NF-κB (p65/RelA) protein expression, SW620 cells were seeded at 1×10⁶ cells/mL into 6-well plates and treated as described above. Following stimulation, the cells were briefly trypsinized, suspended in Leibovitz’s L-15/10% FBS, pelleted, and resuspended in Tris-buffered saline (TBS) at a density of 10⁶ cells/mL. After washing 3 times with TBST, the membranes were blocked with a blocking solution containing 5% defatted milk/Tris-buffered saline/0.05% Tween-20 (TBST) (pH 7.6) for 1 h at room temperature (RT). After being washed 3 times with TBST, the membranes were probed with anti-NF-κB (p65/RelA, 1:1000), anti-caspase-7 (1:1000), anti-phospho-ERK1/2 (1:1000), anti-total-ERK1/2 (1:1000), anti-β-actin (1:2500) or anti-histone H3 (1:500) antibodies with gentle agitation overnight (16 h) at 4 °C. The membrane was then washed with TBST three times and incubated for 1 h at RT with horseradish peroxidase (HRP)-conjugated secondary antibodies (1:2000, Cell Signaling Technology, Billerica, MA, USA). Finally, immunoreactive proteins were visualized using ECL Western blotting detection reagents (GE Healthcare, Buckinghamshire, UK) and quantitated using a Bio-Rad Fluor-S Multi Imager (Typhoon 9400, Amersham, Sweden).

Statistical analysis

The data were expressed as mean±SEM. The statistical significance of the differences was calculated by applying analysis of variance (ANOVA) using SPSS software (version 16.0). P<0.05 was considered statistically significant.

Results

EGCG inhibits PAR2-AP- or factor VIIa-induced proliferation and migration of SW620 cells

Our previous work indicated that PAR2-AP (100 μmol/L) and factor VIIa (10 nmol/L) promoted SW620 cell proliferation and migration[9]. In this study, we first investigated whether EGCG at various concentrations could decrease the effects of PAR2-AP and factor VIIa on cell proliferation (as assayed using the MTT reagent). As shown in Figure 1A, EGCG inhibited PAR2-AP- (100 μmol/L) or factor VIIa- (10 nmol/L) induced growth

![Figure 1](image-url)

**Figure 1.** Effects of EGCG on PAR2-AP- and factor VIIa-induced proliferation and migration of SW620 cells. (A) SW620 cells were plated at 5×10⁴ cells in 96-well plates and pretreated with different concentrations of EGCG (0, 25, 50, 75, and 100 μg/mL) for 15 min, then incubated with PAR2-AP (100 μmol/L) or factor VIIa (10 nmol/L) for 24 h. The above EGCG was kept in media. The cell proliferation was measured using MTT colorimetric assay. n=6. Mean±SEM. *P<0.05, **P<0.01 vs PAR2-AP- or factor VIIa-stimulated alone. (B) The cells were pretreated with or without EGCG (100 μg/mL) for 15 min as described above and then incubated with PAR2-AP (100 μmol/L) or factor VIIa (10 nmol/L) for 8 h. The cell migratory potential was measured by modified Boyden Chambers as described in Methods. n=3. Mean±SEM. *P<0.05; **P<0.01 vs media only; ***P<0.05, ****P<0.01 vs PAR2-AP- or factor VIIa-stimulated alone.
of SW620 cells. The inhibitory effects of EGCG were shown at concentrations as low as 25 μg/mL, with the maximal effects observed at 50 μg/mL (approximately 60% inhibition). Increasing the concentration of EGCG to 75 or 100 μg/mL did not further enhance its effects. In the absence of stimulation by PAR2-AP or factor VIIa, EGCG itself had no significant effects on the proliferation of cells. The inhibitory effects of EGCG were also confirmed with cell cycle analysis using flow cytometry, where a dose-dependent effect was observed from 25 to 100 μg/mL EGCG (Supplemental Figure 1). The differences between the dose effects observed in the MTT and cell cycle analyses may be due to different incubation times (24 h in MTT versus 8 h in the cell cycle analysis). EGCG itself was not toxic, based on a trypan blue exclusion assay (Supplemental Figure 2).

