JOURNAL OF BIOSCIENCE AND BIOENGINEERING
Vol. 105, No. 6, 586–594. 2008
DOI: 10.1263/jbb.105.586

© 2008, The Society for Biotechnology, Japan

Effect of Ascorbic Acid on Bone Marrow-Derived Mesenchymal Stem Cell Proliferation and Differentiation

Kyung-Min Choi,[1] Young-Kwon Seo,[1] Hee-Hoon Yoon,[1] Kye-Yong Song,[2] Soon-Yong Kwon,[3] Hwa-Sung Lee,[3] and Jung-Keug Park[1]*

Department of Chemical and Biochemical Engineering, Dongguk University, 3-26 Pil Dong, Choong-Gu, Seoul 100-715, Korea,[1] Department of Pathology, Chung-Ang University, 221 Dongjak-Gu, Seoul 156-756, Korea,[2] and Department of Orthopedics, Catholic University, 62 Yeongdeungpo-Gu, Seoul 150-713, Korea[3]

Received 22 January 2007/Accepted 27 February 2008

Mesenchymal stem cells (MSCs) derived from bone marrow are an important tool in tissue engineering and cell-based therapies because of their multipotent capacity. Majority of studies on MSCs have investigated the roles of growth factors, cytokines, and hormones. Antioxidants such as ascorbic acid can be used to expand MSCs while preserving their differentiation ability. Moreover, ascorbic acid can also stimulate MSC proliferation without reciprocal loss of phenotype and differentiation potency. In this study, we evaluated the effects of ascorbic acid on the proliferation, differentiation, extracellular matrix (ECM) secretion of MSCs. The MSCs were cultured in media containing various concentrations (0–500 μM) of L-ascorbate-2-phosphate (Asc-2-P) for 2 weeks, following which they were differentiated into adipocytes and osteoblasts. Ascorbic acid stimulated ECM secretion (collagen and glycosaminoglycan) and cell proliferation. Moreover, the phenotypes of the experimental groups as well as the differentiation potential of MSCs remained unchanged. The apparent absence of decreased cell density or morphologic change is consistent with the toxicity observed with 5–250 μM concentrations of Asc-2-P. The results demonstrate that MSC proliferation or differentiation depends on ascorbic acid concentration.

[**Key words:** ascorbic acid, proliferation, differentiation, mesenchymal stem cell, bone marrow]

Mesenchymal stem cells (MSCs) can be derived from specific organs including the gut, lung, liver, and bone marrow. MSCs isolated from the bone marrow have multilineage potential and have been used experimentally in a number of cell-based therapies. MSCs are capable of producing multiple mesenchymal cell lineages such as fibroblasts, osteoblasts, chondrocytes, and adipocytes, under specific culture conditions. In contrast to other adult cells such as ligament cells, chondrocytes, and osteoblasts, MSCs are not rejected and can be easily obtained after bone marrow aspiration and subsequent *in vitro* expansion. However, continued MSC culture for tissue engineering applications requires proper stimulation to prevent premature cell aging, spreading, and inactivity with increasing passage number (1–8). To support and enhance the *in vitro* growth and activity of MSCs, the cell culture medium can be supplemented with various proteins and factors to mimic the physiologic environment, in which the cells demonstrate optimal proliferation and differentiation (9–13).

The cell metabolism in an organized environment is closely related to the intercellular metabolic interactions between the different types of cells. However, when the cells are isolated from their original tissues and cultured, the nutritional requirements of the cells change and vary between the individual cells (14–17). The stimulatory effect of various nutrients, especially ascorbic acid, on the extracellular matrix (ECM) production of cells *in vitro* has been extensively investigated. Ascorbic acid plays a key role as a cofactor in the post-translational modification of collagen molecules and increases collagen production (18–22). An investigation of the ascorbic acid effect on procollagen synthesis in human skin fibroblast cultures revealed an increased production of Type I collagen (20). Furthermore, it is well-known that ascorbic acid stimulates the proliferation of various mesenchyme-derived cell types including osteoblasts, adipocytes, chondrocytes, and odontoblasts *in vitro* (23–31), and modulates cell proliferation *in vitro* (32–39).

Moreover, at specific concentrations in culture medium, ascorbic acid acts as growth promoter to increase the proliferation and DNA synthesis of cells, when supplied to culture medium. However, markedly elevated concentrations are cytotoxic, and can lead to proliferation inhibition and apoptosis. This relationship between cytotoxicity and ascorbic acid concentration is determined by culture-related factors such as the type of medium or incubator condition (CO_2 concentration) (40–42).

Ascorbic acid regulates cell differentiation, particularly of multilineage mesenchymal cells (adipogenesis, osteogenesis, chondrogenesis, and myogenesis). Tsai-Ming Lin *et al.*

* Corresponding author. e-mail: jkpark@dongguk.edu
phone: +82-2-2260-3365 fax: +82-2-2271-3489

cultivated adipose tissue-derived human MSCs using a novel, low calcium cell culture medium supplemented with the antioxidant compounds *N*-acetyl-L-cysteine (NAC) and L-ascorbate 2-phosphate (Asc-2-P) (28). This medium increased cell proliferation and their differentiation into adipocytes, osteoblasts, and chondrocytes.

Many papers have also been published on the effects of ascorbic acid on ECM production, and cell proliferation and differentiation. However, little is known about the effects of various concentrations (5–500 pM) of ascorbic acid on the *in vitro* behaviors of MSCs including proliferation and differentiation, and the compound's cytotoxicity. Presently, we aimed to determine the effect of the ascorbic acid derivative, Asc-2-P, on the *in vitro* behavior of MSCs including proliferation, cytotoxicity, ECM production, and osteogenic and adipogenic differentiation.

