Epigallocatechin-3-gallate (EGCG) downregulates gelatinase-B (MMP-9) by involvement of FAK/ERK/NFκB and AP-1 in the human breast cancer cell line MDA-MB-231

Triparna Sen, Anindita Dutta and Amitava Chatterjee

Epigallocatechin-3-gallate (EGCG) is effective against the initiation, progression, and invasion of carcinogenesis. Matrix-metalloproteinases (MMPs) are a family of endopeptidases that hydrolyze the majority of extracellular proteins. MMP-9 is one of the most important members of the family and we observed the effect of EGCG on MMP-9 in the human breast cancer cell line, MDA-MB-231. The effect of EGCG on MMP-9 was studied by gelatin zymography, western blot, quantitative and semiquantitative real-time RT-PCR, immunoflourescence, cell adhesion assay, enzyme-linked immunosorbent assay, and electrophoretic mobility shift assay. EGCG treatment reduced the activity, protein, and mRNA expression of MMP-9 and enhanced the expression of the tissue inhibitor of MMP 1 (TIMP-1). EGCG downregulated the activation of focal adhesion kinase (FAK) and extracellular regulated kinase (ERK), reduced the adhesion of MDA-MB-231 cells to fibronectin and vitronectin, and reduced the mRNA expression of the integrin receptors α5β1 and αvβ3. The expression of the nuclear factor kappa B (NFκB), and the DNA binding activity of NFκB and activator protein 1 (AP1) to MMP-9 promoter were noticeably reduced on EGCG treatment. Upregulation of TIMP-1 and disruption of the functional status of integrin receptors may indicate decreased MMP-9 activation; inhibition of FAK and ERK activation might indicate disruption in the FAK/ERK-induced MMP-9 secretion and induction. Decreased DNA binding activity of NFκB and AP1 to MMP-9 promoter might indicate transcriptional deregulation of MMP-9 gene on EGCG treatment. We propose EGCG as a potential inhibitor of the expression and activity of MMP-9 by a process involving FAK/ERK and transcription factors in MDA-MB-231. *Anti-Cancer Drugs* 21:632–644 © 2010 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Introduction

Epidemiological studies have shown that the consumption of green tea lowers the risk of developing various cancers including breast cancer [1]. The anticarcinogenic and antiproliferative effects of green tea have been attributed to the polyphenols present in it, the most abundant and biologically active being epigallocatechin-3-gallate (EGCG). EGCG has been reported to be most effective against the initiation, progression, and invasion stages of multistage carcinogenesis [2].

The progression of human tumors involves the matrix metalloproteinase (MMP) family [3,4]. Two members of this 24-member family, gelatinase A and B (MMP-2 and 9), play an important role in tumor invasion and metastasis [4]. MMP-2 and MMP-9 play an important role in the homeostasis of the extracellular matrix (ECM), and hence an imbalance in their expression or activity may have important consequences in various pathologies such as development and progression of cancer [5]. Similar to MMP-2, MMP-9 is a zinc-dependant endopeptidase produced in a proenzyme form and requires activation to perform their activities. MMP-9 can form a complex with the tissue inhibitor metalloproteinase-1 (TIMP-1). Although the exact role of this complex remains to be clearly established, many studies have suggested that the association between the latent and active forms of MMP-9 and TIMP-1 are involved in their stabilization and activation [3–6]. Integrins regulate the expression and activation of MMPs, and guide them to their targets by simultaneous binding of MMPs and ECM molecules. The integrins work in concordance with focal adhesion kinase (FAK), which serves as an important integration point of the growth factors and integrin signaling with respect to cell migration [7]. Postactivation, the function/activity of MMP-9 is regulated by various signaling kinases working in concordance.

Studies have suggested that EGCG inhibits tumor formation through inhibition of various cellular processes involved in cell adhesion [8,9] and invasion. Downregulation of MMPs is one of the principal mechanisms of inhibition of tumor growth and invasion by EGCG [10]. An earlier study from the same group has shown that EGCG downregulates MMP-2 in the breast cancer cell, MCF-7, by involvement of multiple regulatory factors [11].

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The earlier study instigated us to observe the effect of EGCG on MMP-9, another important gelatinase in breast cancer cell. EGCG has been found to inhibit MMP-9 and thereby prevent the invasion and spread of cancer [12]. EGCG is identified as an inhibitor of MMP-9 [13]. The molecular mechanism of MMP-9 inhibition by EGCG is far from being fully explored yet.

This study evaluates the effect of EGCG on the expression and activity of MMP-9 in a highly metastatic human breast cancer cell line, MDA-MB-231, which mainly releases MMP-9 in the culture supernatant. We observed the effect of EGCG on the molecules participating in MMP-9 regulation and elucidated a possible molecular mechanism of its action.

Materials and methods

Materials

Minimal essential medium, fetal bovine serum, fibronectin (440 kDa), and protease inhibitor cocktail tablets (complete, mini, EDTA-free) were purchased from Roche, Germany. Human Vitronectin was purchased from BD Biosciences, San Jose, California, USA. EGCG was obtained from Sigma-Aldrich, St Louis, Missouri, USA. Gelatin Sepharose 4B beads were purchased from GE Healthcare Bio-Sciences AB, Uppsala, Sweden. Anti-MMP-9, anti-NFκB, anti-FAK, anti-phospho FAK (tyr397), anti-TIMP-1, anti-ERK, anti-phospho-ERK, anti-PI-3K (p110), anti-phospho-PI-3K, and anti-actin antibodies were purchased from Santa Cruz Biotechnologies, Santa Cruz, California, USA. Alkaline phosphatase-coupled, HRP-coupled, and fluorescein isothiocyanate-coupled secondary antibodies (both monoclonal and polyclonal) and NBT-BCIP (nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate, western blue-stabilized substrate for alkaline phosphatase) were from Promega, Madison, Wisconsin, USA. Trypan blue (0.4%) was purchased from Gibco-BRL, Gaithersburg, Maryland, USA. SYBR green JumpStart Taq Readymix was purchased from Sigma-Aldrich. Primers and AP1, nuclear factor kappa B (NF-κB) probes were synthesized by Operon, Germany. RNAqueous 4 PCR (Total RNA isolation kit) and Retroscript (RT-PCR Kit) were purchased from Ambion, Austin, Texas, USA. NP-40 (nonidet P-40) was purchased from Amresco, Solon, Ohio, USA.

Methods

Cell culture

MDA-MB-231 (human breast cancer cell line) was obtained from the National Centre for Cell Sciences, Pune, India. Cells were grown and maintained in minimal essential medium containing 10% fetal bovine serum in a 5% CO_2 incubator at 37°C.

Treatment of cells with EGCG

MDA-MB-231 cells were treated with 20 μmol/l EGCG for 48 h in a serum-free culture medium (SFCM). The treated cells and SFCM were collected for further experiments.

Cell viability assay by the Trypan blue dye exclusion method

MDA-MB-231 cells (300 000 cells/ml) were grown in a SFCM in the absence and presence of 5, 10, 20, and 40 μmol/l EGCG for 48 h, and in the presence of 20 μmol/l EGCG for 24, 36, 48, and 60 h. The cells were trypsinised and a uniform cell suspension was made. Twenty microliters of the uniform cell suspension was taken and an equal volume (20 μl) of 0.4% Trypan blue was added, gently mixed, and allowed to stand for 5 min at room temperature. Ten microliters of the mixture was placed in a hemocytometer and the number of viable (unstained) and dead (stained) cells was calculated. The average number of unstained cells in each quadrant was calculated and multiplied by 2×10^4 to find the cells/ml. Cell viability was calculated by the following formula:

$$\text{Cell viability}\,(\%) = \left[\frac{\text{total viable cell (unstained)}}{\text{total cells (unstained and stained)}}\right] \times 100$$

Wound healing assay

MDA-MB-231 cells were cultured in a monolayer in the absence (control) and presence (experimental) of EGCG (20 μmol/l) for 48 h. The monolayer was then scratched with a sterile pipette tip, followed by washing with SFCM to remove cellular debris. The cells were maintained in fresh SFCM and cell migration was observed under a microscope and photographed at different time points (0, 12, and 24 h).

Cell adhesion assay

The microtiter plate wells were coated separately with fibronectin (1.56, 3.13, 6.25, 12.5, and 25 μg/ml fibronectin) and vitronectin (1.25, 2.5, and 5 μg/ml vitronectin) in triplicate. The ligands were allowed to bind for 1.5 h at 37°C. The wells were blocked with buffer C [1% BSA, 1 mmol/l $CaCl_2$ and 1 mmol/l $MgCl_2$, for vitronectin 1 mmol/l $MnCl_2$ (instead of $MgCl_2$) in PBS] for 1 h at 37°C. The cells (both control and experimental) were trypsinized from the culture dishes, washed, suspended in buffer C, and added to the microtiter plates (50 000 cells/well) and were allowed to bind at 37°C for 1.5 h. The wells were washed three times with buffer C. The bound cells were trypsinized, counted on a hemocytometer, and expressed as a percentage of adhesion.

