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Multifunctional effect of epigallocatechin-3-gallate (EGCG) in downregulation of gelatinase-A (MMP-2) in human breast cancer cell line MCF-7

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ABSTRACT

Aims: The tumor inhibiting property of green tea polyphenol epigallocatechin-3-gallate (EGCG) is well documented. Studies reveal that matrix-metalloproteinases (MMPs) play pivotal roles in tumor invasion through degradation of basement membranes and extracellular matrix (ECM). We studied the effect of EGCG on matrixmetalloproteinases-2 (MMP-2), the factors involved in activation, secretion and signaling molecules that might be involved in the regulation of MMP-2 in human breast cancer cell line, MCF-7.
Main methods: MCF-7 was treated with EGCG (20 μM, 24 h), the effect of EGCG on MMP-2 expression, activity and its regulatory molecules were studied by gelatin zymography, Western blot, quantitative and semi-quantitative real time RT-PCR, immunoflourescence and cell adhesion assay.
Key findings: EGCG treatment reduced the activity, protein expression and mRNA expression level of MMP-2. EGCG treatment reduced the expression of focal adhesion kinase (FAK), membrane type-1-matrix metalloproteinase (MT1-MMP), nuclear factor-kappa B (NF-kB), vascular endothelial growth factor (VEGF) and reduced the adhesion of MCF-7 cells to ECM, fibronectin and vitronectin. Real time RT-PCR revealed a reduced expression of integrin receptors α5, β1, αv and β3 due to EGCG treatment.
Significance: Down regulation of expression of MT1-MMP, NF-kB, VEGF and disruption of functional status of integrin receptors may indicate decreased MMP-2 activation; low levels of FAK expression might indicate disruption in FAK-induced MMP-2 secretion and decrease in activation of phosphatidyl-inositol-3-kinase (PI-3K), extracellular regulated kinase (ERK) indicates probable hindrance in MMP-2 regulation and induction. We propose EGCG as potential inhibitor of expression and activity of pro-MMP-2 by a process involving multiple regulatory molecules in MCF-7.

Introduction

Tea (*Camellia sinensis*) with its appealing aroma and flavonoids is one of the most consumed beverages in the world. The beneficial properties of tea polyphenols in inhibition of tumor growth and invasion have been reported in many studies (Taniguchi et al., 1992). A number of epidemiological observations have revealed the consumption of green tea inhibits growth of several tumor types (Katiyar and Elmets, 2001; Ahmad and Mukhtar, 1999). The cancer chemopreventive property of green tea has mainly been attributed to the most prevalent, biologically active polyphenol of green tea, epigallocatechin-3-gallate (EGCG) (Mukhtar and Ahmad, 1999).

Degradation of basal lamina and extracellular matrix (ECM) is crucial for invasion and metastasis of malignant cells (Chiang et al., 2006). Proteolytic enzymes play fundamental role in cancer progression facilitating access of tumor cells to vasculature and lymphatic systems. Studies reveal that matrix metalloproteinases (MMPs) play pivotal roles in tumor cell migration and invasion (Curran and Murray, 1999). MMPs comprise a family of highly conserved zinc-dependant endopeptidases that collectively are capable of degradation of most components of basement membranes and extracellular matrix (Khokha and Denhardt, 1989). Among the currently known 24 human MMPs (Puente et al., 2003), matrix metalloproteinase-2 (MMP-2 or gelatinase A) is most frequently overexpressed in cancer and is instrumental in cutting through basement membrane barriers (Nelson et al., 2000). MMP-2 is secreted from various tumor cells in a pro-enzyme form i.e. an enzymologically inactive zymogen and is activated by processing mainly by MT1-MMP (membrane type-1 matrix metalloproteinase) (Nakamura et al., 1999). Integrins regulate expression and activation of MMPs, guides them to their targets by simultaneous binding of MMPs and ECM molecules. Among known integrins, $\alpha_v\beta_3$ plays a unique functional role in tumor angiogenesis and metastasis (Brooks, 1996; Chattopadhyay et al., 2001; Eliceiri and

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Cheresh, 2000). The integrins work in concert with focal adhesion kinase (FAK), that serves as an important integration point of growth factor and integrin signaling with respect to cell migration (Sieg et al., 2000).

Studies have suggested that EGCG inhibits tumor formation through inhibition of various cellular processes involved in cell adhesion (Isemura et al., 1993; Chung et al., 1999), tumor growth and invasion. Downregulation of MMPs might be the principle mechanism behind the effect of EGCG in the inhibition of tumor growth and invasion (Yang and Wang, 1993). EGCG has been reported to be a potent inhibitor of expression and activity of MMP-2 (Garbisa et al., 2001). Although it has been reported that EGCG inhibits the activity of MMP-2, the molecular mechanisms by which EGCG blocks gelatinolytic activities remain largely unknown.

In the present study, we report the multifunctional effect of EGCG in downregulation of MMP-2 in human breast cancer cell line MCF-7. We observed the effect of EGCG on different molecules playing pivotal role in the activation and secretion of MMP-2 and on different signaling molecules that might be involved in MMP-2 regulation. Our results put forward the possibility that EGCG is a potential natural inhibitor of MMP-2 and this inhibition might occur by a multi-factorial mechanism involving downregulation of MMP-2 expression, activation, secretion and thus the gelatinolytic activity of pro-MMP2 in MCF-7 cells.

Materials and methods

Materials

Minimal Essential Medium (MEM), fetal bovine serum (FBS), fibronectin (440 kDa), Protease Inhibitor Cocktail Tablets (complete, mini, EDTA-free) were purchased from Roche, Germany. Human Vitronectin was purchased from BD Biosciences, San Jose, CA USA. EGCG was obtained from Sigma-Aldrich, USA. Protein G agarose was purchased from Roche, Germany. Gelatin Sepharose 4B beads was purchased from GE Healthcare Bio-Sciences AB, Uppsala, Sweden. Anti-MMP-2, anti-NFκB, anti-FAK, anti-phospho FAK (tyr397), anti-MT1-MMP, anti VEGF, anti-ERK, anti-phospho-ERK, anti-PI-3K (p110) and anti-phospho-tyrosine antibodies were purchased from Santa Cruz, USA. Alkaline phosphatase coupled and Fluorescein isothiocyanate (FITC) 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) was from Promega, Madison, WI, USA. Trypan Blue (0.4%) was purchased from Gibco-BRL, Gaithersburg MD, USA. SYBR Green JumpStart™ Taq Readymix ™ was purchased from Sigma-Aldrich, USA. Primers were synthesized by Operon, Germany. RNAqueous 4 PCR (Total RNA isolation kit) and Retroscript (RT-PCR Kit) were purchased from Ambion, USA. Anti-human IgG was purchased from Bangalore Genei, India. 100 bp DNA ladder was purchased from Fermentas International Inc, Ontario, Canada. NP-40 (nonidet P-40) was purchased from Amresco, Solon, OH.

Methods

Cell culture

MCF-7 (human breast cancer cell line), A375 (human melanoma cell line) and HT-1080 (human fibrosarcoma cell line) was obtained from National Centre for Cell Sciences (NCCS), Pune, India. MCF-7 and HT-1080 cells were grown and maintained in MEM and A375 cells were grown and maintained in DMEM, containing 10% FBS in a 5% CO_2 incubator at 37 °C.

Treatment of cells with EGCG

MCF-7 cells were treated with 20 μM EGCG for 24 h in serum free culture medium (SFCM). The treated cells and SFCM were collected for further experiments.

Cell viability assay by Trypan Blue Dye Exclusion Method

MCF-7 cells (300,000 cells/ml) were grown in serum free culture medium (SFCM) in absence and in presence of 5, 10, 20 and 40 μM EGCG for 24 h, and in presence of 20 μM EGCG for 0, 12, 24, 36 and 48 h. The cells were trypsinised and a uniform cell suspension was made. 20 μl of the uniform cell suspension was taken and equal volume (20 μl) of 0.4% Trypan Blue was added, gently mixed and allowed to stand for 5 min at room temperature. 10 μl of the mixture was placed in a haemocytometer 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:

$$\text{Cell Viability}\,(\%) = \text{total viable cell}\,(\text{unstained}) \div \text{total cells} \times (\text{unstained and stained}) \times 100$$

Cell adhesion assay

The microtitre plate wells were coated separately with fibronectin (1.56 μg/ml, 3.13 μg/ml, 6.25 μg/ml, 12.5 μg/ml and 25 μg/ml fibronectin) and vitronectin (1.25 μg/ml, 2.5 μg/ml and 5 μg/ml vitronectin) in triplicate. The ligands were allowed to bind for 1½ h at 37 °C. Wells were blocked with Buffer C (1% BSA, 1 mM $CaCl_2$ and 1 mM $MgCl_2$, for vitronectin 1 mM $MnCl_2$ [instead of $MgCl_2$] in PBS) for 1 h at 37 °C. Cells (both control and experimental) were trypsinised from culture dishes, washed, suspended in Buffer C and added to microtitre plates (50,000 cells/well) and allowed to bind at 37 °C for 1½ h. The wells were washed ×3 with Buffer C. The bound cells were trypsinised, counted on haemocytometer and expressed as percentage of adhesion.