MATERIALS AND METHODS

Primary culture of bone marrow-derived MSCs The MSCs were isolated from the bone marrow aspirates obtained from the iliac crest of healthy human donors. All donors consented to the procedure, which was also approved by the Institutional Review Board, St. Mary's Hospital, Catholic University. The bone marrow aspirates were collected in a syringe containing 10,000 IU heparin to prevent coagulation. The mononuclear cell fraction was isolated by 0.77 g/ml Ficoll density gradient centrifugation. Mononuclear cells were plated into tissue culture flasks in an expansion medium at a density of 10^5 cells/cm^2. The expansion medium consisted of low glucose defined minimal essential medium (LG-DMEM; Invitrogen, NY, USA) and 10% fetal bovine serum (FBS; BioWhittaker, Lonza, Walkersville, MD, USA). Upon reaching 80% confluency, the cells were trypsinized with 0.25% trypsin-1 mM EDTA (Sigma, St. Louis, MO, USA) and replated at a density of 10^4 cells/cm^2. The cells were expanded for 2 to 6 passages.

Cell proliferation assay Single cell preparations were obtained by incubating the samples with a 0.05% trypsin solution for an additional 10 min at 37°C. Aliquots of the samples were mixed with trypan blue, and the viable cells were counted using a hemocytometer. MSCs obtained following the 3rd passage were seeded at a density of 1×10^4 cells/well and cultured in unsupplemented medium containing 10% FBS/LG-DMEM (A group), or medium supplemented with 5 μM (B group), 50 μM (C group), 250 μM (D group), or 500 μM (E group) Asc-2-P. MSCs were maintained and subcultured three times every 3 d. Cell numbers were determined by using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT; Sigma-Aldrich) assay. MSCs in 24-well tissue culture plates (TCP) were incubated designated times in 0.5 mg/ml MTT-supplemented cell culture medium at 37°C and 5% CO_2 for 2 h. The intense purple-colored formazan derivative formed during active cell metabolism was eluted and dissolved in 95% iso-propanol containing 0.04 N HCl, and the absorbance was measured at 570 nm. The population doubling level (PDL) was calculated using the following equation:

$$PDL = \log (X_1/X_0)/\log 2$$

where X_0 is the initial cell number and X_1 is the final cell number. Phase-contrast light microscopy was used to observe the morphology of MSCs on the tissue culture dish.

Cell surface antigen analysis by fluorescence-activated cell sorting (FACS) Antibodies against human antigens CD73 and CD90 were purchased from BD Biosciences (San Jose, CA, USA) and CD105 antibody was purchased from Ancell (Bayport, MN, USA). A total of 5×10^5 cells were resuspended in 200 μl of phosphate buffered saline (PBS) and incubated with fluorescein isothiocyanate- or phycoerythrin-conjugated antibodies for 20 min at room temperature (or for 45 min at 4°C). The fluorescence intensity of the cells was evaluated using a FACScan flow cytometer (BD Sciences), and the data were analyzed using CellQuest software (BD Biosciences).

***In vitro* osteogenic and adipogenic differentiation of MSCs** After reaching confluence, the MSCs were maintained for three weeks in either an osteogenic or adipogenic medium.

The osteogenic medium consisted of DMEM containing 10% FBS, 10 mM β-glycerophosphate, 50 μM Asc-2-P and 10^{-7} M dexamethasone (all from Sigma), and the medium was changed every 3 to 4 d. After 21 d, von Kossa staining (described below) was performed to analyze the MSCs.

The adipogenic medium consisted of DMEM containing 10% FBS, 1 mM dexamethasone (Sigma), 0.5 mM 1-methyl-3-isobutylxanthin (Sigma), 10 mg/ml h-insulin (WelGENE), and 10 mM indomethacin (Sigma) that was unsupplemented or Asc-2-P-supplemented (B-E groups, as described above).

The differentiation medium was replaced once every 3 d for 21 d, and Oil Red-O staining (described below) was performed to analyze the MSCs.

Immunocytochemical analysis For alpha-smooth muscle actin (α-SMA) staining, α-SMA antibody (clone 1A4; DAKO Cytomation, Heverlee, Belgium) was biotinylated using the DAKO Animal Research Kit before application and then incubated overnight at 4°C. Thereafter, the sections were thoroughly washed with PBS and incubated with streptavidin-horse radish peroxidase (HRP) for 15 min at room temperature. NovaRed (Vector Laboratories, Burlingame, CA, USA) was used for HRP detection. Sections were counterstained with Mayers haematoxylin, mounted, and covered as described below.

The degree of differentiation of the cells into adipocytes and osteocytes was evaluated by the appearance of Oil Red-O stained lipid vacuoles and von Kossa stained mineralization of calcium nodules, respectively. The degree of osteogenic differentiation was evaluated using von Kossa staining to detect mineral deposition and calcification. For von Kossa staining, the cells were fixed with 10% formalin for 30 min and washed three times with 10 mM Tris–HCl, pH 7.2. The fixed cells were incubated with 5% silver nitrate for 5 min in daylight, washed twice with hydrogen peroxide (H_2O_2), and then treated with 5% sodium thiosulfate. For Oil Red-O staining, the slides were immersed in propylene glycol for 5 min, and the procedure was repeated twice. Oil Red-O was slowly dissolved in propylene glycol (7 mg/*l*) by heating to 100°C, but not over 110°C, with constant stirring.

The solution was filtered-through Whatman no. 5 filter paper, cooled, and filtered again. The slide was then dipped in Oil Red-O for 7 min with agitation, dipped in 85% propylene glycol for 3 min, and rinsed in distilled water. The stained samples were allowed to react with hematoxylin for 1 min, washed in water, and then mounted with glycerol.

