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Research Article

(–)-Epigallocatechin-3-gallate interferes with mast cell adhesiveness, migration and its potential to recruit monocytes

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Abstract. Mast cells are multipotent effector cells of the immune system. They are able to induce and enhance angiogenesis *via* multiple pathways. (–)-Epigallocatechin-3-gallate (EGCG), a major component of green tea and a putative chemopreventive agent, was reported to inhibit tumor invasion and angiogenesis, processes that are essential for tumor growth and metastasis. Using the human mast cell line HMC-1 and commercial cDNA macroarrays, we evaluated the effect of EGCG on the expression of angiogenesis-related genes. Our data show that among other effects, EGCG treatment reduces expression of two integrins (α5 and β3) and a chemokine (MCP1), resulting in a lower adhesion of mast cells associated with a decreased potential to produce signals eliciting monocyte recruitment. These effects on gene expression levels are functionally validated by showing inhibitory effects in adhesion, aggregation, migration and recruitment assays.

Keywords. Mast cells, adhesion, chemokines, integrin α5, integrin β3, monocyte chemoattractant protein-1.

Introduction

Mast cells accumulate in many angiogenesis-dependent situations, including tumor growth, rheumatoid arthritis, ovulation, wound healing, and tissue repair [1]. Mast cells can produce, store, and release many kinds of chemical mediators, including histamine, tryptase, chymase, heparin, proteoglycans, various growth factors, and cytokines [2]. Most of these mediators contribute to the involvement of mast cells in angiogenesis [3]. In addition, the most severe forms of human mastocytosis can be considered as leukemias that, as any other neoplastic tissue, require new vessel formation for growth [4]. The balance or imbalance of angiogenesis modulators (either inducers or inhibitors) caused by mast cells and other inflammatory cells may favor either tumor progression or regression. However, the circumstances in which mast cells critically contrib-

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ute as a source of angiogenic factors, and the biosignaling involved in the regulation of their production and secretion, are far from being completely elucidated [5].

Green tea contains a unique set of catechins that possess biological activity in antioxidant, anti-angiogenesis, and anti-proliferative assays potentially relevant to the prevention and treatment of various forms of cancer [6]. The major constituent of green tea is (–)-epigallocatechin-3-gallate (EGCG), a compound with pleiotropic effects by itself. EGCG has been shown to have potent antitumor effects over a wide range of tumor cell types, not only by inhibiting their proliferation but also by induction of apoptosis through p53 activation and survival pathway inhibition [7–9]. One facet of the antitumor activities exerted by EGCG seems to be attributed to platelet-derived growth factor (PDGF) receptor blocking [10]. A recent study has demonstrated that EGCG inhibits focal adhesion kinase activity, indicating that it interferes with cancer cell adhesion and movement processes [11, 12]. Moreover, EGCG has been described to inhibit matrix metalloproteinases (MMP)-2 and -9, resulting in a significant reduction of the invasive behavior of gelatinase-expressing cancer cells [13]. EGCG also inhibits tumoral telomerase activity [14], affects epigenetic responses by inhibition of DNA methyltransferases [15] and inhibits metastasis [16]. On the other hand, EGCG has also anti-angiogenic effects, including inhibition of endothelial cell differentiation and inhibition of the extracellular matrix remodeling potential [17–22]. Interestingly, EGCG is also a potent anti-inflammatory compound able to inhibit the proinflammatory NF-κB pathway [23], the activity of histidine decarboxylase (the enzyme responsible for the synthesis of histamine, a potent elicitor of inflammatory responses) [24], and the migratory potential of monocytes and neutrophils [25, 26].

The anti-angiogenic and anti-inflammatory effects of EGCG suggest that mast cells are probably a target for it. Some very recent results show that this is, indeed, the case [27]. In the present study, we examine the effects of EGCG treatment on the expression of angiogenesis-related genes by mast cells, and we demonstrate that EGCG targets genes related to mast cell adhesiveness and its potential to produce signals eliciting monocyte recruitment.

Materials and methods

Cell culture and treatment. The human mast cell line HMC-1, kindly supplied by Dr., J. Butterfield (Mayo Clinic, Rochester), was cultured at 37 °C in a humidified atmosphere containing 5 % CO_2 at a starting density of 10^5 cells/ml in Iscove's medium (Cambex) supplemented with 10 % calf serum (PAA), 2 mM L-glutamine, penicillin (Cambex), streptomycin (Cambex), Fungizone (Gibco), iron supplement (Sigma) and 1.2 mM α-thioglycerol (Sigma). The medium was renewed every 7 days. HMC-1 cells (8×10^5 cells/ml) were treated with a fresh solution of EGCG (100 μM final concentration). After 8 h, total RNA was isolated or conditioned medium was stored at –80 °C until used. The human monocyte cell line THP-1, kindly supplied by Dr. Rodríguez-Agudo (Department of Medicine, Veterans Affairs Medical Center and Virginia Commonwealth University), was cultured under identical atmosphere conditions at a starting density of 3×10^5 cells/ml in RPMI medium (Cambex) supplemented with 10 % fetal calf serum (PAA), 2 mM L-glutamine, penicillin (Cambex), streptomycin (Cambex), Fungizone (Gibco) and 50 μM 2-mercaptoethanol (Sigma). The medium was renewed every 3 days.

Apoptosis assay. The effects of EGCG on the induction of apoptosis both in HMC-1 and THP-1 cell lines were examined by flow cytometry with the annexin V-PE apoptosis kit (BD Biosciences). Cells were prepared and cultured, as previously described, in the presence or absence of EGCG. After incubation, cells were washed and stained with phycoerythrin (PE)-labeled annexin V (AN) and 7-aminoactinomycin D (7AAD) following the protocol provided with the kit (BD Biosciences). Cells (10^4) were analyzed by flow cytometry (FACScan, BD Biosciences), and the $AN^-/7AAD^-$, $AN^+/7AAD^-$, $AN^+/7AAD^+$ populations were enumerated. The three populations, $AN^-/7AAD^-$, $AN^+/7AAD^-$ and $AN^+/7AAD^+$, have been found to correspond to live cells, early apoptotic cells, and necrotic cells, respectively.

