ARTICLE
Journal of Cellular Biochemistry 114:1163–1173 (2013)

Journal of Cellular Biochemistry

Polyphenols Suppress Hydrogen Peroxide-Induced Oxidative Stress in Human Bone-Marrow Derived Mesenchymal Stem Cells

Haruyo Yagi, Jian Tan, and Rocky S. Tuan*

Center for Cellular and Molecular Engineering, Department of Orthopaedic Surgery, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania 15219

ABSTRACT

Human mesenchymal stem cells (hMSCs) are considered a highly promising candidate cell type for cell-based tissue engineering and regeneration because of their self-renewal and multi-lineage differentiation characteristics. Increased levels of reactive oxygen/nitrogen species (ROS/RNS) are associated with tissue injury and inflammation, impact a number of cellular processes, including cell adhesion, migration, and proliferation, and have been linked to cellular senescence in MSCs, potentially compromising their activities. Naturally occurring polyphenolic compounds (polyphenols), epigallocatechin-3-gallate (EGCG), and curcumin, block ROS/RNS and are potent inflammation-modulating agents. However, their potential protective effects against oxidative stress in hMSCs have not been examined. In this study, we carried out a systematic analysis of the effects of polyphenols on hMSCs in their response to oxidative stress in the form of treatment with H_2O_2 and *S*-nitroso-*N*-acetylpenicillamine (SNAP), respectively. Parameters measured included colony forming activity, apoptosis, and the levels of antioxidant enzymes and free reactive species. We found that polyphenols reversed H_2O_2-induced loss of colony forming activity in hMSCs. In a dose-dependent manner, polyphenols inhibited increased levels of ROS and NO, produced by H_2O_2 or SNAP, respectively, in MSCs. Notably, polyphenols rapidly and almost completely blocked H_2O_2-induced ROS in the absence of significant direct effect on H_2O_2 itself. Polyphenols also protected the antioxidant enzymes and reduced apoptotic cell death caused by H_2O_2 exposure. Taken together, these findings demonstrate that EGCG and curcumin are capable of suppressing inducible oxidative stress in hMSCs, and suggest a possible new approach to maintain MSC viability and potency for clinical application. J. Cell. Biochem. 114: 1163–1173, 2013.

KEY WORDS: MESENCHYMAL STEM CELL; POLYPHENOL; REACTIVE OXYGEN SPECIES

Mesenchymal stem cells (MSCs) are adult stem cells, first identified in bone marrow stroma, with capacity for self-renewal and multi-lineage differentiation such as adipogenesis, chondrogenesis, and osteogenesis [Pittenger et al., 1999]. MSCs with similar characteristics are also found in virtually all tissues of the body. From a therapeutic perspective, because of their easy preparation and immunologic privilege, MSCs have emerged as a highly promising therapeutic agent for tissue regeneration and repair, as evidenced by the large number of active clinical trials involving MSCs (http://clinicaltrials.gov/).

Reactive oxygen species (ROS), including hydroxyl radical, superoxide anion radical, hydrogen peroxide (H_2O_2), and peroxide, have been implicated in a number of diseases such as cancer [Van de Bittner et al., 2010], heart failure [Melov et al., 1998], osteoarthritis [Tiku et al., 2000], and neurodegenerative disorders [Wu et al., 2003]. Although excessive levels of ROS are deleterious, a basal level

Abbreviation used: EGCG, epigallocatechin gallate; NAC, *N*-acetyl-L-cysteine; DMSO, dimethyl sulfoxide; DPPH, 2, 2-diphenyl-1-picrylhydrazyl; H_2O_2, hydrogen peroxide; DMEM, Dulbecco's modified eagle medium; FBS, fetal bovine serum; PBS, phosphate buffered saline; H_2DCFDA, 2′,7′-dichlorodihydrofluorescein diacetate; DAF-FM, 4-amino-5-methylamino-2′,7′-difluorofluorescein; L-NAME, N_ω-Nitro-L-arginine methyl ester hydrochloride; CAT, catalase; SNAP, *S*-nitroso-*N*-acetylpenicillamine; GPX, glutathione peroxidase; ROS, reactive oxygen species; NO, nitric oxide.

The authors have no conflict of interest to declare.

Grant sponsor: Commonwealth of Pennsylvania Department of Health.

*Correspondence to: Rocky S. Tuan, PhD, Center for Cellular and Molecular Engineering, Department of Orthopaedic Surgery, University of Pittsburgh School of Medicine, 450 Technology Drive, Bridgeside Point II, Room 221, Pittsburgh, PA 15219. E-mail: rst13@pitt.edu

Manuscript Received: 2 November 2012; Manuscript Accepted: 7 November 2012

Accepted manuscript online in Wiley Online Library (wileyonlinelibrary.com): 28 November 2012

DOI 10.1002/jcb.24459 • © 2012 Wiley Periodicals, Inc.

of ROS is required for multiple cellular functions. Cells can normally defend themselves against ROS damage through the use of specific ROS reducing mechanisms that can be enzymatic (involving dismutases, catalases, and peroxidases) or non-enzymatic (involving vitamins A, C and E, urate, and bilirubin). For example, superoxide dismutase (SOD) catalyzes the dismutation of O_2^- (superoxide anion radical) into H_2O_2, which in turn is converted to water and oxygen by glutathione peroxidase (GPX) and catalase. In this manner, the levels of different ROS are buffered to regulate intracellular redox balance while avoiding excessive oxidation of cellular components [Giorgio et al., 2007]. Oxidative stress is known to play a role in stem cell self-renewal and differentiation. Several groups have shown the relationship between oxidative stress level and the proliferative capacity of stem cells and increased number of differentiated cells by the presence of antioxidants such as tocopherol [Geissler et al., 1994], and epicathechin gallate [Chen et al., 2005]. However, the response of stem cells to oxidative stress is not well understood.

Polyphenolic compounds, or polyphenols, are widely distributed in plants from the roots to the seeds. These compounds are widespread in nature and range from simple structures with one aromatic ring to highly complex polymeric substances such as tannins and lignins. Polyphenols are classified as flavonoids and non-flavonoids, according to their chemical composition [Quideau et al., 2011]. Polyphenols have been shown to exhibit a variety of biological properties, including anticarcinogenic [Lu et al., 2002], anti-inflammatory [Ahmed et al., 2004; Ghanim et al., 2011], and estrogenic activities [Gehm et al., 1997], as well as cardiovascular protection [Morimoto et al., 2008], free radical scavenging [Katiyar et al., 2001; Kao et al., 2010], DNA damage protection [Anderson et al., 2001], inhibition/induction of apoptosis [Townsend et al., 2004; Hastak et al., 2005], and inhibition of platelet aggregation [Freedman et al., 2001]. Also, polyphenols are generally considered pharmacologically safe in humans [Cheng et al., 2001; Chow et al., 2001; Boocock et al., 2007]. Therefore, polyphenols may act as powerful modulators of MSCs via their antioxidant activities. Such applications would require characterization of the effect of polyphenols on oxidative stress in hMSCs, which is poorly understood.

In this report, we have selected epigallocatechin gallate (EGCG, from green tea) and curcumin (from turmeric), in view of their popularity as daily foods, to study their effects on oxidative stress in hMSCs. Our results showed that EGCG and curcumin reversed H_2O_2-induced loss of colony forming number of MSCs in bone marrow environment. Polyohenols also suppressed the production of both intracellular ROS and NO in hMSCs, and protected the cells from antioxidant enzymes and apoptosis resulting from the action of H_2O_2-induced oxidative stress.