Quantitative analysis of calcium and Oil Red-O Calcium deposition was measured using the Quantichrom calcium assay kit (Bioassay Systems, Hayward, CA, USA). Briefly, the supernatants of the MSC cultures were prepared and mixed with working reagent. The mixtures were incubated for 3 min and the amount of calcium in the supernatants was quantified using an ELISA at 612 nm. The amount of oil produced was measured using a general quantification of Oil Red-O staining. Briefly, the MSC cultures were mildly rinsed and fixed with 10% (v/v) buffered formalin in Dulbecco's PBS for 24 h. Following fixation, they were added to a filtered 60% Oil Red-O stock solution in distilled water and incubated for 1 h. Thereafter, they were rinsed with 70% alcohol, and isopropanol was added. The mixtures were centrifuged at 50,000 rpm for 5 min and the supernatants were assayed using an ELAIA at 540 nm.

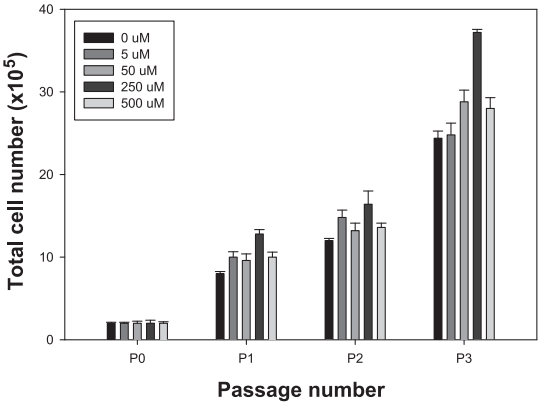

FIG. 1. Effect of L-ascorbate 2-phosphate (Asc-2-P) on growth and proliferation of mesenchymal stem cells (MSCs) according to passage number and concentration of Asc-2-P during the 3rd subculture.

Intercellular collagen and glycosaminoglycan analysis
Total intracellular soluble collagen was measured using the Sircol-collagen assay kit (Bioassay Systems). Briefly, the collagen samples were prepared from the MSC cultures containing various concentrations of Asc-2-P. The samples were then mixed with Sircol dye reagent (Bioassay Systems). After centrifugation (10,000×*g* for 10 min), the mixtures were dissolved in alkali reagent and the absorbance was measured at 540 nm.

The total intracellular sulfated glycosaminoglycan (GAG) content was measured using the Blyscan sulfated glycosaminoglycans assay kit (Bioassay, UK). The GAG samples were prepared from the MSC cultures containing various concentrations of Asc-2-P. The samples were mixed with Blyscan Dye Reagent and incubated for 30 min.After centrifugation (10,000×*g* for 10 min), visual inspection revealed a dark purple residue in the sample test tubes. The samples were dissolved in a dissociation reagent and the absorbance was measured at 656 nm.

RESULTS

Effect of ascorbic acid concentration on MSC proliferation To examine the effects of concentration of Asc-2-P on MSC proliferation, the cell numbers in each culture were estimated by a MTT assay after 1st, 2nd, and 3rd subcultures. The initial seeding cell number in each subculture was the same (1×10^4 cells/well) in each group. As shown in Fig. 1, the cell growth was significantly enhanced in proportion to the increasing Asc-2-P concentration. The stimulating effect of Asc-2-P on the proliferation remained unchanged during cell passage. Asc-2-P significantly increased cell growth. Especially, MSCs cultured in 250 μM Asc-2-P showed the highest cell proliferation activity, while addition of 500 μM Asc-2-P produced decreased rate of cell proliferation in comparison with 250 μM Asc-2-P. It is believed that 500 μM Asc-2-P might have induced cellular damage during culture.

Figure 2 shows the morphology of MSCs cultivated for 3 d (Fig. 2a, c, e, g, i) and 14 d (Fig. 2b, d, f, h, j) after five subcultures. There was no morphological change and the cells became confluent after 14 d of culture, with the exception of the control group.

To investigate the effects of Asc-2-P concentration on MSC proliferation after the third subculture, the cell numbers in each culture were counted on the 3rd, 6th, and 9th day after seeding using the MTT assay. The cell number was determined to be about 2.76×10^4 cells/well in the A group, 3.70×10^4 cells/well in the B group, 4.82×10^4 cells/well in the C group, 6.96×10^4 cells/well in the D group, and 5.76×10^4 cells/well in the E group on the 9th day after seeding (Fig. 3). In this case, the PDL values were 1.46 (A), 1.89 (B), 2.27 (C), 2.80 (D), and 2.53 (E), respectively. This result showed that the appropriate concentration of Asc-2-P (B, C, D groups) enhanced the proliferation of MSCs, while excess of Asc-2-P (500 μM) significantly reduced MSC proliferation. The D group in particular showed significant improvement in the

