Green Tea Extract and (−)-Epigallocatechin-3-Gallate Inhibit Mast Cell-Stimulated Type I Collagen Expression in Keloid Fibroblasts via Blocking PI-3K/Akt Signaling Pathways

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Keloid, a chronic fibro-proliferative disease, exhibits distinctive histological features characterized by an abundant extracellular matrix stroma, a local infiltration of inflammatory cells including mast cells (MCs), and a milieu of enriched cytokines. Previous studies have demonstrated that co-culture with MCs stimulate type I collagen synthesis in fibroblasts, but the signaling mechanisms remain largely unknown. In this study, we investigated the signaling pathways involved in MC-stimulated type I collagen synthesis and the effects of green tea extract (GTE) and its major catechin, (−)-epigallocatechin-3-gallate (EGCG), on collagen homeostasis in keloid fibroblasts. Our results showed that MCs significantly stimulated type I collagen expression in keloid fibroblasts, and the upregulation of type I collagen was significantly attenuated by blockade of phosphatidylinositol-3-kinase (PI-3K), mammalian target of rapamycin (mTOR), and p38 MAPK signaling pathways, but not by blockade of ERK1/2 pathway. Furthermore, GTE and EGCG dramatically inhibited type I collagen production possibly by interfering with the PI-3K/Akt/mTOR signaling pathway. Our findings suggest that interaction between MCs and keloid fibroblasts may contribute to excessive collagen accumulation in keloids and imply a therapeutic potential of green tea for the intervention and prevention of keloids and other fibrotic diseases.

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INTRODUCTION

Keloids are fibrous overgrowth induced by cutaneous injury (Tuan and Nichter, 1998; Niessen *et al.*, 1999). Clinically, keloids behave like benign dermal fibro-proliferative tumors as they continue to grow and extend beyond the confines of the original wound margins, without evidence of spontaneous regression as observed in hypertrophic scars (Ehrlich *et al.*, 1994). Histologically, keloids and hypertrophic scars differ from normal skin and normal scars by their rich vasculature (Rockwell *et al.*, 1989; Lee *et al.*, 2004), a high density of mesenchymal cells, a thickened epidermal cell layer, an increased infiltration of inflammatory cells including lymphocytes, mast cells (MCs), and macrophages (Amadeu *et al.*, 2003), and an abundant deposition of extracellular matrix (ECM) (Niessen *et al.*, 1999).

Human type I collagen, the major component of ECM in skin, bone, and ligament, comprises of two α1 (I) chains and one α2 (I) chain, which are derived from pro-*COL1A1* and pro-*COL1A2* genes, respectively (Ghosh, 2002). Abnormality in collagen synthesis leading to an imbalance in ECM metabolism has been recognized as an essential factor in the pathogenesis of several fibrotic diseases including keloids (Niessen *et al.*, 1999; Myllyharju and Kivirikko, 2001). However, the molecular mechanisms underlying the overproduction of type I collagen in keloids still remain largely unknown.

MCs are tissue dwelling cells containing prominent cytoplasmic granules. MC hyperplasia and activation have been implicated in the pathogenesis of several chronic inflammatory fibrotic diseases such as aberrant wound healing, idiopathic lung fibrosis, scleroderma, liver fibrosis,

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Abbreviations: ECM, extracellular matrix; EGCG, (−)-epigallocatechin-3-gallate; GTE, green tea extract; MC, mast cell; mTOR, mammalian target of rapamycin; PI-3K, phosphatidylinositol-3-kinase

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inflammatory bowel diseases such as Crohn's disease, and rheumatoid arthritis (Benoist and Mathis, 2002; Puxeddu *et al.*, 2003; Nigrovic and Lee, 2005). Previous studies have shown that MCs undergo significant qualitative and quantitative changes during abnormal wound healing, which may correlate with a prolonged inflammatory state, an increase in fibroblast proliferation, and an aberrant collagen production (Artuc *et al.*, 1999; Huttunen *et al.*, 2000; Abe *et al.*, 2002). Such changes in MCs have also been observed in keloids and hypertrophic scars, thus implicating an important role of MCs and their mediators in keloid formation (Craig *et al.*, 1986; Smith *et al.*, 1987; Lee and Vijayasingam, 1995).

