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Inhibition of HuR and MMP-9 expression in macrophage-differentiated HL-60 myeloid leukemia cells by green tea polyphenol EGCg

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Abstract

Matrix metalloproteinase (MMP)-9 expression is linked with myeloid cell differentiation, as well as inflammation and angiogenesis processes related to cancer progression. MMP-9 secretion and macrophage-like HL-60 myeloid leukemia cells differentiation were triggered by the tumor-promoting agent PMA. The chemopreventive effects of green tea catechins epigallocatechin-gallate, catechin-gallate, and epicatechin-gallate, but not those catechins that lack a 3′-galloyl group, inhibited in a time- and dose-dependent manner MMP-9 secretion. The gene and protein expression of MMP-9 and of the mRNA stabilizing factor HuR were also inhibited, while that of the 67 kDa laminin receptor remained unaffected. Specific catechins may help optimize current chemotherapeutic treatment protocols for leukemia.

Keywords: Leukemia; Chemoprevention; Chemotherapy; Green tea; MMP-9; HuR

1. Introduction

Chemotherapy for the treatment of several types of neoplastic disease has been one of the success stories of medicine. Unfortunately, the chemotherapeutic treatment outcome of various hematological disorders, including most adult acute promyelocitic leukemia (APL) and acute myeloid leukemia (AML), remains unacceptable [1]. Among the latter, the development of resistance to a wide spectrum of cytotoxic drugs frequently impedes AMLs successful treatment [2]. Hence, novel avenues for the treatment of leukemia are required. Considerable attention has recently been focused on identifying naturally occurring chemopreventive substances capable of inhibiting, retarding, or reversing multi-stage carcinogenesis [3]. In fact, it has been demonstrated that some edible phytochemicals alter gene expression, directly or indirectly, thereby regulating carcinogenic processes.

(−)-Epigallocatechin gallate (EGCg), a principal antioxidant derived from green tea, is one of the most extensively investigated chemopreventive phytochemicals [4]. EGCg has been shown to block each stage of carcinogenesis by modulating the signal transduction pathways involved in cell proliferation, transformation, inflammation, apoptosis, metastasis and invasion [5]. Moreover, its anti-angiogenic properties make it a good candidate for targeting tumor-associated neovascularization [6]. Since the inclusion of antiangiogenic drugs into treatment protocols for leukemia and for hematologic malignancies is becoming an important task for future clinical studies [7,8], we sought to investigate the *in vitro*, molecular effects of the antiangiogenic and chemopreventive properties of green tea catechins on the macrophage/monocyte differentiation processes in myeloid leukemia cells.

Among the several leukemic cell lines that have been established over the years, the human promyelocytic HL-60 leukemia cells were derived from the peripheral blood

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of a patient with APL [9], and have proven useful in understanding the process whereby immature cells differentiate into cells of distinct mature myelomonocytic lineages. In particular, HL-60 cells can be induced to differentiate into mature functional macrophage-monocyte-like cells by 1α,25-dihydroxyvitamin D_3 and by cytosine arabinoside (Ara-C) [10]. Tumor-promoting agents, particularly the protein kinase C (PKC) activator phorbol 12-myristate 13-acetate (PMA, also known as TPA), have also long been known to induce HL-60 cell macrophage differentiation [11] via signal transduction processes in which PKC-β plays an essential role as a binding site for PMA [12]. Macrophage differentiation has also been shown to involve secretion and activation of collagenase MMP-9 [13], a crucial matrix metalloproteinase involved in extracellular matrix (ECM) degradation during tumor metastasis and in inflammatory disorders [14]. Although most published studies have focused on the transcriptional control of MMP-9 expression, there is increasing evidence that its expression can also be regulated at the levels of mRNA stability, translation and protein secretion. The ability to modulate MMP-9 expression at multiple steps through distinct signaling pathways may be particularly important during malignant conversion and metastasis, when tumor cells need to induce or maintain MMP-9 levels in response to changing environmental cues. Among the nuclear factors shown to stabilize and augment the expression of MMP-9 mRNA [15], HuR has been ascribed a pivotal role in the development of tumors [16] and been found to be a key mediator during macrophage activation in PMA-differentiated HL-60 cells [17].