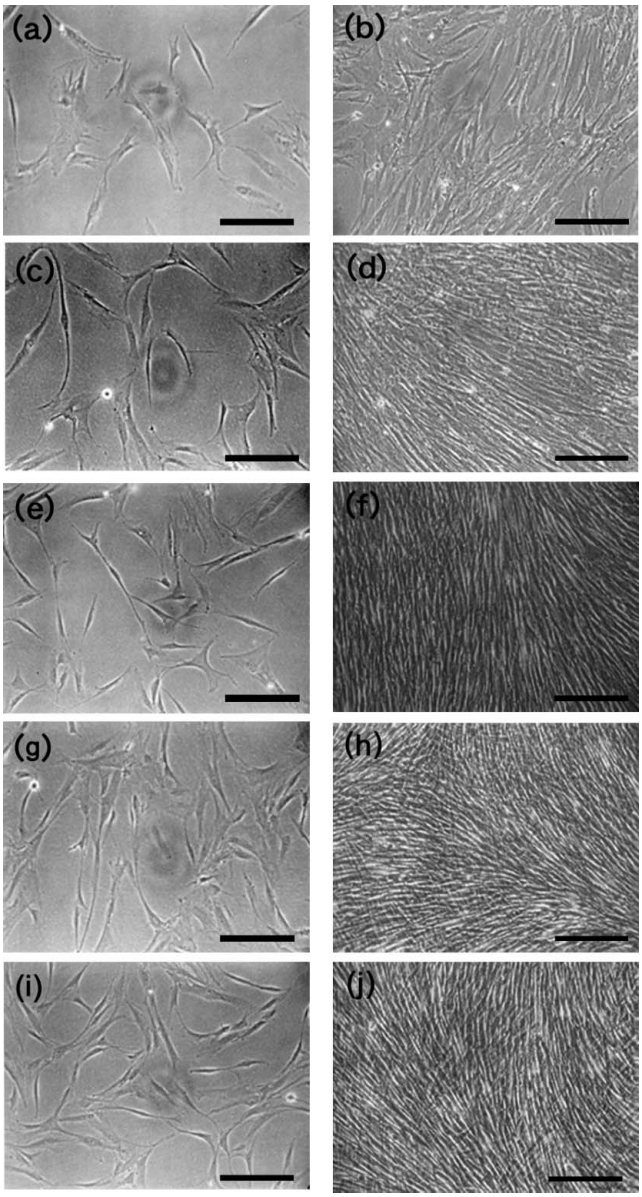

FIG. 2. Phase-contrast photograph of mesenchymal stem cells (MSCs) (P5) cultivated for 3 d (a, c, e, g, i) and 14 d (b, d, f, h, j) after five subcultures. (a, b) A group (0 μM L-ascorbate 2-phosphate [Asc-2-P]): (c, d) B group (5 μM Asc-2-P): (e, f) C group (50 μM Asc-2-P): (g, h) D group (250 μM Asc-2-P): (i, j) E group (500 μM Asc-2-P). Bars: 100 μm.

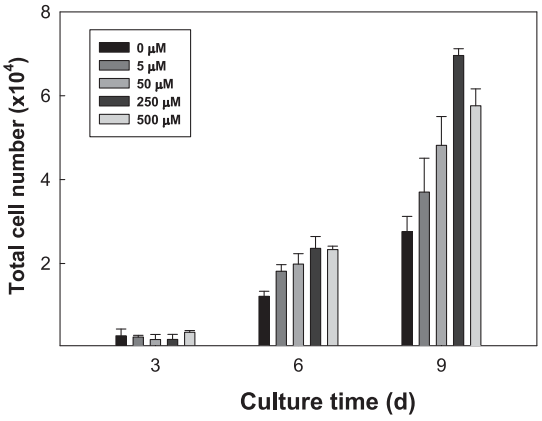

FIG. 3. Proliferation of mesenchymal stem cells (MSCs) (P5) depending on the concentration of L-ascorbate 2-phosphate (Asc-2-P). The cell number was determined by a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay at the 3rd, 6th, and 9th day of culture.

MSC proliferation activity in comparison with the A group having conventional MSC culture condition.

Effect of ascorbic acid concentration on MSC surface antigen expression To determine whether the Asc-2-P concentration altered MSC surface antigen expression, FACS analysis was performed for MSC markers CD73, CD90, and CD105. The MSCs in the A group were cultured using a conventional culture condition, and they served as the control group. There was no difference in the MSC surface antigen expression between the groups (Fig. 4), suggesting that the various Asc-2-P concentrations did not alter MSCs' surface antigen expression.

Effect of ascorbic acid on α-SMA expression in MSCs Continued *in vitro* culture of MSCs induces cell aging or differentiation depending on the environmental parameters. In generally, a high concentration of Asc-2-P induces α-SMA expression when MSCs differentiate into smooth muscle cells (43–46). Thus, to examine the aging or differentiation of MSCs in Asc-2-P-supplemented culture medium, immunocytochemical analysis of α-SMA was performed after 9 days of culture. The expression of α-SMA in MSCs was not observed in any group (Fig. 5). These data suggest that the addition of Asc-2-P did not induce MSC differentiation.

Effect of ascorbic acid concentration during culture following osteogenic and adipogenic MSC differentiation To investigate whether the Asc-2-P concentration during culture affected the capacity of MSCs for osteogenic and adipogenic differentiation, calcium and oil staining was performed after proliferation culture.

Generally speaking, the osteogenic medium is usually supplemented with 50 μM Asc-2-P and conventional adipogenic medium is usually free of Asc 2-P; however, we supplemented the differentiation medium with 0, 5, 50, 250, or 500 μM of Asc-2-P and induced MSC differentiation into osteoblasts and adipocytes for three weeks. Calcium and oil deposition was detected by von Kossa and Oil Red-O staining, respectively. As shown in Fig. 6, there were osteoblast-like morphological changes in the MSCs, and many mineral depositions were observed on the surface in the experimental group (Fig. 6c, e, g, i) in comparison with the control group (Fig. 6a). MSCs cultured in media containing 0 to 5 μM Asc-2-P (Fig. 6b and d, respectively) showed weak staining and those cultured in the media containing 50 to 500 μM Asc-2-P (Fig. 6f, h, j) showed strong staining. According to the quantitative assay for calcium (Fig. 8a), the MSCs cultured in 50 μM ascorbic acid showed the greatest amount of calcium deposition. The calcium content per cell in the group with over 50 μM of Asc-2-P was higher (0.35 μg/cells) than that in the control group (0.25 μg/cells).

As shown in Fig. 7, the MSCs cultured in media containing Asc-2-P at concentrations over 250 μM (Fig. 7g, i) showed more oil droplets than the control group (Fig. 7a). The MSCs cultured in adipocyte differentiation medium containing Asc-2-P at concentrations of 0, 5, and 50 μM (Fig. 7b, d, and f, respectively) showed weak staining and those cultured in the media containing Asc-2-P at concentrations of 250 to 500 μM (Fig. 7h and j, respectively) showed strong staining. According to the quantitative assay for oil staining (Fig. 8b), the MSCs cultured in 500 μM Asc-2-P showed the highest number of oil droplets. The oil content per cell in the group of MSCs cultured in media containing Asc-2-P at concentrations over 250 μM was higher (0.3 μg/cells) than that of the control group (0.25 μg/cells). The addition of As-2-P at a concentration of 50 μM or less did not differ from control (Asc-2-P-free). This result showed that the concentration of Asc-2-P affected the osteogenic and adipogenic capacity of the MSCs regardless of the differentiation condition.