Gelatin zymography

MCF-7 cells (300,000 cells/ml) and A375 cells (300,000 cells/ml) were grown in absence and in presence of EGCG in SFCM for the required time period. To obtain conditioned SFCM containing MMP-2 and MMP-9 as standard, HT-1080 cells were grown in SFCM for 24 h. The culture supernatant was collected by centrifugation. The gelatinases were separated from SFCM using Gelatin Sepharose 4B beads shaking for overnight at 4 °C. The beads were washed ×3 with Tris-buffered saline with (0.02%) Tween-20 (TBST) and suspended in 50 μl of 1× sample buffer (0.075 gm Tris, 0.2 gm SDS in 10 ml water, pH 6.8) for 30 min at 37 °C. The extract was then subjected to zymography on 7.5% SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) co-polymerized with 0.1% gelatin. Gel was washed in 2.5% Triton-X-100 for 30 min to remove SDS and was then incubated overnight in reaction buffer (50 mM Tris–HCl pH 7, 4.5 mM $CaCl_2$, 0.2 M 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.

Immunoblot assay of MMP-2, focal adhesion kinase (FAK), membrane type-1-matrix metalloproteinase (MT1-MMP), nuclear factor-kappa B (NFκB) and vascular endothelial growth factor (VEGF)

MCF-7 cells (300,000 cells/ml) were grown in serum free culture medium (SFCM) in absence and in presence of 20 μM EGCG for 24 h. The cells were collected, extracted with cell extraction buffer (Tris—37.7 mM, NaCl—75 mM, Triton X-100—0.5%, protease inhibitor cocktail and pH adjusted to 7.5) and the protein content of the extracts were estimated by Lowry's method. 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 was subjected to electrophoresis on 7.5% SDS-PAGE. The proteins were electrophoretically transferred on to nitrocellulose membranes. The membranes were blocked with 1% BSA and subsequently washed ×3 with TBST. The membranes were

reacted with anti-MMP-2, anti FAK, anti MT1-MMP, anti-NFκB, anti-VEGF and anti-IgG antibodies at 1/1000 dilution each and kept at 37 °C for 1 h 30 min and subsequently washed ×3 with TBST. The blots were developed using respective alkaline phosphatase coupled second antibodies at 1/1000 dilution and kept at 37 °C for 1 h 30 min, the blots were then thoroughly washed ×6 with TBST. Bands were visualized using NBT-BCIP (nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate) as substrate.

Immunoblot assay of extracellular regulated kinase (ERK), phospho-ERK, phosphatidyl inositol 3 kinase (PI-3K) and phospho-PI-3K

MCF-7 cells (300,000 cells/ml) were grown in serum free culture medium (SFCM) in absence and in presence of 20 μM EGCG for 24 h. The respective cells were collected and extracted in NP-40 Lysis buffer (150 mM NaCl, 1% NP-40, 50 mM Tris, protease inhibitor cocktail; pH 8). The protein content of the extracts was estimated Lowry's method. Equal amount of protein (100 μg each) was taken in duplicate and PI-3K and ERK were immunoprecipitated using anti-PI-3K and anti-ERK antibodies and protein-G agarose beads and shaking overnight at 4 °C. The resultant immune-complex was washed ×3 in PBS, suspended in 1× sample buffer, heated with 0.1 volumes β-mercaptoethanol for 5–8 min at 80–90 °C and subjected to electrophoresis on 7.5% SDS-PAGE. The proteins were electrophoretically transferred on to nitrocellulose membrane. The membranes were blocked with 1% BSA and subsequently washed ×3 with TBST. The membranes were reacted with anti-ERK antibody; anti-phospho-ERK antibody, anti-PI-3K antibody and anti-phospho-tyrosine antibody and anti-IgG antibody at 1/1000 dilution and kept at 37 °C for 1 h 30 min, and subsequently washed ×3 with TBST. The immunoblots were reacted with alkaline phosphatase coupled respective second antibodies 1/1000 dilution and kept at 37 °C for 1 h 30 min followed by an extensive washing×6 with TBST. Bands were visualized using NBT/BCIP as substrate.

Semi-quantitative RT-PCR

RNA was extracted from 1×10^6 cells/ml MCF-7 cells grown in absence and in presence of 20 μM EGCG for 24 h. Cells were washed in PBS and total RNA was extracted (RNaqeous, Ambion, USA) from the cell. 2 steps RT-PCR (Retroscript, Ambion, USA) was done with equal amounts of total RNA, using specific primers for PCR. 20 μl of each PCR products were run on a 2.5% agarose gel and bands 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. 2 μl of cDNA was subjected to real-time quantitative RT-PCR using the real time PCR (ABI-7500, USA) with SYBR Green as a fluorescent reporter using the SYBR Green JumpStart™ Taq Readymix ™ (Sigma, USA). The specific gene primers (MMP-2, FAK, MT1-MMP, α5, β1, αv, β3 and the internal control gene G3PDH were amplified in separate reaction tubes. Threshold cycle number (C_T), of triplicate reactions, was determined using the ABI-7500 software and the mean C_T of 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 MCF-7 cells and the samples are the EGCG treated (20 μM, 24 h) MCF-7 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 melt curve analysis. The specificity of the products was also confirmed by melt curve analysis. The reaction conditions and the primer sequences are tabulated below. The PCR cycles in all cases were started with Taq activation at 94 °C for 5 min and followed by final extension of 72 °C for 7min.

PCR cycles and conditions

cDNA	Primer sequence	PCR cycles (40 cycles)	Amplicon size (bp)
hMMP-2	5′-GTATTTGATGGCATCGCTCA-3′ (forward) 5′-CATTCCCTGCAAAGAACACA-3′ (reverse)	94 °C—30 s 56 °C—90 s 72 °C—30 s 94 °C—30 s	198
hFAK	5′-GCGCTGGCTGGAAAAAGAGGAA-3′ (forward) 5′-TCGGTGGGTGCTGGCTGGTAGG-3′ (reverse)	60 °C—90 s 72 °C—30 s 94 °C—30 s 56 °C—90 s	475
hMT1-MMP	5′-CCCTATGCCTACATCCGTGA-3′ (forward) 5′-TCCATCCATCACTTGGTTAT-3′ (reverse).	72 °C—30 s 94 °C—30 s 58 °C—30 s 72 °C—90 s	569
α5	5′-CATTTCCGAGTCTGGGCCAA-3′ (forward) 5′-TGGAGGCTTGAGCTGAGCTT-3′ (reverse).	94 °C—30 s 58 °C—30 s 72 °C—90 s 94 °C—30 s	324
β1	5′-TGTTCAGTGCAGAGCCTTCA-3′ (forward) 5′-CCTCATACTTCGGATTGACC-3′ (reverse)	58 °C—30 s 72 °C—90 s 94 °C—30 s 58 °C—30 s	452
αV	5′-GTTGGGAGATTAGACAGAGGA-3′ (forward) 5′-CAAAACAGCCAGTAGCAACAA-3′ (reverse)	72 °C—90 s	288
β3	5′-GGGGACTGCCTGTGTGACTC-3′ (forward) 5′-CTTTTCGGTCGTGGATGGTG-3′ (reverse)		544
G3PDH	5′-CGGAGTCAACGGATTTGGTCGTAT-3′ (forward) 5′-AGCCTTCTCCATGGTGGTGAAGAC-3′ (reverse)		454

Immunocytochemical analysis of p-FAK

MCF-7 cells were grown on coverslips in absence (control) and in presence of 20 μM EGCG for 24 h (experimental). Cells on coverslips were fixed with 3.5% formaldehyde and treated with 0.5% Triton X-100. 1% BSA solution was used for blocking and cells were incubated with anti-p-FAK primary antibody (1:1000 dilution for 1½ h at 37 °C), washed×3 with PBS and incubated with FITC coupled secondary antibody (1:1000 dilution for 11/2 h at 37 °C). Cells were washed thoroughly×6 in PBS and the coverslips were mounted on glass slides and observed under fluorescence microscope.