Gelatin zymography

MDA-MB-231 cells (300 000 cells/ml) were grown in the absence and presence of EGCG in SFCM for the required time period. The culture supernatant was collected by centrifugation. The gelatinases were separated from

SFCM using Gelatin Sepharose 4B beads by shaking overnight at 4°C. The beads were washed three times with Tris-buffered saline with (0.02%) Tween-20 (TBST) and suspended in 50 µl of 1X sample buffer (0.075 g Tris, 0.2 g SDS in 10 ml water, pH 6.8) for 30 min at 37°C. The extract was then subjected to zymography on a 7.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis copolymerized with 0.1% gelatin. The gel was washed in 2.5% Triton-X-100 for 30 min to remove SDS and was then incubated overnight in a reaction buffer (50 mmol/l Tris-HCl pH 7, 4.5 mmol/l $CaCl_2$, 0.2 mol/l NaCl). After incubation, the gel was stained with 0.5% Coomassie blue in 30% methanol and 10% glacial acetic acid. The bands were visualized by destaining the gel with 30% methanol and 10% glacial acetic acid.

ELISA of MMP-9 in a serum-free culture medium

MDA-MB-231 cells (300 000 cells/ml) were grown in the absence and presence of 20 µmol/l EGCG for 48 h. For estimation of MMP-9 expression in SFCM, equal volumes (75 µl/well) of SFCM from both the control and experimental sets were added to the microtiter plate well in triplicate and kept overnight at 4°C. For estimation from the whole-cell extract (WCE), the cells were collected, extracted and the protein was estimated. Then 50 µg of protein was added to each well in triplicate and kept overnight at 4°C. Enzyme-linked immunosorbent assay (ELISA) was performed using anti-MMP-9 antibody (1 : 1000 dilutions for 1 h) as described earlier [14].

Immunoblot assay of MMP-9, focal adhesion kinase (FAK), phospho-FAK, tissue inhibitor of metalloproteinase-1 (TIMP-1), NFκB, extracellular regulated kinase (ERK), phospho-ERK, phosphatidyl inositol 3 kinase (PI-3K), and phospho-PI-3K:MDA-MB-231 cells (300 000 cells/ml) were grown in a serum-free culture medium (SFCM) in the absence and presence of 20 µmol/l EGCG for 48 h. In the case of MMP-9, the SFCM was collected and gelatinase was extracted from it by the gelatin sepharose beads and then eluted at 37°C for 30 min. The cells were collected, extracted with the cell extraction buffer (Tris – 37.7 mmol/l, NaCl – 75 mmol/l, Triton X-100 – 0.5%, protease inhibitor cocktail and pH adjusted to 7.5), and the protein content of the extracts was estimated by Lowry's method. An equal amount of protein (100 µg each) was taken and heated with 0.1 volumes β-mercaptoethanol for 5–8 min at 80–90°C, and then subjected to electrophoresis on a 7.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis. The proteins were electrophoretically transferred on to nitrocellulose membranes. The membranes were blocked with 1% BSA and subsequently washed thrice with TBST. The membranes were reacted with the respective primary antibodies at 1/1000 dilution each and kept at 37°C for 1 h 30 min and subsequently washed thrice with TBST. The blots were developed using the respective alkaline phosphatase-coupled secondary antibodies at 1/1000 dilution and kept at 37°C for 1 h 30 min; the blots were then thoroughly washed six times with TBST. The bands were visualized using NBT-BCIP as the substrate.

Semiquantitative RT-PCR

RNA was extracted from 1×10^6 cells/ml MDA-MB-231 cells grown in the absence and presence of 20 µmol/l EGCG for 48 h. The cells were washed in PBS and total RNA was extracted (RNaqeous, Ambion, USA) from the cells. A two-step RT-PCR (Retroscript, Ambion, USA) was performed with equal amounts of total RNA, using specific primers for PCR. Twenty microliters of each PCR product were run on a 2.5% agarose gel and bands were visualized under UV. Glyceraldehyde phosphate dehydrogenase (GAPDH) primers were used as control to normalize for mRNA integrity and equal loading. The primer sequences and PCR cycles/conditions for each primer are tabulated below.

Quantitative real-time RT-PCR

Real-time quantitative RT-PCR using relative quantitation by the comparative C_T method was used to determine mRNA expression. Two microliters of cDNA were subjected to real-time quantitative RT-PCR using the real-time PCR (ABI-7500, Foster City, California, USA) with SYBR green as a fluorescent reporter using the SYBR Green JumpStart Taq Readymix (Sigma, St Louis, Missouri, USA). The specific gene primers (MMP-9, FAK, TIMP-1, α5, β1, αv, β3 and the internal control gene G3PDH) were amplified in separate reaction tubes. The threshold cycle number (C_T) of the triplicate reactions was determined using the ABI-7500 software and the mean C_T of the triplicate reactions was determined. The levels of specific gene expression were normalized to G3PDH levels using the formula $2^{-\Delta\Delta C}$T, where $\Delta\Delta C_T = \Delta C_T$ (sample)–ΔC_T (calibrator) and ΔCT is the C_T of the housekeeping gene (G3PDH) subtracted from the C_T of the target genes. The calibrator used in our experiments is the control MDA-MB-231 cells and the samples are the EGCG-treated (20 µmol/l, 48 h) MDA-MB-231 cells, the ΔC_T value being inversely proportional to the mRNA expression of the samples. No primer dimers were obtained for either the target genes or G3PDH as assessed by the melt curve analysis. The specificity of the products was also confirmed by the melt curve analysis. The reaction conditions and the primer sequences are tabulated below. The PCR cycles in all the cases were started with Taq activation at 94°C for 5 min and followed by final extension of 72°C for 7 min.

Immunocytochemical analysis of FAK, p-FAK, and NFκB

MDA-MB-231 cells were grown on coverslips in the absence (control) and in the presence of 20 µmo/l EGCG for 48 h (experimental). The cells on the coverslips were fixed with 3.5% formaldehyde and treated with 0.5% Triton X-100. BSA (1%) solution was used for blocking

PCR cycles and conditions

cDNA	Primer sequence	PCR cycles (40 cycles)	Amplicon size (bp)
hMMP-9	5′-TTGAGTCCGGCAGACAATCC-3′ (forward) 5′-CTTATCCACGCGAATGACG-3′ (reverse)	94°C-30 s 56°C-30 s 72°C-90 s	198
hFAK	5′-GCGCTGGCTGGAAAAAGAGGAA-3′ (forward) 5′-TCGGTGGGTGCTGGCTGGTAGG-3′ (reverse)	94°C-30 s 60°C-90 s 72°C-30 s	475
hTIMP-1	5′-CACCCACAGACGGCCTTCTGCAAT-3′ (forward) 5′-AGTGTAGGTCTTGGTGAAGCC-3′ (reverse)	94°C-30 s 58°C-30 s 72°C-90 s	256
α5	5′-CATTTCCGAGTCTGGGCCAA-3′ (forward) 5′-TGGAGGCTTGAGCTGAGCTT-3′ (reverse)	94°C-30 s 58°C-30 s 72°C-90 s	324
β1	5′-TGTTCAGTGCAGAGCCTTCA-3′ (forward) 5′-CCTCATACTTCGGATTGACC-3′ (reverse)	94°C-30 s 58°C-30 s 72°C-90 s	452
αV	5′-GTTGGGAGATTAGACAGAGGA-3′ (forward) 5′-CAAAACAGCCAGTAGCAACAA-3′ (reverse)	94°C-30 s 58°C-30 s 72°C-90 s	288
β3	5′-GGGGACTGCCTGTGTGACTC-3′ (forward) 5′-CTTTTCGGTCGTGGATGGTG-3′ (reverse)	94°C-30 s 58°C-30 s 72°C-90 s	544
G3PDH	5′-CGGAGTCAACGGATTTGGTCGTAT-3′ (forward) 5′-AGCCTTCTCCATGGTGGTGAAGAC-3′ (reverse)		454

and the cells were incubated with the anti-FAK, anti-p-FAK, and anti-NFκB primary antibody (1 : 1000 dilution for 1.5 h at 37°C), washed three times with PBS, and incubated with the fluorescein isothiocyanate-coupled secondary antibody (1 : 1000 dilution for 1.5 h at 37°C). The cells were washed thoroughly six times in PBS and the coverslips were mounted on glass slides and observed under the fluorescence microscope.