Effect of ascorbic acid concentration on collagen and GAG production Collagen and GAG are the main components of ECM involved in both cell proliferation and differentiation. Thus, analysis of the intracellular collagen and GAG content was performed after 21 d of differentiation culture (Fig. 9). According to the quantitative assay for intercellular collagen (Fig. 9a), total collagen production in the experimental group 5–500 μM Asc-2-P was significantly increased in comparison to the control group. However, the collagen content per cell in the other experimental group (50–500 μM Asc-2-P) did not differ from that of the control group, and 5 μM Asc-2-P significantly improved the amount of collagen production per cell. It is believed that energy is required for MSC differentiation. The total intercellular GAG production (Fig. 9b) in the cells cultured in 5–250 μM Asc-2-P was similar to that of the control group; however, it decreased at higher concentrations of Asc-2-P (500 μM). The GAG content per cell in the experimental group (5–50 μM Asc-2-P) did not differ from that of the control group, and Asc-2-P concentrations of 250 and 500 μM showed decreased total intercellular GAG production in comparison with the control group. These results showed that the appropriate level of Asc-2-P significantly increased collagen and GAG production; however, over-addition of Asc-2-P led to reduced GAG production.

Correlation between cell proliferation and differentiation The appropriate level of Asc-2-P enhanced MSC proliferation; however, excess of Asc-2-P (250 μM) reduced MSC proliferation. Although some MSC surface antigens (CD73, CD90, and CD105) showed similar expression levels regardless of the Asc-2-P concentration, they are not exactly MSC-specific and do not represent enough MSC stemness

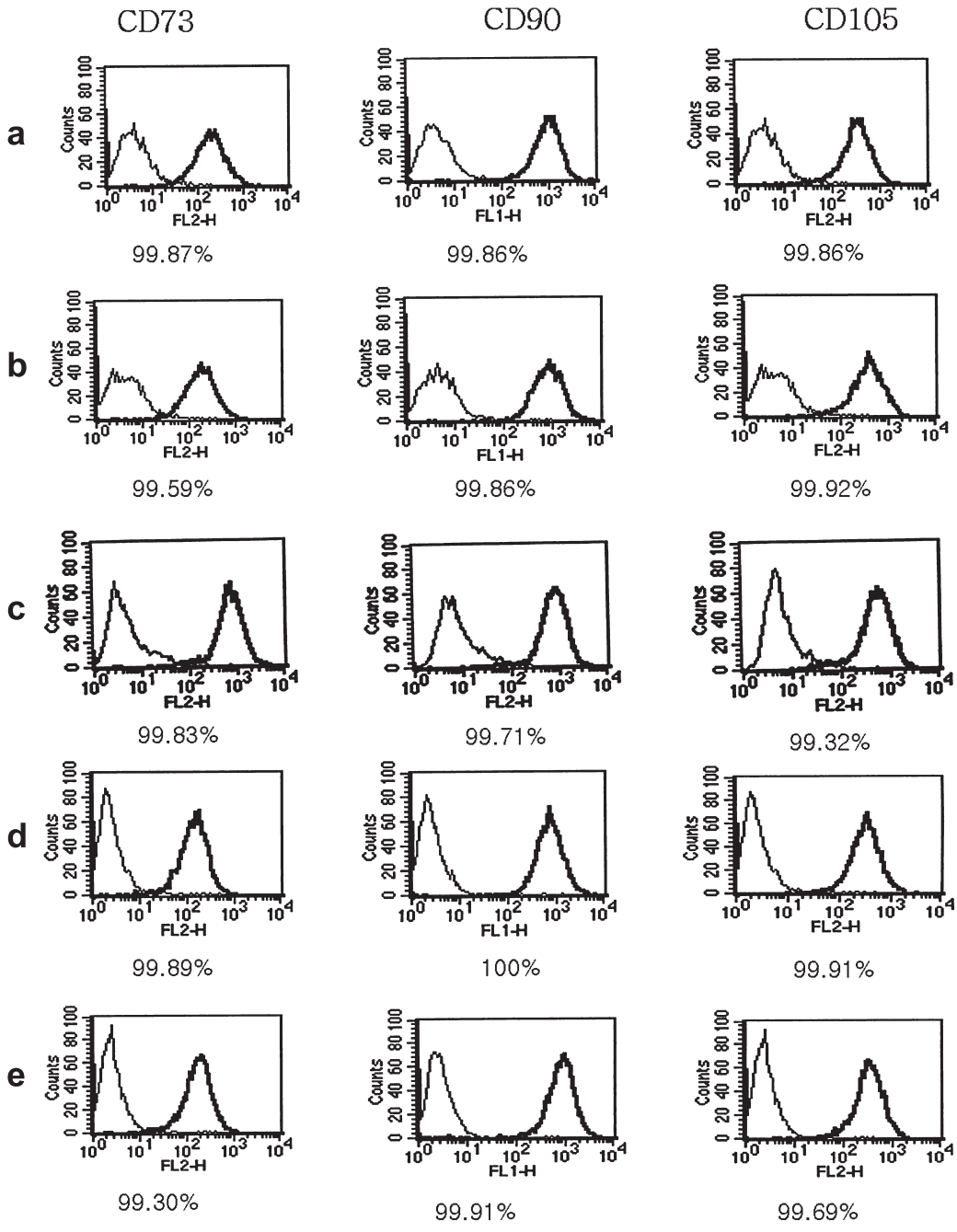

FIG. 4. Fluorescence-activated cell sorting (FACS) analysis of the surface marker after culture with various concentrations of L-ascorbate 2-phosphate (Asc-2-P). Mesenchymal stem cells (MSCs) (P5) were labeled with fluorescein isothiocyanate- or phycoerythrin-conjugated antibodies and then analyzed in a flow cytometer: (a) 0 μM (A group), (b) 5 μM (B group), (c) 50 μM (C group), (d) 250 μM (D group), (e) 500 μM (E group) Asc-2-P.