Green tea, derived from dried leaves of the plant *Camelia sinensis,* harbors several polyphenolic components known as catechins, including (−)-epigallocatechin-3-gallate (EGCG), (−)-epigallocatechin, (−)-epicatechin gallate, and (−)-epicatechin. A wide range of pharmacological effects of green tea and its major catechins has been reported with promising therapeutic potentials in cancer, cardiovascular, and neurological diseases (Shimizu and Weinstein, 2005; Zaveri, 2006). In addition, several recent studies have shown that polyphenols from green tea significantly improved the quality of wound healing and scar formation in a rat model (Kapoor *et al.*, 2004), remarkably suppressed both collagen production and collagenase activity in hepatic stellate cells (Nakamuta *et al.*, 2005), and dramatically attenuated experimental cholestasis-induced liver fibrosis in rats (Zhong *et al.*, 2003). In this study, we explored whether GTE and EGCG had any effect on type I collagen synthesis in keloid fibroblasts co-cultured with human MCs. We reported for the first time that both GTE and EGCG significantly suppressed MC-stimulated type I collagen expression in keloid fibroblasts. Our results also indicated that the inhibitory effects of GTE and EGCG on type I collagen expression in keloid fibroblasts appeared to be mediated via the phosphatidylinositol-3-kinase (PI-3K)/Akt/mTOR signaling pathways. These unique findings provided further understanding of the molecular mechanisms underlying the antifibrogenic effects of GTE and EGCG, and helped to further delineate the targets of therapeutic intervention and prevention of keloids and other fibrotic diseases.

RESULTS

MCs stimulate type I collagen expression in keloid fibroblasts *in vitro*

To investigate the effects of MCs on type I collagen expression in keloid fibroblasts, a fixed density of normal skin or keloid fibroblasts was co-cultured with different numbers of HMC-1 cells (a human leukemic mast cell line) under the condition where direct cell–cell contact is allowed. Our results showed that co-culture with HMC-1 cells led to a substantial increase in type I collagen synthesis in both normal and keloid fibroblasts, and such increase was dependent on the number of HMC-1 cells (Figure 1a and b). Similar results were obtained by immunofluorescence studies (Figure 1c).