Total RNA isolation. Total RNA was isolated from HMC-1 cells (8×10^5 cells per extraction) following the protocol provided with the GenElute Mammalian Total RNA Miniprep Kit (Sigma). RNA yield and purity were assessed spectrophotometrically at 260 and 280 nm.

RT-PCR. The first-strand cDNA synthesis from extracted RNA (1 μg) was performed using the iScript cDNA Synthesis Kit (Bio-Rad) in a final volume of 20 μl according to the recommendations of the manufactured.

The semiquantitative PCRs were carried out in a final volume of 20 μl containing 1–3 μl cDNA synthesized as above, 1× reaction buffer, dNTP mixture (0.2 mM each), 1 μM forward and reverse primers, and 1 U Taq polymerase (Bio-Rad or EuroTaq EuroLone). Pri-

Table 1. Primers, amplicon size and program used for PCR amplification.

Gen	Primers	Size	Program
ITGA5	F: 5′-GGCAGCTATGGCGTCCCACTGT-3′ R: 5′-GGCATCAGAGGTGGCTGGAGGC-3′	171 bp	28× (95 °C 30″; 60 °C 30″; 72 °C 30″)
ITGB3	F: 5′-GTGCTGACGCTAACTGACC-3′ R: 5′-AGTCTTGGCATCAGTGGTAA-3′	182 bp	34× (95 °C 20″; 60 °C 15″; 72 °C 20″)
MCP1	F: 5′-GCCTTAAGTAATGTTAATTCTTAT-3′ R: 5′-GGTGTAATAGTTACAAAATATTCA-3′	241 bp	32× (95 °C 30″; 51 °C 30″; 72 °C 40″)
β-Actin	F. 5′-ACCTCATGAAGATCCTGAC-3′ R: 5′-ACTCCTGCTTGCCGATCC-3′	524 bp	28× (95 °C 30″; 57.4 °C 30″; 72 °C 60″)

ITGA5: integrin α5; ITGB3: integrin β3; MCP1: monocyte chemoattractant protein-1; ″: seconds.

mers, amplicon size, number of cycles and PCR conditions for each gene are shown in Table 1. The number of cycles for each reaction was chosen to ensure a linear relationship between the quantity of input RNA and the final product during PCR amplification. Amplified products were visualized by ethidium bromide fluorescence in 1 % agarose gels and were confirmed by sequencing. At least three different experiments were carried out.

DNA array. Total RNA was isolated from HMC-1 control and treated with 100 μM EGCG as described before. Total RNA (3 μg) was employed as a template for [^{32}P]cDNA probe synthesis using a GE-Array AmpoLabeling-LPR Kit (Superarray), according to the manufacturer's instructions. ^{32}P-labeled cDNA was used for screenings of GE-Array Q Series Human Angiogenesis Gene Array (SuperArray). Data were collected using a Phosphoimager FujiBass 1500 (Fujifilm) and analyzed using the ImageGauge software (Fujifilm). Each array membrane contained 96 marker genes in quadruplicates, including four positive controls [β-actin, glyceraldehyde-3-phosphate dehydrogenase, peptidylprolyl isomerase A (cyclophilin A, PPIA), and ribosomal protein L13a] and a negative control (bacterial plasmid pUC18). Relative expression levels of different genes were estimated by comparing their signal intensities with that of the PPIA internal control, whereas negative values were transformed into zeros. Two independent experiments were carried out.

Enzyme-linked immunosorbent assay. HMC-1 cells were treated or not with 100 μM EGCG for 8 h. Monocyte chemoattractant protein-1 (MCP1) in culture supernatants after the treatment was measured using a commercial MCP1 Human Biotrak Easy ELISA kit (GE Healthcare) according to the manufacturer's instructions. The absorbance at 450 nm was determined using a microplate reader 680 (Bio-Rad). The MCP1 protein expression levels were normalized to total protein as pg/mg protein.

Flow cytometry for integrin expression determination. Flow cytometric analysis of integrin α5 (ITGA5) and integrin β3 (ITGB3) expression in HMC-1 cells was performed essentially as described elsewhere [28]. Before staining cell surface receptors, HMC-1 cells were treated or not as described above (100 μM EGCG for 8 h). Cells were then incubated for 30 min with anti-CD49e (ITGA5) or anti-CD61 (ITGB3) (BD Biosciences) on ice, washed twice in PBS and incubated for 30 min with Alexa Fluor 488 goat anti-mouse IgG (Invitrogen) on ice. Specific fluorescence was detected with a FACScan flow cytometer (BD Biosciences).

Adhesion assay. Adhesion assays were carried out in 24-well plates. Wells were coated overnight at 4 °C with 10 ng/ml fibronectin (Sigma). Plates were then gently washed and remaining binding sites were blocked by adding 3 % BSA in PBS for 1 h at 37 °C. HMC-1 cells were treated or not with 100 μM EGCG for 8 h; then cells were suspended at 3×10^4 cells/ml, and 300 μl of the cell suspension were added to each pre-coated well. Plates were further incubated for 1 h at 37 °C. Unbound cells were removed by gentle washing with PBS. The remaining cells were counted under light microscope. Alternatively, adhesion assays were performed in 96-well plates. Briefly, wells were coated and blocked as above. A volume of 100 μl of (control or treated) HMC-1 cells was added to each pre-coated well, and plates were further incubated for 1 h at 37 °C. Unbound cells were removed by gentle washing with PBS. The adherent cells were grown for 3 days, and the number of cells was then estimated by the MTT assay, as described elsewhere [24].

Spontaneous aggregation assay. Control or treated HMC-1 cells were washed with PBS and seeded into 6-well plates with fresh medium, at 3×10^5 cells/well. After 3 days, aggregation was determined, using ten pictures per well, with the option "analyze particles" of ImageJ software (NIH). Counted particles bigger than 60 pixel square were grouped by size

(less than 120, 120–300, 300–700, more than 700 pixel square).