or multipotency. Under the same osteogenic and adipogenic condition, the degree of calcium deposition was different among the MSCs after they were cultured under different concentrations of Asc-2-P. Moreover, other capacities like that for chondrogenesis, remain unexplored. Therefore, further investigation is necessary. Notably, the D group (250 μM Asc-2-P) showed significant improvement in MSC proliferation and significant reduction in cellular damage without the reduction of differentiation capacity in comparison with the A group (control).

DISCUSSION

Many studies have shown that ascorbic acid induces various cellular responses in a variety of cells and that the dose- and environmental parameter-dependent ascorbic acid effects also modulate various cellular responses (ECM synthesis, proliferation, and differentiation). Although these ascorbic

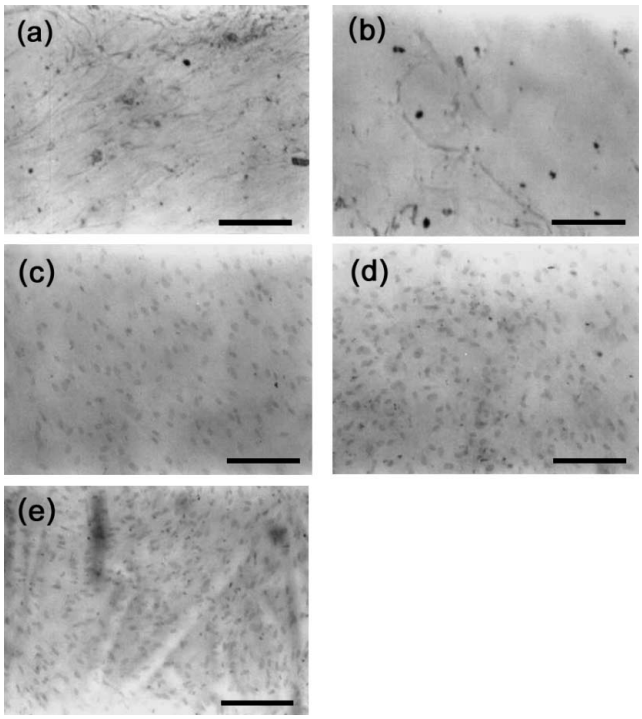

FIG. 5. Immunocytochemical analysis of mesenchymal stem cells (MSCs) (P5) in α-smooth muscle actin (α-SMA). MSCs were stained with α-SMA stain: (a) 0 μM (A group), (b) 5 μM (B group), (c) 50 μM (C group), (d) 250 μM (D group), and (e) 500 μM (E group) Asc-2-P. Alpha-SMA was not expressed on the MSC culture plates at any concentration of L-ascorbate 2-phosphate (Asc-2-P). Bars: 100 μm.

acid-induced cellular responses in many types of cells are not completely understood, many studies have shown that the *in vitro* proliferation and differentiation of MSCs into skeletal tissues requires ascorbic acid as an essential medium component.

Generally, a low concentration of ascorbic acid increases cell proliferation; this effect is more pronounced in malignant cells than in normal cells (47–51). Malicev *et al*. (52) investigated the potential cytotoxic effect of ascorbic acid on human articular chondrocytes and found that ascorbic acid can induce morphological changes and apoptosis in a cell culture of chondrocytes after 18 h of cultivation. Apoptotic changes were found to be minimal in cells cultivated in the absence of ascorbic acid or with low concentrations (0.05 to 0.2 mg/ml) (52).

Growth inhibition should not be generally considered as a cytotoxic effect, a inhibition of growth by low concentrations of ascorbic acid (200 μg/ml) was observed in the murine melanoma cell line BL6 without any sign of obvious toxicity (53). Furthermore, various mesenchymal cells forming connective tissues can proliferate at various medium concentrations of ascorbic acid, whereas embryonic fibroblasts react with growth arrest to ascorbic acid at concentrations as low as 0.05 mM (22). This pronounced susceptibility of embryonic fibroblast cells can be tentatively attributed to an immature antioxidant defense system, since it can be prevented by catalase and other antioxidants. Particularly, when investigating cells forming connective tissues, the possibility that growth inhibition might also mark the beginning