Electrophoretic mobility shift assay

Nuclear extraction from cells: MDAMB231 cells (300 000 cells/ml) were grown in absence (C) and presence (E) of EGCG (20 μmol/l) for 48 h in SFCM. The cells were collected and resuspended in 1 ml of hypotonic buffer [10 mmol/l Hepes pH 7.9, 1.5 mmol/l $MgCl_2$, 10 mmol/l KCl, 0.5 mmol/l phenylmethylsulfonylfluoride (PMSF), 0.5 mmol/l DTT]. The cells were pelleted and lysed in hypotonic buffer containing 0.5% NP-40. The nuclear pellet was lysed in a lysis buffer (20 mmol/l Hepes (pH 7.9), 420 mmol/l NaCl, 1.5 mmol/l $MgCl_2$, 0.2 mmol/l EDTA, 0.5 mmol/l PMSF, 25% v/v glycerol). The nuclear extract was removed into a storage buffer (10 mmol/l Hepes pH 7.9, 50 mmol/l KCl, 0.2 mmol/l EDTA, 20% v/v glycerol, 0.5 mmol/l PMSF, 0.5 mmol/l DTT). The protein content of the nuclear extract was determined using Lowry's method. Labeling of probes – the probes of the double-stranded oligonucleotides for NF-κB, Sp1 and AP1 – was based on the human MMP-9 promoter sequence as follows: NF-κB (5′-TGG AAT TCC CAG), AP1 (5′-CCT GAG TCA GCA). The complementary oligonucleotides were annealed using the annealing buffer (10 mmol/l Tris pH 8, 50 mmol/l NaCl, 1 mmol/l EDTA) by heating at 90°C for 2 min. NF-κB and AP1 oligonucleotides were end-labeled with [γ^{-32}P]-ATP using T4 polynucleotide kinase (Promega) by incubating for 1 h at 37°C. Five micrograms of the nuclear protein from the control and treated cells were incubated with ^{32}P-labeled oligonucleotide probes using 2X binding buffer [25 mmol/l Hepes (pH7.6), 1 mmol/l EDTA, 0.5 mmol/l DTT, 5 mmol/l $MgCl_2$, 75 mmol/l KCl, 10% glycerol] for 30 min at room temperature in a final volume of 20 μl. After binding, the protein–DNA complexes were electrophoresed on a native 5% polyacrylamide gel using 0.5X TBE buffer. Each gel was then dried and subjected to autoradiography at –80°C.

Quantification of the results

The bands of zymography, western blots, and RT-PCR were quantitated using the Image J Launcher (version 1.4.3.67, Research Services Branch, NIMH, Bethesda, Maryland, USA).

Results

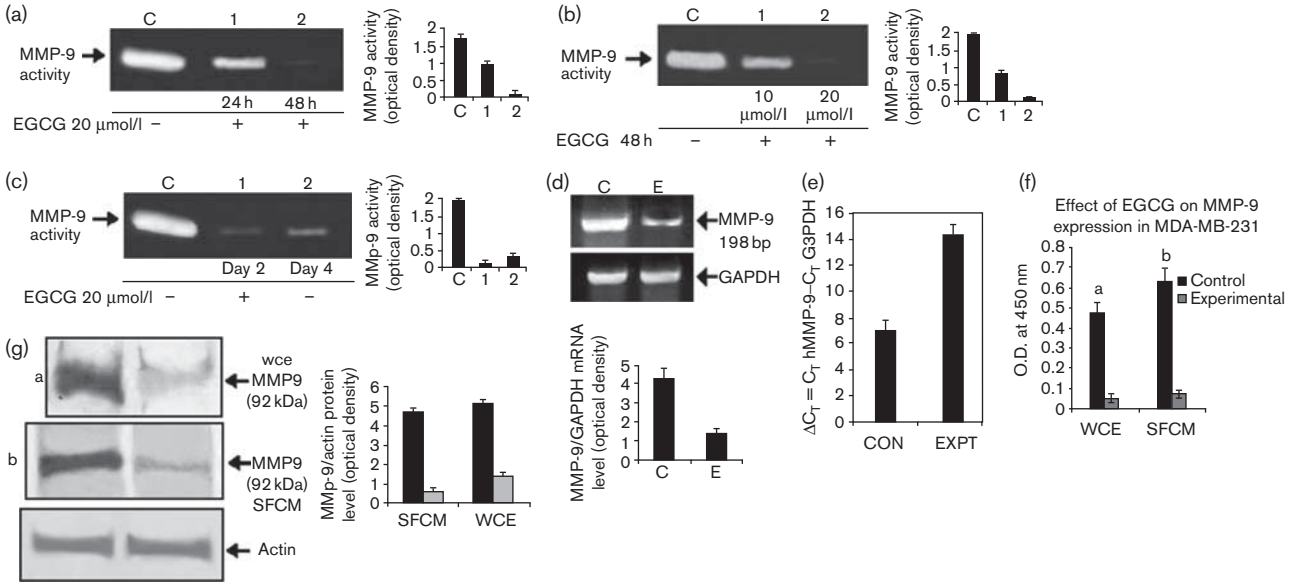
EGCG downregulates the gelatinolytic activity of MMP-9 in a dose and time-dependant manner in MDA-MB-231 cells

Figure 1a MDA-MB-231 cells (300 000 cells/ml) were grown in the absence (lane C) and presence of 20 μmol/l EGCG for 24 h (lane 1) and 48 h (lane 2). The zymographic analysis of MMP-9 activity in SFCM clearly shows that EGCG treatment causes a time-dependant inhibition of MMP-9 activity with an appreciable inhibition with 20 μmol/l EGCG treatment for 48 h (lane 2). Figure 1b MDA-MB-231 cells (300 000 cells/ml) were grown in the absence (lane C) and presence of 10 μmol/l EGCG (lane 1) and 20 μmol/l EGCG (lane 2) for 48 h. The comparative zymographic analysis showed a dose-dependent decrease in MMP-9 activity with the treatment of EGCG and appreciable inhibition was observed with the treatment of 20 μmol/l EGCG for 48 h (lane 2). The accompanying arrays in both the figures represent the comparative densitometric/quantitative analysis of the band intensities using the Image J Launcher (version 1.4.3.67).

EGCG downregulates the mRNA expression level of MMP-9 and the protein expression level of MMP-9 in the whole-cell extract and culture supernatant in MDA-MB-231 cells

The effect of EGCG on the mRNA expression of MMP-9 was determined in the MDA-MB-231 cells

Fig. 1



Effect of epigallocatechin-3-gallate (EGCG) on the gelatinolytic activity and expression of MMP-9 in MDA-MB-231 cells: Zymographic analysis of MMP-9. (a) MDA-MB-231 cells (300 000 cells/ml) were grown in a serum-free culture medium (SFCM) in the absence (lane C) and presence of 20 μmol/l EGCG for 24 h (lane 1) and 48 h (lane 2). (b) MDA-MB-231 cells (300 000 cells/ml) were grown in a serum-free culture medium (SFCM) in the absence (lane C) and presence of 10 μmol/l EGCG (lane 1) and 20 μmol/l EGCG (lane 2) for 48 h. The gelatinases in all the cases were separated from SFCM by mixing Gelatin Sepharose 4B beads and subjected to gelatin zymography. The accompanying graph represents the comparative densitometric/quantitative analysis of the band intensities using Image J Launcher (version 1.4.3.67). Data are means ± SEM of three experiments. Determination of gene expression of MMP-9 by semiquantitative and Quantitative Real-Time RT-PCR: MDA-MB-231 cells were grown in the absence (lane C) and presence of 20 μmol/l EGCG (lane E) for 48 h in SFCM. Total RNA was extracted from the control and fibronectin-treated MDA-MB-231 cells (1×10^6 cells). (c) A two-step RT-PCR was performed with equal amounts of total RNA, using specific primers for PCR (MMP 9). GAPDH primers were used to confirm equal loading. The array represents the comparative densitometric/quantitative analysis of the band intensities using Image J Launcher (version 1.4.3.67). Data are means ± SEM of three experiments. (d) 2 μl of cDNA was subjected to real-time quantitative RT-PCR using SYBR Green as a fluorescent reporter. The calibrator used in our experiments is the control untreated (CON) MDA-MB-231 cells and the samples are the EGCG treated (20 μmol/l, 48 h) (EXPT) MDA-MB-231 cells. In the given graph, the C_T value is inversely proportional to the mRNA expression of the samples. Determination of protein expression of MMP-9 by enzyme-linked immunosorbent assay (ELISA) and immunoblot analysis: MDA-MB-231 cells (300 000 cells/ml) were grown in A serum-free culture medium (SFCM) in the absence and presence of 20 μmol/l EGCG for 48 h in SFCM. For ELISA (e) the SFCM (50 μl each well) and whole-cell extract (WCE) (50 μg protein each well) was coated on the ELISA plate and developed with MMP-9 antibody. The culture supernatants (SFCM) (f-b) were collected and the gelatinases were separated from SFCM using Gelatin Sepharose 4B beads shaking for overnight at 4°C. In case of the whole-cell extract (WCE) (f-a), the cells were collected; extracted and equal protein (100 μg) was subjected to western blot analysis with anti-MMP-9 antibody (1 : 1000 dilution for 1.5 h at 37°C). (g) Actin was used as an internal control and carried out in parallel with all the blots. The accompanying graphs represent the comparative densitometric/quantitative analysis of the band intensities using Image J Launcher (version 1.4.3.67). Data are means ± SEM of three experiments. O.D., optical density.