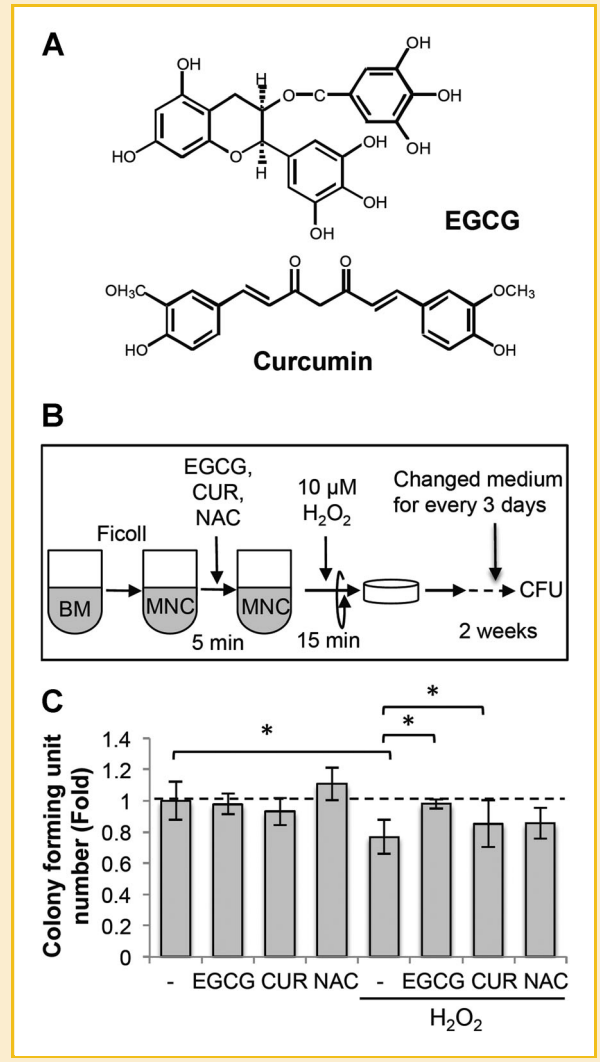

Fig. 1. EGCG and curcumin inhibited H_2O_2-induced failure of CFU ability in BM-MNCs. A: Chemical structures of EGCG and curcumin (CUR). B: Experimental protocol for CFU assay. C: BM-MNCs (1×10^6 cell/ml) were treated with 5 μM polyphenols or 100 μM NAC for 5 min and then exposed to 10 μM H_2O_2 for 15 min, and then analyzed for colony forming activity as described in Materials and Methods Section. Relative fold value was calculated polyphenol untreated groups designated as 1. Results are expressed as means ± SD (N = 4). Significant differences relative to untreated controls: $^*P < 0.05$. MSCs from two patients were used.

MATERIALS AND METHODS

CHEMICALS

EGCG and curcumin (Fig. 1A) were purchased from LKT Laboratories, Inc (St. Paul, MN). *N*-acetyl-L-cysteine (NAC) as a ROS inhibitor, dimethyl sulfoxide (DMSO) and 2,2-diphenyl-1-picrylhydrazyl (DPPH), and hydrogen peroxide (H_2O_2) were obtained from Sigma–aldrich (St. Louis, MO). Dulbecco's modified eagle medium (DMEM), α-minimum essential medium (MEM), antibitotic–antimycotic (anti–anti), ethylenediaminetetraacetic acid (EDTA), penicillin–streptomycin (pen–strep), fetal bovine serum (FBS), phosphate buffered saline (PBS), 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA), 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate (DAF-FM diacetate), Amplex Red Catalase activity assay kit, N_ω-Nitro-L-arginine methyl ester hydrochloride (L-NAME) as a NO inhibitor, and *S*-nitroso-*N*-acetylpenicillamine (SNAP) were purchased from Invitrogen (Carlsbad, CA). Fibroblast growth factor-2 (FGF-2) was obtained from RayBiotech, Inc. (Norcross, GA). HT Titer TACS was obtained from R&D System (Minneapolis, MN).

Glutathione peroxidase assay kit was purchased from BioVision (Milpitas, CA). Bradford protein assay reagent was purchased from Bio-Rad (Hercures, CA). PeroXOquant™ Quantitative Peroxide Assay kit was obtained from Pierce (Rockford, IL). Ficoll-Paque was obtained from GE Healthcare Biosciences (Pittsburgh, PA).

Stock solutions of 100 mM EGCG and 100 mM curcumin were prepared in DMSO and stored at −20°C until use. One molar solution of NAC and L-NAME were prepared in DMSO and stored at −20°C until use. H_2DCFDA was prepared as a stock solution in DMSO at 10 mM, and was diluted in PBS to give H_2DCFDA at 20 μM prior to use. DAF-FM diacetate was prepared as a stock solution in DMSO at 5 mM, and was diluted in PBS to 10 μM prior to use. DPPH was dissolved in methanol at 250 mM prior to use.

CELL CULTURE

Human bone marrow was obtained from femoral heads of patients undergoing total hip arthroplasty with Institutional Review Board (IRB) approval (University of Washington School of Medicine) as tissue culture plastic adherent cell populations [Lin et al., 2012]. Human hips from five different patients, ages of 51 (Female; F), 57 (F), 60 (Male; M), 61 (F), and 63 (F), were obtained. Trabecular bone was cored out using curette or rongeur and flushed with rinsing medium (α-MEM, 1% antibiotic/antimycotic) using 18-gauge hypodermic needles. After mincing with scissors, the bone chips were flushed, and the flushed medium was passed through 40 μm mesh filters to remove debris, and cells pelleted by centrifugation for 5 min at 300*g*. Cell pellets were washed twice with rinsing medium and resuspended in MSC growth medium (GM, α-MEM) containing 10% fetal bovine serum, 1% Anti–anti (Invitrogen) and 1 ng/ml FGF-2 and plated into two 150 cm^2 tissue culture flasks. On Day 4, cells were washed with PBS and fresh GM was added. GM was changed every 3–4 days. Once 70–80% confluence was reached, cells were removed with 0.25% trypsin containing 1 mM EDTA and passaged by re-plating. Populations of MSCs isolated from each patient were routinely validated as capable of differentiating into bone, fat, and cartilage (data not shown). All experiments were performed with passage three to five MSCs from at least two patients indicated above.

Primary human bone marrow mononuclear cells (BM-MNCs) were harvested from the hips of consenting patients (following IRB approval, University of Washington School of Medicine; age 53 and age 57, male) using Ficoll-Paque density gradient separation according to the manufacturer's protocol. BM-MNCs were washed twice with PBS and the total number of viable cells counted using trypan blue dye exclusion, and then resuspended in basal medium (DMEM) containing 10% fetal bovine serum, 1% Penicillin–streptomycin (Invitrogen, Carlsbad, CA). Immediately after resuspension, cells were co-treated with polyphenols and H_2O_2 for CFU assay.

DPPH RADICAL QUENCHING ASSAY IN VITRO

2,2-Diphenyl-1-Picrylhydrazyl (DPPH) radical quenching activity of polyphenols was determined according to the method of Yoshida et al. [1989]. Aliquots (10 μl) of different concentrations of polyphenols were incubated with 0.25 mM DPPH in 90 μl methanol for 10 min at room temperature. DPPH products were measured by spectrophotometrically based on A_{520} using a Bio-Tek microplate reader (Winooski, VT).

COLONY FORMING UNIT (CFU) ASSAY

CFU potential of BM-MNCs was determined by plating aliquots of cells (1×10^6 cells/10 cm^2 dish) in basal medium (10% FBS in DMEM) at 37°C with 5% humidified CO_2 and grown under previously described conditions [Baksh and Tuan, 2007]. Briefly, BM-MNCs were co-treated with EGCG or curcumin for 5 min followed by exposure to H_2O_2 for 15 min, and then incubated for 2 weeks without exposure to polyphenol and H_2O_2. Basal medium was changed every 3 days. After 2 weeks, CFU cultures were terminated and stained with 0.5% crystal violet solution (in methanol), and number of colonies counted.

DETECTION OF REACTIVE OXYGEN SPECIES (ROS) PRODUCTION IN MSCs

Production of cellular ROS was evaluated by analyzing changes in fluorescence intensity resulting from the oxidation of the intracellular fluoroprobe H_2DCFDA. H_2DCFDA enters cells passively and is de-acetylated by esterase to the non-fluorescent dichloro-dihydrofluorescein (DCFH). DCFH reacts with ROS to form dichlorofluorescein (DCF), a fluorescent product. 1.0×10^4 MSC cells were seeded on 96-well plates. Cells were co-incubated with different concentrations of polyphenols and 10 μM H_2O_2 for 30 min. H_2DCFDA was added 25 min before treatment with polyphenols and H_2O_2. NAC was used for positive control as a ROS inhibitor. The fluorescence was read using a Bio-Tek microplate reader (Ex: 485 nm; Em: 528 nm). For cell imaging, 1.0×10^5 MSCs were plated on 12-well plates, and then co-treated with 10 μM H_2O_2 in combination with EGCG, curcumin, or 1 mM NAC for 30 min. H_2DCFDA was added 25 min before adding H_2O_2 and polyphenols. Cell images under phase contrast and fluorescence optics were taken with a 40× objective on an OLYMPUS CKX41 microscope (Olympus, Tokyo, Japan) in combination with a LEICA DFC320 digital camera (LEICA Microsystems, Buffalo Grove, IL) and processed with Adobe Photoshop (Adobe System).