Since an important aspect in inflammation and tumor progression is the involvement of the inflammatory response mediated by tumor-associated macrophages (TAM) [18], and since polyphenols have been suggested to regulate the antitumorigenic properties of TAM [19], we investigated the chemopreventive effects of green tea catechins on the mechanisms regulating MMP-9 secretion in PMA-induced macrophage-like HL-60 myeloid leukemia cells.

2. Materials and methods

2.1. Materials

Sodium dodecylsulfate (SDS) and bovine serum albumin (BSA) were purchased from Sigma (Oakville, Ont.). Cell culture media was obtained from Life Technologies (Burlington, Ont.). Electrophoresis reagents were purchased from Bio-Rad (Mississauga, Ont.). The enhanced chemiluminescence (ECL) reagents were from Amersham Pharmacia Biotech (Baie d'Urfé, Que.). Micro bicinchoninic acid protein assay reagents were from Pierce (Rockford, IL). The polyclonal antibodies against the 37 kDa laminin receptor precursor (37LRP) were from Santa Cruz Biotechnology (Santa Cruz, CA) and HuR was from Jackson ImmunoResearch Laboratories (West Grove, PA). All the green tea-derived catechins tested and other reagents were from Sigma–Aldrich Canada.

2.2. Cell culture

The HL-60 promyelocytic cell line was purchased from American Type Culture Collection (Manassas, VA) and maintained in Iscove's modified Dulbecco's medium (Gibco Invitrogen Cell Culture Systems, Burlington, ON) containing 20% (v/v) fetal bovine serum (FBS) (HyClone Laboratories, Logan, UT), 2 mM glutamine, 100 unit/ml penicillin, 100 μg/ml streptomycin, and were cultured at 37 °C under a humidified atmosphere containing 5% CO_2. Slides of PMA-treated HL-60 cells were mounted for light microscopy and air-dried, stained with Diff-Quick (Baxter Healthcare Corp., Miami, FL) and examined for morulae.

2.3. Total RNA isolation and reverse transcriptase-polymerase chain reaction (RT-PCR) analysis

Total RNA was extracted from cultured HL-60 cells using the TRIzol reagent (Invitrogen, Burlington, ON). One microgram of total RNA was used for first strand cDNA synthesis followed by specific gene product amplification with the One-Step RT-PCR Kit (Invitrogen). Primers for 67LR (forward: 5′-TGCAACAACAAGGGAGCTCAC-3′, reverse: 5′-TCCATCAACCATTTTTCCAT-3′), for HuR (forward: 5′-TCGCAGCTGTACCACTCGCCAG-3′, reverse: 5′-CCAAACATCTGCCAGAGGATC-3′) and for MMP-9 (forward: 5′-AAGATGCTGCTGTTCAGCGGG-3′, reverse: 5′-GTCCTCAGGGCACTGCAGGAT-3′) were all derived from human sequences. PCR conditions were optimized so that the gene products were examined during the exponential phase of their amplification and the products were resolved on 1.5% agarose gels containing 1 μg/ml ethidium bromide.

2.4. Immunoblotting procedures and zymography

Proteins from control and treated cells were separated by SDS-polyacrylamide gel electrophoresis (PAGE), electrotransferred to polyvinylidene difluoride membranes which were then blocked with 5% non-fat dry milk. Immunoreactive material was visualized by enhanced chemiluminescence (Amersham Biosciences, Baie d'Urfée, QC). Gelatin zymography was used to assess the extent of MMP-9 activity. Briefly, an aliquot (40 μl) of the culture medium was subjected to SDS-PAGE in a gel containing 0.1 mg/ml gelatin. The gels were then renatured and stained with 0.1% Coomassie Brilliant blue R-250. Gelatinolytic activity was detected as unstained bands on a blue background.