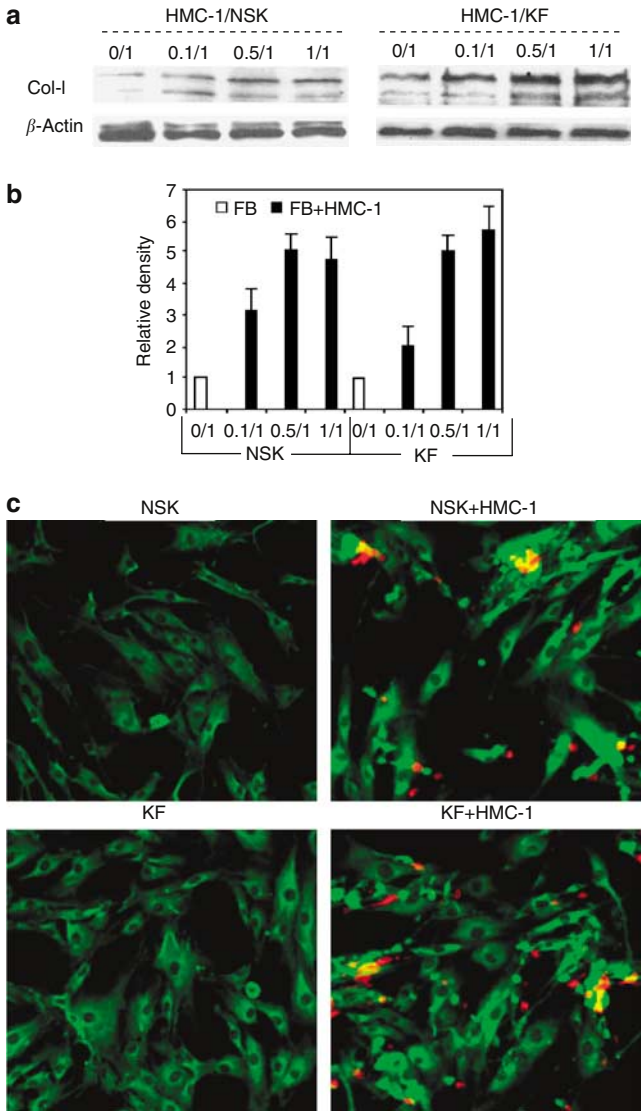

Figure 1. HMC-1 cells stimulated type I collagen expression in fibroblasts derived from keloids and their corresponding peripheral normal skins. (**a**) Keloid fibroblasts (KF) or normal skin fibroblasts (NSK) (1×10^5/well) were directly co-cultured with increasing number of HMC-1 cells in a six-well plate under normal culturing condition for 24 hours. Equal amount of cell lysates (100 μg) was subjected to Western blot analysis with a specific antibody against type I collagen (Col-I). (**b**) Densitometric analysis of the results of (**a**). The relative density ratio of the two bands of type I collagen to β-actin in fibroblasts (FB) cultured alone was arbitrarily set as 1.0. (**c**) Dual-color immunofluorescent staining of type I collagen (Green) in keloid fibroblasts and c-kit on MCs (Red). Co-cultured cells (cell density ratio of 1:1) were fixed in cold methanol:acetone (1/1) and stained with mouse monoclonal antitype I collagen antibody and rabbit polyclonal anti-c-kit antibody followed by Alexa Fluor®568 conjugated goat anti-rabbit IgG and Alexa Fluor®488 conjugated goat anti-mouse IgG. Representative results from three independent experiments are shown. Bar: 200 μM.

MCs stimulate type I collagen expression in keloid fibroblasts through activation PI-3K/Akt and p38 MAPK signaling pathways

Most recently, we have demonstrated that co-culture of keloid fibroblasts and HMC-1 cells under the condition of direct cell–cell contact led to the activation of both ERK1/2 and PI-3K/Akt signaling pathways (Zhang *et al.*, 2006).

Consistently, herein we demonstrated a time-dependent increase in both phosphorylated ERK1/2 and Akt levels in the co-cultured keloid fibroblasts and HMC-1 cells under normal conditions, with maximal activity at 1–2 hours (Figure 2). Likewise, we also observed a time-dependent increase in the phosphorylated levels of p38 MAPK, eukaryotic initiation factors (eIFs) binding protein (p-4E-BP)-1 and p70S6K1 in the co-culture (Figure 2), implying the involvement of several important signaling pathways.

Next, we ask whether these activated signaling pathways are involved in MC-induced upregulation of type I collagen expression. Keloid fibroblasts at about 80% confluence were pretreated with different concentrations of various protein kinase inhibitors for 1 hour followed by co-culture with the same cell density of HMC-1 cells for 24 hours under normal culturing conditions. Cell viability of both fibroblasts and HMC-1 cells was more than 95% at all concentrations tested as determined by trypan blue exclusion (data not shown). Our results showed that pretreatment of keloid fibroblasts with LY294002, a specific inhibitor of PI-3K, dramatically decreased HMC-1-stimulated type collagen expression in a dose-dependent manner ($P<0.05$) (Figure 3a and b). Similar findings were obtained with wortmannin, an alternative and structurally different inhibitor of PI-3K ($P<0.05$) (Figure 3c and d). Meanwhile, pretreatment of keloid fibroblasts with different concentrations of rapamycin, a specific inhibitor of mammalian target of rapamycin (mTOR), or SB203580, a specific inhibitor of p38 MAPK, also led to a dose-dependent inhibition of type-I collagen expression stimulated by HMC-1 cells ($P<0.05$) (Figure 3c–f). However, blocking ERK1/2 signaling pathway by pretreatment of keloid fibroblasts with PD98059 or U0126 had no obvious inhibitory effects on HMC-1-stimulated type I collagen expression ($P>0.05$) (Figure 3a, e, and f). To rule out the possibility that the suppression of MCs stimulated type I collagen synthesis was secondary to drug toxicity, keloid fibroblasts were exposed to LY294002, rapamycin, or SB203580 at maximal concentrations for 24 hours in the absence of MCs. The results indicated that mere exposure to these inhibitors had no obvious effects on type I collagen levels in keloid fibroblasts in the absence of HMC-1 cells (Figure S1). Taken together, these observations indicated that both PI-3K/Akt/mTOR and p38 MAPK signaling pathways are involved in MC-stimulated upregulation of type I collagen expression in keloid fibroblasts.