Quantification of the results

Bands of zymography, Western blots and RT-PCR were quantitated using Image J Launcher (version 1.4.3.67).

Results

EGCG downregulates the gelatinolytic activity of pro-MMP-2 in a dose and time-dependant manner in MCF-7 cells

MCF-7 cells (300,000 cells/ml) were grown in absence (Lane C) and in presence of 5 μM (Lane 1), 10 μM (Lane 2) and 20 μM (Lane 3) EGCG for 24 h (Fig. 1A). The zymographic analysis of MMP-2 activity in SFCM clearly shows that EGCG treatment causes a dose-dependant inhibition of pro-MMP-2 activity with an appreciable inhibition with 20 μM EGCG treatment for 24 h (Lane 3). MCF-7 cells (300,000 cells/ml) were grown in absence (Lane C) and in presence of 20 μM EGCG for 12 h (Lane 1) and 24 h (Lane 2) (Fig. 1B). The comparative zymographic analysis showed a time-dependant decrease in pro-MMP-2 activity with treatment of 20 μM EGCG. Appreciable inhibition was observed with treatment of 20 μM EGCG for 24 h (Lane 2). MCF-7 cells

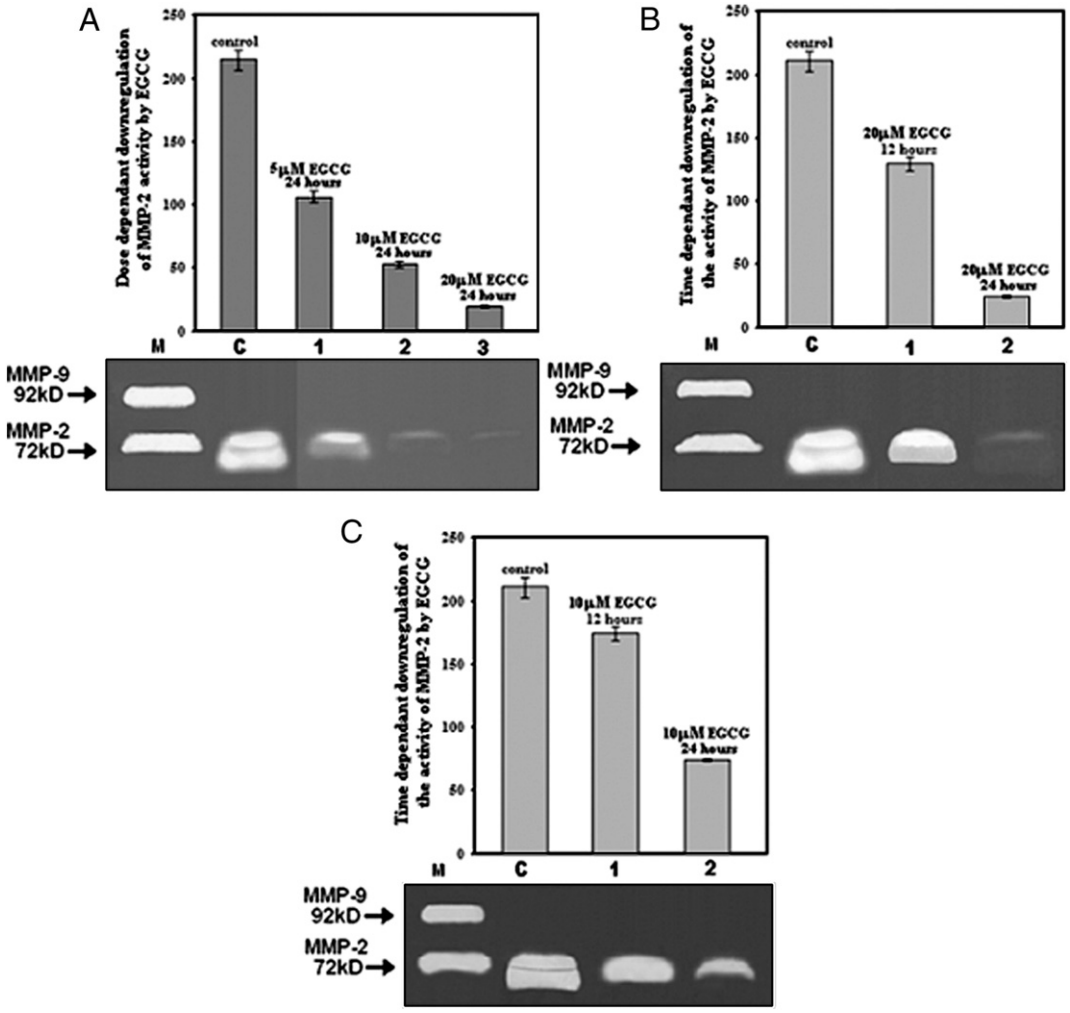

Fig. 1. Comparative zymographic analysis of MMP-2: ■ A. MCF-7 cells (300,000 cells/ml) were grown in serum free culture medium (SFCM) in absence (Lane C) and in presence of 5 μM EGCG (Lane 1), 10 μM EGCG (lane 2) and 20 μM EGCG (Lane 3) for 24 h. ■ B. MCF-7 cells (300,000 cells/ml) were grown in serum free culture medium (SFCM) in absence (Lane C) and in presence of 20 μM EGCG for 12 h (Lane 1) and 24 h (Lane 2). ■ C. MCF-7 cells (300,000 cells/ml) were grown in serum free culture medium (SFCM) in absence (Lane C) and in presence of 10 μM EGCG for 12 h (Lane 1) and 24 h (Lane 2). Lane M in all the zymograms is MMP-9/MMP-2 marker lane (SFCM of HT-1080 cells grown for 24 h). The gelatinases in all the cases were separated from SFCM by mixing Gelatin Sepharose 4B beads. The beads were suspended in 50 μl of 1× sample buffer 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.

(300,000 cells/ml) were grown in absence (Lane C) and in presence of 10 μM EGCG for 12 h (Lane 1) and 24 h (Lane 2) (Fig. 1C). The comparative zymographic analysis showed a gradual time-dependant decrease in pro-MMP-2 activity with treatment of 10 μM EGCG. Lane M is MMP-9/MMP-2 marker (culture supernatant of HT-1080 cells grown for 24 h in SFCM). The accompanying arrays in both the figures represent the comparative densitometric/quantitative analysis of the band intensities using Image J Launcher (version 1.4.3.67).

EGCG downregulates the mRNA expression level of MMP-2 and protein expression level of MMP-2 in the whole cell extract as well as culture supernatant in MCF-7 cells

The effect of EGCG on the expression of MMP-2 mRNA was determined in MCF-7 cells by semiquantitative RT-PCR followed by real time RT-PCR. In Fig. 2A the semi-quantitative RT-PCR profile of MCF-7 cells clearly indicates that the treatment of MCF-7 cells with 20 μM EGCG for 24 h (lane E) downregulates MMP-2 (198 bp) at mRNA level as compared to 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-2 was also confirmed by real time quantitative RT-PCR (Fig. 2B). In each reaction the threshold cycle number (C_T) was determined for both the target (MMP-2) and the housekeeping (G3PDH) gene using the real time PCR software and the mean C_T for three reactions were calculated. The ΔC_T or the C_T of the housekeeping gene subtracted from the C_T of the target gene was plotted. ΔC_T value is inversely proportional to the expression of MMP-2. The expression of MMP-2 decreased 32 fold in the EGCG treated MCF-7 cells as compared to control untreated set. These data suggest effective prevention of MMP-2 expression by EGCG treatment.

The comparative immunoblot of Fig. 2C shows that treatment of 20 μM EGCG for 24 h causes an appreciable inhibition of the expression of pro-MMP-2 (72 kDa) in the whole cell extract in the experimental set (Lane E) as compared to control (Lane C). In Fig. 2D the comparative immunoblot analysis shows that the expression of pro-MMP-2 (72 kDa) in the serum free culture medium is also appreciably reduced with the treatment of 20 μM EGCG for 24 h in the experimental set (Lane E) as compared to control (Lane C). Human IgG was used as internal control and done in parallel to the MMP-2 blots.