3. Results

3.1. PMA induces an invasive phenotype in macrophage-differentiated HL-60 cells

Among the agents who are well documented as inducing differentiation of HL-60 cells, the tumor-promoting agent

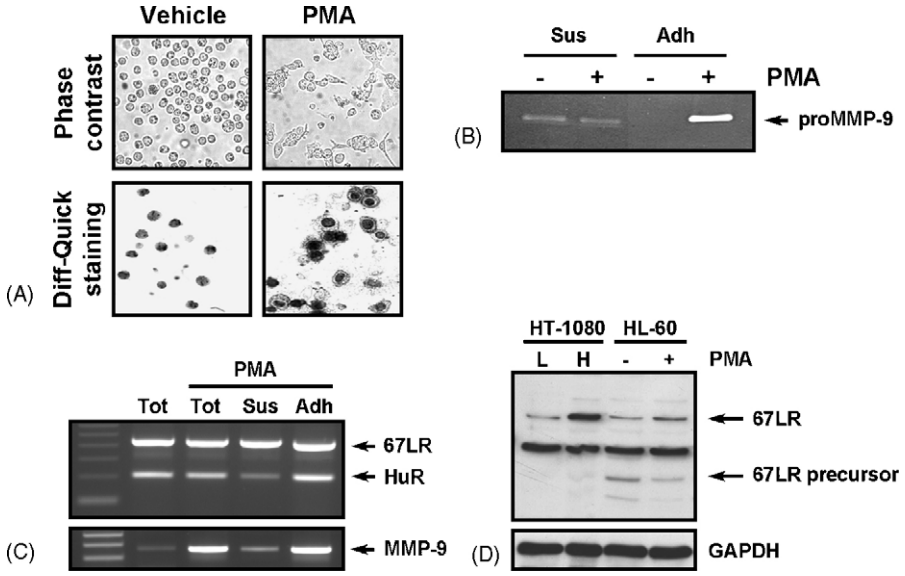

Fig. 1. PMA induces an invasive phenotype in macrophage-like HL-60 cells. HL-60 cells were cultured in suspension as described in Section 2. Differentiation was induced in serum-starved HL-60 cells by the presence of 50 nM PMA for 18 h. (A) Phase contrast microscopy shows that PMA induced HL-60 cell adhesion (phase contrast, upper right panel) characterized by flattening and cell extensions once the floating cells were discarded. In contrast, untreated control cells remain uniformly round and in suspension (upper left panel). Diff-Quick staining (lower panels) shows morphological characteristics of mononuclear, adherent macrophages. (B) Conditioned media was extracted from adherent (Adh) and floating (Sus) cells in untreated and PMA-treated HL-60 cells, and subjected to gelatin zymography as described in Section 2. (C) Total RNA was isolated from cells treated as in (B) and RT-PCR performed in order to evaluate the gene expression of 67LR, HuR, and MMP-9. (D) Western blotting was performed with total cell lysates of HT-1080 fibrosarcoma and HL-60 cells and immunodetection was performed with an anti-67LR antibody as described in Section 2 (the 67LR precursor has a M_W of 37 kDa).