DETECTION OF NO PRODUCTION IN MSC

Production of cellular NO was evaluated by analyzing changes in fluorescence intensity resulting from the oxidation of the intracellular fluoroprobe DAF-FM-diacetate. DAF-FM-diacetate enters cells passively and is de-acetylated by esterase to the weakly fluorescent DAF-FM. DAF-FM reacts with NO to form benzotriazole derivative, a fluorescent product. 1.0×10^4 MSCs were seeded on 96-well plates and incubated with different concentrations of polyphenols and 500 μM SNAP for 30 min. DAF-FM were added 25 min before treatment with polyphenols and SNAP. L-NAME was used for positive control as a NO inhibitor. Fluorescence intensity was read at 485 nm (Ex) and 528 nm (Em). For cell imaging, 1.0×10^5 MSCs were plated on 12-well plates, and then co-treated with 10 μM H_2O_2 in combination with EGCG, curcumin or 1 mM NAME for 30 min. DAF-FM was added 25 min before adding SNAP and polyphenols. Cell images under phase contrast and fluorescence optics were taken with a 40× objective on an OLYMPUS CKX41 microscope (Olympus) in combination with a LEICA DFC320 digital

camera (LEICA Microsystems) and processed with Adobe Photoshop (Adobe System).

PEROXIDE ASSAY

Peroxide assay was performed using PeroXOquant™ Quantitative Peroxide Assay kit (Pierce) according to the manufacturer's protocol. Briefly, 10 μl of samples, containing different concentrations of polyphenols with or without 10 μM H_2O_2, were incubated with 100 μl of reaction mixture (25 mM ammonium ferrous (II) sulfate, 100 mM sorbitol, 125 μM xylenol orange) for 20 min, and A_{560} was measured.

DNA DAMAGE BASED APOPTOSIS ANALYSIS

1.0×10^4 MSCs were seeded on 96-well plates. Cells were co-incubated 10 μM H_2O_2 and polyphenols or ROS inhibitor (0.5–1 mM NAC) for 3 days. Apoptosis was quantified as the percent of cells with hypodiploid DNA, as assessed using a colorimetric quantitative assay, the HT titer TACS assay (R&D System).

CATALASE ACTIVITY

0.5×10^5 MSCs were seeded on 6-well plates, and then co-incubated with 10 μM H_2O_2 and polyphenols or ROS inhibitor (0.5–1 mM NAC) for 3 days. Cells were extracted with 0.5% Triton X-100 solution in PBS for 20 min at room temperature (r.t.) [Yokota and Fahimi, 1978], and the supernatant after centrifugation at 10,000*g* for 10 min at 4°C was used for catalase assay using the Amplex Red Catalase Assay Kit (Invitrogen) according to the manufacturer's protocol. In brief, reaction mixtures containing 50 μl of 100 μM Amplex red/0.4 U/ml HRP working solution, 25 μl of 40 μM H_2O_2, 0.2 units/ml horseradish peroxidase, and 25 μl of cell lysate were incubated at 37°C for 30 min. Catalase activities were determined by spectrophotometrically based on A_{570} and normalized with respect to protein concentration of each sample.

GPX ACTIVITY

0.5×10^5 MSCs were seeded on 6-well plates. Then, cells were co-incubated with 10 μM H_2O_2 and polyphenols or ROS inhibitor (0.5–1 mM NAC) for 3 days. The activity of GPX was measured with the glutathione peroxidase activity assay Kit (BioVision, Milpitas, CA) according to the manufacturer's protocol. GPX activity was determined by spectrophotometrically based on A_{340} and normalized with respect to protein concentration of each sample.

STATISTICAL ANALYSIS

Significant differences was assessed with two-tailed Student's *t*-test for two-group comparisons. $^{*}P < 0.05$ and $^{**}P < 0.1$.

RESULTS

EGCG AND CURCUMIN INHIBITED H_2O_2-INDUCED FAILURE OF COLONY FORMING ABILITY IN BM-MNCs

Oxidative stress has been previously shown to affect growth and differentiation potential of MSCs [Stolzing and Scutt, 2006]. In this study, we employed a suspension culture approach, simulating the bone marrow (BM) cellular environment [Baksh and Tuan, 2007], to study the effects of polyphenols on BM-MNCs immediately after isolation, prior to adherent culture on tissue culture plastic (Fig. 1B). The ROS that are generated by mitochondrial respiration, including H_2O_2, are potent inducers of oxidative damage. Therefore we used H_2O_2 as an ROS to examine the effects on BM-MNCs. We observed that cultures grown in the presence of 10 μM H_2O_2 for 15 min and then incubated for 2 weeks without exposure to H_2O_2, showed a 35% loss of total colony numbers (Fig. 1C). To test whether polyphenols are able to reverse the loss of colony numbers induced by H_2O_2, we treated BM-MNCs with EGCG or curcumin for 5 min followed by exposure to H_2O_2 for 15 min, and then incubation for 2 weeks without exposure to polyphenol and H_2O_2 (Fig. 1B). A significant reversal of H_2O_2-induced loss of colony numbers was observed when stressed cells were treated with EGCG and/or curcumin (Fig. 1C). These effects are comparable to treatment with *N*-acetyl-L-cysteine (NAC) at 500 μM. As control, incubation with EGCG and curcumin as well as NAC, did not affect the activity of unstressed cells.

EGCG AND CURCUMIN SUPPRESSED H_2O_2-INDUCED INTRACELLULAR ROS IN MSCs

The accumulation of ROS in cells leads to various forms of reversible and irreversible oxidative modifications of proteins (carbonylation or nitro-modifications), lipids (hydroperoxide lipid derivatives), and DNA (adducts and breaks) that eventually lead to loss of biological functions [Giorgio et al., 2007]. To investigate whether EGCG and curcumin can inhibit intracellular ROS, hMSCs were co-treated with EGCG or curcumin at varying concentrations, and then exposed to H_2O_2. A fluorescence protocol based on H_2DCFDA was used to measure intracellular ROS. Both EGCG and curcumin suppressed H_2O_2-induced intracellular ROS accumulation in a dose-dependent manner (Fig. 2A–C), with complete suppression upon co-treatment with polyphenols at 25 μM for 30 min. Again, in comparison, these effects were similar to those seen with 1 mM NAC treatment (Fig. 2A–C). Cell viability was not affected with these treatments.

LACK OF OR MINIMAL DIRECT INTERACTION BETWEEN POLYPHENOLS AND HYDROGEN PEROXIDE

In order to investigate whether the effects observed above could have resulted from direct interactions between polyphenols and H_2O_2, ROS levels were measured after adding polyphenols in the absence of cells. The results in Figure 3A,B clearly showed that the rapid and almost complete ROS blocking effect of EGCG and curcumin was a cell-dependent effect, as there was minimal direct effect on H_2O_2 itself, with only curcumin observed to scavenge 10–20% of H_2O_2 directly. Interestingly, EGCG itself produced peroxide in a dose-dependent manner (Fig. 3A).

EGCG AND CURCUMIN SUPPRESSED SNAP-INDUCED INTRACELLULAR NO IN MSCs

We next investigated whether EGCG and curcumin could inhibit NO accumulation. hMSCs were co-treated with EGCG or curcumin at varying concentrations, and then exposed to SNAP as an NO donor. Intracellular NO was measured using DAF-FM in a fluorescence-based protocol. Our results showed that EGCG and curcumin suppressed SNAP-induced intracellular NO in a dose-dependent