by semiquantitative RT-PCR and confirmed by real-time RT-PCR. In Fig. 1c the semiquantitative RT-PCR profile of MDA-MB-231 cells clearly indicates that the treatment of MDA-MB-231 cells with 20 μmol/l EGCG for 48 h (lane E) downregulates MMP-9 (198 bp) at the mRNA level as compared with the control cells (lane C). GAPDH were used as control to normalize for mRNA integrity and equal loading. The accompanying array represents the comparative densitometric/quantitative analysis of the band intensities using Image J Launcher (version 1.4.3.67). The expression of MMP-9 was also confirmed by real-time quantitative RT-PCR (Fig. 1d). In each reaction, the threshold cycle number (C_T) was determined for both the target (MMP-9) and the housekeeping (G3PDH) genes using the real-time PCR software and the mean C_T for the three reactions were calculated. ΔC_T value is inversely proportional to the expression of MMP-9. The expression of MMP-9 decreased 16-fold in the EGCG-treated MDA-MB-231 cells as compared with the untreated control set. These data suggest effective prevention of MMP-9 expression by EGCG treatment. The comparative ELISA profile of Fig. 1e shows that EGCG treatment of 20 μmol/l for 48 h appreciably downregulates the protein expression of MMP-9, both in the WCE and in the serum-free culture supernatant. This inhibition was further confirmed by an immunoblot analysis. The comparative immunoblot of Fig. 1f-a shows that the treatment of 20 μmol/l EGCG for 48 h causes an appreciable inhibition of the expression of MMP-9 (92 kDa) in the WCE in the experimental set (lane E) as compared with the control (lane C). In Fig. 1f-b the comparative immunoblot analysis shows that the treatment of 20 μmol/l EGCG for 48 h appreciably decreases the expression of MMP-9 (92 kDa) in the serum-free culture medium and the experimental set (lane E) as compared with the control (lane C). Actin was used as an internal control and was

carried out in parallel with the MMP-9 blots. The accompanying array represents the comparative densitometric/quantitative analysis of the band intensities using Image J Launcher (version 1.4.3.67).

Effect of EGCG on viability of MDA-MB-231 cells

In Fig. 2 the viability of the MDA-MB-231 cells after the treatment of EGCG at different concentrations (Fig. 2a) and different time points (Fig. 2b) was calculated by the Trypan blue dye exclusion assay. The viability of the MDA-MB-231 cells after 20 μmol/l EGCG treatment for 48 h was found to be about 95%. The cells were trypsinised and replated as well to ascertain the viability of the EGCG-treated cells.

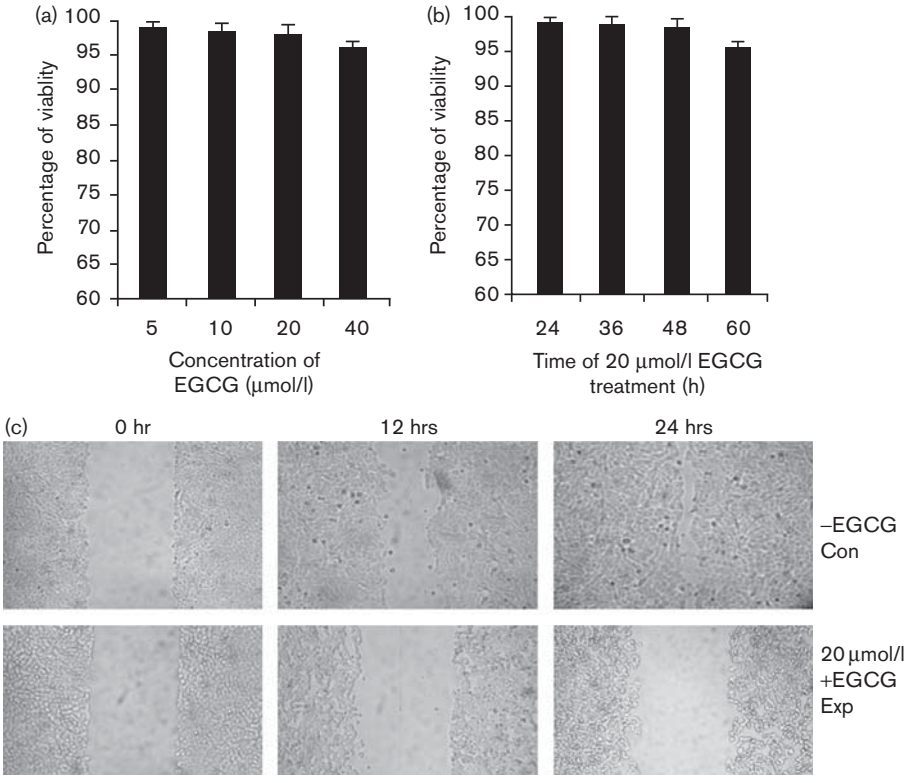
EGCG inhibits the motility/migration of MDA-MB-231 cells

Figure 2c the in-vitro wound healing assay showed that EGCG considerably decreased the migration of the MDA-MB-231 cells as compared with the untreated control cells. The EGCG-treated hindrance in motility was measured from 0 to 24 h.

EGCG upregulates the expression of TIMP-1 in MDA-MB-231 cells

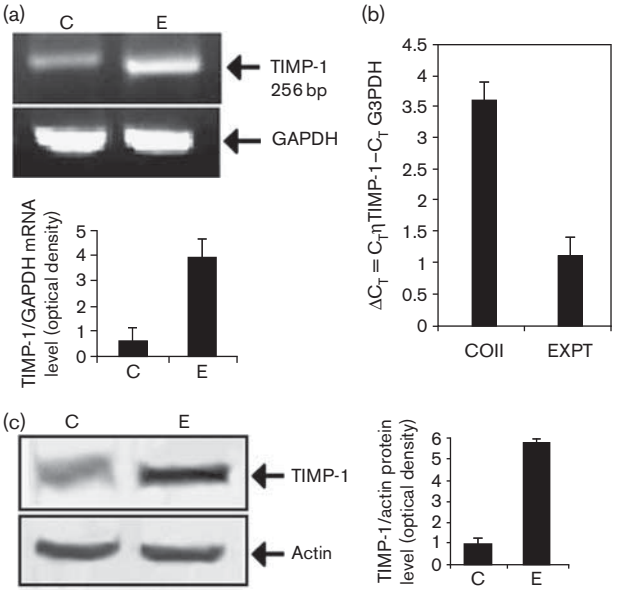
The effect of EGCG on the expression of TIMP-1 mRNA in MDA-MB-231 cells was determined by a semiquantitative RT-PCR followed by a real-time RT-PCR. In Fig. 3a the semiquantitative RT-PCR profile of TIMP-1 in MDA-MB-231 cells clearly indicates that the treatment of MDA-MB-231 cells with 20 μmol/l EGCG for 48 h (lane E) appreciably upregulates TIMP-1 (256 bp) at the mRNA level as compared with the control cells (lane C). GAPDH were used as control to normalize for mRNA integrity and equal loading. The accompanying array represents the comparative densitometric/quantitative analysis of the band intensities using Image J Launcher (version 1.4.3.67). The expression of TIMP-1 was also confirmed by real-time quantitative RT-PCR (Fig. 3b). In each reaction, the threshold cycle number (C_T) was determined for both the target (TIMP-1) and the housekeeping (G3PDH) genes using the real-time PCR software and the mean C_T for the three reactions were calculated. The expression of TIMP-1 increased 32-fold in the EGCG-treated MDA-MB-231 cells as compared with the untreated control set. The data suggest effective upregulation of TIMP-1

Fig. 2



Effect of epigallocatechin-3-gallate (EGCG) on the viability of MDA-MB-231 cells: MDA-MB-231 cells (300 000 cells/ml) were grown in the absence and presence of 5, 10, 20, and 40 μmol/l EGCG for 48 h (a) and in the presence of 20 μmol/l EGCG for 24, 36, 48, and 60 h (b) and the viability of the cells were determined by Trypan Blue Dye Exclusion Assay. (c) Wound healing assay: MDA-MB-231 cells were cultured in a monolayer in the absence (–EGCG) and presence (+EGCG) of EGCG (20 μmol/l) for 48 h. The monolayer was scratched with a sterile pipette tip, followed by washing with SFCM to remove cellular debris. The cells were maintained in fresh SFCM and cell migration was observed at 0, 12, and 24 h.