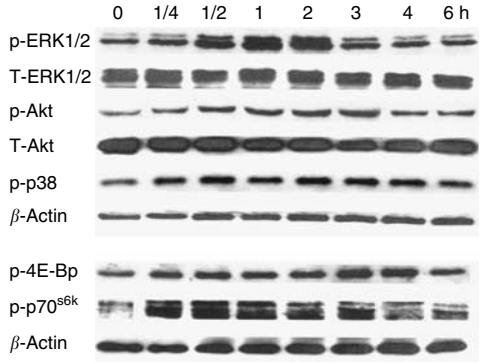

Figure 2. Activation of multiple signaling pathways in the co-cultured keloid fibroblasts and HMC-1 cells. Keloid fibroblasts were directly co-cultured with the same density of HMC-1 cells for different time intervals, and equal amounts of cell lysates were subjected to Western blot analysis with various specific antibodies. Representative results from three independent experiments are shown.

GTE and EGCG suppressed HMC-1-stimulated type I collagen expression in keloid fibroblasts by interfering with PI-3K/Ak signaling pathway

Previous studies have shown that green tea and its major catechins not only have inhibitory effects on MC activation (Li *et al.*, 2005), but also possess antifibrogenic activity in some animal models (Nakamuta *et al.*, 2005). To explore whether green tea extract (GTE) and (−)-epigallocatechin-3-gallate (EGCG) had any effects on MC-stimulated type I collagen expression, keloid fibroblasts were pretreated with different concentrations of GTE or EGCG for 1 hour followed by co-culture with the same cell density of HMC-1 cells for additional 24 hours under normal culturing conditions. Our results showed that pretreatment with GTE or EGCG led to a dose-dependent reduction in HMC-1-stimulated type I collagen protein production in keloid fibroblasts ($P<0.05$) (Figure 4a and b). Immunofluorescence studies also illustrated that treatment with GTE significantly attenuated type I collagen signals in keloid fibroblasts stimulated by HMC-1 cells (Figure 4c, the right panel *vs* the middle panel). To rule

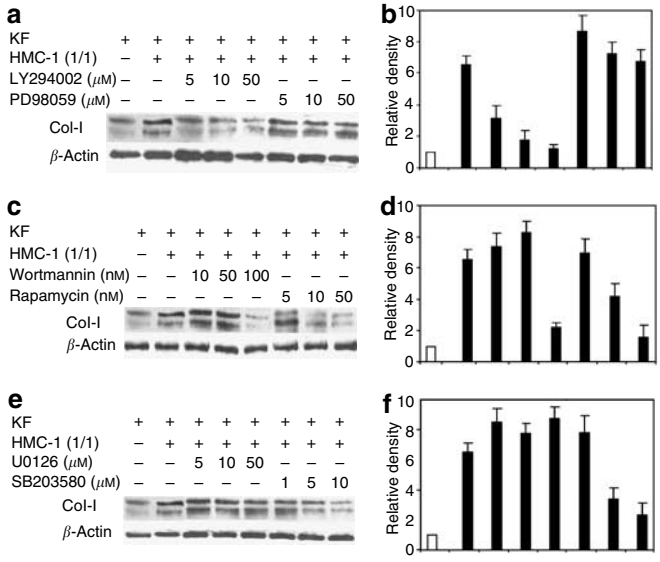

Figure 3. Involvement of PI-3k/Akt/mTOR and p38 MAPK pathways in MC-stimulated type I collagen production in keloid fibroblasts. (**a**, **c**, and **e**) Keloid fibroblasts (KF) were pretreated with different concentrations of various inhibitors followed by direct co-culture with the same density of HMC-1 cells under normal culturing condition for 24 hours. Equal amounts of cell lysates (100 μg) were subjected to Western blot analysis with specific antibody against type I collagen (Col-I). (**b**, **d**, and **f**) Densitometric analysis of results from (**a**, **c**, and **e**), respectively. The relative density ratio of the two bands of type I collagen (Col-I) to β-actin in keloid fibroblast (KF) cultured alone was arbitrarily set as 1.0. The results represent three independent experiments and expressed as the mean ± SD.