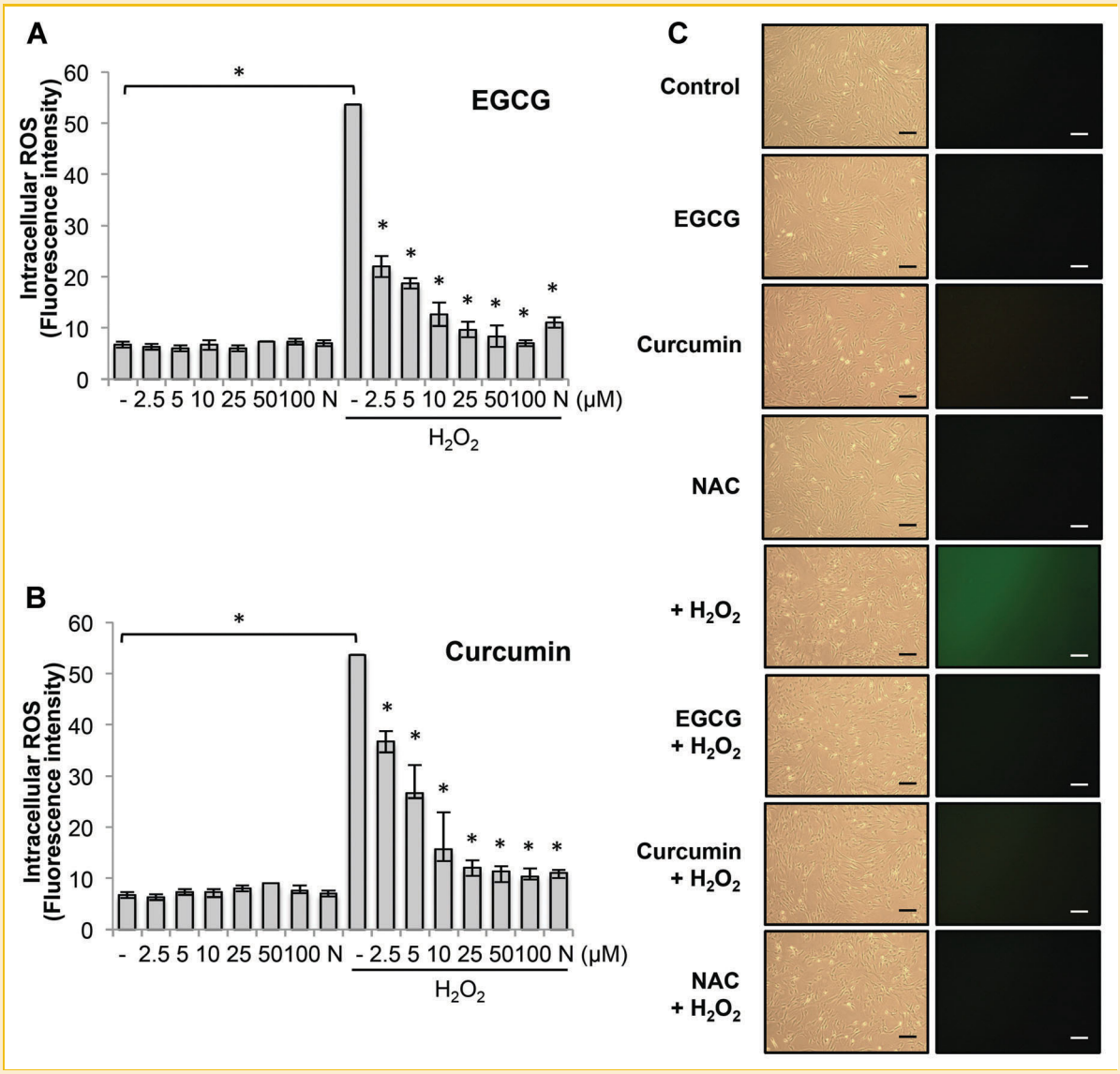

Fig. 2. EGCG and curcumin reduced H_2O_2-induced intracellular ROS in MSCs. 1.0×10^4 hMSCs were plated on 96-well plates, and co-treated with 10 μM H_2O_2 in combination with EGCG (A), curcumin (B), or 1 mM NAC (N) for 30 min, and intracellular ROS measured with H_2DCFDA as described in Materials and Methods Section. Results are expressed as means ± SD (N = 3). Significant differences relative to untreated controls: $^*P < 0.05$, $^{**}P < 0.1$. hMSCs from three patients were used and showed similar results. C: Cell images under phase contrast (left panels) and fluorescence optics (right panels) to visualize intracellular ROS levels. Bar = 200 μm. hMSCs from two patients were used and showed similar results.

manner (Fig. 4A–C). Complete suppression was observed with 10 μM EGCG and 100 μM curcumin after 30 min treatment. However, in comparison, 1 mM L-NAME treated cells failed to show suppression after 30 min treatment (Fig. 4A–C). Cell viability was not affected by these treatments.

EGCG AND CURCUMIN PROTECTED H_2O_2-INDUCED DNA DAMAGE IN MSCs

If DNA damage, such as that produced upon ROS exposure, is not properly repaired, cells will progress to cellular senescence or apoptosis. Indeed, recent reports showed that H_2O_2 induced apoptosis of MSCs in a dose- and time-dependent manner [Wei et al., 2010]. Therefore, we next investigated whether EGCG and curcumin could act to protect MSCs from apoptotic cell death induced by H_2O_2. Cells were co-treated with 10 μM H_2O_2 and different concentrations of polyphenols for 3 days and then analyzed for DNA fragmentation. We observed that H_2O_2 induced apoptosis of MSCs at a two-fold higher level compared to vehicle control cells (Fig. 5). Upon polyphenol treatment, an inhibitory effect on H_2O_2-induced apoptotic cell death in hMSCs was seen, based on comparison between treated control cells and polyphenols treated cells under H_2O_2 (Fig. 5A,B). Curcumin and H_2O_2 co-treated cells maintained ROS level as in the curcumin control after 3 days, but EGCG did not (data not shown). NAC suppressed apoptosis at 0.5–1 mM (Fig. 5C). It should be noted that polyphenol treatment alone showed slightly elevated apoptotic cell death, especially with

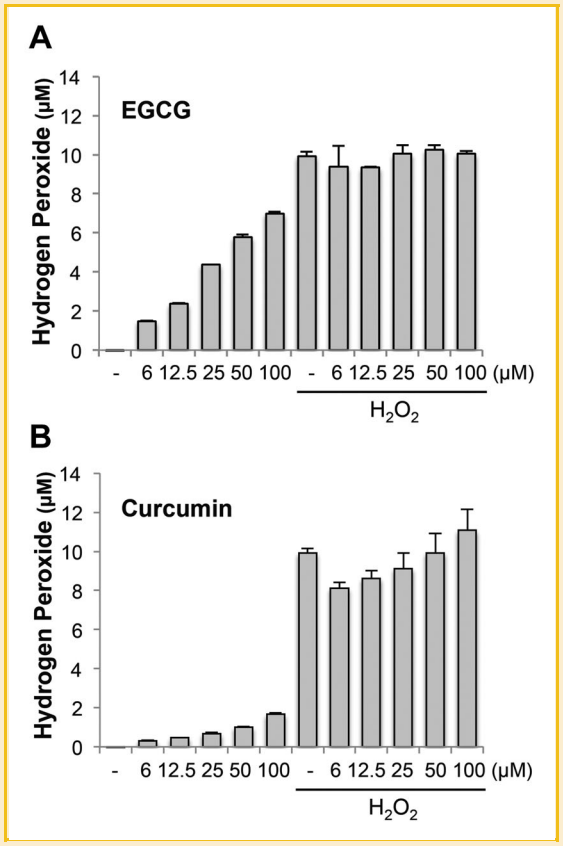

Fig. 3. Lack of or minimal direct interaction between polyphenols and hydrogen peroxide. Ten microliters of samples, containing different concentrations of polyphenols with or without 10 μM H_2O_2, were incubated and then assayed for ROS level. Results are expressed as means ± SD (N = 3). Significant differences relative to untreated controls: $^*P < 0.05$.

EGCG, compared to vehicle control cells (data not shown). The results reported here are based on comparison with polyphenol treated control cells.

EGCG AND CURCUMIN SUPPRESSED H_2O_2-INDUCED CYTOSOLIC CATALASE ACTIVITY IN MSCs

To assess the accumulated damage to MSCs upon ROS elevation, we next measured activities of antioxidative enzymes responsible for ROS deactivation, such as catalase and GPX [Yu, 1994]. Cells were co-treated with H_2O_2 and EGCG or curcumin for 3 days and cytosolic catalase activities were measured. Because polyphenols alone suppressed catalase activity, the results we obtained were compared to the polyphenol-treated control cells instead of vehicle control cells. Our results showed that both EGCG and curcumin suppressed H_2O_2-induced cytosolic catalase activity in hMSCs (Fig. 6A,B). As a control, treatment with NAC also suppressed catalase activity at 0.5–1 mM (Fig. 6C).

CO-TREATMENT WITH EGCG AND H_2O_2 ENHANCED GPX ACTIVITY IN H_2O_2 TREATED MSCs

To investigate the effect of EGCG and curcumin on GPX in hMSCs, cells were co-treated with H_2O_2 and EGCG or curcumin for 3 days. We observed that exposure to H_2O_2 slightly reduced GPX activity in hMSCs compared to vehicle control cells. Because polyphenols alone suppressed GPX activity, the results were compared to the polyphenol-treated control cells instead of vehicle control cells. EGCG and curcumin treatment reversed the effect of H_2O_2 and resulted in enhanced GPX activity in H_2O_2 treated hMSCs (for curcumin, effect observed at 5–10 μM; Fig. 7A,B). As a control, treatment with NAC at 0.5–1 mM also reversed the effect of H_2O_2, and enhanced GPX activity (Fig. 7C).