phorbol-12-myristate-13-acetate (PMA) is thought to trigger a terminal differentiated monocytic/macrophage phenotype [11]. PMA treatment of serum-starved HL-60 cells partially induced an adhesive phenotype in these cells which originally remain in suspension (Fig. 1A). This adhesive phenotype was accompanied by macrophage differentiation (Diff-quick staining, Fig. 1A), and so was further investigated in light of the capacity of PMA to trigger MMP-9 secretion. HL-60 cells were treated (or not) with PMA in order to induce cell adhesion as described in Section 2. The remaining cells, which did not adhere upon PMA treatment, were transferred into a fresh dish and MMP-9 secretion was monitored by gelatin zymography. While MMP-2 levels were very low to undetectable (not shown), those for MMP-9 were detected in basal secretion by HL-60 cells and were specifically induced by PMA in cells that adopted an adherent phenotype (Fig. 1B). Total RNA was next isolated from untreated and from PMA-treated HL-60 cells and RT-PCR was performed in order to monitor MMP-9, the 67 kDa laminin receptor (67LR) and HuR gene expression levels. The results show that the 67LR gene expression was unaffected by PMA treatment and can thus be considered as an internal loading control (Fig. 1C). In contrast, although HuR seemed to be unaffected in combined floating and adherent cells from control and PMA-treated cells (Tot, Fig. 1C), it was significantly increased in the cells that adopted an adhesive phenotype upon PMA treatment. This is further confirmed by the increase in MMP-9 gene expression following PMA treatment that is shown in both the combined total floating and adherent cells as well as in the PMA-induced adherent HL-60 cells (Fig. 1C). Finally, although no modulation in the 67LR gene expression was observed, we found that maturation of the 67LR protein from its 37 kDa laminin receptor precursor occurred upon PMA treatment of HL-60 cells, as was similarly observed in HT-1080 fibrosarcoma cells (Fig. 1D). Altogether, these observations suggest that PMA triggers an invasive phenotype in HL-60 cells that is reflected by the increased capacity of the adherent cells to secrete MMP-9.

3.2. *EGCg inhibits HuR and MMP-9 gene expression during HL-60 cell transformation by PMA*

Recent reports have shown that EGCg, a green tea-derived catechin with chemopreventive properties, inhibited MMP-9 secretion in lung carcinoma and gastric cancer cells [20,21]. This prompted us to investigate whether EGCg also inhibited MMP-9 secretion in PMA-treated HL-60 cells. Serum-starved HL-60 cells were treated with various doses of EGCg for 18 h in the presence of PMA. Conditioned media was then harvested, and gelatin zymography performed to determine whether MMP-9 secretion was inhibited by EGCg (Fig. 2A). Complete inhibition was observed at 10 μM EGCg with an IC50 constant inhibition of 3.2 ± 0.7 μM (Fig. 2B). In order to evaluate the respective contributions and sensitivity to EGCg of the macrophage-like adherent cells and of cells that remained in suspension after PMA treatment, total RNA was isolated from these two cell populations. Gene expression of MMP-9, HuR, and of the 67LR was assessed by RT-PCR.

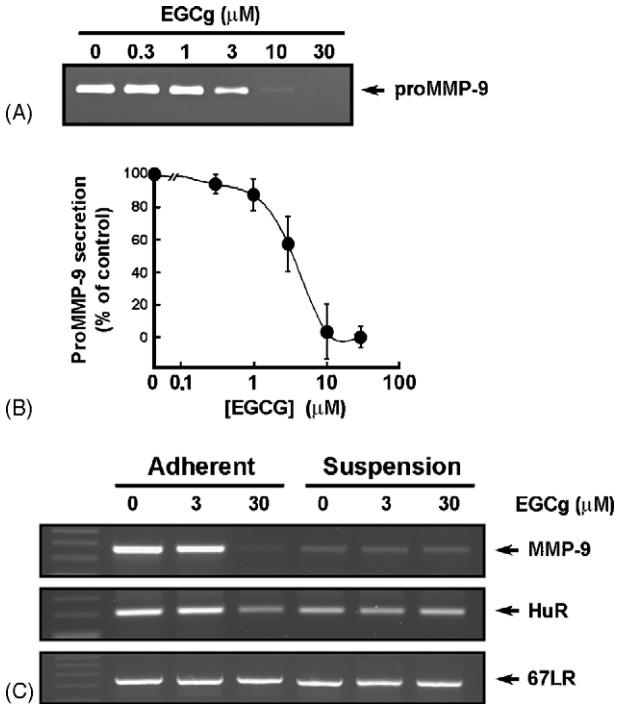

Fig. 2. EGCg inhibits HuR and MMP-9 gene expression in adherent HL-60 cells. (A) Serum-starved HL-60 cells were treated with 50 nM PMA in the presence of increasing concentrations of EGCg for 18 h. Conditioned media were collected and gelatin zymography performed. (B) Scanning densitomery was used to quantify the extent of gelatin hydrolysis, and the mean ± S.D. are shown for three independent experiments. (C) Total RNA was extracted from PMA-treated HL-60 cells and RT-PCR performed to assess the extent of 67LR, HuR, and MMP-9 gene expression in adherent cells and cells that remained in suspension.