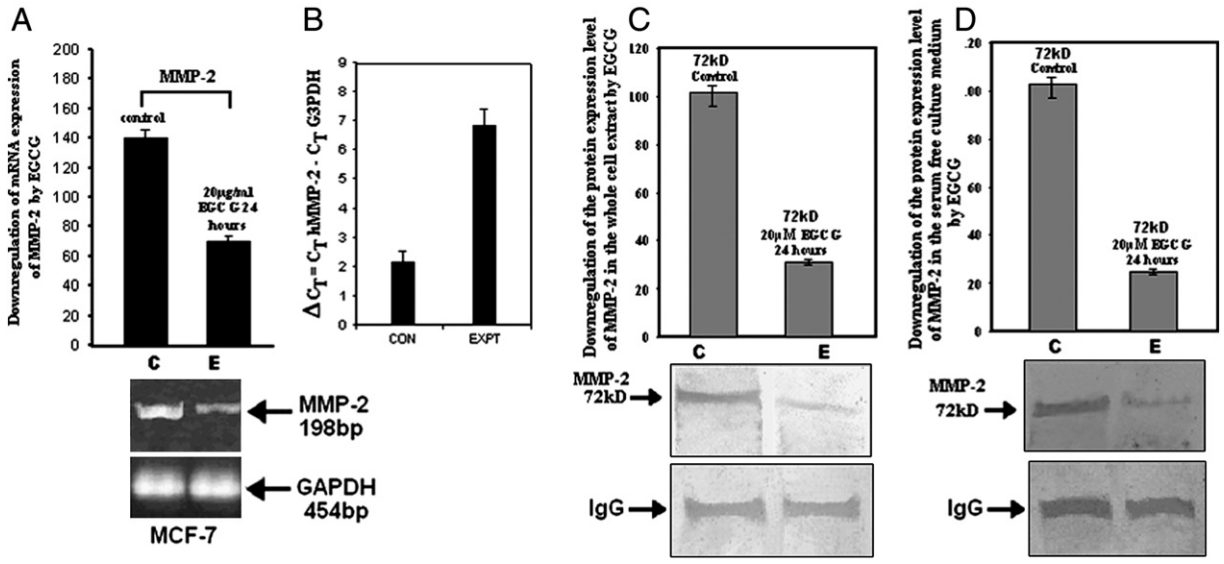

Fig. 2. Determination of gene expression of MMP-2 by semi-quantitative and quantitative real-time RT-PCR: MCF-7 cells (300,000 cells/ml) were grown in absence (lane C) and in presence of 20 μM EGCG (lane E) for 24 h in SFCM. Total RNA was extracted from control and EGCG treated MCF-7 cells (1×10^6 cells). A. 2 steps RT-PCR was done with equal amounts of total RNA, using specific primers for PCR (MMP 2). 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. 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. B. 2 μl of cDNA was subjected to real-time quantitative RT-PCR using the real time PCR instrument (ABI7500, USA) with SYBR Green as a fluorescent reporter. The specific gene primer (MMP-2) and the internal control gene G3PDH were amplified in separate reaction tubes. Relative levels of expression of *MMP-2* and the control G3PDH in control and EGCG treated (20 μM for 24 h) MCF-7 cells as measured by quantitative real time RT-PCR by calculating the C_T value. The calibrator used in our experiments is the control untreated (CON) MCF-7 cells and the samples are the EGCG treated (20 μM, 24 h) (EXPT) MCF-7 cells. In the given graph the C_T value is inversely proportional to the mRNA expression of the samples. Immunoblot analysis of the effect of EGCG on MMP-2: MCF-7 cells (300,000 cells/ml) were grown in serum free culture medium (SFCM) in absence (control) and in presence of 20 μM EGCG for 24 h (experimental). C. The respective cells were collected, extracted in cell extraction buffer. D. The SFCM was collected; the gelatinase was separated from SFCM by mixing Gelatin Sepharose 4B beads. The beads were suspended in 50 μl of 1× sample buffer. The cell lysate and SFCM elute were subjected to immunoblot analysis with anti-MMP-2 antibody. Human IgG was used as internal control and done in parallel to the MMP-2 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.

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 MCF-7 cells

In Fig. 3 the viability of MCF-7 cells after treatment of EGCG at different concentrations (Fig. 3A) and different time points (Fig. 3B) was calculated by Trypan Blue Dye Exclusion Assay. The viability of MCF-7 cells after 20 μM EGCG treatment for 24 h was found to be about 91%.

EGCG downregulates the expression of MT1-MMP in MCF-7 cells

The effect of EGCG on the expression of MT1-MMP mRNA in MCF-7 cells was determined by semi quantitative RT-PCR followed by real time RT-PCR. In Fig. 4A the semi-quantitative RT-PCR profile of MT1-MMP in MCF-7 cells clearly indicates that the treatment of MCF-7 cells with 20 μM EGCG for 24 h (lane E) downregulates MT1-MMP (569 bp) at mRNA level as compared to 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 MT1-MMP was also confirmed by real time quantitative RT-PCR (Fig. 4B). In each reaction the threshold cycle number (C_T) was determined for both the target (MT1-MMP) and the housekeeping (G3PDH) gene using the real time PCR software and the mean C_T for three reactions were calculated. The ΔC_T or the C_T of the housekeeping gene subtracted from the C_T of the target gene was plotted. ΔC_T value is inversely proportional to the expression of MT1-MMP. The expression of MT1-MMP decreased 40 fold in the EGCG treated MCF-7 cells as compared to control untreated set. These data suggest effective inhibition of MT1-MMP expression by EGCG

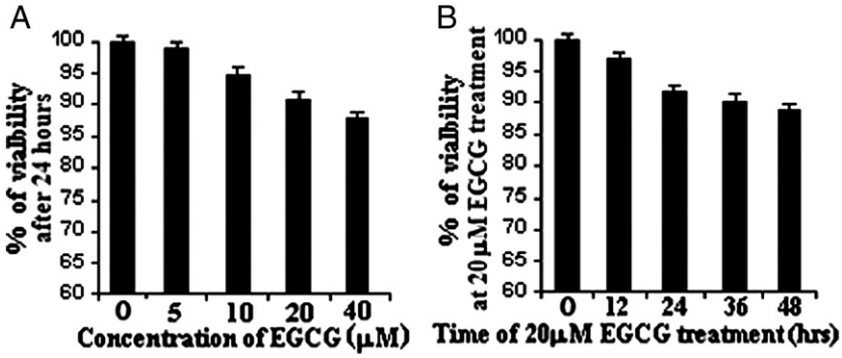

Fig. 3. Effect of EGCG on the viability of MCF-7 cells: MCF-7 cells (300,000 cells/ml) were grown in absence and in presence of 5, 10, 20 and 40 μM EGCG for 24 h (A.) and in presence of 20 μM EGCG for 0, 12, 24, 36 and 48 h (B.) and the viability of the cells were determined by Trypan Blue Dye Exclusion Assay.