Chemotaxis assay. Mast cell migration was examined using a 24-well microchemotaxis assay. Control and treated HMC-1 mast cells (resuspended in medium without FBS at 2×10^6–5×10^6 cells/ml) were placed in the upper compartment. Medium supplemented with FBS was placed in the lower compartment of a 24-well microchemotaxis chamber. The upper and lower compartments of the chamber were separated by an 8-μm polycarbonate filter coated with fibronectin (Sigma). The chambers were incubated for 4 h at 37 °C. Filters were then scraped, washed, fixed with methanol, and stained with toluidine blue. Cell migration was measured by counting the number of cells attached to the lower surface of the filter. The results were expressed as the average of the number of migrating cells per high-magnification field and million of seeded cells (HMF). The chemotaxis assay for the THP-1 monocytic cell line was carried out using conditioned medium of HMC-1 cells treated or not for 8 h with 100 μM EGCG in the lower compartment as chemoattractant.

Results

Evaluation of the potential toxicity of EGCG treatment for HMC-1 mast cells. Since EGCG has been described to have toxic effects, we initially examined the effect of EGCG treatment on HMC-1 by cell counting. At 8 h after addition of 100 μM EGCG, HMC-1 cell number (860000 ± 62149) was not significantly different from that of control, non-treated HMC-1 cells (942500 ± 57263).

In short-term treatments, total cells counting might not change even in the cases in which a relevant portion of cell population has entered in early apoptosis. The effects of EGCG on the induction of apoptosis in HMC-1 cell line 8 h after addition of 100 μM EGCG were examined by flow cytometry. Two-color flow cytometric analysis using AN and 7AAD can discriminate three populations, viable ($AN^-/7AAD^-$), early apoptotic ($AN^+/7AAD^-$) and both late apoptotic and necrotic cells ($AN^+/7AAD^+$). The percentage of early apoptotic cells was similar in control (11.98 %) and treated cells (10.10 %) (Fig. 1). Furthermore, the small portion of necrotic cells in the control situation was not increased at all with the treatment (data not shown).

Expression of angiogenesis-related genes in HMC-1. We investigated the expression of angiogenesis-related genes in HMC-1 mast cells using a commercial cDNA macroarray containing probes for 96 genes, the GE-Array Q Series Human Angiogenesis Gene Array (SuperArray). Table 2 lists, as a percentage of PPIA expression, those genes with detectable expression levels on HMC-1 cells grouped according to their functional role.

The highest expression levels were found for transforming growth factor receptor-3 (TGFBR3) and the hypoxia-inducible factor 1 alpha subunit (HIF1A) with 240 % and 234 % of PPIA expression, respectively. Five genes presented moderate expression levels: tissue inhibitor of metalloproteinase-1 (TIMP1) (27.4 % of PPIA), ephrin-A2 (EFNA2) (16.9 %), v-erb-b2 erythroblast leukemia viral oncogene homolog 2 (ERBB2) (10.3 %), fms-related tyrosine kinase 1 (FLT1) (8.9 %) and ITGA5 (7.8 %). Nine genes were found with a low level

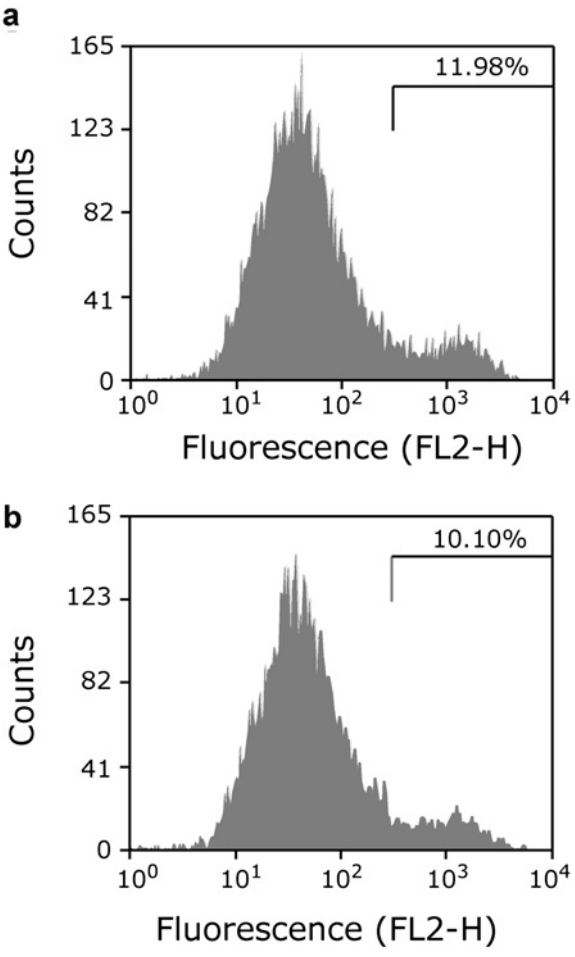

Figure 1. Annexin V apoptosis assay. HMC-1 cells treated (*b*) or not (*a*) with 100 μM (–)-epigallocatechin-3-gallate (EGCG) for 8 h were stained with phycoerythrin (PE)-labeled annexin V (AN) and 7-amino-actinomycin D (7AAD). The percentage of $AN^+/7AAD^-$ (early apoptotic) cells is showed. Two independent experiments were carried out, both showing no apoptotic effect of EGCG treatment on HMC-1 cells.

Table 2. Angiogenesis related genes expressed by HMC-1 cells grouped by function.