DISCUSSION

In this investigation, our findings showed that treatment of hMSCs with polyphenols, specifically EGCG and curcumin, inhibited intracellular ROS/NO, protected the cells from H_2O_2-induced DNA damage, enhanced antioxidant enzyme activities in hMSCs and reversed the H_2O_2-induced loss of colony forming of hMSCs induced in a stimulated bone marrow microenvironment.

An important characteristic of MSCs that renders them a promising cell types for therapeutic applications is their capacity to home to target tissues and proliferate. However, the functional applications of MSCs are limited by a variety of factors, including the resident tissue microenvironment, that affect their proliferative capacity [Izadpanah et al., 2006, 2008; Kretlow et al., 2008; Neuhuber et al., 2008]. To improve cell therapy efficacy, it is therefore necessary to modulate MSCs in a manner to enhance their survival upon environmental insults such as oxidative stress.

We initiated our studies by first examining the antioxidant capacity for several polyphenols (EGCG from green tea, curcumin from turmeric, resveratrol from grapes, genistein from soybean) against inducible ROS production in hMSCs (data not shown) and DPPH radical quenching activity in vitro (Table I). DPPH is a stable free radical at room temperature and its quenching assay is easy to perform to show in vitro antioxidant capacity [Yoshida et al., 1989]. In both studies, EGCG, curcumin, and resveratrol acted as antioxidants; however, genistein did not, and most likely, acted as pro-oxidant. Therefore, we selected the polyphenols, curcumin, and EGCG, which showed strong effects, for further investigation.

In this study, we employed a suspension culture approach to simulate the three-dimensional BM cellular environment [Baksh and Tuan, 2007] to study the effects of polyphenols on MSCs directly after isolation from BM. Our results showed that EGCG and curcumin reversed the H_2O_2-induced loss of colony formation ability in BM-MNCs in vitro (Fig. 1C), suggesting that the presence of EGCG and curcumin in vivo could be effective in protecting MSCs from oxidative stress in situ. The rapidity of the effects of these polyphenols is remarkable: a 5 min incubation was sufficient to suppress oxidative stress in BM-MNCs (Fig. 1C). In previous studies, approximately 75% of exogenously added [^{3}H]-EGCG was found in the cytosolic fraction in HT-29 human colon adenocarcinoma cells when the cells were incubated with 0.5–20 μM [^{3}H]-EGCG for 15 min [Hong et al., 2002]. Wahlang's group also found that curcumin was accumulated in Caco-2 cells [Wahlang et al., 2011]. Other studies on polyphenol–cell interactions also showed rapid incorporation into cells and binding of polyphenols to cell

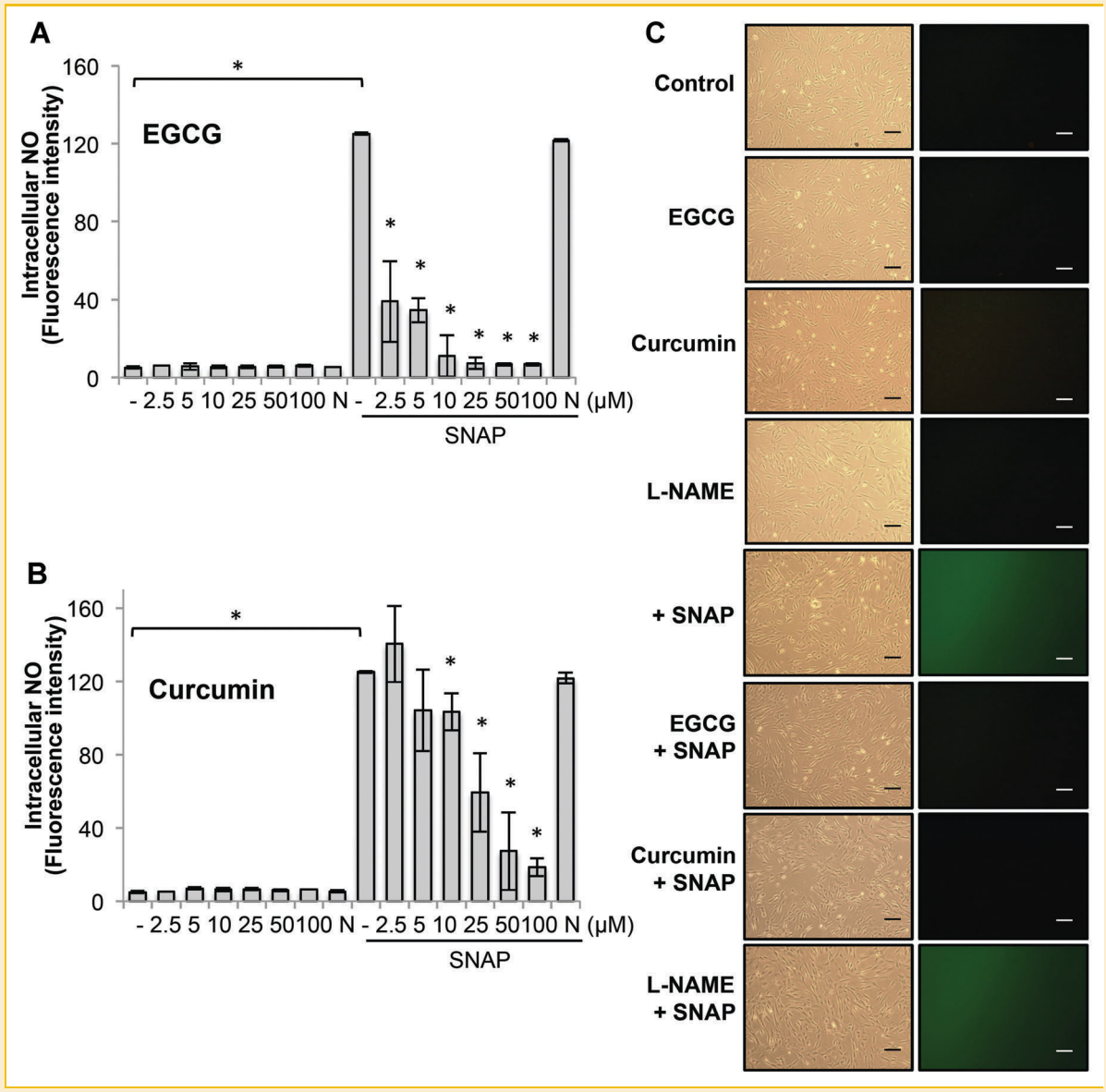

Fig. 4. EGCG and curcumin suppressed SNAP-induced intracellular NO in MSCs. 1.0×10^4 hMSCs were seeded on 96-well plates. Cultures were co-treated with 500 μM SNAP in combination with EGCG (A), curcumin (B), or 1 mM L-NAME (N) for 30 min and intracellular NO measured using DAF-FM as described in Materials and Methods Section. Results are expressed as means ± SD (N = 3). Significant differences relative to untreated controls: $^{*}P < 0.05$, $^{**}P < 0.1$. hMSCs from three patients were used and showed similar results. C: Cell images under phase contrast (left panels) and fluorescence optics (right panels) to visualize intracellular NO levels. Bar = 200 μm. hMSCs from two patients were used and showed similar results.

membrane with concomitant and anti-oxidative effects [Zenda et al., 1997; Jaruga et al., 1998; Yano et al., 2007]. However, we also observed that colony forming activity was significantly reduced when the polyphenol treatment was carried out at 10 μM (data not shown). The relevance of polyphenols on cell cycle has been reported previously: (1) EGCG treatment resulted in G(0)/G(1)-phase arrest of the cell cycle in human prostate carcinoma [Gupta et al., 2000, 2003] and G2/M arrest in PC-9 lung cancer cells [Okabe et al., 1997]; and (2) curcumin caused G0/G2 arrest in vascular smooth muscle cells [Chen and Huang, 1998] and G2/M phase arrest in HCT-116 colon cancer cells [Jaiswal et al., 2002]. Thus, high concentrations of polyphenols appear to cause cell cycle arrest in hMSCs.