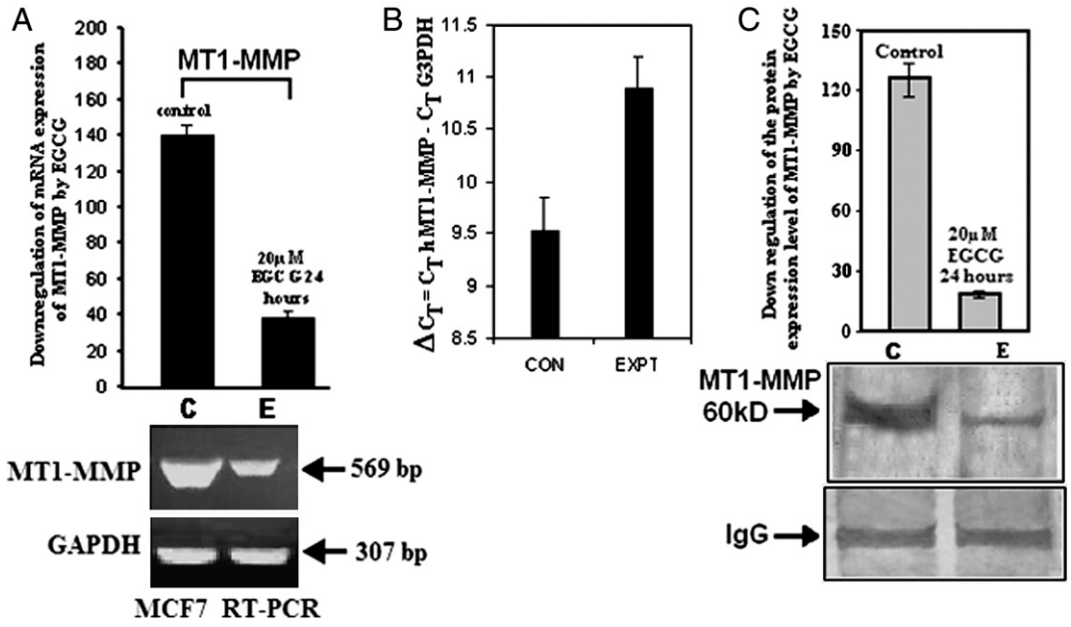

Fig. 4. Effect of EGCG on MT1-MMP: MCF-7 cells (300,000 cells/ml) were grown in serum free culture medium (SFCM) in absence (Lane C) and in presence of 20 μM EGCG for 24 h (Lane E). A. Total RNA was extracted from control and EGCG treated (1×10^6) MCF-7 cells. 2 steps RT-PCR was done with equal amounts of total RNA, using specific primer for PCR (MT1-MMP). 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 the real time PCR instrument (ABI7500, USA) with SYBR Green as a fluorescent reporter. The specific gene primer (MT1-MMP) and the internal control gene G3PDH were amplified in separate reaction tubes. Relative levels of expression of *MT1-MMP* and the control G3PDH in control and EGCG treated (20 μM for 24 h) MCF-7 cells as measured by quantitative real time RT-PCR by calculating the C_T value. The calibrator used in our experiments is the control untreated (CON) MCF-7 cells and the samples are the EGCG treated (20 μM, 24 h) (EXPT) MCF-7 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 control and experimental cells were subjected to immunoblot analysis with anti-MT1-MMP antibody. Human IgG was used as internal control and done in parallel to the MT1-MMP 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.

treatment. In Fig. 4C the comparative immunoblot shows that the treatment of 20 μM EGCG for 24 h appreciably decreases the expression of MT1-MMP (60 kDa) in experimental (lane E) as compared to control (lane C) in MCF-7 cells. Human IgG was used as internal control and done in parallel to the MT1-MMP 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 MCF-7 cells to extracellular matrix proteins fibronectin, vitronectin and inhibits the mRNA expression of the integrin receptors

In Fig. 5A the graphical representation of cell adhesion assay shows that control MCF-7 cells can efficiently bind to ECM ligand fibronectin. The treatment of MCF-7 cells with 20 μM EGCG for 12 h and 24 h appreciably downregulates the binding of the cells to fibronectin as compared to the control cells in time and concentration dependant manner. In Fig. 5B the graphical representation of cell adhesion assay shows that control MCF-7 cells can efficiently bind to ECM ligand vitronectin. The treatment of MCF-7 cells with 20 μM EGCG for 12 h and 24 h appreciably downregulates the binding of the cells to vitronectin as compared to the control cells in time and concentration dependant manner.

The effect of EGCG on the mRNA expression of integrin receptors like α5, β1, αv and β3 in MCF-7 cells was determined by real time RT-PCR. In each reaction the threshold cycle number (C_T) was determined for both the target αv and β3 (Fig. 5C) and α5 and β1 (Fig. 5D) and the housekeeping (G3PDH) gene using the real time PCR software and the mean C_T for three reactions were calculated. The ΔC_T or the C_T of the housekeeping gene subtracted from the C_T of the target gene was plotted. ΔC_T value is inversely proportional to the expression of the target genes. The expression of α5, β1, αv and β3 decreased 32 fold, 64 fold, 16 fold and 8 fold respectively in the EGCG treated MCF-7 cells as compared to control untreated set. These data suggest effective inhibition of mRNA expression integrin receptors by EGCG treatment.

EGCG downregulates the expression of FAK in MCF-7 cells

The effect of EGCG on the mRNA expression of FAK in MCF-7 cells was determined by semiquantitative RT-PCR followed by real time RT-PCR. In Fig. 6A the RT-PCR profile of FAK in MCF-7 cells clearly indicates that the treatment of MCF-7 cells with 20 μM EGCG for 24 h (lane E) downregulates FAK (475 bp) at mRNA level as compared to 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. In each reaction the threshold cycle number (C_T) was determined for both the target (FAK) and the housekeeping (G3PDH) gene using the real time PCR software and the mean C_T for three reactions were calculated (Fig. 6B). The ΔC_T or the C_T of the housekeeping gene subtracted from the C_T of the target gene was plotted. ΔC_T value is inversely proportional to the expression of FAK. The expression of FAK decreased 64 fold in the EGCG treated MCF-7 cells as compared to control untreated set. These data suggest effective inhibition of FAK expression by EGCG treatment. In Fig. 6C the comparative immunoblot with FAK antibody shows that the control MCF-7 cells (lane C) show an appreciable level of FAK expression (125 kDa), the treatment of 20 μM EGCG for 24 h (lane E) causes a noticeable decrease in the expression of FAK. Human IgG was used as internal control and done in parallel to the FAK blot. The accompanying array represents the comparative densitometric/

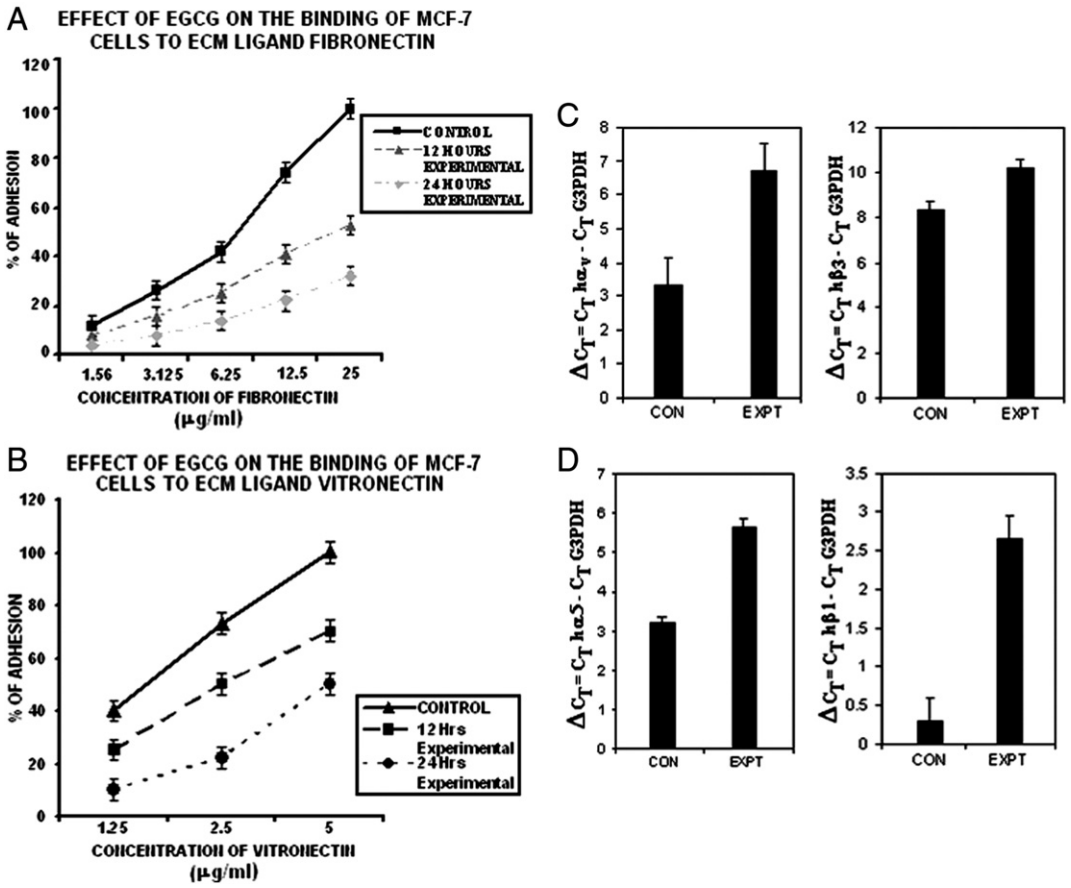

Fig. 5. Effect of EGCG on binding of MCF-7 cells to ECM ligands fibronectin and vitronectin and mRNA expression of α5β1 and αvβ3 integrin receptors in MCF-7 cells: MCF-7 cells (300,000 cells/ml) were grown in complete medium in absence (control) and in presence of 20 μM EGCG for 12 h and 24 h. The ligand fibronectin (A.) and vitronectin (B.) in different concentrations (in triplicate), was allowed to bind to the wells for 1½ h at 37 °C. Wells were blocked for 1 h at 37 °C. MCF-7 cells of both control and experimental sets were added to microtitre plates (50,000 cells/well) and allowed to bind to the ligands at 37 °C for 1½ h. The wells were washed and the bound cells were trypsinised, counted on haemocytometer and expressed as % of adhesion. C. and D. 2 μl of cDNA was subjected to real-time quantitative RT-PCR using the real time PCR instrument (ABI7500, USA) with SYBR Green as a fluorescent reporter. The specific gene primer (α5, β1, αv, β3) and the internal control gene G3PDH were amplified in separate reaction tubes. Relative levels of expression of αvβ3 (C.), α5β1 (D.) and the control G3PDH in control and EGCG treated (20 μM for 24 h) MCF-7 cells as measured by quantitative real time RT-PCR by calculating the C_T value. The calibrator used in our experiments is the control untreated (CON) MCF-7 cells and the samples are the EGCG treated (20 μM, 24 h) (EXPT) MCF-7 cells. In the given graphs the C_T value is inversely proportional to the mRNA expression of the samples.

quantitative analysis of the band intensities using Image J Launcher (version 1.4.3.67). In Fig. 6D the immunocytochemical localization of p-FAK shows that the expression of p-FAK in cells treated with 20 μM EGCG for 24 h (exp) was much reduced as compared to the control untreated set (con).