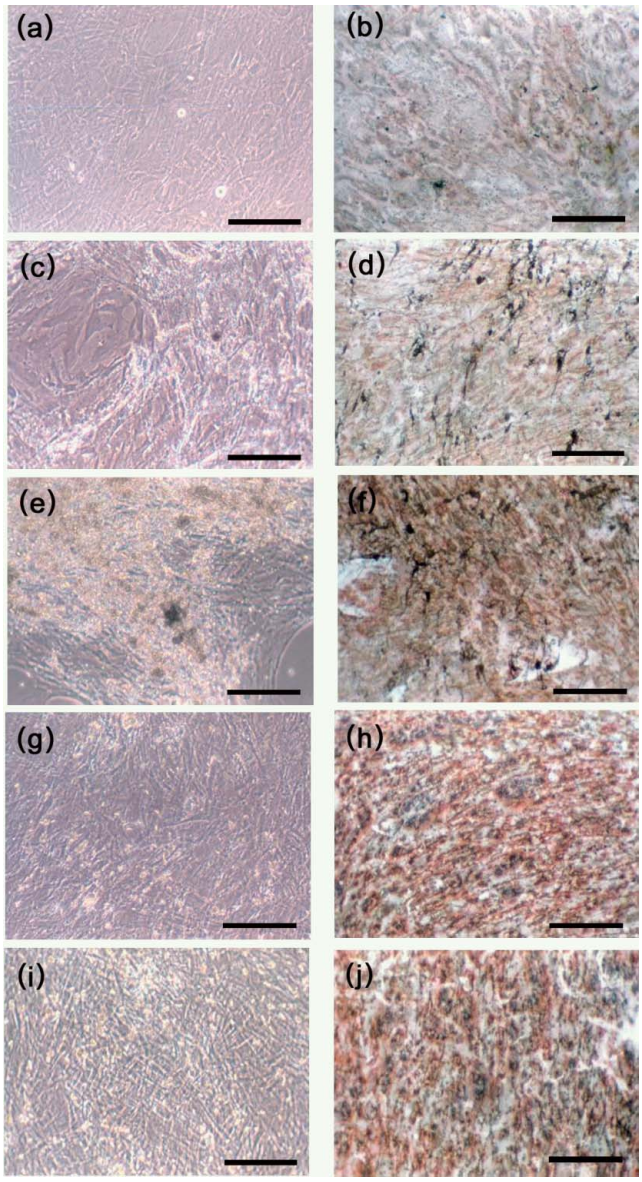

FIG. 6. Morphological examination of mesenchymal stem cells (MSCs) (P5) that had differentiated into adipocytes (a, c, e, g, i) and osteoblasts (b, d, f, h, j) for 21 d. (a, b) A group (0 μM L-ascorbate 2-phosphate [Asc-2-P]); (c, d) B group (5 μM Asc-2-P); (e, f) C group (50 μM Asc-2-P); (g, h) D group (250 μM Asc-2-P); (i, j) E group (500 μM Asc-2-P). Bars: 100 μm.

of a differentiation process that can be induced by ascorbic acid should not be overlooked. In some cell culture conditions, the ascorbic acid concentrations of 0.01–0.5 mM inhibit or stimulate proliferation of various kinds of cells including HEP2, KB, ascites, bone marrow and melanoma cells.

Ascorbic acid is especially essential for the expression of osteoblastic markers and for mineralization in various osteoblast culture systems. Takamizawa *et al.* (27) studied the effect of ascorbic acid and the Asc 2-P derivative on the proliferation and differentiation of human osteoblast-like cells. Both ascorbic acid and Asc-2-P were shown to stimulate nascent cell growth and expression of various osteo-

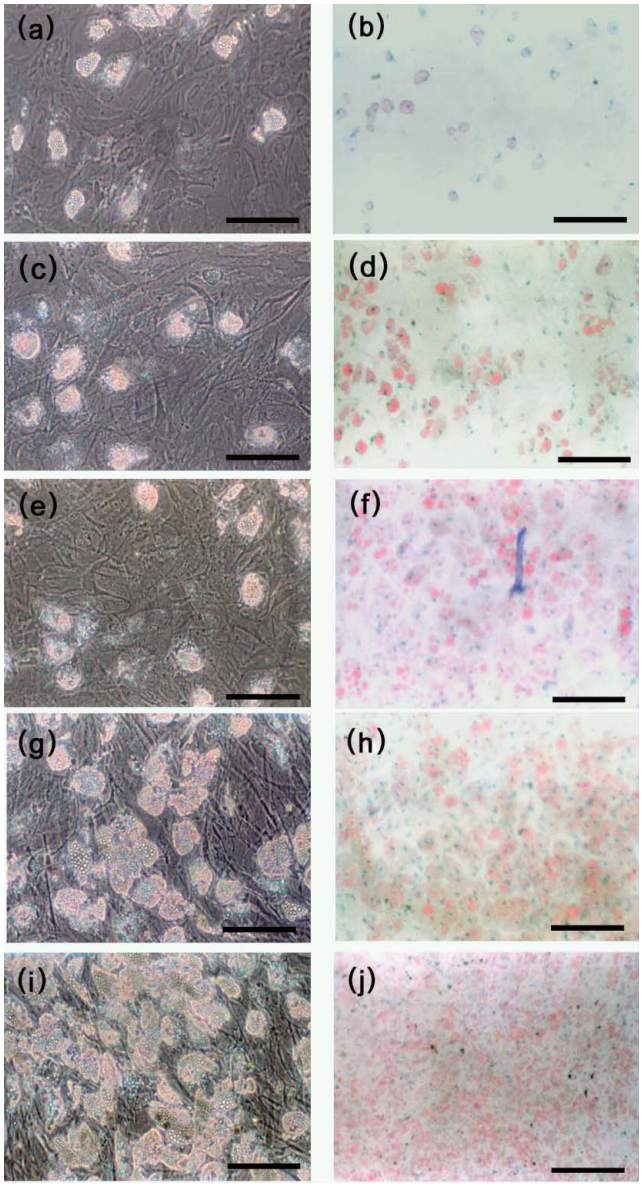

FIG. 7. Immunocytochemical analysis of mesenchymal stem cells (MSCs) (P5) maintained in adipogenic and osteogenic medium for 21 d. (a, c, e, g, i) Oil Red-O staining; (b, d, f, h, j) von-Kossa staining. (a, b) A group (0 μM L-ascorbate 2-phosphate [Asc-2-P]); (c, d) B group (5 μM Asc-2-P); (e, f) C group (50 μM Asc-2-P); (g, h) D group (250 μM Asc-2-P); (i, j) E group (500 μM Asc-2-P). Bars: 100 μm.