To assess the mechanistic pathway of polyphenol effect on MSC colony formation under oxidative stress, we examined changes in intracellular ROS/NO levels, DNA damage, and antioxidant enzyme activity. Our results showed that co-incubation with polyphenols suppressed levels of both intracellular ROS/NO in hMSCs induced upon H_2O_2 treatments, in a dose dependent manner. Specially, EGCG inhibited intracellular ROS/NO significantly compared to curcumin (Figs. 2 and 4). The effects of polyphenols were seen after 30 min (Figs. 2 and 4) and earlier (data not shown). Although the exact target of this rapid effect of polyphenols remains to be identified, one candidate may be the enzyme, NADPH (nicotinamide adenine dinucleotide phosphate) oxidase (NOX), a major source of

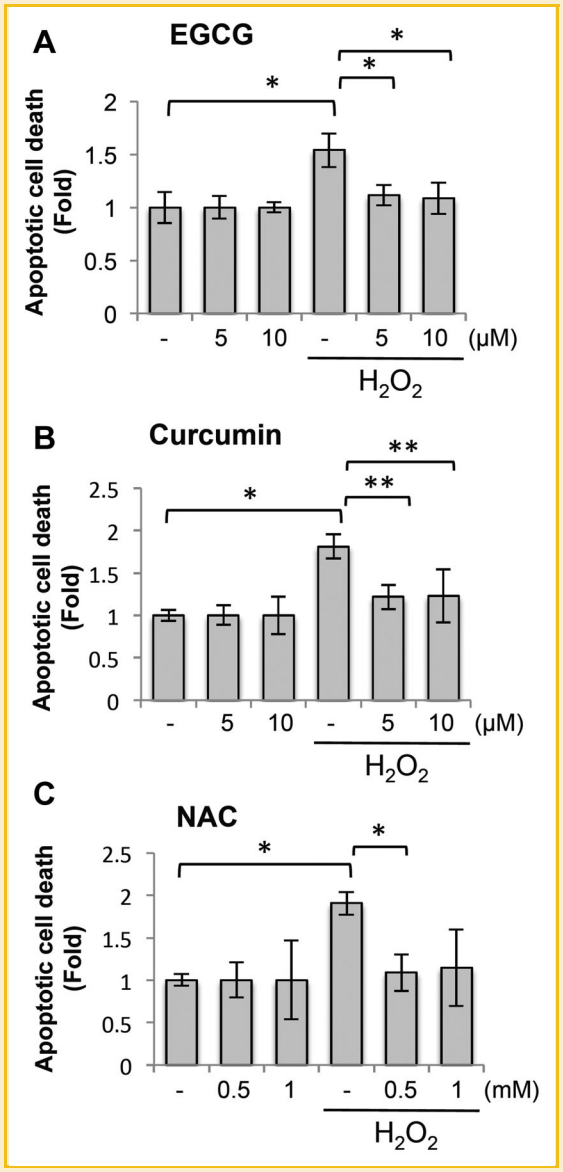

Fig. 5. EGCG and curcumin protected H_2O_2-induced DNA damage. 1.0×10^4 hMSCs were seeded on 96-well plates, and co-treated with 10 μM H_2O_2 in combination with EGCG (A), curucumin (B), or NAC (C) for 3 days. Apoptosis was quantified as percentage of cells with hypodiploid DNA, as assessed using a HT titer TACS assay kit. Fold changes were calculated between (1) untreated cells (value designated as 1) and H_2O_2 treated cells, (2) polyphenol treated cells (value designated as 1) and polyphenol + H_2O_2 treated cells. Results are expressed as means ± SD. Indicated results were averages from four patients for EGCG and curcumin treated groups, three patients for NAC treated group. Significant differences relative to untreated controls: $^{*}P < 0.05$, $^{**}P < 0.1$.

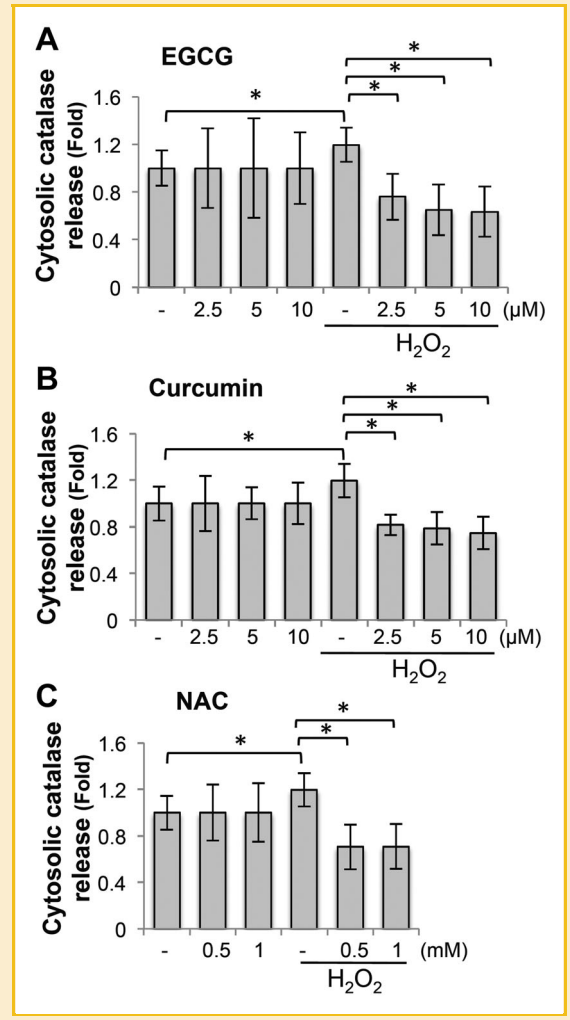

Fig. 6. EGCG and curcumin protected H_2O_2-induced cytosolic catalase activity. 0.5×10^5 hMSCs were seeded on 6-well plates, and co-treated with 10 μM H_2O_2 in combination with EGCG (A), curcumin (B), or NAC (C) for 3 days. Catalase activity was measured using the Amplex Red Catalase Assay Kit. Fold changes were calculated between (1) untreated cells (value designated as 1) and H_2O_2 treated cells, and (2) polyphenol treated cells (value designated as 1) and polyphenol + H_2O_2 treated cells. Results are expressed as means ± SD. Indicated results were averages from two patients. Significant differences relative to untreated controls: $^{*}P < 0.05$, $^{**}P < 0.1$. MSCs from five patients were used and showed similar results.

intracellular ROS production [Babior, 1999; Bedard and Krause, 2007]. We are currently examining the possibility that EGCG and curcumin suppress ROS production thorough rapid suppression of NOX and members of its protein family in hMSCs.

Co-incubation with polyphenols also affected H_2O_2-induced apoptotic cell death in hMSCs (Fig. 5). Specifically, curcumin treatment for 3 days protected the cells from apoptosis. On the other hand, our preliminary results showed that MSCs treated with EGCG resulted in enhanced intracellular ROS after 6 h, suggesting pro-oxidant activity (data not shown). EGCG is generally considered as an antioxidant; however, there is increasing evidence that this tea constituent can be cell damaging and pro-oxidant under certain conditions. These effects were suggested to be due to spontaneous H_2O_2 generation by polyphenols in solution [Elbling et al., 2005; Li et al., 2010]. These reports support our results from DNA damage and enzyme activity assays. Therefore, care should be exercised when applying EGCG to animal models, clinical trials, and cell culture models in the future.