Symbol	% of PPIA expression[a]	GenBank accession no.	Description
Specific promoters and inhibitors			
KIAA1071	1.20 ± 0.55	NM_133265	Angiomotin
Growth factors and receptors			
EFNA2	16.92 ± 1.56	NM_001405	Ephrin-A2
EGFR	1.41 ± 1.36	NM_005228	Epidermal growth factor receptor
FGF6	1.74 ± 1.16	NM_020996	Fibroblast growth factor 6
FLT1	8.82 ± 6.94	NM_002019	Fms-related tyrosine kinase 1
GRO1	5.46 ± 0.82	NM_001511	Chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha)
PDGFA	1.18 ± 0.19	NM_002607	Platelet-derived growth factor alpha polypeptide
PF4	3.01 ± 0.13	NM_002619	Platelet factor 4 (chemokine (C-X-C motif) ligand 4)
TGFB1	3.14 ± 0.61	NM_000660	Transforming growth factor, beta 1 (Camurati-Engelmann disease)
TGFBR1	240 ± 16	NM_004612	Transforming growth factor, beta receptor I
Cytokines and chemokines			
MDK	1.92 ± 0.02	NM_002391	Midkine (neurite growth-promoting factor 2)
NRP1	1.49 ± 0.69	NM_003873	Neuropilin 1
MCP1	2.75 ± 1.16	NM_002982	Chemokine (C-C motif) ligand 2, monocyte chemoattractant protein-1
TNFA	4.05 ± 1.03	NM_000594	Tumor necrosis factor (TNF superfamily, member 2)
Adhesion molecules			
CDH5	2.16 ± 0.55	NM_001795	Cadherin 5, type 2, VE-cadherin (vascular epithelium)
ITGA5	7.76 ± 2.49	NM_002205	Integrin, alpha 5 (fibronectin receptor, alpha polypeptide)
ITGB3	0.28 ± 0.16	NM_000212	Integrin, beta 3 (platelet glycoprotein IIIa, antigen CD61)
Matrix proteins, proteases and inhibitors			
THBS3	3.09 ± 1.07	NM_007112	Thrombospondin 3
TIMP1	27.41 ± 8.86	NM_003254	TIMP metallopeptidase inhibitor 1
Transcription factors			
ERBB2	10.26 ± 0.29	NM_004448	V-erb-b2 erythroblast leukemia viral oncogene homolog 2
ETS1	4.04 ± 1.65	NM_005238	V-ets erythroblastosis virus E26 oncogene homolog 1 (avian)
HIF1A	234 ± 19	NM_001530	Hypoxia-inducible factor 1, alpha subunit (basic helix-loop-helix transcription factor)
MADH 1	4.71 ± 2.63	NM_005900	SMAD, mothers against DPP homolog 1 (Drosophila)
Other related genes			
RSN	3.52 ± 1.57	NM_002956	Restin (Reed-Steinberg cell-expressed intermediate filament-associated protein)

[a] Percentage relative to expression level of peptidylprolyl isomerase A (PPIA).

(between 5 % and 3 %): melanoma growth stimulating activity alpha (GRO1) (5.5 %), Smad transcription factor (MADH1) (4.7 %), tumor necrosis factor-alpha (TNFA) (4.1 %), inhibitor of DNA binding 3 (ID3) (3.7 %), transforming growth factor-beta 1 (TGFB1) (3.5 %), thrombospondin 3 (THBS3) (3.1 %) and platelet factor 4 (PF4) (3.0 %). At very low level (<3 %) HMC-1 cells express the monocyte chemoattractant protein-1 (MCP1) > cadherin 5 (CDH5) > midkine (MDK) > osteopontin (SPP1) > fibroblast growth factor 6 (FGF6) > neuropilin 1 (NRP1) > epidermal growth factor receptor (EGFR) > angiomotin (KIAA1071) > platelet-derived growth factor alpha (PDGFA) > ITGB3.

Regulation of mRNA expression of HMC-1 by EGCG treatment. We identified angiogenesis-related genes that are regulated by EGCG through comparison of the data obtained from treated samples with controls. Figure 2a shows the expression of angiogenesis-related genes on HMC-1 cells treated or not with 100 μM EGCG for 8 h. We found that nine genes decreased their expression levels by more than 1.3-fold in response to EGCG treatment (Fig. 2b). Five of these genes encode for growth factors (EFNA2, FGF6, GRO1, PDGFA, and PF4), two for adhesion molecules (ITGA5 and ITGB3), one for a chemokine (MCP1) and another one for a transcription factor (ERBB2).

Validation of expression data by RT-PCR. To validate the changes observed using macroarrays, we set up a semiquantitative RT-PCR for selected genes. Decreased expression of ITGA5, ITGB3 and MCP1 was confirmed by this technique (Fig. 3).

Analysis of the protein expression levels of ITGA5 and ITGB3. Our results also showed that EGCG decreased the protein expression levels of ITGA5 and ITGB3 on HMC-1 cells. We tested the protein expression levels of these integrins by flow cytometry and calculated the median fluorescence intensity for treated and not treated cells. Figure 4 shows that EGCG treatment, lightly but consistently, decreases the levels of both integrins on the surface of the cells.

Analysis of the secretion of MCP1. Since our results have shown that EGCG decreased the mRNA level of