We next asked if EGCG was capable of inhibiting PAR2-AP- or factor VIIa-induced SW620 cell migration, as determined by Transwell chamber assays. The data showed that pre-incubation of cells with EGCG (100 μg/mL) prior to PAR2-AP (100 μmol/L) or factor VIIa (10 nmol/L) treatment resulted in decreases of 63% and 62% in the number of migratory cells, compared with the corresponding control (PAR2-AP or factor VIIa treatment without EGCG pre-treatment). EGCG alone had no significant effects on the migration of cells (Figure 1B) (cell migration images are shown in Supplemental Figure 3). These results demonstrated that EGCG inhibited both PAR2-AP- and factor VIIa-mediated migration of SW620 cells.

**Effects of EGCG on the actin cytoskeleton in SW620 cells**

Phalloidin staining, which labels actin filaments, was used to assess changes in the cytoskeleton following PAR2-AP and factor VIIa treatment in the presence and absence of EGCG. The cytoskeleton of untreated cells was found to have some homogeneous actin fibers, which were stained a moderate red color (Figure 2A). However, after exposure to PAR2-AP (100 μmol/L) or factor VIIa (10 nmol/L) for 1 h, the actin filaments were present at the cell cortex, in filopodia and pseudopodia and stained a strong red color (Figures 2B, 2C). Pre-treatment of cells with EGCG (100 μg/mL) significantly rescued the aberrant actin organization that was induced by PAR2-AP or factor VIIa (Figures 2E, 2F). EGCG alone had no effect on the actin cytoskeleton in SW620 cells (Figure 2D).

**Effects of EGCG on the expression of caspase-7 in SW620 cells**

Caspase-7 is one of the key effector caspases that induce cell apoptosis. In a previous study, we showed that caspase-7 was down-regulated by PAR2-AP or factor VIIa[9]. To examine whether EGCG could affect the regulatory effects of PAR2-AP or factor VIIa on caspase-7 expression in SW620 cells, the caspase-7 mRNA and protein levels in these cells were evaluated under different conditions. We found that both PAR2-AP (100 μmol/L) and factor VIIa (10 nmol/L) decreased the level of caspase-7 mRNA (Figure 3A, white column). However, pre-incubation of SW620 cells with EGCG (100 μg/mL) significantly increased caspase-7 mRNA expression, even though the cells were treated with similar concentrations of PAR2-AP and factor VIIa (Figure 3A, black column). Compared with the corresponding controls (PAR2-AP or factor VIIa treatment alone), EGCG increased caspase-7 mRNA levels to approximately 4.3- and 4-fold (Figure 3A). Similarly, the caspase-7 protein level also increased with treatment of EGCG prior to PAR2-AP or factor VIIa stimulation (Figure 3B). EGCG alone had a slight effect on the regulation of caspase-7 release compared with media alone, but it was not statistically significant.

**Effects of EGCG on the expression of TF and MMP-9 in SW620 cells**

It has been shown that TF and MMP-9 are closely associated with tumor growth, invasion and metastasis[12, 13]. Our previous data also showed that TF levels were elevated in SW620 cells stimulated with PAR2-AP or factor VIIa[9]. In this study, we found that both PAR2-AP (100 μmol/L) and factor VIIa (10 nmol/L) enhanced the expression of TF and MMP-9 (Figure 4A and 4B, white column). When the cells were pre-incubated with EGCG (100 μg/mL), PAR2-AP- or factor VIIa-mediated TF activity and MMP-9 expression were markedly decreased (P<0.01 vs PAR2-AP or factor VIIa alone), as shown in the black column in Figures 4A and 4B. EGCG alone seemed to...
have a slight inhibitory effect on the MMP-9 level ($P<0.05$ vs media only).