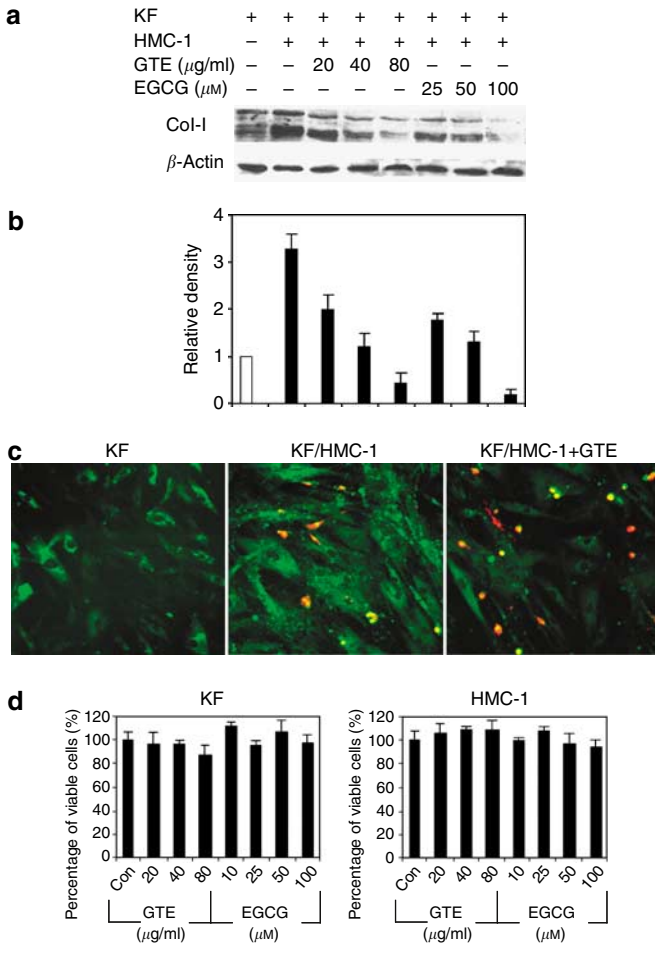

Figure 4. GTE and EGCG inhibited MC-stimulated type I collagen production in keloid fibroblasts. (**a**) Keloid fibroblasts (KF) were pretreated with different concentrations of GTE or EGCG for 1 hour followed by direct co-culture with the same number of HMC-1 cells under normal culturing condition for 24 hours. Equal amounts of cell lysates (100 μg) were subjected to Western blot analysis with specific antibody against type I collagen. (**b**) Densitometric analysis of results from (**a**). The relative density ratio of the two bands of type I collagen (Col-I) to β-actin in KF cultured alone was arbitrarily set as 1.0. (**c**) Dual-color immunofluorescent staining of type I collagen (Green) in KF and c-kit on HMC-1 cells (Red). Keloid fibroblasts (KF) were pretreated with 80 μg/ml GTE for 1 hour followed by co-cultured with HMC-1 (cell density ratio of 1:1) for 16 hours. The co-cultured cells were fixed in cold methanol:acetone (1:1) and stained with mouse monoclonal antibody against type I collagen and rabbit polyclonal antibody against c-kit, followed by Alexa Fluor®568 conjugated goat anti-rabbit IgG and Alexa Fluor®488 conjugated goat anti-mouse IgG. (**d**) KF or HMC-1 ells were treated with various concentrations of GTE or EGCG for 24 hours under normal culturing conditions and cell viability was assayed using MTT method. The percentage of viable cells represented the mean ± SD from three replicate experiments. The results represent three independent experiments and expressed as the mean ± SD. Bar: 200 μM.