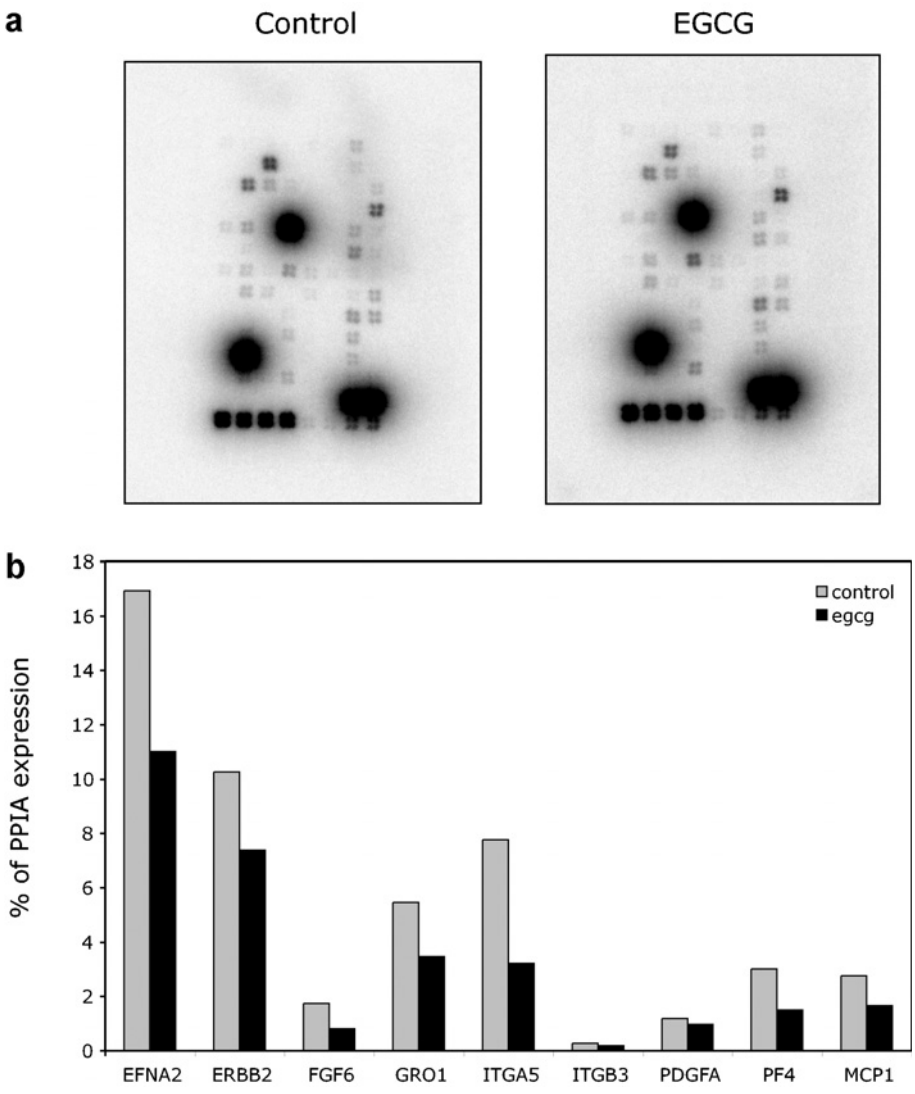

Figure 2. Expression of angiogenesis-related genes by HMC-1 cells treated or not with 100 μM EGCG for 8 h. Total RNA (3 μg) were used as template for [^{32}P]cDNA probe synthesis and hybridization of GE-Arrays Q Series Human Angiogenesis Gene Arrays. (*a*) Representative expression profile of angiogenesis-related genes in HMC-1 cells detected by cDNA macroarrays. (*b*) Results for genes whose expression, as a percentage of peptidylprolyl isomerase A (PPIA) housekeeping gene, was decreased in response to EGCG treatment. Data shown are the mean of two independent experiments.

MCP1 on HMC-1, we examined the MCP1 protein secretion. ELISA showed significantly lower MCP1 levels in the medium cultures of the cells treated with EGCG as compared to non-treated cells, 75.8 ± 6.9 % (means ± SEM of six independent duplicated experiments).

Functional validation (I): EGCG decreases mast cell adhesiveness and migration. Since a decreased expression of ITGA5 and ITGB3 was observed after EGCG treatment, we studied the effect of EGCG on adhesion and migration of HMC–1 cells. Figure 5 shows that EGCG totally inhibited HMC-1 cell adhesion to fibronectin.

Alteration of adhesive interaction of cells is one of the most important steps in an inflammatory response. The histological hallmark of primary mast cell diseases is the formation of mast cell aggregates in different tissues [29]. We therefore performed a spontaneous aggregation assay to examine the homotypical adhesion among mast cells. Figure 6 shows that the clustering of HMC-1 cells significantly decreased after EGCG treatment. Image analysis of HMC-1 aggregates reveals that EGCG-treated cells presented three times more small particles (< 120 pixels square, isolates cells) and the half as many big aggregates (particles > 700 pixels square).

We also investigated if EGCG affects *in vitro* HMC-1 cell migration. Figure 7 shows that treated cells presented a 37 % inhibition in migration compared with non-treated cells.

Functional validation (II): EGCG decreases monocyte recruitment by mast cells. Our results also showed that EGCG decreased the expression level of MCP1 on HMC-1 cells. Since MCP1 is a key factor for monocyte recruitment, we tested whether EGCG-treated HMC-1-conditioned medium could influence

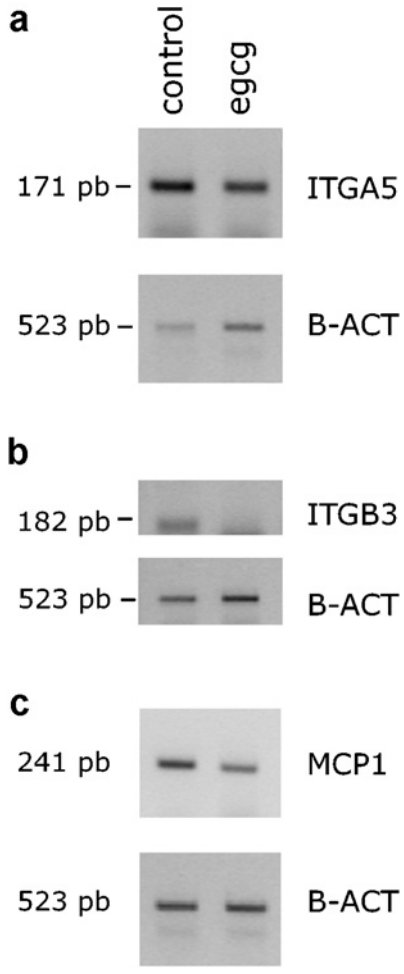

Figure 3. RT-PCR semiquantitative expression validation. RNA extracted from control and EGCG-treated HMC-1 cells was reverse transcribed into cDNA and amplified using conditions listed in Table 1. Amplification was performed to ensure a linear relationship between the quantity of input RNA and the final product during PCR amplification. Relative expression was assessed after normalization on β-actin amplification. Amplifications were carried out at least in triplicates. (*a*) Integrin α5 (ITGA5) expression; (*b*) integrin β3 (ITGB3) expression; (*c*) monocyte chemoattractant protein-1 (MCP1) expression.

THP-1 cell migration *in vitro*. The migration of THP-1 induced by conditioned medium from EGCG-treated mast cells was 2.7-fold less than that induced by conditioned medium from the control cells (Fig. 8). EGCG alone did not produce any cytotoxic effect on THP-1 cells compared with the non-treated cells, as assayed by counting the cell numbers after 4 h of treatment (control $2.48 \pm 0.08 \times 10^6$ cells *vs* 100 μM EGCG $2.61 \pm 0.17 \times 10^6$ cells; mean ± SEM). Furthermore, we determined the percentage of $AN^+/7AAD^-$ (early apoptotic) cells as described in Materials and methods for HMC-1 in the apoptosis assay. We found no pro-apoptotic effect of the treatment on THP-1 cells (data not shown).