While the internal control 67LR gene expression remained unchanged, PMA induced HuR and MMP-9 expression significantly in adherent cells in comparison to the floating cells (Fig. 2C). Furthermore, EGCg diminished the PMA-induced expression of both MMP-9 and HuR in adherent cells while it did not affect HuR or MMP-9 gene expression in floating cells (Fig. 2C). Collectively, EGCg clearly targets the transformed cells that acquired adhesive and invasive properties.

3.3. EGCg inhibits MMP-9 secretion in adherent, macrophage-like HL-60 cells

While EGCg inhibition of MMP-9 gene expression was mostly attributable to the adherent transformed HL-60 cells, we assessed whether EGCg also targeted the terminally differentiated HL-60 cells which now exhibit an adhesive and macrophage-like phenotype. PMA-induced differentiation was performed and the adherent cells were serum-starved in the presence or absence of EGCg for 12 h. As shown by the gelatin zymography of the respective conditioned media (Fig. 3A), the extent of MMP-9 secretion was significantly lowered by EGCg and represented only ∼10% of the maximal secretion observed at 12 h in untreated cells (Fig. 3B). This shows that EGCg efficiently targets MMP-9 secretion in macrophage-differentiated HL-60 cells.

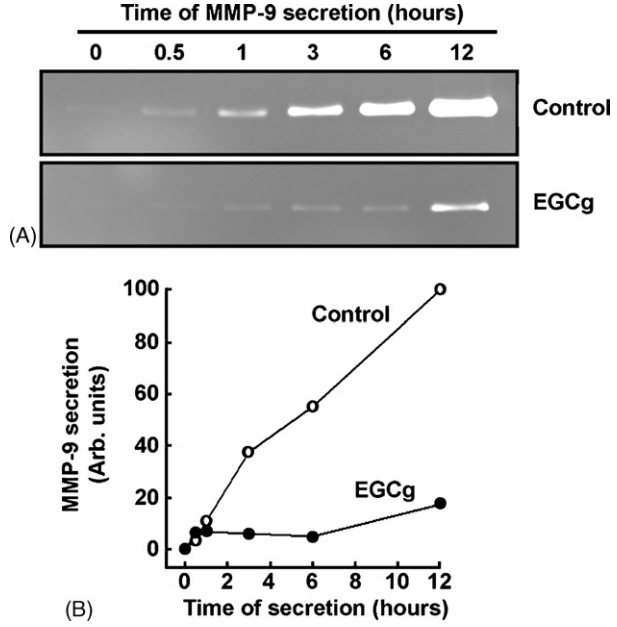

Fig. 3. EGCg inhibits MMP-9 secretion in adherent macrophage-like HL-60 cells. (A) HL-60 cells were treated with PMA for 18 h as described in Section 2. Cells that remained in suspension were discarded and the adherent macrophage-differentiated cells were serum-starved in the presence or absence of 30 μM EGCg for an additional 12 h. Conditioned media were collected and gelatin zymography performed as described in Section 2. (B) Scanning densitometry was performed to assess the extent of gelatinolytic activity in control (open circles) and EGCg (closed circles)-treated cells, and is representative of three different experiments.