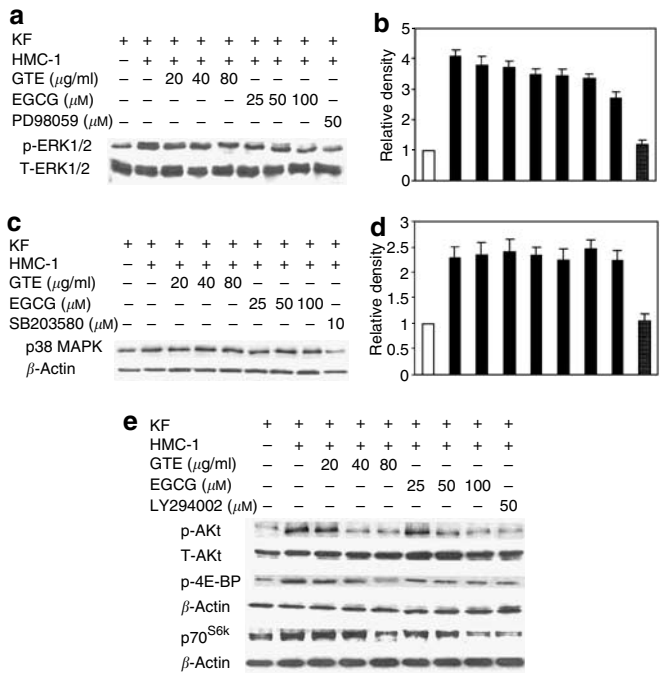

Figure 5. Effects of GTE and EGCG on the activation of signaling pathways in the co-cultured keloid fibroblasts and HMC-1 cells. Keloid fibroblasts (KF) were pretreated with different concentrations of GTE or EGCG, or various kinase inhibitors for 1 hour, followed by direct co-culture with the same cell number of HMC-1 cells under normal culturing condition for 1 hour. Equal amounts of cell lysates were subjected to Western blot analysis with specific antibody against the phosphorylated form of ERK1/2 (**a**), p38 MAPK (**c**), Akt, p70S6K, and 4E-BP1 (**e**), respectively. (**b** and **d**) Densitometric analysis of results from (**a**) and (**c**). The relative density ratio of the p-ERK1/2 or p-Akt to total (T-) ERK1/2 or total (T-) Akt in KF cultured alone was arbitrarily set as 1.0. Data presented are representative of results from three independent experiments.

out the possibility that the inhibitory effect of GTE and EGCG on type I collagen expression was due to cellular toxicity, MTT assay was performed to determine the cell viability following treatment with different concentrations of GTE and EGCG under normal conditions for 24 hours. No obvious changes in cell viability were observed in both keloid fibroblasts and HMC-1 cells (Figure 4d).

Next, we explored the signaling mechanisms underlying the inhibitory effects of GTE and EGCG on HMC-1-stimulated type I collagen expression in keloid fibroblasts. As expected, pretreatment of keloid fibroblasts with PD98059 (50 μM) and SB203580 (10 μM) significantly inhibited the activation of ERK1/2 and p38 MAPK, respectively ($P<0.05$; Figure 5a and b). However, exposure of keloid fibroblasts to GTE or EGCG only led to a moderate decrease in the phosphorylated ERK1/2 levels, but had no obvious inhibitory effects on the activation of p38 MAPK ($P>0.05$, Figure 5a–d). On the other hand, similar to the inhibitory effects of LY294002, treatment with GTE or EGCG resulted in a dose-dependent reduction in the increased levels of phosphorylated Akt, 4E-BP, and p70S6K in the co-cultured keloid fibroblasts and HMC-1 cells (Figure 5e), which paralleled their inhibitory effects on HMC-1-stimulated upregulation of type I collagen expression (Figure 4). To rule out drug toxicity, keloid fibroblasts were exposed to increased concentrations of LY294002, PD98059, and SB203580 for 1 hour. Our results showed no obvious changes in the levels of these phosphorylated signaling components (Figure S2). Collectively, these data suggest that GTE and EGCG inhibited MC-stimulated type I collagen

expression in keloid fibroblasts mainly by interfering with the PI-3K/Akt signaling pathways.

DISCUSSION

MCs have been attributed to several patho-physiological conditions via their ability to respond to a composite range of stimuli, and release of a wide array of biologically active mediators (Mekori and Metcalfe, 2000; Ribatti *et al.*, 2004; Nigrovic and Lee, 2005). Several studies have shown that co-culture with MCs promote type I collagen synthesis in fibroblasts (Abe *et al.*, 2000; Garbuzenko *et al.*, 2002). Consistently, our findings demonstrated that co-culture with MCs substantially stimulated type I collagen synthesis in keloid fibroblasts (Figure 1).

Most recently, our group has reported the activation of ERK1/2 and PI-3K/Akt signaling pathways in the co-cultured keloid fibroblasts and HMC-1 cells (Zhang *et al.*, 2006). The current study has extended our previous observation and found that co-culture with HMC-1 cells activated not only ERK1/2 and PI-3K/Akt signaling pathways but also p38 MAPK pathway (Figure 2). This finding is consistent with previous report that co-culture of MC and lung fibroblast with direct cell–cell interaction led to the activation of p38 MAPKs (Fitzgerald *et al.*, 2004). In addition, our results also indicated that co-culture with HMC-1 cells upregulated both phosphorylated 4E-BP and p70S6K (Figure 2), two important regulatory components of protein translational machinery that are downstream targets of PI-3K/Akt/mTOR signaling pathways. The involvement of ERK1/2, PI-3K/Akt/mTOR, and p38 MAPK signaling pathways have been described in the upregulation of type I collagen expression in dermal fibroblasts (Lim *et al.*, 2003; Asano *et al.*, 2004; Ihn *et al.*, 2005). In this study, we have demonstrated for the first time that treatment with specific inhibitors of PI-3K, mTOR and p38 MAPK significantly inhibited MC-stimulated type I collagen production in keloid fibroblasts, while treatment with specific inhibitors of ERK1/2 had no obvious inhibitory effects (Figure 3). These findings suggest that MCs stimulate type I collagen expression in keloid fibroblasts via activating both PI-3K/Akt/mTOR and p38 MAPK signaling pathways.