**Effects of EGCG on activation of ERK1/2 and NF-κB in SW620 cells**

Previously, we found that PAR2-AP and factor VIIa stimulated the activation of ERK1/2 and NF-κB (p65/RelA) in SW620 cells within 30 min of treatment$^{[14, 15]}$. In this study, we further investigated whether EGCG could influence the effects of PAR2-AP and factor VIIa on the levels of p-ERK1/2 and NF-κB (p65/RelA) in the cells. As the concentrations of EGCG increased (0–100 µg/mL), the levels of p-ERK1/2 (Figure 5A) and NF-κB (p65/RelA) (Figure 5B) in SW620 cells stimulated with factor VIIa (10 nmol/L) gradually decreased, indicating a dose-dependent inhibitory effect of EGCG. Similarly, the stimulatory effects of PAR2-AP (100 µmol/L) on p-ERK1/2 and NF-κB (p65/RelA) were also blocked by EGCG (100 µg/mL).

**Discussion**

It is well known that factor VII can bind to TF on the cell surface and then activated by cleavage to generate factor VIIa. The formation of the TF/factor VIIa complex triggers the blood coagulation cascade. As a trypsin-like serine protease, factor VIIa by itself or in the TF/factor VIIa complex can trigger the cell signal transduction pathway in certain cells. It has been reported that factor VIIa or TF/factor VIIa might activate other receptors on the cell surface and cause a series of changes within the cell$^{[16]}$. It was found that PAR2 was activated by factor VIIa and that TF/factor VIIa/PAR2 was involved in tumor growth and invasion in colorectal cancer and breast carcinoma. We previously demonstrated that both TF and PAR2 are highly expressed in the colon cancer cell line SW620. Factor VIIa can activate PAR2 and promote SW620 cell proliferation and migration in a TF-dependent manner. Some intracellular signaling molecules, such as ERK1/2 and NF-κB, are activated in these processes$^{[9, 14, 15]}$. Lim et al found that EGCG effectively inhibited HGF-induced invasion and metastasis of hypopharyngeal carcinoma cells via several intracellular signaling pathways$^{[18]}$. In the current study, we investigated whether EGCG was able to interfere...
involved in apoptosis induced by various stimuli, and it is nec-
mediated effects on cell proliferation and migration. This result may partly elucidate the mechanism(s) of EGCG-
induced cytoskeleton reorganization, as indicated by increased
nal transduction [19]. Indeed, both PAR2-AP and factor VIIa
were closely associated with cell adhesion, invasion and sig-
in the actin cytoskeleton and to determine if these changes
of EGCG on PAR2-AP- and factor VIIa
63% or 62%, respectively (Figure 1). These inhibitory effects
were stimulated by PAR2-AP or factor VIIa by approximately
µg/mL, EGCG decreased the number of migratory cells that
dose-dependent manner by EGCG. At a concentration of 100
or factor VIIa-stimulated alone.

Our results showed that PAR2 agonist- (PAR2-AP) or fac-
tor VIIa-induced SW620 cell proliferation was inhibited in a
dose-dependent manner by EGCG. At a concentration of 100
µg/mL, EGCG decreased the number of migratory cells that
were stimulated by PAR2-AP or factor VIIa by approximately
63% or 62%, respectively (Figure 1). These inhibitory effects of EGCG on PAR2-AP- and factor VIIa-induced proliferation and migration of SW620 cells led us to explore the changes in the actin cytoskeleton and to determine if these changes were closely associated with cell adhesion, invasion and sig-
F

Figure 5. Effects of EGCG on activation of ERK1/2 and NF-κB pathway
in SW620 cells. The cells were pretreated with or without the indicated
concentrations of EGCG for 15 min and then incubated with PAR2-AP
(100 μmol/L) or factor VIIa (10 nmol/L) for indicated time. The above
EGCG was kept in media. The cytoplasmic and nuclear lysates of the cells
were collected and subjected to Western analysis with antibodies to total
and phosphorylated ERK1/2 (A) and to NF-κB/p65 as well as Histon H3
(B). n=3. Mean±SEM. *P<0.05, **P<0.01 vs control; &P>0.05,
&<0.05, &P<0.01 vs PAR2-AP- or factor VIIa-stimulated alone.

with the effects of the TF/factor VIIa/PAR2 axis on SW620
cells.