3.4. EGCg inhibition of MMP-9 and HuR gene expression is synchronized in adherent, macrophage-like HL-60 cells

In order to evaluate the potential contribution of HuR in the regulation of MMP-9 gene expression, we isolated total RNA from control and from EGCg-treated adherent HL-60 cells after different treatment periods. We performed RT-PCR and semi-quantitative analysis to assess the levels of HuR and MMP-9 gene expression. We show that MMP-9 and HuR gene expression remained relatively constant up to 12 h (Fig. 4, white circles). In contrast, EGCg treatment induced a synchronized and time-dependent decrease in both HuR and MMP-9 gene expression which started as soon as 3 h after the treatment, and resulted in a 30–35% inhibition at 12 h (Fig. 4, black circles). This observation supports the possibility that the decrease in HuR expression may affect the stability of MMP-9, which would then lead to diminished synthesis and secretion of MMP-9.

3.5. The galloyl group in the 3′ position of green tea-derived catechins provides specificity in the inhibition HuR and MMP-9 expression

Structure–function relationships have yet to be determined for the anti-cancer properties of several of the green tea-derived catechins. Interestingly, among the catechins present

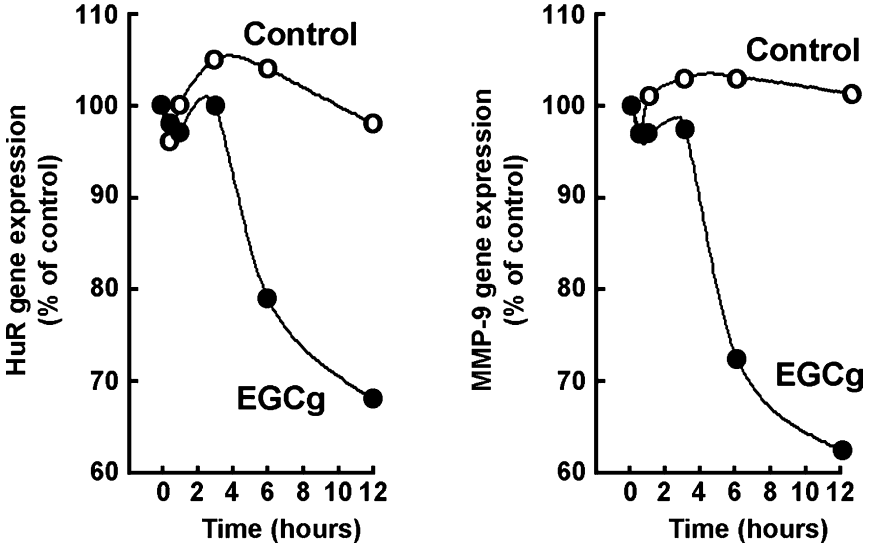

Fig. 4. EGCg inhibition of MMP-9 and HuR gene expression is synchronized in adherent macrophage-like HL-60 cells. HL-60 cells were treated with PMA for 18 h as described in Section 2. Cells that remained in suspension were discarded and the adherent macrophage-differentiated cells were serum-starved in the presence or absence of 30 μM EGCg for an additional 12 h. Total RNA was isolated at the indicated times and RT-PCR performed as described in Section 2. Scanning densitometry was performed on the amplicons to assess the extent of HuR and MMP-9 gene expression and is presented as the percent of control untreated cells. Control (open circles) and EGCg (closed circles)-treated cells are data representative of three different experiments.

in green tea plyphenols, EGCg, epicatechin gallate (ECg) and catechin gallate (Cg) were found to be responsible for inhibiting P-gp which is involved in the multidrug resistance phenotype of cancer cells [22]. Furthermore, the most potent inhibitors of MMP catalytic activities were EGCg and ECg [23]. We have tested whether any three dimensional structural specificity could be found within the catechins with respect to inhibiting PMA-induced MMP-9 secretion in adherent HL-60 cells. We show that EGCg, Cg, and ECg all inhibited PMA-induced MMP-9 secretion in HL-60 cells, while epigallocatechin (EGC), catechin (C), and epicatechin (EC) had no effect (Fig. 5A). The catechins which inhibited MMP-9 secretion also affected MMP-9 gene expression, but not that of the internal loading control 67LR (Fig. 5B). The gallate moiety of the catechins tested thus seems to provide some specificity as to their MMP-9 secretion inhibition capacity. Cell lysates from adherent cells and from HL-60 cells that remained in suspension were also assessed for protein expression of HuR by Western blotting and immunodetection (Fig. 5C). Similar inhibition pattern to the effect of the catechins bearing