Discussion

This study demonstrates that EGCG treatment down-regulates the expression of several angiogenesis-related genes in mast cells, three of which are directly related to adhesion and migration features: ITGA5, ITGB3 and MCP1. These three genes showed low or very low expression levels in control HMC-1 cells (Fig. 2); despite this, the inhibitory effect of EGCG on their expression could be clearly demonstrated by semiquantitative RT-PCR (Fig. 3). In addition, down-regulation of ITGA5 and ITGB3 on the surface was observed by flow cytometry and down-regulation of MCP1 secretion by ELISA, respectively. Moreover, these effects on gene and protein expression levels were functionally validated by showing inhibitory effects in adhesion, aggregation, migration and recruitment assays (the rest of the results are shown). Integrins are noncovalently associated heterodimeric cell surface adhesion molecules that are involved in cell-matrix and cell-cell interactions [30]. The ITGB3 subunit dimerizes with integrin αv giving rise to the functional integrin $\alpha_v\beta_3$, which has been shown to be up-regulated on certain tumor vessels [31] and, hence, has been described as a pro-angiogenic integrin [32]. The ITGA5 dimerizes with integrin β1 giving rise to the functional integrin $\alpha_5\beta_1$, which is clearly pro-angiogenic, along with its ligand, fibronectin [33–35]. However, the precise role of the integrins expressed on mast cells in angiogenesis remains to be clarified. In any case, if the inhibitory effects of EGCG on both ITGA5 and ITGB3 at mRNA and protein levels were biologically relevant, clear effects on adhesive and migratory potential of mast cells could be expected. This was, in fact, the case, as demonstrated in the present study by showing that EGCG treatment produces potent inhibitory effects on the adhesion of HMC-1 cells to fibronectin (Fig. 5), the homotypic adhesion of HMC-1 cells in culture (Fig. 6) and the HMC-1 migration capabilities (Fig. 7). Although the contribution of other EGCG targets to these functional effects cannot be ruled out, these functional responses are consistent with the observed inhibitory effect on integrin expression.

MCP1 is a member of the C-C class of the β chemokine family with inflammatory properties [36]. Although expressed at very low levels in HMC-1 cells (Fig. 2), the inhibitory effect of EGCG on its mRNA and its protein secretion was evident by RT-PCR (Fig. 3) and ELISA, respectively, suggesting an inhibitory role of EGCG on monocyte recruitment. Our results (Fig. 8) clearly show that this was the case. Previously, it has been shown that EGCG can inhibit fibroblast adhesion and migration through multiple mechanisms [37]. EGCG can also inhibit the migratory potential of

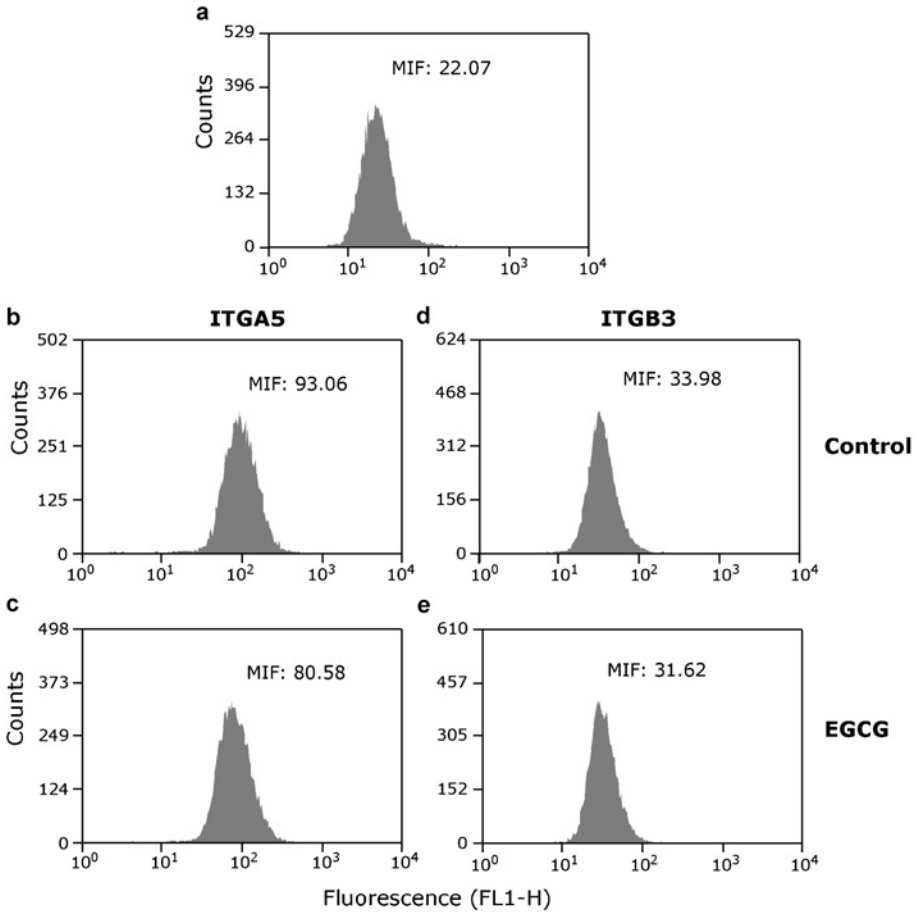

Figure 4. Expression of ITGA5 and ITGB3 by HMC-1 cells treated or not with 100 μM EGCG for 8 h. Cells were incubated with anti-CD49e (ITGA5) or anti-CD61 (ITGB3) mouse IgGs and then with Alexa Fluor 488 goat anti-mouse IgG. Median fluorescence intensity (MIF) was calculated for (*a*) unstained cells, (*b*) control cells stained with anti-CD49e, (*c*) treated cells stained with anti-CD49e, (*d*) control cells stained with anti-CD61, and (*e*) treated cells stained with anti-CD61. A typical experiment is shown. Three independent experiments were carried out, showing the same tendencies.

