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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

Triparna Sen [a], Shuvojit Moulik [a], Anindita Dutta [a], Paromita Roy Choudhury [a], Aniruddha Banerji [a], Shamik Das [a], Madhumita Roy [b], Amitava Chatterjee [a,*]

[a] Department of Receptor Biology & Tumor Metastasis, Chittaranjan National Cancer Institute, 37, S.P. Mukherjee Road, Kolkata-700 026, India
[b] Department of Environmental Carcinogenesis and Toxicology, Chittaranjan National Cancer Institute, 37, S.P. Mukherjee Road, Kolkata-700 026, India

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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

* Corresponding author. Department of Receptor Biology & Tumor Metastasis, Chittaranjan National Cancer Institute, 37, S.P. Mukherjee Road, Kolkata-700 026, West Bengal, India. Tel.: +91 33 9830128320; fax: +91 33 2475 7606.
E-mail address: amitava_chatter@yahoo.co.in (A. Chatterjee).

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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