Fig. 3



Effect of epigallocatechin-3-gallate (EGCG) on MT1-MMP: MDA-MB-231 cells (300 000 cells/ml) were grown in a serum-free culture medium (SFCM) in the absence (lane C) and presence of 20 μmol/l EGCG for 48 h (lane E). (a) Total RNA was extracted from the control and EGCG-treated (1×10^6) MDA-MB-231 cells. A two-step RT-PCR was performed with equal amounts of total RNA, using specific primer for PCR (TIMP-1). 20 μl of each PCR products were run on a 2.5% agarose gel and bands visualized under UV. GAPDH primers were used to confirm equal loading. (b) 2 μl of cDNA was subjected to real-time quantitative RT-PCR using SYBR Green as a fluorescent reporter and specific primer for TIMP-1. The calibrator used in our experiments is the control EGCG-untreated (CON) MDA-MB-231 cells and the samples are the EGCG treated (20 μmol/l, 48 h) (EXPT) MDA-MB-231 cells. In the given graph, the C_T value is inversely proportional to the mRNA expression of the samples. (c) The respective cells were collected, extracted in cell extraction buffer and the cell lysates of the control and experimental cells were subjected to immunoblot analysis with anti-TIMP-1 antibody. Actin was used as an internal control and carried out in parallel with the blot. The accompanying array represents the comparative densitometric/quantitative analysis of the band intensities using Image J Launcher (version 1.4.3.67). Data are means ± SEM of three experiments.

mRNA expression by EGCG treatment. In Fig. 3c the comparative immunoblot shows that the treatment of 20 μmol/l EGCG for 48 h appreciably enhances the protein expression of TIMP-1 (22 kDa) in experimental set (lane E) as compared with the control (lane C) in MDA-MB-231 cells. Actin was used as an internal control and carried out in parallel with the TIMP-1 blot. The accompanying array represents the comparative densitometric/quantitative analysis of the band intensities using Image J Launcher (version 1.4.3.67).

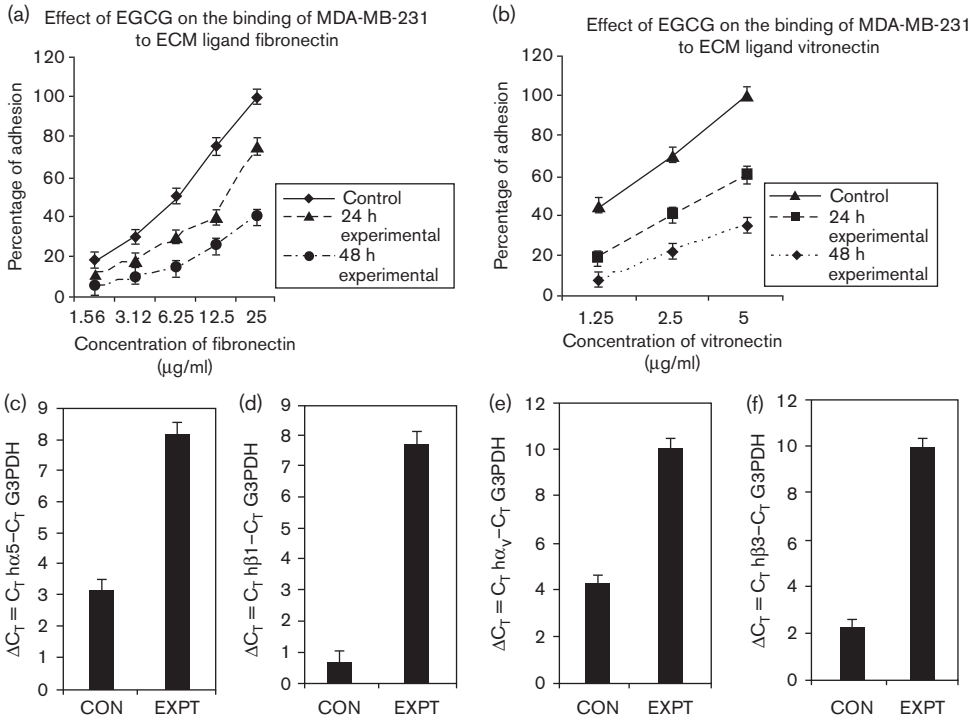
EGCG inhibits the binding of MDA-MB-231 cells to the extracellular matrix proteins, fibronectin and vitronectin, and inhibits the mRNA expression of the integrin receptors

In Fig. 4a the graphical representation of the cell adhesion assay shows that the control MDA-MB-231 cells efficiently bind to the ECM ligand fibronectin. The treatment of the MDA-MB-231 cells with 20 μmol/l EGCG for 24 and 48 h appreciably downregulates the binding of the cells to fibronectin as compared with the control cells in a time-dependant manner. In Fig. 4b the graphical representation of the cell adhesion assay shows that the control MDA-MB-231 cells efficiently bind to the ECM ligand vitronectin. The treatment of the MDA-MB-231 cells with 20 μmol/l EGCG for 24 and 48 h appreciably inhibits the binding of the cells to vitronectin as compared with the control cells in a time-dependant manner. The effect of EGCG on the mRNA expression of integrin receptors such as α5, β1, αv, and β3 in MDA-MB-231 cells was determined by real-time RT-PCR. In each reaction, the threshold cycle number (C_T) was determined for both the target α5 (Fig. 4c), β1 (Fig. 4d), αv (Fig. 4e), and β3 (Fig. 4f) and the housekeeping (G3PDH) genes using the real-time PCR software and the mean C_T for the three reactions were calculated. ΔC_T value is inversely proportional to the expression of the target genes. The expression of α5, β1, αv, and β3 decreased 40-fold, 64-fold, 40-fold, and 32-fold, respectively, in the EGCG-treated MDA-MB-231 cells as compared with the untreated control set. The data suggest effective inhibition of mRNA expression integrin receptors by EGCG treatment.

EGCG downregulates the expression and activation of FAK in MDA-MB-231 cells

In Fig. 5a the comparative immunoblot shows that the treatment of 20 μmol/l EGCG for 48 h inhibits the protein expression of FAK (upper panel) and also appreciably decreases the activation/phosphorylation of FAK (lower panel) in MDA-MB-231 cells. The inhibition is evident from the appreciable decrease in the 125 kDa FAK bands in the EGCG-treated sets (lane E) as compared with the untreated control set (lane C). Actin was used as an internal control and carried out in parallel with the blots. The accompanying array represents the comparative densitometric/quantitative analysis of the band intensities using Image J Launcher (version 1.4.3.67). The effect of EGCG on the mRNA expression of FAK in the MDA-MB-231 cells was determined by semiquantitative RT-PCR and confirmed by real-time RT-PCR. In Fig. 5b the RT-PCR profile of FAK in MDA-MB-231 cells clearly indicates that the treatment of MDA-MB-231 cells with 20 μmol/l EGCG for 48 h (lane E) downregulates FAK (475 bp) at the mRNA level as compared with the control cells (lane C). GAPDH were used as control to normalize for mRNA integrity and equal loading. The accompanying array represents the comparative densitometric/quantitative analysis of the band intensities using Image J Launcher (version 1.4.3.67). The mRNA expression of FAK was also confirmed by real-time quantitative RT-PCR (Fig. 5c). In each reaction, the threshold cycle number (C_T) was determined for both the target (FAK) and the housekeeping (G3PDH) genes using the real-time PCR software and the mean C_T for the three reactions were calculated. The ΔC_T value is inversely proportional to the expression of FAK. The expression of FAK decreased 32-fold in the EGCG-treated MDA-MB-231

Fig. 4



Effect of epigallocatechin-3-gallate (EGCG) on binding of MDA-MB-231 cells to the ECM ligands, fibronectin and vitronectin, and mRNA expression of α5β1 and αvβ3 integrin receptors in MDA-MB-231 cells: MDA-MB-231 cells (300 000 cells/ml) were grown in a complete medium in the absence (control) and presence of 20 μmol/l EGCG for 24 and 48 h. The ligands fibronectin (a) and vitronectin (b) in different concentrations (in triplicate), were allowed to bind to the wells for 1.5 h at 37°C. Cell adhesion assay was performed for both the control and experimental sets. Two microliters of cDNA was subjected to real-time quantitative RT-PCR using SYBR Green as a fluorescent reporter. Relative levels of expression of α5 (c) β1 (d) αv (e) β3 (f) and the control G3PDH in control and EGCG treated (20 μmol/l for 48 h) MDA-MB-231 cells as measured by quantitative real-time RT-PCR by calculating the C_T value. The calibrator used in our experiments is the untreated control (CON) MDA-MB-231 cells and the samples are the EGCG treated (20 μmol/l, 48 h) (EXPT) MDA-MB-231 cells. In the given graphs, the C_T value is inversely proportional to the mRNA expression of the samples.

cells as compared with the untreated control set. These data suggest effective inhibition of FAK expression by EGCG treatment. The immunocytochemical analysis of FAK (Fig. 5d) and p-FAK (Fig. 5e) shows that the expression of FAK and p-FAK in the cells treated with 20 μmol/l EGCG appreciably decreases as compared with the EGCG-untreated set (control) in a time-dependant manner with appreciable reduction after 48 h of EGCG treatment (48 h EGCG).