Effect of EGCG on signaling molecules like ERK/p-ERK, PI-3K/p-PI-3K, NF-kB and VEGF in MCF-7 cells

In Fig. 7A the comparative immunoblot shows that the treatment of MCF-7 cells with 20 μM EGCG for 24 h (lane E) does not cause an appreciable change in the protein expression level of PI-3K as compared to the control MCF-7 cells (lane C). The comparative immunoblot of Fig. 7B clearly shows that the treatment of MCF-7 cells with 20 μM EGCG for 24 h (lane E) causes a manifold inhibition in the phosphorylation of PI-3K as compared to control cells (lane C). The comparative immunoblot in Fig. 7C indicates that the treatment of MCF-7 cells with 20 μM EGCG for 24 h (lane E) does not cause any appreciable change in the protein expression of ERK as compared to control (lane C) MCF-7 cells untreated with EGCG. However, the comparative immunoblot of Fig. 7D shows that the treatment of 20 μM EGCG for 24 h causes an appreciable decrease in the phosphorylation level of ERK in EGCG treated MCF-7 cells (lane E) as compared to the control (lane C). In Fig. 7E the comparative immunoblot clearly indicates that the treatment of 20 μM EGCG for 24 h appreciably downregulates the expression of NF-kB in MCF-7 cells in the experimental (lane E) set as compared to the control (lane C). In Fig. 7F the comparative immunoblot clearly indicates that the treatment of 20 μM EGCG for 24 h appreciably downregulates the expression of VEGF in MCF-7 cells as indicated by the decrease in the band intensity of VEGF (45 kDa) in the experimental (lane E) set as compared to the control (lane C). Human IgG was used as internal control and done in parallel to 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 gelatinolytic activity of pro-MMP-2 in a dose and time-dependant manner in A-375 cells

In order to see whether the downregulation of MMP-2 in MCF-7 cells by EGCG is cell type specific or not, we examined the effect of EGCG treatment at different concentrations and time points on another cell line from a different tissue of origin, A375. A375 cells (300,000 cells/ml) were grown in absence (Lane C) and in presence of 10 μM (Lane 1) and 20 μM (Lane 2) EGCG for 24 h (Fig. 8A). The zymographic analysis of MMP-2 activity in SFCM clearly shows that EGCG treatment causes a dose-dependant inhibition of pro-MMP-2 activity with a noticeable inhibition with 20 μM EGCG treatment for 24 h (Lane 2). A375 cells (300,000 cells/ml) were grown in absence

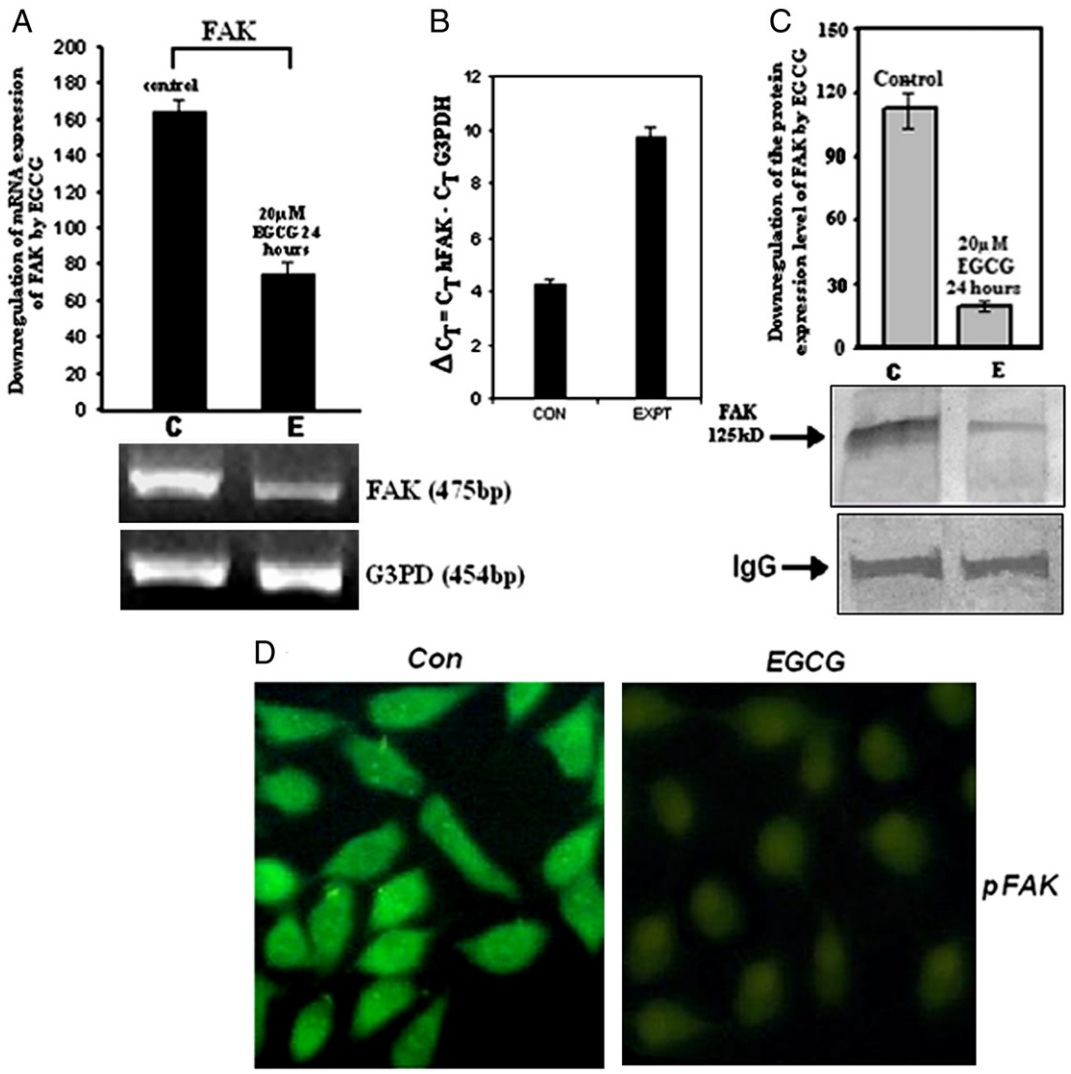

Fig. 6. Effect of EGCG on focal adhesion kinase (FAK): MCF-7 cells (300,000 cells/ml) were grown in serum free culture medium (SFCM) in absence (Lane C) and in presence of 20 μM EGCG for 24 h (Lane E). A. Total RNA was extracted from control and EGCG treated (1×10^6) MCF-7 cells. 2 steps RT-PCR was done with equal amounts of total RNA, using specific primer for PCR (FAK). 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 the real time PCR instrument (ABI7500, USA) with SYBR Green as a fluorescent reporter. The specific gene primer (FAK) and the internal control gene G3PDH were amplified in separate reaction tubes. Relative levels of expression of *FAK* and the control G3PDH in control and EGCG treated (20 μM for 24 h) MCF-7 cells as measured by quantitative real time RT-PCR by calculating the C_T value. The calibrator used in our experiments is the control untreated (CON) MCF-7 cells and the samples are the EGCG treated (20 μM, 24 h) (EXPT) MCF-7 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 control and experimental cells were subjected to immunoblot analysis with anti-FAK antibody. Human IgG was used as internal control and done in parallel to the FAK 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. D. MCF-7 cells were grown on coverslips in absence (control) and in presence of 20μM EGCG for 24 h (experimental). The control and EGCG treated cells were then subjected to Immunocytochemical analysis with anti-p-FAK primary antibody and then incubated with FITC-labeled secondary antibody. Coverslips were mounted on glass slides and observed under a fluorescence microscope.