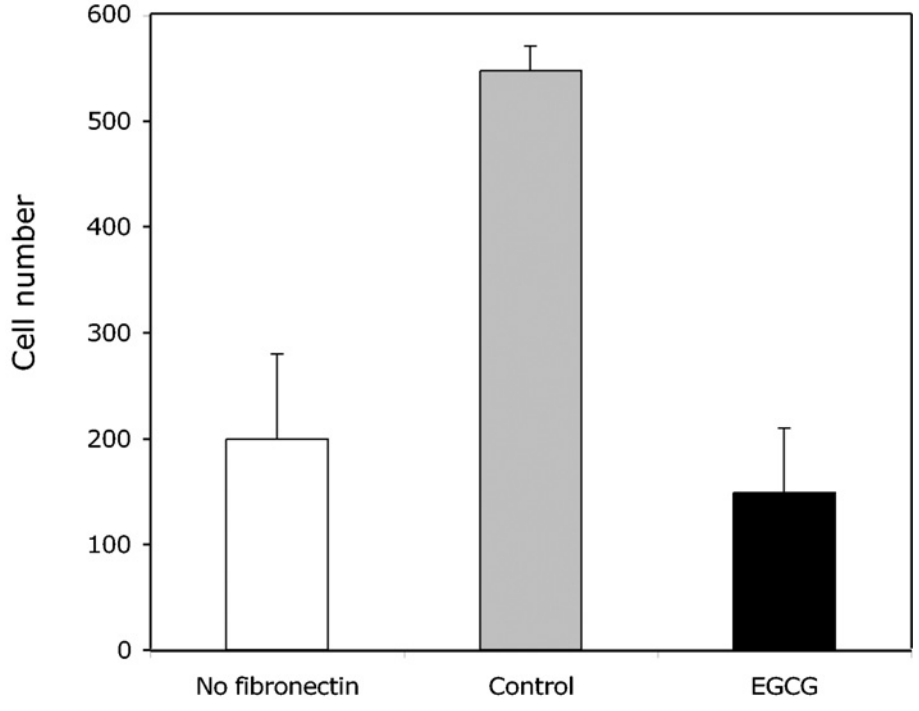

Figure 5. HMC-1 cell adhesion to fibronectin. HMC-1 cells (3×10^4) treated or not with 100 μM EGCG for 8 h were seeded on 24-well-plates, incubated for 1 h and nonadherent cells were removed by gently washing with PBS. The remaining cells were counted. Empty bar, cells counted on wells without fibronectin treatment; gray bar, control cells; black bar, EGCG-treated cells.

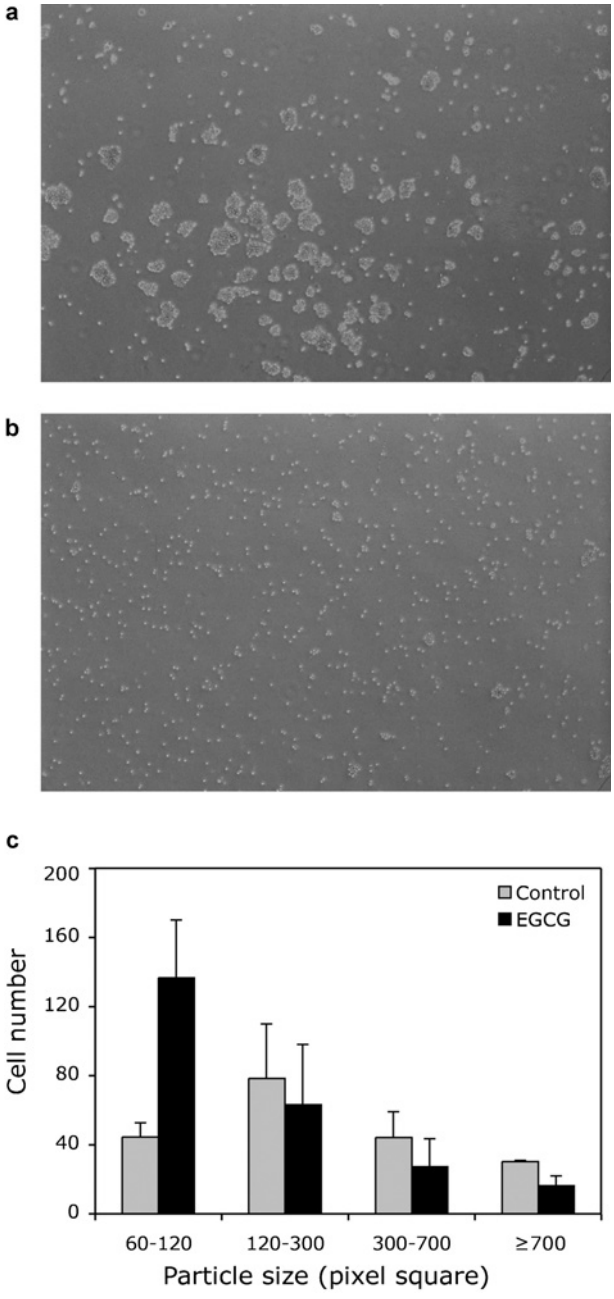

Figure 6. Spontaneous aggregation of HMC-1 cells. After 8 h of EGCG treatment, cells were washed and seeded on 6-well plates in the absence of EGCG, and incubated for 3 more days. Spontaneous aggregation was determined by image analysis of ten pictures per well using ImageJ software. Particles counted were grouped by size: <120, 120–300, 300–700 and >700 pixel square. (*a*) Control cells; (*b*) EGCG-treated cells; (*c*) particles distribution, gray bar, control cells; black bar, EGCG-treated cells.

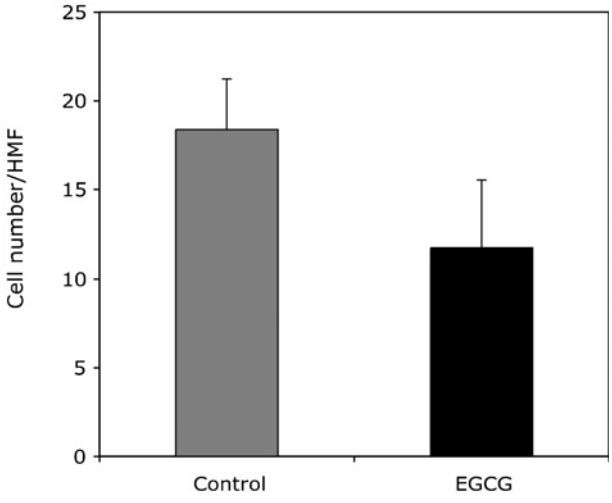

Figure 7. *In vitro* HMC-1 migration. Mast cell (HMC-1), treated or not with 100 μM EGCG for 8 h, were suspended in medium without serum and places in a migration chamber. Complete medium with FBS was used as chemoattractant. Cells were incubated for 4 h and migration was measured after cell staining with toluidine blue under light microscopy. The results were expressed as the average of the number of migrating cells per high-magnification field and million of seeded cells (HMF). Gray bar, untreated cells; black bar, EGCG-treated cells. Data represent the mean ± SEM of three independent experiments.