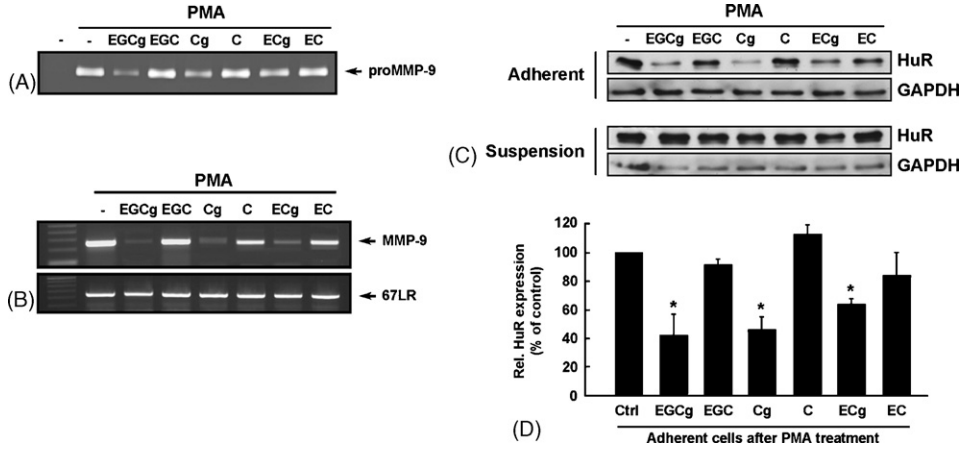

Fig. 5. The gallate moiety of green tea-derived catechins provides the specificity in the inhibition of HuR and MMP-9 expression. (A) Serum-starved adherent macrophage-like HL-60 cells were treated with 3 μM of green tea-derived catechins for 18 h. Conditioned media were collected and subjected to gelatin zymography. (B) Total RNA was extracted from adherent macrophage-like HL-60 cells and RT-PCR performed to assess the extent of 67LR and MMP-9 gene expression. (C) Cell lysates from adherent cells and from cells that remained in suspension after PMA treatment were harvested and 20 μg of protein loaded onto SDS gels. Western blotting followed by an immunodetection of HuR or GAPDH was performed. (D) Scanning densitometry was performed on the bands reflecting green tea catechins effect on adherent cells only and the values represent the mean values ± S.D. of three experiments.

the gallate moiety on MMP-9 gene expression, HuR protein expression was also found to specifically decrease by 40–50% in adherent HL-60 cells treated with these catechins (Fig. 5D). In contrast, HuR in those cells that remained in suspension was unaffected.

4. Discussion

Antiangiogenic agents which target the neovascularization process are being intensively investigated against various highly vascularized solid tumors [24]. In contrast, the rationale for the use of angiosuppressive therapy in hematological malignancies, and the status of novel antiangiogenic agents in clinical trials, particularly in leukemia, remain much less documented. The aim of our study was to assess the molecular impact of green tea-derived catechins on terminally differentiated macrophage-like HL-60 myeloid leukemia cells, as several of these catechins have already been characterized for their anti-angiogenic properties [6,25] and for their potential radiosensitizing effects [26,27]. Here, we show that green tea catechins specifically containing a galloyl group in the 3′ position effectively inhibited secretion of MMP-9, a process correlated with the acquisition of an invasive phenotype. Furthermore, we provide evidence that this inhibition mechanism involves destabilization of the MMP-9 transcript through reduced gene and protein expression of the MMP-9 mRNA stabilizing nuclear factor HuR.