GTE and its major polyphenolic components have long been reported to possess various pharmacological activities with a broad variety of health benefits (Yang *et al.*, 2002). Recent studies have shown that some components of green tea can inhibit MC activation (Yamamoto *et al.*, 1998; Li *et al.*, 2005), and affect ECM metabolism/remodeling in several experimental models (Zhong *et al.*, 2003; Kapoor *et al.*, 2004; Nakamuta *et al.*, 2005). In this study, we reported that GTE and EGCG had strong inhibitory effects on MC-stimulated type I collagen production in keloid fibroblasts (Figure 4). Moreover, our results indicated that treatment with GTE or EGCG suppressed the phosphorylated levels of Akt, 4E-BP-1, p70S6K1, and ERK1/2, but had no obvious inhibitory effects on p38 MAPK in the co-cultured keloid fibroblasts and HMC-1 cells (Figure 5). These results suggest that GTE or EGCG harbor potential antifibrogenic activity by inhibiting MC-stimulated type I collagen production mainly via the PI-3K/Akt/mTOR signaling pathways. However, further studies are required to explore whether the inhibitory effects of GTE and EGCG on MC-stimulated type I collagen expression were due to their direct effects either on the activation of MCs or keloid fibroblasts, or both.

In summary, the present study has demonstrated that MCs can stimulate type I collagen production via activating multiple signaling pathways in keloid fibroblasts. Keloids, a chronic fibroproliferative disease, are characterized by excessive collagen deposition, and, notoriously prone to recurrence. Despite several treatment approaches, no single modality has proven successful (Kelly, 2004; Burd and Huang, 2005). Herein, we have demonstrated for the first time that both GTE and EGCG significantly inhibited MC-stimulated type I collagen expression by suppressing activation of the PI-3k/Akt/mTOR signaling pathways in keloid fibroblasts. Our unique findings have provided further understanding of the molecular mechanisms of keloid pathogenesis and identify a therapeutic potential of green tea for the intervention and prevention of keloids and other fibrotic diseases.

MATERIALS AND METHODS

Reagents

GTE (Pharmanex Inc., Provo, UT) and EGCG (Sigma, St Louis, MO) were dissolved in distilled water and stored at −80°C as stock solutions. The purity and components of GTE were previously described (Lu *et al.*, 2005). PD98059, LY294002, U0126, wortmannin, SB203580, and rapamycin were purchased from Calbiochem (La Jolla, CA). All inhibitors were dissolved in DMSO. Cell viability using trypan blue exclusion was determined for both keloid fibroblasts and HMC-1 cells at the highest concentrations of inhibitors used. Mouse monoclonal antibodies against human type I collagen and β-actin were obtained from Sigma. Mouse monoclonal antibodies against human total or phosphorylated ERK1/2 (Thr^{202}/Tyr^{204}) or Akt (Ser^{473}), p38 MAPK were from New England Biolabs Inc. (Beverly, MA). Antibodies for phosphorylated Mr 70,000 ribosomal protein S6 kinase 1 (p70S6K) (Thr^{421}/Ser^{424}) and eukaryotic initiation factor 4E (eIF)-binding protein 1 (4E-BP1) (Ser^{65}/Thr^{70}) were from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal antibodies against human c-kit and type I collagen were from Oncogene™ Research Products (San Diego, CA) and Rockland Immunochemicals (Gilbertsville, PA), respectively. Horseradish peroxidase conjugated secondary antibodies were from PIERCE (Rockford, IL). Alexa Fluor®568 conjugated rabbit anti-goat IgG and Alexa Fluor®488 conjugated goat anti-mouse IgG were from Molecular Probes Inc. (Eugene, OR). All reagents used were analytical grade.