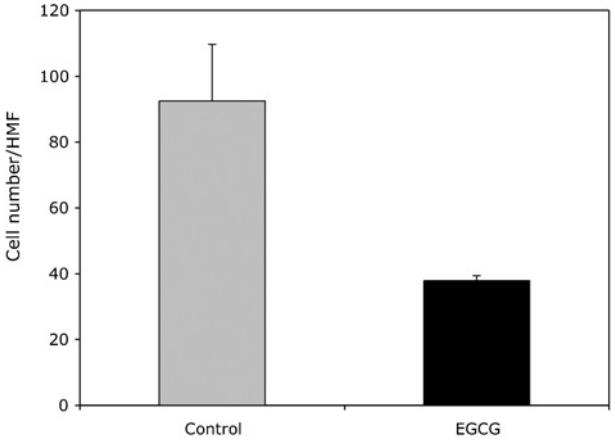

Figure 8. EGCG treatment of HMC-1 reduces recruitment of monocytes. Supernatants were collected from human mast cells (HMC-1) that were either left untreated, or incubated (8 h) with EGCG and were used as chemoattractant. THP-1 cells were placed in a migration chamber and migration was measured by counting cells under light microscopy after staining with toluidine blue. Gray bar, untreated cells; black bar, EGCG-treated cells. Data represent the mean ± SEM of three independent experiments.

other cell types involved in inflammatory processes, such as monocytes and neutrophils [25, 26]. It has also been shown that EGCG could suppress the PMA-induced MCP1 expression in human endothelial ECV304 cells at the transcriptional level by blockade of p38 mitogen-activated protein kinase (MAPK) and NF-κB [38]. Very recently, EGCG has been shown to inhibit cytokine secretion through ERK and NF-κB attenuation in HMC-1 [27]. Human mast cells, including HMC-1 cells used in the present study, express MCP1 along with an array of other chemokines [39]. Furthermore, MCP1 expression by monocytes, other stromal cells and tumor cells has been

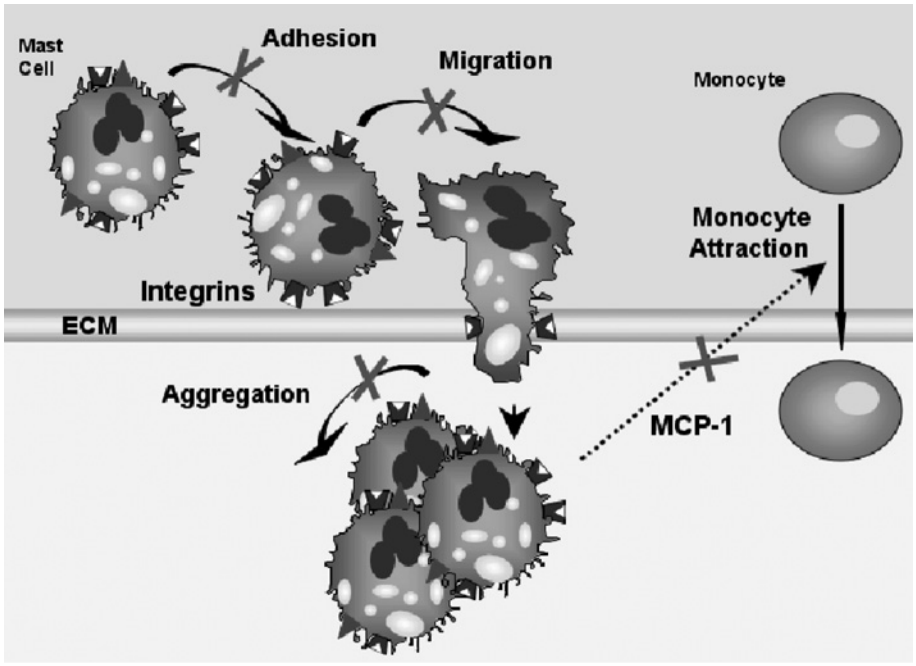

Figure 9. Scheme of the effects of EGCG on mast cell aggregation, migration and recruitment of monocytes.

demonstrated in a wide range of neoplastic diseases, including melanoma, glioma, osteosarcoma, leukemia, breast cancer, and ovarian cancer [39–42]. Up-regulation of MCP1 is related to macrophage recruitment, angiogenesis and survival in human breast cancer [42] and MCP1 transfection has been shown to induce angiogenesis and tumorigenesis in gastric carcinoma [43]. All together these observations reinforce the relevance of our results, stressing a new therapeutical potential for EGCG based on its specific targeting of MCP1. Furthermore, since MCP1 is one of the chemokines most deeply involved in atherogenesis [44], our results provide new insight into the potential pharmacological action of EGCG not only on angiogenesis and cancer, but also on atherosclerosis. This suggestion deserves further studies in the near future.

Figure 9 is a scheme of the effects of EGCG on mast cell adhesion, migration, aggregation and monocyte chemoattractant activity. On the other hand, based on our results and the well-characterized anti-inflammatory nature of EGCG [23–26], we suggest that EGCG could also have therapeutical applications on the treatment of systemic mastocytosis, an unusual disease characterized by variable mast cell hyperplasia associated with pathological changes in specific tissues, including skin, bone marrow, liver, spleen, lymph nodes, and gastrointestinal tract [45]. Conventional therapy of mastocytosis is based on agents that antagonize mediators released from mast cells, drugs inhibiting this release and agents modulating mast cell proliferation. Both steroidal and non-steroidal anti-inflammatory drugs are included in the panel of therapeutical agents for the treatment of mastocystosis [46]. In this point, the anti-inflammatory effects of EGCG should be underscored. To reinforce our suggestion, systemic mastocytosis is also characterized by overexpression of several surface antigens related to adhesion [29, 47], including ITGA5 (CD49e), one of the targets down-regulated by EGCG treatment as revealed in the present study.

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