Protection of cells against oxidative stress depends on an antioxidant system consisting of enzymes such as superoxide dismutase (SOD), CAT, and GPX, that are capable of scavenging

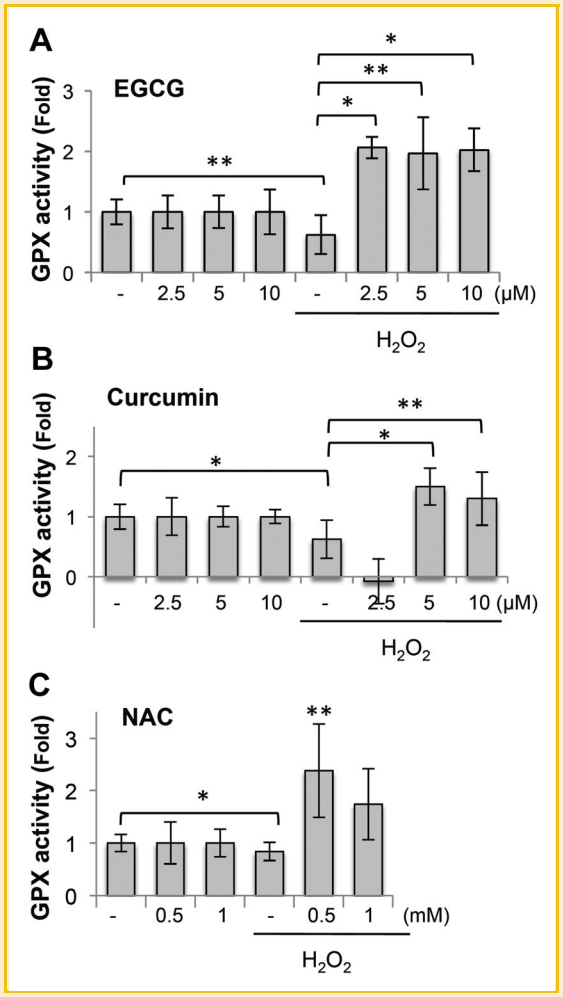

Fig. 7. Co-treatment with H_2O_2 and EGCG enhanced GPX activity. 0.5×10^5 hMSCs were seeded on 6-well plates, and co-treated with 10 μM H_2O_2 in combination with EGCG (A), curcumin (B), or NAC (C) for 3 days. The activity of GPX was measured using the glutathione peroxidase activity assay Kit. Fold changes were calculated between (1) untreated cells (value designated as 1) and H_2O_2 treated cells, and (2) polyphenol treated cells (value designated as 1) and polyphenol + H_2O_2 treated cells. Results are expressed as means ± SD. Indicated results were averages from four patients for EGCG and curcumin treated groups, three patients for NAC treated group. Significant differences relative to untreated controls: $^*P < 0.05$, $^{**}P < 0.1$.

different kinds of free radicals, including lipid peroxide, hydroxyl radical, and superoxide anion radical. These scavengers are not only distributed in the cytosol but are also localized in the mitochondria, where most of the intracellular free radicals are produced [Laroff et al., 1972; Ji et al., 1988]. In our study, both EGCG and curcumin inhibited H_2O_2-induced catalase activity. Interestingly, EGCG enhanced the activity of GPX, whereas curcumin did not show marked effect, indicating that EGCG and curcumin have different effects on H_2O_2-induced oxidative stress in hMSCs. Taken together, these results suggest that treatment with polyphenols may act on the defense system of the cell to avoid any accumulation of cell damage.

In our study, NAC and L-NAME were used as positive control. Our results showed that polyphenols (<10 μM) exhibited significant

TABLE I. EGCG Inhibited DPPH Quenching In Vitro*

Compounds	IC_{50} (μM)
EGCG	2.5
Curcumin	100
Resveratrol	250
Genistein	0
N-acetylcysteine	N/A ($IC_{10} = 500$)

*10 μl of different concentrations of EGCG, curcumin, resveratrol, genistein, or NAC were incubated with 0.25 mM DPPH in 90 μl methanol for 10 min at room temperature. DPPH products was measured as A_{520}. Polyphenol concentrations at 50% inhibition (IC_{50}) are presented.

effects at considerably lower concentrations compared to the positive controls (NAC and L-NAME, both at 1 mM).

In conclusion, we report here that EGCG and curcumin effectively reversed H_2O_2-induced loss of colony forming activity of hMSCs in stimulated bone marrow environment. The polyphenols inhibited both intracellular ROS and NO in hMSCs and protected antioxidant enzymes and reduced apoptotic cell death in hMSCs caused by H_2O_2-induced oxidative stress. These findings demonstrate that EGCG and curcumin are capable of suppressing inducible oxidative stress in hMSCs, and suggest a possible new approach to maintain the viability and potency of hMSCs for clinical application.

REFERENCES

Ahmed S, Wang N, Lalonde M, Goldberg VM, Haqqi TM. 2004. Green tea polyphenol epigallocatechin-3-gallate (EGCG) differentially inhibits interleukin-1 beta-induced expression of matrix metalloproteinase-1 and -13 in human chondrocytes. J Pharm Exp Ther 308(2):767–773.

Anderson RF, Fisher LJ, Hara Y, Harris T, Mak WB, Melton LD, Packer JE. 2001. Green tea catechins partially protect DNA from (•)OH radical-induced strand breaks and base damage through fast chemical repair of DNA radicals. Carcinogenesis 22(8):1189–1193.

Babior BM. 1999. NADPH oxidase: An update. Blood 93(5):1464–1476.

Baksh D, Tuan RS. 2007. Canonical and non-canonical Wnts differentially affect the development potential of primary isolate of human bone marrow mesenchymal stem cells. J Cell Physiol 212(3):817–826.

Bedard K, Krause KH. 2007. The NOX family of ROS-generating NADPH oxidases: Physiology and pathophysiology. Physiol Rev 87(1):245–313.

Boocock DJ, Faust GE, Patel KR, Schinas AM, Brown VA, Ducharme MP, Booth TD, Crowell JA, Perloff M, Gescher AJ, Steward WP, Brenner DE. 2007. Phase I dose escalation pharmacokinetic study in healthy volunteers of resveratrol, a potential cancer chemopreventive agent. Cancer Epidemiol Biomarkers Prev 16(6):1246–1252.

Chen HW, Huang HC. 1998. Effect of curcumin on cell cycle progression and apoptosis in vascular smooth muscle cells. Br J Pharmacol 124(6):1029–1040.

Chen CH, Ho ML, Chang JK, Hung SH, Wang GJ. 2005. Green tea catechin enhances osteogenesis in a bone marrow mesenchymal stem cell line. Osteoporos Int 16(12):2039–2045.

Cheng AL, Hsu CH, Lin JK, Hsu MM, Ho YF, Shen TS, Ko JY, Lin JT, Lin BR, Ming-Shiang W, Yu HS, Jee SH, Chen GS, Chen TM, Chen CA, Lai MK, Pu YS, Pan MH, Wang YJ, Tsai CC, Hsieh CY. 2001. Phase I clinical trial of curcumin, a chemopreventive agent, in patients with high-risk or pre-malignant lesions. Anticancer Res 21(4B):2895–2900.

Chow HH, Cai Y, Alberts DS, Hakim I, Dorr R, Shahi F, Crowell JA, Yang CS, Hara Y. 2001. Phase I pharmacokinetic study of tea polyphenols following single-dose administration of epigallocatechin gallate and polyphenon E. Cancer Epidemiol Biomarjers Prev 10(1):53–58.

Elbling L, Weiss RM, Teufelhofer O, Uhl M, Knasmueller S, Schulte-Hermann R, Berger W, Micksche M. 2005. Green tea extract and (−)-epigallocatechin-3-gallate, the major tea catechin, exert oxidant but lack antioxidant activities. FASEB J 19(7):807–809.

Freedman JE, Parker C III, Li L, Perlman JA, Frei B, Ivanov V, Deak LR, Iafrati MD, Folts JD. 2001. Select flavonoids and whole juice from purple grapes inhibit platelet function and enhance nitric oxide release. Circulation 103(23):2792–2798.

Gehm BD, McAndrews JM, Chien PY, Jameson JL. 1997. Resveratrol, a polyphenolic compound found in grapes and wine, is an agonist for the estrogen receptor. Proc Natl Acad Sci USA 94(25):14138–14143.

Geissler RG, Ganser A, Ottmann OG, Gute P, Morawetz A, Guba P, Helm EB, Hoelzer D. 1994. In vitro improvement of bone marrow-derived hematopoietic colony formation in HIV-positive patients by alpha-D-tocopherol and erythropoietin. Eur J Haematol 53(4):201–206.

Ghanim H, Sia CL, Korzeniewski K, Lohano T, Abuaysheh S, Marumganti A, Chaudhuri A, Dandona P. 2011. A resveratrol and polyphenol preparation suppresses oxidative and inflammatory stress response to a high-fat, high-carbohydrate meal. J Clin Endocrinol Metab 96(5):1409–1414.

Giorgio M, Trinei M, Migliaccio E, Pelicci PG. 2007. Hydrogen peroxide: A metabolic by-product or a common mediator of ageing signals? Nature Rev Mol Cell Biol 8(9):722–728.

Gupta S, Ahmad N, Nieminen AL, Mukhtar H. 2000. Growth inhibition, cell-cycle dysregulation, and induction of apoptosis by green tea constituent (−)-epigallocatechin-3-gallate in androgen-sensitive and androgen-insensitive human prostate carcinoma cells. Toxicol Appl Pharmacol 164(1):82–90.