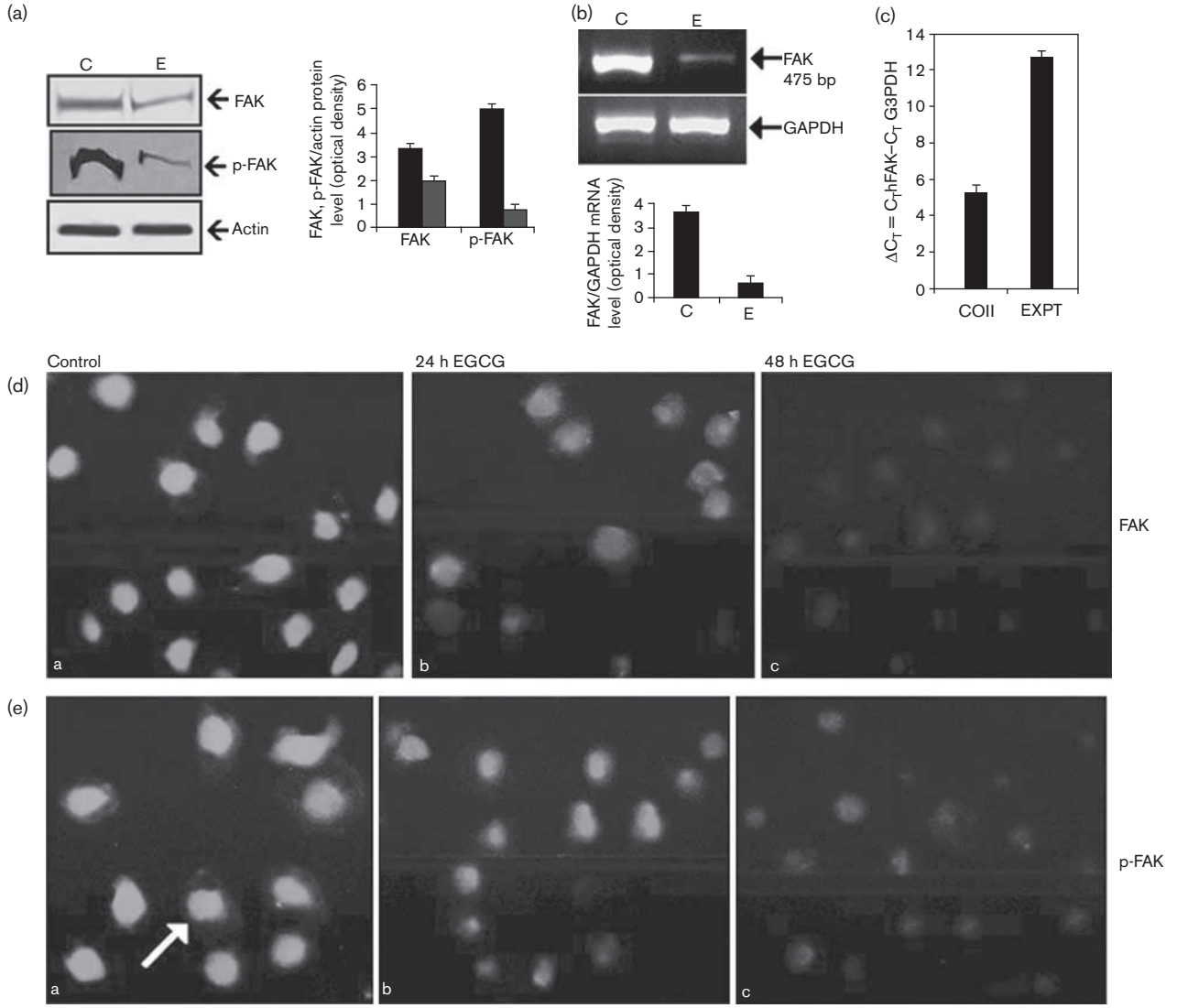
Effect of EGCG on signaling molecules such as ERK/p-ERK and PI-3K/p-PI-3K in MDA-MB-231 cells

In Fig. 6a-a the comparative immunoblot shows that the treatment of MDA-MB-231 cells with 20 μmol/l EGCG for 48 h (lane E) does not cause any appreciable change in the protein expression level of PI-3K as compared with the control MDA-MB-231 cells (lane C). The comparative immunoblot of Fig. 6a-b shows that the treatment of MDA-MB-231 cells with 20 μmol/l EGCG for 48 h (lane E) causes a minor inhibition in the phosphorylation of PI-3K as compared with the control cells (lane C). The comparative immunoblot in Fig. 6b-a indicates that the treatment of MDA-MB-231 cells with 20 μmol/l EGCG for 24 h (lane E) does not cause any appreciable change in the protein expression of ERK as compared with the control (lane C) MDA-MB-231 cells without EGCG treatment. However, the comparative immunoblot of Fig. 6b-b shows that the treatment of 20 μmol/l EGCG for 24 h causes an appreciable decrease in the phosphorylation level of ERK in the EGCG-treated MDA-MB-231 cells (lane E) as compared with the control cells (lane C). Actin was used as an internal control and carried out in parallel with all the blots. The accompanying arrays of the figures represent the respective comparative densitometric/quantitative analysis of the band intensities using Image J Launcher (version 1.4.3.67).

EGCG downregulates the expression of NFκB in MDA-MB-231 cells

In Fig. 7a the comparative immunoblot shows that the treatment of MDA-MB-231 cells with 20 μmol/l EGCG for 48 h (lane E) causes an appreciable reduction in the protein expression level of NFκB as compared with the untreated control set (lane C). Actin was used as an internal control and carried out in parallel with the blot. The accompanying array of the figure represents the

Fig. 5



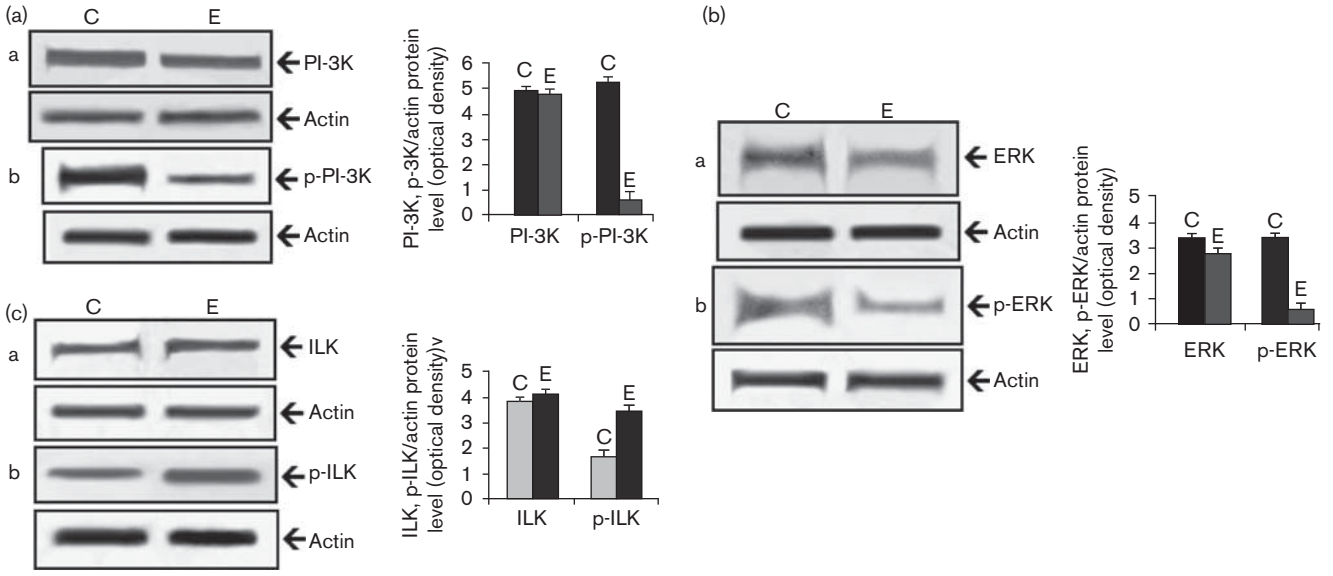
Effect of epigallocatechin-3-gallate (EGCG) on focal adhesion kinase (FAK): MDA-MB-231 cells (300 000 cells/ml) were grown in a serum-free culture medium (SFCM) in the absence (lane C) and presence of 20 μmol/l EGCG for 48 h (lane E). (a) The respective cells were collected, extracted in cell extraction buffer and the cell lysates of the control and experimental cells were subjected to immunoblot analysis with anti-FAK (upper panel) and anti-p-FAK (lower panel) antibody. Actin was used as an internal control and carried out in parallel with the blots. The accompanying array represents the comparative densitometric/quantitative analysis of the band intensities using Image J Launcher (version 1.4.3.67). Data are means ± SEM of three experiments. (b) Total RNA was extracted from the control and EGCG-treated MDA-MB-231 (1×10^6) cells. A two-step RT-PCR was performed with equal amounts of total RNA, using specific primer for PCR (FAK). GAPDH primers were used to confirm equal loading. (c) 2 μl of cDNA was subjected to real-time quantitative RT-PCR using SYBR Green as a fluorescent reporter. The calibrator used in our experiments is the untreated control (CON) MDA-MB-231 cells and the samples are the EGCG treated (20 μmol/l, 48 h) (EXPT) MDA-MB-231 cells. In the given graph, the C_T value is inversely proportional to the mRNA expression of the samples. MDA-MB-231 cells were grown on coverslips in the absence (control) and presence of 20 μmol/l EGCG for 24 and 48 h. The control and EGCG-treated cells were then subjected to immunocytochemical analysis with anti-FAK (d) and anti-p-FAK (e) primary antibody and then incubated with a fluorescein isothiocyanate-labeled secondary antibody. The coverslips were mounted on glass slides and observed under a fluorescence microscope.