One major impact of MMP secretion inhibition is in neuroinflammation, since MMP-9 secretion by macrophages and neutrophils could affect the integrity and lead to the breakdown of the blood–brain barrier [28]. We believe that the green tea catechin-mediated decrease in MMP-9 secretion from terminally macrophage-differentiated HL-60 cells might consequently impede their ability to migrate across the endothelium and may lead to decreased inflammatory infiltration. Interestingly, green tea catechins containing a galloyl group in the 3′ position have also been shown to inhibit tissue factor-induced thrombin generation [29], and to differentially regulate platelet aggregation [30]. Noteworthy, whether the 67 kDa laminin receptor (67LR), which was in fact found to be the EGCg cell surface receptor [31], provides the specificity and transduces the effects of the other catechins containing the galloyl group in the 3′ position remains to be confirmed. In light of our results, it is however tempting to hypothesize that the interaction between the galloyl groups in the 3′ position of catechins with the cell surface 67LR would be responsible for the specific downstream signaling.

Expression of the 67LR in AML cells is associated with monocytic differentiation since the 67LR expression strongly increased after PMA-induced monocytic differentiation of HL-60 cells [32]. Moreover, while the expression of the 67LR was undetectable in normal bone marrow hematopoietic cells, in precursor-B acute lymphoblastic leukemia, in chronic lymphocytic leukemia, and in chronic myeloid leukemia in chronic phase, it was significantly enhanced in 40% of 53 *de novo* AML, which frequently exhibited monocytic or myelomonocytic morphology and expressed CD14 and CD11a [32]. The involvement of the 67LR in transducing the intracellular signaling that regulates MMP-9 mRNA stability was highlighted in immortalized keratinocytes [33] and in prostatic adenocarcinoma [34]. In support to these findings, inhibition of MMP-9 secretion by PCK3145, an anti-angiogenic [35] and anti-metastatic [36] PSP94-derived peptide in phase IIa clinical trial against prostate cancer, was also recently found to require cell surface laminin receptor activity in order to inhibit HuR and MMP-9 expression [37]. Interestingly, the 67LR role in binding EGCg has led to the conclusion that the catechins C, EC, and EGC were unable to bind to the 67LR [31]. Unfortunately, the authors of that study failed to report any effects of those catechins that possessed an additional gallate moiety. It is thus unknown whether EGCg's alternate structural analogs that contain the gallate moiety also bind to the EGCg receptor.

Our finding that the 3′ galloyl group of the green tea-derived catechins would provide interaction specificity and be responsible for the 67LR-mediated signaling responsible for the specific inhibition of HuR has relevance to several other HuR-dependent cellular processes. For instance, HuR levels are elevated in cancer [38], and have a pivotal role in promoting angiogenesis [39,40]. Finally, HuR is thought to be a key mediator of posttranscriptional regulation and expression of the SLC11A1 gene, a solute carrier family 11 member 1 gene that plays an important role in macrophage activation in PMA-differentiated HL-60 cells [17]. It is tempting to suggest that these cellular processes, and the HuR-regulated expression of these markers, could be potential targets of green tea catechins. Whether any lowering of HuR by green tea catechins also contributes to cell death has yet to be investigated.

The role of angiogenesis in the growth and survival of leukemic cells has been recently demonstrated by showing that the progression of several forms of leukemia is related to the degree of angiogenesis [41]. Given that circulating inflammatory cells such as tumor associated macrophages (TAM), neutrophils and activated T lymphocytes can contribute to tumor angiogenesis by releasing MMP, and because the relationship between inflammatory cells and the process of cancer is well accepted [42], we believe that the anti-angiogenic and chemopreventive effects of specific green tea catechins may prove to be beneficial in targeting macrophage-like leukemia cells, help reduce tumor-associated inflammation to ultimately optimize current chemotherapeutic interventions.

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Contributions: Annabi designed the research, collected, analyzed the data and wrote the paper. Currie performed experiments, collected the data and wrote the paper. Moghrabi analyzed the data and wrote the paper. Béliveau designed the research, analyzed the data and wrote the paper.

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