Gupta S, Hussain T, Mukhtar H. 2003. Molecular pathway for (−)-epigallocatechin-3-gallate-induced cell cycle arrest and apoptosis of human prostate carcinoma cells. Arch Biochem Biophys 410(1):177–185.

Hastak K, Agarwal MK, Mukhtar H, Agarwal ML. 2005. Ablation of either p21 or Bax prevents p53-dependent apoptosis induced by green tea polyphenol epigallocatechin-3-gallate. FASEB J 19(7):789–791.

Hong J, Lu H, Meng X, Ryu JH, Hara Y, Yang CS. 2002. Stability, cellular uptake, biotransformation, and efflux of tea polyphenol (−)-epigallocatechin-3-gallate in HT-29 human colon adenocarcinoma cells. Cancer Res 62(24):7241–7246.

Izadpanah R, Trygg C, Patel B, Kriedt C, Dufour J, Gimble JM, Bunnell BA. 2006. Biologic properties of mesenchymal stem cells derived from bone marrow and adipose tissue. J Cell Biochem 99(5):1285–1297.

Izadpanah R, Kaushal D, Kriedt C, Tsien F, Patel B, Dufour J, Bunnell BA. 2008. Long-term in vitro expansion alters the biology of adult mesenchymal stem cells. Cancer Res 68(11):4229–4238.

Jaiswal AS, Marlow BP, Gupta N, Narayan S. 2002. Beta-catenin-mediated transactivation and cell–cell adhesion pathways are important in curcumin (diferuylmethane)-induced growth arrest and apoptosis in colon cancer cells. Oncogene 21(55):8414–8427.

Jaruga E, Sokal A, Chrul S, Bartosz G. 1998. Apoptosis-independent alterations in membrane dynamics induced by curcumin. Exp Cell Res 245(2):303–312.

Ji LL, Stratman FW, Lardy HA. 1988. Antioxidant enzyme systems in rat liver and skeletal muscle. Influences of selenium deficiency, chronic training, and acute exercise. Arch Biochem Biophys 263(1):150–160.

Kao CL, Chen LK, Chang YL, Yung MC, Hsu CC, Chen YC, Lo WL, Chen SJ, Ku HH, Hwang SJ. 2010. Resveratrol protects human endothelium from H(2)O(2)-induced oxidative stress and senescence via SirT1 activation. J Atheroscler Thromb 17(9):970–979.

Katiyar SK, Afaq F, Perez A, Mukhtar H. 2001. Green tea polyphenol (−)-epigallocatechin-3-gallate treatment of human skin inhibits ultraviolet radiation-induced oxidative stress. Carcinogenesis 22(2):287–294.

Kretlow JD, Jin YQ, Liu W, Zhang WJ, Hong TH, Zhou G, Baggett LS, Mikos AG, Cao Y. 2008. Donor age and cell passage affects differentiation potential of murine bone marrow-derived stem cells. BMC Cell Biol 9:60.

Laroff GP, Fessenden RW, Schuler RH. 1972. The electron spin resonance spectra of radical intermediates in the oxidation of ascorbic acid and related substances. J Am Chem Soc 94(26):9062–9073.

Li GX, Chen YK, Hou Z, Xiao H, Jin H, Lu G, Lee MJ, Liu B, Guan F, Yang Z, Yu A, Yang CS. 2010. Pro-oxidative activities and dose-response relationship of (−)-epigallocatechin-3-gallate in the inhibition of lung cancer cell growth: A comparative study in vivo and in vitro. Carcinogenesis 31(5):902–910.

Lin H, Yang G, Tan J, Tuan RS. 2012. Influence of decellularized matrix derived from human mesenchymal stem cells on their proliferation, migration and multi-lineage differentiation potential. Biomaterials 33(18): 4480–4489.

Lu YP, Lou YR, Xie JG, Peng QY, Liao J, Yang CS, Huang MT, Conney AH. 2002. Topical applications of caffeine or (−)-epigallocatechin gallate (EGCG) inhibit carcinogenesis and selectively increase apoptosis in UVB-induced skin tumors in mice. Proc Natl Acad Sci USA 99(19):12455–12460.

Melov S, Schneider JA, Day BJ, Hinerfeld D, Coskun P, Mirra SS, Crapo JD, Wallace DC. 1998. A novel neurological phenotype in mice lacking mitochondrial manganese superoxide dismutase. Nat Genet 18(2):159–163.

Morimoto T, Sunagawa Y, Kawamura T, Takaya T, Wada H, Nagasawa A, Komeda M, Fujita M, Shimatsu A, Kita T, Hasegawa K. 2008. The dietary compound curcumin inhibits p300 histone acetyltransferase activity and prevents heart failure in rats. J Clin Invest 118(3):868–878.

Neuhuber B, Swanger SA, Howard L, Mackay A, Fischer I. 2008. Effects of plating density and culture time on bone marrow stromal cell characteristics. Exp Hematol 36(9):1176–1185.

Okabe S, Suganuma M, Hayashi M, Sueoka E, Komori A, Fujiki H. 1997. Mechanisms of growth inhibition of human lung cancer cell line, PC-9, by tea polyphenols. Jpn J Cancer Res 88(7):639–643.

Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S, Marshak DR. 1999. Multilineage potential of adult human mesenchymal stem cells. Science 284(5411): 143–147.

Quideau S, Deffieux D, Douat-Casassus C, Pouysegu L. 2011. Plant polyphenols: Chemical properties, biological activities, and synthesis. Angew Chem Int Ed Engl 50(3):586–621.

Stolzing A, Scutt A. 2006. Effect of reduced culture temperature on antioxidant defences of mesenchymal stem cells. Free Radic Biol Med 41(2): 326–338.

Tiku ML, Shah R, Allison GT. 2000. Evidence linking chondrocyte lipid peroxidation to cartilage matrix protein degradation. Possible role in cartilage aging and the pathogenesis of osteoarthritis. J Biol Chem 275(26): 20069–20076.

Townsend PA, Scarabelli TM, Pasini E, Gitti G, Menegazzi M, Suzuki H, Knight RA, Latchman DS, Stephanou A. 2004. Epigallocatechin-3-gallate inhibits STAT-1 activation and protects cardiac myocytes from ischemia/reperfusion-induced apoptosis. FASEB J 18(13):1621–1623.

Van de Bittner GC, Dubikovskaya EA, Bertozzi CR, Chang CJ. 2010. In vivo imaging of hydrogen peroxide production in a murine tumor model with a chemoselective bioluminescent reporter. Proc Natl Acad Sci USA 107(50): 21316–21321.

Wahlang B, Pawar YB, Bansal AK. 2011. Identification of permeability-related hurdles in oral delivery of curcumin using the Caco-2 cell model. Eur J Pharm Biopharm 77(2):275–282.

Wei H, Li Z, Hu S, Chen X, Cong X. 2010. Apoptosis of mesenchymal stem cells induced by hydrogen peroxide concerns both endoplasmic reticulum stress and mitochondrial death pathway through regulation of caspases, p38 and JNK. J Cell Biochem 111(4):967–978.

Wu DC, Teismann P, Tieu K, Vila M, Jackson-Lewis V, Ischiropoulos H, Przedborski S. 2003. NADPH oxidase mediates oxidative stress in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine model of Parkinson's disease. Proc Natl Acad Sci USA 100(10):6145–6150.

Yano S, Fujimura Y, Umeda D, Miyase T, Yamada K, Tachibana H. 2007. Relationship between the biological activities of methylated derivatives of (−)-epigallocatechin-3-O-gallate (EGCG) and their cell surface binding activities. J Agric Food Chem 55(17):7144–7148.

Yokota S, Fahimi HD. 1978. The peroxisome (microbody) membrane: Effects of detergents and lipid solvents on its ultrastructure and permeability to catalase. Histochem J 10(4):469–487.

Yoshida T, Mori K, Hatano T, Okumura T, Uehara I, Komagoe K, Fujita Y, Okuda T. 1989. Studies on inhibition mechanisms of antioxidation by tannins and flavonoids. V. Radical scavenging effects of tannins and related polyphenols on 1,1,-diphenyl-2-picrylhydrazyl radical. Chem Pharm Bull 37:2016–2021.

Yu BP. 1994. Cellular defenses against damage from reactive oxygen species. Physiol Rev 74(1):139–162.

Zenda N, Okubo S, Hu ZQ, Hara Y, Shimamura T. 1997. Erythrocyte-dependent mitogenic activity of epigallocatechin gallate on mouse splenic B cells. Int J Immunopharmacol 19(7):399–403.