respective comparative densitometric/quantitative analysis of the band intensities using Image J Launcher (version 1.4.3.67). The immunocytochemical analysis of Fig. 7b shows that the treatment with 20 μmol/l EGCG appreciably decreases the expression of NFκB as compared with the EGCG-untreated set (control) in a time-dependent manner with appreciable reduction after 48 h of EGCG treatment (48 h EGCG). There was no observable nuclear translocation of NFκB as well.

EGCG inhibits the DNA binding activity of NFκB and Ap-1 to the MMP-9 promoter

EMSA showed that EGCG treatment appreciably reduced the DNA binding activity of NF-κB (Fig. 7c, lane E) and

Fig. 6



Effect of epigallocatechin-3-gallate (EGCG) on different signaling molecules involved in the regulation of MMP-9 in MDA-MB-231 cells. Western blot analysis of PI-3K, p-PI-3K, ERK, and p-ERK in EGCG-treated MDA-MB-231 cells: MDA-MB-231 cells (300 000 cells/ml) were grown in a serum-free culture medium (SFCM) in the absence (lane C) and presence of 20 μmol/l EGCG for 48 h (lane E). The cells were collected; extracted and equal amount of protein (100 μg) was subjected to western blot analysis with anti-PI3K (a-a), anti-p-PI-3K (a-b), anti-ERK (b-a) and anti-p-ERK (b-b), antibodies (1 : 1000 dilution for 1.5 h at 37°C). (c) Actin was used as an internal control and carried out in parallel with all the blots. The accompanying graphs represent the comparative densitometric/quantitative analysis of the band intensities using Image J Launcher (version 1.4.3.67). Data are means ± SEM of three experiments.

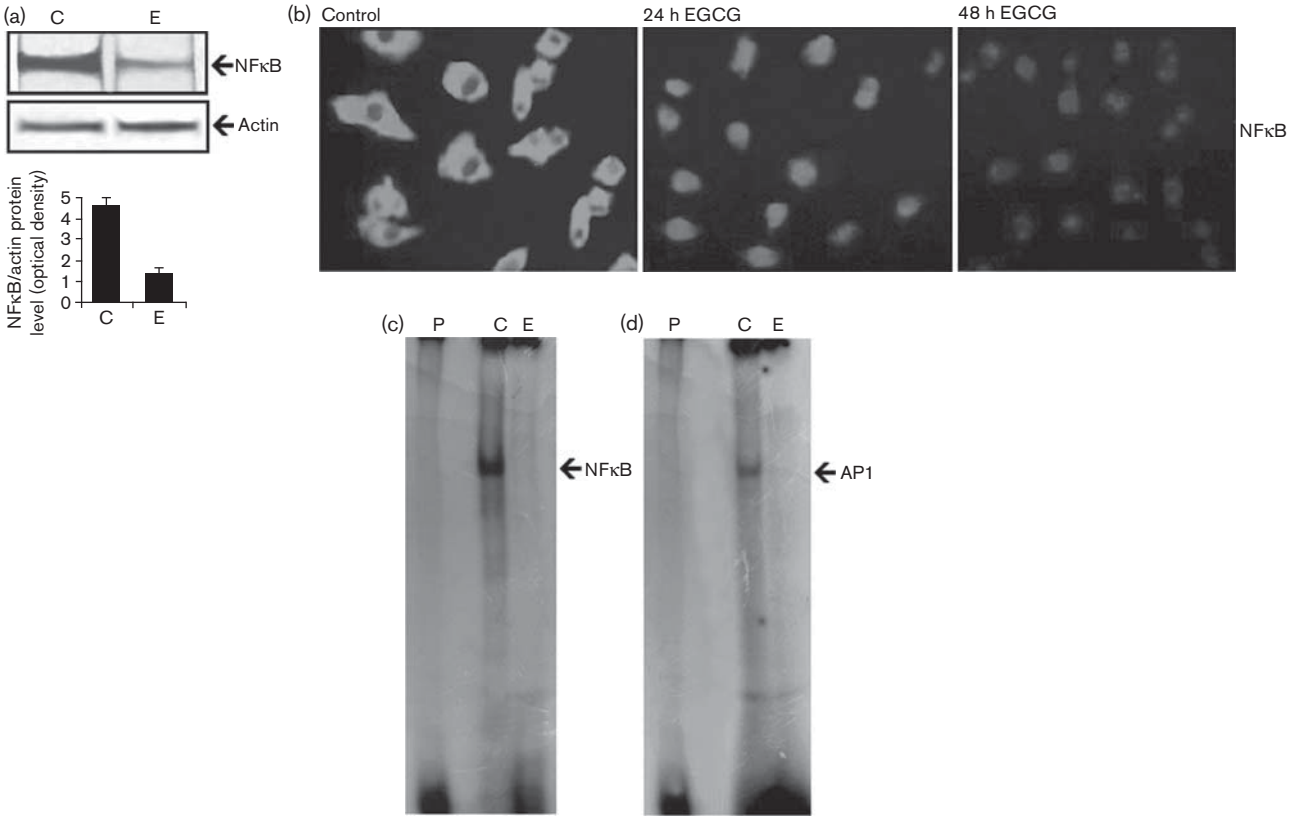
AP-1 (Fig. 7d lane E) to the nuclear protein as compared with the untreated controls (lane C).

Discussion

EGCG has been shown to possess anticarcinogenic and chemopreventive effects in various types of cancer, including breast cancer [1,15]. In our earlier study we have shown the inhibitory effect of EGCG on a very important ECM-degrading metalloproteinase, MMP-2, in the human breast cancer cell line, MCF-7 [11]. In this study we show that the treatment of EGCG (20 μmol/l, 48 h) has a strong inhibitory effect on MMP-9, another pivotal endopeptidase, in a highly metastatic human breast cancer cell line, MDA-MB-231. EGCG in the present experimental conditions caused a strong inhibition of the gelatinolytic activity of MMP-9 in a dose and time-dependent manner. This is in accord with earlier evidence showing the inhibitory effect of EGCG on MMP-9 activity [13]. EGCG treatment caused an appreciable inhibition of MMP-9 in mRNA level as is evident from the semiquantitative and quantitative real-time RT-PCR profiles. Western blot analysis and ELISA confirmed the inhibitory effect of EGCG on the protein expression of MMP-9 in both the WCE and culture supernatant. Earlier reports provide evidence of inhibition of MMP-9 expression by EGCG [16], but the molecular mechanism of the inhibition was not fully elucidated. The viability of cells after EGCG treatment was well over 95%. In this study, an in vitro wound healing assay confirmed that EGCG treatment considerably decreased the migration of MDA-MB-231 cells as compared with the EGCG-untreated cells. Hence, we show that the treatment of EGCG causes an appreciable inhibition of MMP-9 activity, mRNA, and protein expression in MDA-MB-231 cells. The inhibition of MMP-9 activity and expression might be because of the downregulation of MMP-9 at the transcriptional level.