Cell origin and cell culture

All studies have been approved by the Institutional Review Board of the University of Southern California and were conducted with strict adherence to the Declaration of Helsinki Principles. Primary cultures of human dermal fibroblasts were isolated by enzymatic digestion of keloid tissues obtained from patients at King Drew Medical Center (Zhang *et al.*, 2003). All keloidal tissues were from untreated, primary lesions. Fibroblasts were maintained in DMEM (Gibco, Rockville, MD) supplemented with 10% fetal bovine serum. Cells from passage 2 through 8 were used for experiments and routinely

monitored for cell proliferation, morphology, and phenotype. HMC-1, a human MC leukemia cell line (a generous gift of Dr JH Butterfield, Mayo Clinic, Rochester, MN), was cultured in Iscove's medium (Gibco) supplemented with 10% fetal bovine serum, antibiotics, 2 mmol/l L-glutamine, 1.2 mmol/l α-thioglycerol (Sigma, St Louis, MO). All cultures were maintained at 37°C, 5% CO_2, and 20% O_2.

Co-culture of HMC-1 and keloid fibroblasts

Keloid fibroblasts (1×10^5/well) were seeded on the bottom of a six-well plate and maintained at normal culturing conditions (37°C, 5% CO_2, and 20% O_2) for at least 24 hours. Different densities of HMC-1 cells were seeded on top of the monolayer of fibroblasts and co-cultured for different time intervals based on experimental purposes. Fibroblasts at about 80% confluence were pretreated with different concentrations of GTE, EGCG, or various kinase inhibitors for 1 hour followed by co-culturing with the same cell density of HMC-1 cells (cell density ratio of 1:1) under normal culturing conditions for indicated time periods. Cell lysates were prepared for Western blot analysis.

Western blot analyses

To determine type I collagen, and phosphorylated ERK1/2 and Akt levels, cells were solubilized in lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 200 μM Na_3VO_4, 50 mM NaF, 0.5% Triton X-100) supplemented with 10 mM dithiothreitol, 200 μM phenylmethylsulfonyl fluoride and protease inhibitor cocktails (Sigma). Equal protein amounts of cell lysates were electrophoresed on 7.5–10% SDS-polyacrylamide gels and electroblotted onto nitrocellulose membranes (Hybond ECL, Amersham Pharmacia). After blocking with TBS/5% skim milk, the membranes were incubated with specific primary antibodies, followed by a horseradish peroxidase conjugated secondary antibodies (1:2,000) (Pierce, Rockford, IL), and visualized using an enhanced chemiluminescent (ECL) detection.

Immunofluorescence studies

Keloid fibroblasts were seeded on four-well Lab-Tek®II Chamber Slide™ System (Nalge Nunc Int., Naperville, IL) (1×10^4) and cultured for 24 hours under normal condition, followed by co-culture with the same number of HMC-1 cells under the condition of direct cell–cell contact. After incubation under normoxia for 24 hours, the medium was removed, and cells were fixed with cold methanol:acetone (1:1) for 15 minutes. The fixed cells were washed, and incubated at 4°C overnight with a mouse monoclonal anti-human type I collagen antibody (1:100) and a rabbit polyclonal anti-human c-kit antibody (1:200). Cells were washed, incubated at room temperature for 1 hour with Alexa Fluor®568 conjugated goat anti-rabbit IgG (1:2,000; 0.5 μg/ml), and Alexa Fluor®488 conjugated goat anti-mouse IgG (1:2,000; 0.5 μg/ml). Slides were then viewed and photographed under a fluorescence microscope. Type I collagen expression appeared green and c-kit-positive HMC-1 cells were stained red. Cells incubated with fluoroscein-conjugated secondary antibodies in the absence of primary antibodies were used as negative control.

Statistical analysis

Data are presented as the mean ± SD of duplicate experiments carried out for at least three separate runs. One representative data set from these three independent experiments is presented where appropriate. Error bars represent SD. A paired Student's test was employed for statistical analysis, with significant differences determined as $P < 0.05$.

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

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SUPPLEMENTARY MATERIAL

Figure S1. Effects of protein kinase inhibitors on type I collagen expression in keloid fibroblasts (KF) in the absence of HMC-1 cells.

Figure S2. Effects of protein kinase inhibitors on the activation of different protein kinases in keloid fibroblasts (KF) in the absence of HMC-1 cells.

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