Over the past few years, several target molecules, including
angiogenic growth factor, chemotactic factors, and some anti-
apoptotic molecules, were found to be regulated by the activa-
tion of the TF/factor VIIa/PAR2 complex. Furthermore, TF
has been implicated in angiogenesis, growth and metastasis of cancers [22]. Matrix metalloproteinases (MMPs) are closely
related to cell invasion and migration. Our data shows that
EGCG significantly suppressed both TF activity and MMP-9
secretion in SW620 cells induced by PAR2 activation with PAR2-AP or factor VIIa stimulation. These results are largely
consistent with other reports. For example, Annabi et al demonstrated that EGCG directly and indirectly decreased
MMP-9 activity in macrophage-like HL-60 cells [23]. Lim et
al showed that EGCG was able to inhibit the HGF-induced
MMP-9 activity in hypopharyngeal carcinoma cells [28]. Taken
together, these results suggest that EGCG is capable of blocking
the expression of some key molecules in tumor cells and,
therefore, it contributes to the suppression of cell proliferation,
migration and metastasis.

It is well known that the ERK1/2 and NF-κB signaling
pathways play a pivotal role in many cellular processes, such
as proliferation, apoptosis, and differentiation [24–27]. PAR2
activation induced by the TF/factor VIIa complex can trigger
cell signal transduction through different pathways, including
ERK1/2 and NF-κB [15, 20]. This study investigated whether
EGCG could reverse the activation of ERK-1/2 as well as
NF-κB by PAR2 activation. As shown in Figure 5, PAR2-AP,
and factor VIIa activated ERK-1/2, as indicated by increased
ERK-1/2 phosphorylation and NF-κB (p65/RelA) expression
in the nuclear fraction. However, EGCG dose-dependently
inhibited both ERK-1/2 and NF-κB activation by factor VIIa.
Similarly, EGCG at a concentration of 100 μg/mL obviously
inhibited PAR2-AP-induced ERK-1/2 and NF-κB activation.
These results indicate that the blockade of ERK-1/2 and NF-κB
activation is the major mechanism for the inhibitory effects of
EGCG on TF/factor VIIa/PAR2 axis-mediated cell prolifera-
tion and migration.

In summary, the present data, together with our previous
studies, strongly indicate that PAR2 activation by PAR2-AP
or the TF/factor VIIa complex triggers the phosphorylation of
ERK1/2 and the activation of the NF-κB signaling path-
way, thereby regulating the expression of caspase-7, TF, and

Caspase-7 has been defined as one of the key executioners
involved in apoptosis induced by various stimuli, and it is nec-
nessary for apoptosis-associated nuclear changes, such as chroma-
itin condensation [20]. It has been demonstrated that EGCG
can mediate the activation of caspases by inhibition of the
NF-kB pathway and subsequently induce apoptosis in human
epidermoid carcinoma A431 cells [21]. In our present study, we
further demonstrated that activation of PAR2 in SW620 cells
by PAR2-AP or factor VIIa suppressed caspase-7 expression at
both the mRNA and protein levels. EGCG reversed the down-
regulating effects of PAR2-AP or factor VIIa on the expression
of caspase-7 (Figure 3). These results suggested that EGCG
might enhance cell apoptosis and thereby reduce cell prolif-
eration. Whether EGCG can affect the expression of other
molecules related to cell apoptosis (such as caspase-3) remains
to be answered.
MMP-9, which further contributes to the proliferation and migration of colon cancer cells. EGCG, the major polyphenolic constituent in green tea, can target the axis of TF/factor VIIa/PAR2 and the ERK1/2/NF-κB signaling pathways and may serve as a preventive and therapeutic agent for colon cancers.

Acknowledgements
This project was supported by the Provincial Science Foundation of Jiangsu (No BK2010336) and the Student’s Scientific Research of Jiangsu University (Ng 09A080).

Author contribution
Hong ZHOU designed the research; Fang ZHOU performed the research and analyzed the data; Ting WANG and Ying WU contributed new reagents; Dong-lin GUO and Xiao-mei ZHANG contributed to the experiments; Yuan MU and Biao WU contributed analytic tools; and Fang ZHOU and Hong ZHOU wrote the paper.

Supplementary information
Supplementary tables are available at Acta Pharmacologica Sinica website of NPG.

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