Although most of the published studies have focused on the transcriptional control of MMP-9 expression, there is increasing evidence that MMP-9 activity and expression can also be regulated at the levels of activation, regulation and protein secretion. Similar to other enzymes, the activation of MMP-9 is crucial for its function. MMP-9 is synthesized and secreted as a zymogen of a proenzyme that remains inactive unless it is activated by the removal of a peptide. TIMPs, specifically TIMP-1, play an interesting role in MMP-9 activation. TIMP-1 is a 22 kDa stable glycoprotein that binds with high affinity to MMP-9. Inhibition of MMP-9 activation occurs through an interaction between the N-terminus domain of TIMP-1 and the active site of MMP-9 [17]. In this study, we show that EGCG treatment appreciably upregulates the mRNA and protein expression level of TIMP-1 in MDA-MB-231 cells. When TIMP-1 is present at a higher concentration then MMP-9 is inhibited and no activation occurs, but when TIMP-1 is present at a lower concentration then MMP-9 becomes fully activated [18]. Thus, the upregulation of both the mRNA

Fig. 7



Effect of epigallocatechin-3-gallate (EGCG) on the expression of nuclear factor kappa B (NFκB) and the DNA binding of NFκB and AP-1 in the MMP-9 promoter in MDA-MB-231 cells: MDA-MB-231 cells (300 000 cells/ml) were grown in a serum-free culture medium (SFCM) in the absence (lane C) and presence of 20 μmol/l EGCG for 48 h (lane E). The respective cells were collected and extracted in a cell extraction buffer and the cell lysates (100 μg protein each) of the control and experimental cells were subjected to immunoblot analysis with anti-NF-κB antibody (a). Actin was used as an internal control and carried out in parallel with the blot. The accompanying array represents the representative comparative densitometric/quantitative analysis of the band intensities using Image J Launcher (version 1.4.3.67). Data are means ± SEM of three experiments. MDA-MB-231 cells were grown on coverslips in the absence (control) and presence of 20 μmol/l EGCG for 24 and 48 h. The control and EGCG-treated cells were then subjected to immunocytochemical analysis with anti-NFκB (b) primary antibody and then incubated with a fluorescein isothiocyanate-labeled secondary antibody. The coverslips were mounted on glass slides and observed under a fluorescence microscope. Effect of EGCG on NFκB (c) and AP-1 (d) binding activities was determined by electrophoretic mobility shift assay. Oligonucleotides containing the NFκB and AP-1 sites were end labeled with [γ^{-32}P] ATP and incubated with nuclear extracts (5 μg) from MDA-MB-231 cells grown in the absence (C) and presence of EGCG (20 μmol/l, 48 h) (E). Lane P in each case denotes the lane for free probe (without the nuclear protein).

and protein expression of TIMP-1 by EGCG treatment might be an important mechanism by which EGCG blocks MMP-9 activation in MDA-MB-231 cells.

As a main link between a cell and the ECM, integrins have an essential role in the invasion process. Some integrins, such as α5β1 and αvβ3, seem to promote tumor progression and metastasis [19,20]. Integrins are needed in cell movement but they might have other roles in cancer invasion; importantly, they induce the expression of proteases such as MMP-2 and MMP-9. Earlier reports have shown that the interaction of αvβ3 with fibronectin and vitronectin [21] and α5β1 with its primary ligand fibronectin [22] induces the activity and expression of MMP-9. In this study, we have shown that EGCG treatment appreciably inhibits the binding of MDA-MB-231 cells to ECM ligands fibronectin and vitronectin in a time-dependant manner. An earlier study from our laboratory has shown similar results in another human breast cancer cell line, MCF-7 [11]. Other reports have also shown that EGCG inhibits the adhesion of fibrosarcoma HT1080 cells to fibronectin by involving the fibronectin receptor α5β1 [23]. We have also shown that EGCG treatment considerably reduces the mRNA expression of the integrin receptors, α5β1 and αvβ3, in MDA-MB-231 cells. Therefore, EGCG may hamper the mRNA level of integrin receptors, and thereby hinder the heterodimer formation of the receptor, leading to decreased binding of MDA-MB-231 cells to ECM ligands. As, these receptors actively participate not only in tumor invasion but also in MMP-9 expression and activity, hence this may be a probable pathway of MMP-9 inhibition by EGCG in MDA-MB-231 cells.

Formation of focal adhesion sites and clustering of integrins are required for ligand binding and signal transduction to the cell nucleus [24,25]. Signaling cascades may be activated when the cytoplasmic tails of the integrin subunits bind to specific proteins inside the cell. The activation of FAK within focal adhesion sites requires ligand binding. FAK can bind to the β1 and β3 subunits of the integrins [26]. Integrin-ligand binding results in FAK autophosphorylation. Phosphorylated tyrosine 397 acts in turn as a binding site for kinases such as Src [27]. These events may lead to the induction of downstream signaling kinases, and among other things to MMP-2 and MMP-9 production. In this study, we show that EGCG treatment downregulates the mRNA expression of FAK. It also decreases the protein expression and phosphorylation of FAK in MDA-MB-231 cells. Earlier reports have shown that when lung carcinoma cells were treated with FAK siRNA to block the expression of endogenous FAK then fibronectin-induced MMP-9 expression was greatly reduced [28], indicating the pivotal role of FAK in MMP-9 regulation. As FAK participates in MMP-9 expression, hence, inhibition of FAK expression and activation might be a mechanism by which EGCG downregulates MMP-9 in breast cancer cells.

Phosphorylation of FAK leads to the activation and induction of PI3K/ERK pathways, which in turn may lead to the induction of MMP-9. The ability to modulate MMP-9 expression at multiple steps through distinct signaling pathways may be particularly important during malignant conversion and metastasis, when tumor cells need to induce or maintain MMP-9 level in response to the changing environmental cues. PI3K on activation by phosphorylation can promote tumor cell invasion and migration by the upregulation of MMP-9, and PI3K in turn can influence integrin affinity and avidity [29]. In this study, we show that EGCG treatment downregulates the phosphorylation of PI-3K to a certain extent, but does not have any appreciable effect on the protein expression of PI-3K. Earlier reports have shown that EGCG inhibits PI-3K pathway in transgenic adenocarcinoma of the mouse prostrate model system [30]. Activation of FAK leads to the activation ERK1/2 signaling cascade. ERK has received increasing attention as a target molecule for cancer invasion. Once activated, ERK can activate a variety of transcription factors such as NFκB and AP1, thus, leading to changes in the expression of MMP-9 at the transcription level. In this study, the treatment of EGCG caused an appreciable downregulation of the phosphorylation/activation of ERK, but did not cause any appreciable changes in its protein expression. This is in agreement with earlier reports showing that EGCG inhibited the phosphorylation of ERK in human fibrosarcoma cell line HT1080 [31]. Other reports show that EGCG has the ability to inhibit UVB-induced MMP production by interfering with the ERK responsive pathway [32].

MMP-9 is crucially regulated at the gene expression/transcriptional level by important transcription factors such as NFκB and AP1. The MMP-9 gene has putative binding sites for NFκB and AP1 [33]. Deregulation of these transcription factors plays an important role in the deregulation of MMP-9 expression. ERK on activation activates NFκB through the activation of IKKβ [34] and also activates AP1 [35]. In this study, we show that EGCG treatment downregulates the protein expression of NFκB considerably. EMSA showed that EGCG downregulates the DNA binding activity of both AP1 and NFκB to the MMP-9 promoter. Earlier reports have shown that EGCG exerts anti-invasive effect in gastric cancer by controlling MMP expression through the suppression of ERK-mediated AP-1 activation [36] and MAPK-mediated NFκB activation in human prostrate cancer cells [37]. According to another report, EGCG inhibited NFκB and AP1 DNA binding activity in human colon cancer cells [38]. Thus, here we show that EGCG inhibited the DNA binding activity of NFκB and AP1 in MDA-MB-231 cells. This inhibition by EGCG results in the suppression of MMP-9 transcription, which corresponds to the downregulation of MMP-9 activity and expression. Thus, in this study we elucidate the possible molecular mechanism by which EGCG exerts its inhibitory effect on the activity and expression of MMP-9 in MDA-MB-231 cells.

Conclusion

EGCG treatment causes considerable inhibition of gelatinolytic activity, mRNA and protein expression of MMP-9. Upregulation of TIMP-1 and the disruption of the functional status of integrin receptors such as α5β1 and αvβ3 on EGCG treatment indicate the possible inhibitory role of EGCG in MMP-9 activation. Downregulation of FAK and ERK activation indicates the possible pathway by which EGCG exerts its inhibitory effect on MMP-9 regulation. Downregulation of DNA binding activity of NFκB and AP1 to the MMP-9 promoter is an interesting finding indicating the role of EGCG in the inhibition of the transcriptional regulation of MMP-9. EGCG downregulates MMP-9 in MDA-MB-231 cells by inhibition of MMP-9 expression and involvement of FAK and ERK-mediated signaling pathway, leading to the decreased DNA binding activity of NFκB and AP1, disrupting the transcription of the MMP-9 gene. The findings of this study re-emphasize the role of EGCG as an anti-invasive agent and also expound the molecular mechanism of its action.

Acknowledgements

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