(Lane C) and in presence of 20 μM EGCG for 12 h (Lane 1) and 24 h (Lane 2) (Fig. 8B). The comparative zymographic analysis showed a time-dependant decrease in pro-MMP-2 activity with treatment of 20 μM EGCG. Lane M is MMP-9/MMP-2 marker (culture supernatant of HT-1080 cells grown for 24 h in SFCM). The accompanying arrays in both the figures represent the comparative densitometric/quantitative analysis of the band intensities using Image J Launcher (version 1.4.3.67).

Discussion

In the present communication we put forward evidences to demonstrate that EGCG is a strong natural inhibitor of the activity and expression of pro-MMP-2 in human breast cancer cell line, MCF-7. The present report clearly indicates by comparative zymographic analysis that EGCG treatment noticeably downregulates the gelatinolytic activity of pro-MMP-2 in MCF-7 cells in a dose and time-dependant manner. This downregulation might not be a cell-specific type behavior by EGCG, which has been shown in the present study by the downregulation of MMP-2 in another cell line of different tissue of origin, A375 by EGCG treatment. This is in conformity with a large body of evidence which suggests that EGCG suppresses the activity and expression of MMP-2 (Maeda-Yamamoto et al., 2003). The optimum time and concentration of EGCG treatment for efficient downregulation of pro-MMP-2 activity in MCF-7 was determined to be 24 h and 20 μM respectively. EGCG treatment was shown to appreciably reduce the MMP-2 mRNA expression as evident from the semi-quantitative and quantitative real time RT-PCR profile. This might suggest that EGCG restricts the activity of MMP-2 in MCF-7 cells by reducing the production of MMP-2 in the transcriptional level. Western blot analysis further indicated that EGCG treatment (20 μM, 24 h) causes an appreciable downregulation of the protein expression level of MMP-2 in both whole cell extract and culture

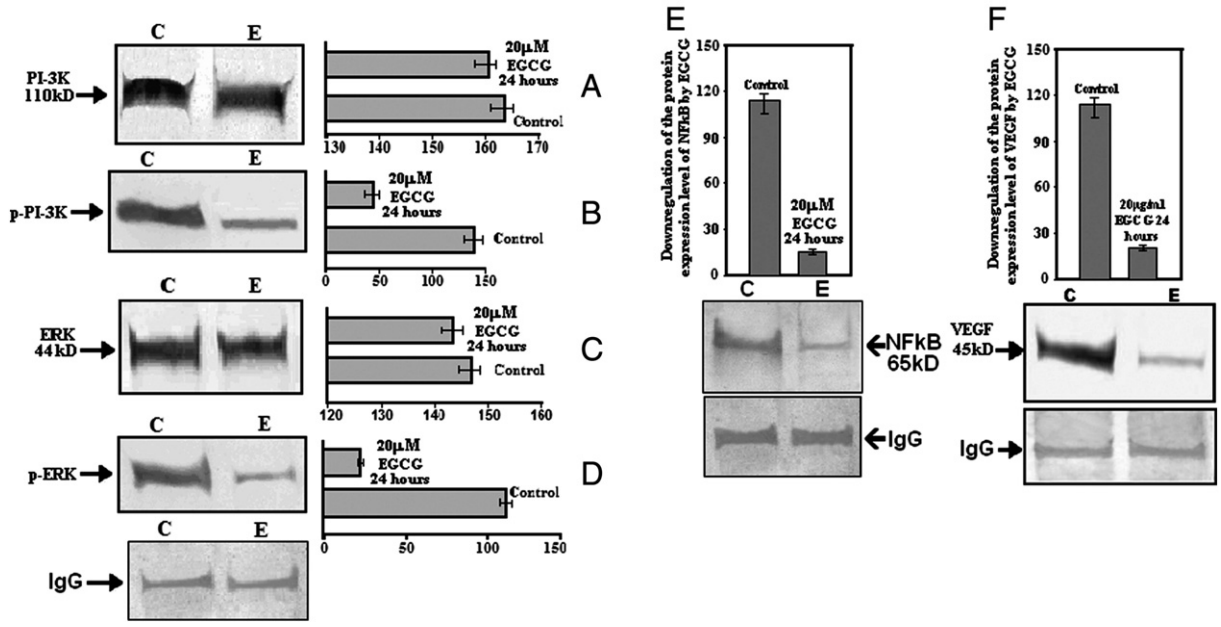

Fig. 7. Effect of EGCG on PI-3K, p-PI-3K, ERK, p-ERK, NF-κB and VEGF: MCF-7 cells (300,000 cells/ml) were grown in serum free culture medium (SFCM) in absence (lane C) and in presence of 20 μM EGCG for 24 h (lane E). The respective cells were collected and extracted in NP 40 Lysis buffer or cell extraction buffer. ■ A and B. Effect of EGCG on PI-3K and p-PI-3K: The cell lysates were immunoprecipitated with anti-PI3K antibody and were subjected to immunoblot analysis with anti-PI-3K antibody (panel 7.A.) and anti-phospho-tyrosine antibody (for phospho-PI3-K, panel 7.B). ■ C and D. Effect of EGCG on ERK/p-ERK: The cell lysates were immunoprecipitated with anti-ERK antibody and were subjected to immunoblot analysis with anti-ERK antibody (panel 7.C) and anti-phospho-ERK antibody (panel 7.D). E Effect of EGCG on NF-κB: The respective cells were collected, extracted in cell extraction buffer and the cell lysates of control and experimental cells were subjected to immunoblot analysis with anti-NF-κB antibody. F Effect of EGCG on VEGF: The respective cells were collected, extracted in cell extraction buffer and the cell lysates of control and experimental cells were subjected to immunoblot analysis with anti-VEGF antibody. Human IgG was used as internal control and done in parallel to the blots. The accompanying arrays represent 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.

supernatant of MCF-7 cells. Thus EGCG inhibits the gelatinolytic activity and the mRNA and protein expression level of MMP-2 in MCF-7 cells. The viability of MCF-7 cells in the present treatment conditions remained more than about 91%.

The activity of MMP-2 is regulated at the level of transcription, activation of the precursor zymogens, interaction with specific ECM components and other related signaling molecules. Activation of gelatinases is one of the crucial steps leading to ECM degradation and can be achieved by different proteases that remove the N-terminal pro-peptide (Mazzieri et al., 1997). The activation pathway of pro-MMP-2 is initiated by membrane type-MMPs (MT-MMPs), mainly MT1-MMP (Okada et al., 1995). These findings make the MT1-MMP/MMP-2 system a probable target for anti-tumor therapy (Brooks et al., 1996, 1998; Chattopadhyay et al., 2001). The initial cleavage of MMP-2 generates the activation intermediate which further auto-catalytically converts to the mature MMP-2 enzyme (Strongin et al., 1993, 1995). In the present report we have shown that EGCG treatment (20 μM, 24 h) appreciably downregulates the mRNA as well as protein expression

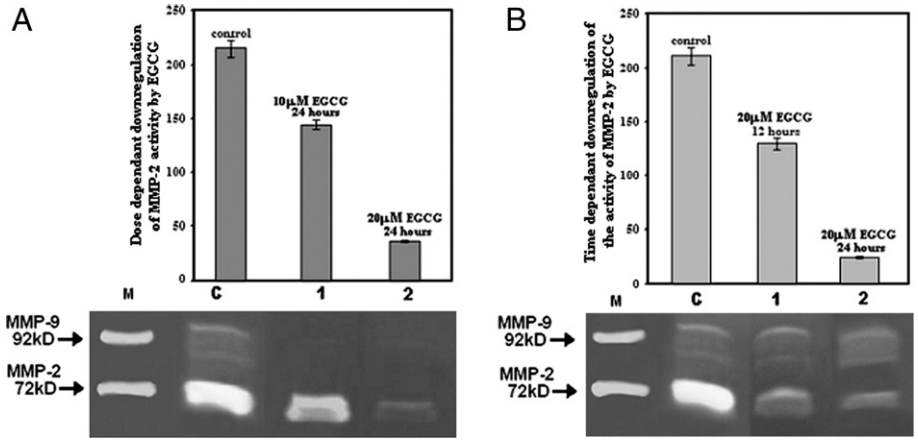

Fig. 8. Comparative zymographic analysis of MMP-2 in A375 cells: ■ A. A375 cells (300,000 cells/ml) were grown in serum free culture medium (SFCM) in absence (Lane C) and in presence of 10 μM EGCG (Lane 1) and 20 μM EGCG (Lane 2) for 24 h. ■ B. A375 cells (300,000 cells/ml) were grown in serum free culture medium (SFCM) in absence (Lane C) and in presence of 20 μM EGCG for 12 h (Lane 1) and 24 h (Lane 2). Lane M in both the zymograms is MMP-9/MMP-2 marker lane (SFCM of HT-1080 cells grown for 24 h). The gelatinases in all the cases were separated from SFCM by mixing Gelatin Sepharose 4B beads. The beads were suspended in 50 μl of 1× sample buffer 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.

level of MT1-MMP in cultured MCF-7 cells. This is in agreement with earlier findings which report that MT1-MMP belongs to a class of proteinases of which members are inhibited by EGCG (Demeule et al., 2000; Maeda-Yamamoto et al., 1999). Thus EGCG disrupts the MT1-MMP mediated activation of MMP-2 by downregulating the expression of MT1-MMP.

Integrin receptor, mainly $\alpha_v\beta_3$, has been shown to associate with active MMP-2 via the C-terminal hemopexin like domain (PEX) of the enzyme (Deryugina et al., 1997) and plays an important role in MMP-2 activation and modulation (Seftor et al., 1992, 1993). It has been reported that integrin $\alpha_v\beta_3$ may cooperate with MT1-MMP in MMP-2 maturation in MCF-7 cells (Brakebusch et al., 2002). Adhesive interactions between tumor cells and extracellular matrix proteins such as fibronectin and vitronectin are of importance in tumor growth, invasion and metastasis (Liotta et al., 1986). Fibronectin and vitronectin through interaction with their corresponding integrin receptors $\alpha_5\beta_1$ and $\alpha_v\beta_3$ respectively, regulate the activity and expression of MMP-2 (Chakrabarti et al., 2006; Mitra et al., 2006). According to a recent report from our laboratory, rapid expression and activation of MMP-2 and MMP-9 takes place upon exposure of human breast cancer cells (MCF-7) to fibronectin in serum free medium (Das et al., 2008). In the present study we demonstrated that EGCG treatment (20 μM, 24 h) considerably reduces the binding of MCF-7 cells to fibronectin and vitronectin in a time dependant manner. This is in agreement with earlier studies which reports that EGCG impair adhesion and/or spreading of mouse lung carcinoma 3LL and melanoma B16F10 cells to fibronectin (Ogata et al., 1995). It was further observed by quantitative real-time RT-PCR that EGCG treatment reduces the mRNA expression of the important integrin receptors like $\alpha_v\beta_3$ and $\alpha_5\beta_1$. So, EGCG by effectively reducing the mRNA level of these receptors might prohibit the heterodimer formation of these receptors on the cell surface leading to the downregulation of the rate of effective binding of the cancer cells to the ligands. Thus, EGCG also interferes or disrupts the functional status of integrin receptors like $\alpha_v\beta_3$, $\alpha_5\beta_1$ etc. and thus might interfere with integrin mediated activation and function of MMP-2.

Binding of ECM proteins to trans-membrane integrins transmits signals into the cell that regulate migration events through outside-in signaling pathway by inducing tyrosine phosphorylation and activation of cytoplasmic tyrosine kinases (Giancotti and Ruoslahti, 1999). Focal adhesion kinase (FAK) is such non-receptor tyrosine kinase that indirectly localizes to sites of integrin receptors clustering through C-terminal domain mediated interactions (Hildebrand et al., 1993) with integrin associated proteins. FAK becomes phosphorylated at seven to eight different tyrosine residues in vivo after engagement of integrins with matrix proteins (Schlaepfer and Hunter, 1996). The activation of FAK seems to be the pre-requisite for cell migration. FAK is seen to play an important role in the activation of MMP-2 via MT1-MMP (Sieg et al., 1999). Recent observations regarding MMP-2 have supported the importance of FAK signaling in the hyaluronan and Con-A dependant secretion of MMP-2 (Zhang et al., 2002). In the present investigation, EGCG treatment (20 μM, 24 h) is shown to downregulate the mRNA and protein expression of FAK in cultured MCF-7 cells. So, EGCG not only might disrupt the activation mechanism but also the secretion mechanism of MMP-2 in MCF-7 cells.

Cell attachment to ECM serves as scaffolds for integrin-mediated signal transduction by recruiting various signaling molecules (Mitra et al., 2005). The phosphorylation of Tyr397 of FAK correlates with increased catalytic activity of the protein (Calalb et al., 1995). This multi-functional phosphorylation site on FAK is the binding site for the SH2 domains of Src family PTKs like the p85 subunit of PI-3K. FAK can promote tumor cell migration by activating PI-3K and the binding of FAK and PI-3K can in turn influence integrin affinity and avidity (Kiosses et al., 1999). The PI-3K activation in turn leads to ERK/MAP kinase activation that has been shown to be important components of integrin stimulated cell migration events. The phosphorylation and activation of ERK is crucial for transcriptional regulation of genes involved in cell migration. In the present report we already demonstrate that treatment of EGCG (20 μM, 24 h) appreciably reduces the phosphorylation of PI-3K and ERK. EGCG has been shown to inhibit the phosphorylation of ERK as well as c-Jun (Chung et al., 1999, 2001). EGCG was also shown to inhibit PI-3K pathway by decreasing the levels of PI-3K phosphorylation and Akt phosphorylation in two human prostrate cancer cell lines, DU145 and LNCap cells (Siddiqui et al., 2004). The suppression of phosphorylation of ERK and PI-3K might be an important finding which might indicate that EGCG not only downregulates the expression, activation and secretion of MMP-2 but also has an inhibitory effect on the downstream signaling cascade.

In the present study we have found an appreciable decrease in the expression of NF-kB upon treatment of MCF-7 cells with EGCG. It has been shown previously that EGCG mediates T cellular NF-κB inhibition by intercellular accumulation of IκBα (Aktas et al., 2004) so our finding confirms a similar observation of NF-kB inhibition in a human breast cancer cell line. NF-κB mediates MT1-MMP induction and MMP-2 activation. Thus, this might be an important pathway of the inhibition of MMP-2 activation by EGCG in MCF-7 cells. Furthermore, constitutive activation of NF-κB may very well play an anti-apoptotic role in the breast and prostate carcinoma cells (Liu et al., 2005). The inhibition of constitutive NF-κB activation by reducing NF-κB expression in EGCG treated MCF-7 cells may therefore enhance the basal apoptotic rate in these cells.

Vascular endothelial growth factor (VEGF) plays an instrumental role in angiogenesis and along with some of the pro angiogenic factors is known to have contribution in the activation of MMP-2 (Ispanovic and Haas, 2006). Upon ligand (VEGF) binding to its receptor VEGFR-2 tyrosine kinase signaling cascade gets stimulated and migration factors like MMPs are produced (Tammela et al., 2005). In the present study we demonstrated that EGCG treatment causes an inhibition in the expression of VEGF in MCF-7 cells. The finding is in agreement with the recent finding that shows that EGCG inhibits VEGF induction in human breast cancer cell MDA-MB-231 (Sartippour et al., 2002). Other studies also suggest that EGCG inhibits VEGF angiogenic signaling by disrupting the formation of a receptor complex essential for VEGF function (Rodriguez et al., 2006; Zhu et al., 2007). Down regulation of VEGF expression in EGCG treated MCF-7 cells may contribute to the reduced MMP-2 activity in treated cells.

Conclusion

EGCG has an appreciable inhibitory effect on the expression and activity of pro-MMP-2 and interferes in the activation and secretion mechanism of MMP-2 by downregulating MT1-MMP, VEGF, NF-kB, FAK and the functional status and expression of $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrin receptors. The downregulation of both the mRNA and protein expression level of FAK by EGCG might be an important finding considering its role as a critical intermediate mediator of signaling between cells and their extracellular matrix. EGCG also disrupts the activation of downstream signaling molecules of MMP-2 like PI-3K and ERK which may have an important role in MMP-2 regulation, induction and thus ECM degradation. Thus, EGCG has a multifactorial effect on the inhibition of MMP-2 by interfering with the activation, secretion and regulation of the molecule in human breast cancer cell line, MCF-7. The novel findings of the present study re-emphasize the potential of EGCG as an anti-tumor agent.

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