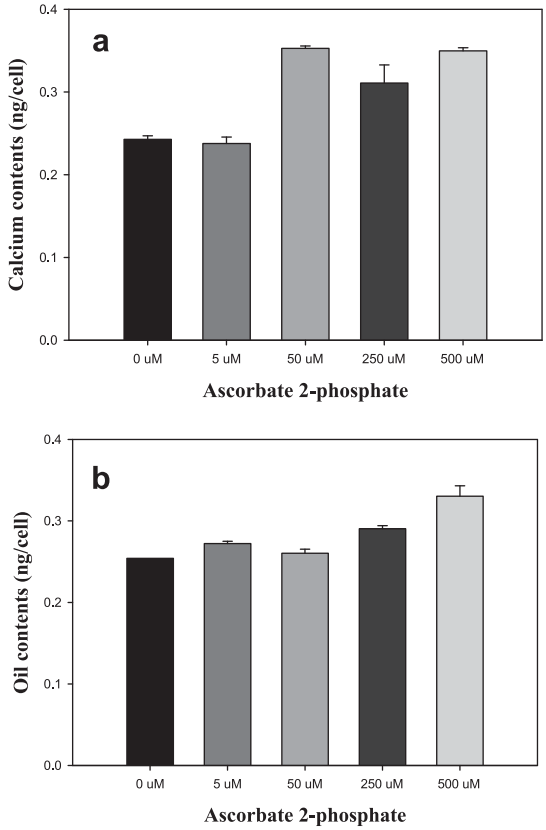

FIG. 8. Quantitative assay: (a) calcium content, (b) oil content on mesenchymal stem cells (MSCs) (P5) in adipogenic and osteogenic differentiation induction plates depending on the concentration of L-ascorbate 2-phosphate (Asc-2-P).

blast differentiation markers, such as collagen synthesis and alkaline phosphatase activity. When they used Asc-2-P, a long-acting ascorbic acid derivative, as a supplement in the culture medium of MG-63 cells, it stimulated nascent growth of cells regardless of the concentration used. These data show that the Asc-2-P-supplemented culture system might be useful for analyzing the process of osteoblast differentiation and for investigating the effects of various hormones and other factors on the differentiation and metabolism of osteoblastic cells (27). Ishikawa *et al.* (31) investigated the role of ascorbic acid on the early osteoblastic differentiation of periodontal ligament cells. Both type I collagen production and alkaline phosphatase activity were upregulated when periodontal ligament cells were cultured in the presence of ascorbic acid (200 pM), and the cell surface expression of $\alpha_2\beta_1$ integrin (a major receptor of type I collagen) was increased in ascorbic acid-stimulated periodontal ligament cells (31).

The present study reveals that the Asc-2-P concentration during culture can modulate MSC proliferation and differentiation, and that specific levels of ascorbic acid serve as potent positive modulators of MSC proliferation without causing a loss in the differentiation capacity of the cells. Asc-2-P can be dephosphorylated to ascorbic acid by alkaline phosphatase and then oxidized to dehydroascorbate (27). However, it is not clear which molecular mechanisms are related to the response and which concentrations of ascorbic acid induce selective response. Thus, further investigation is necessary to understand the molecular biological mechanism underlying the effects of ascorbic acid concentration on MSC proliferation and differentiation.

ACKNOWLEDGMENT

This work was supported by a grant from the Korean Health 21 R&D Project, Ministry of Health and Welfare, Republic of Korea (0405-BO01-0204-0006).

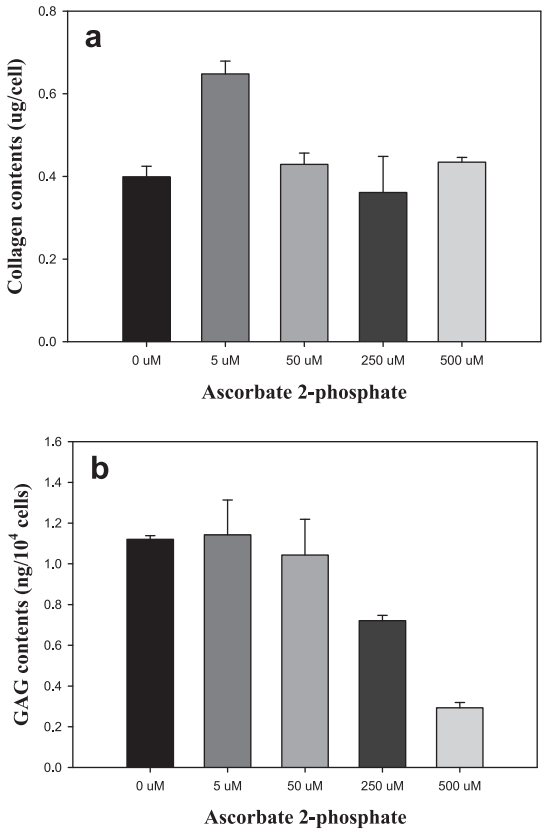

FIG. 9. Intercellular collagen and glycosaminoglycan (GAG) analysis of mesenchymal stem cells (MSCs) (P5) depending on the type of media: (a) Total collagen content, (b) Total GAG content in the MSCs cultures depending on the concentration of L-ascorbate 2-phosphate (Asc-2-P) (* $P<0.001$).

REFERENCES

1. **Bruder, S. P., Jaiswal, N., and Haynesworth, S. E.:** Growth kinetics, self-renewal, and the osteogenic potential of purified human mesenchymal stem cells during extensive subcultivation and following cryopreservation. J. Cell Biochem., **64**, 278–294 (1997).
2. **Jiang, Y., Jahagirdar, B. N., Reinhardt, R. L., Schwartz, R. E., Keene, C. D., Ortiz-Gonzalez, X. R., Reyes, M., Lenvik, T., Lund, T., Blackstad, M., Du, J., Aldrich, S., Lisberg, A., Low, W. C., Largaespada, D. A., and Verfaillie, C. M.:** Pluripotency of mesenchymal stem cells derived from adult marrow. Nature, **428**, 41–49 (2002).
3. **Grove, J. E., Bruscia, E., and Krause, D. S.:** Plasticity of bone marrow-derived stem cells. Stem Cells, **22**, 487–500 (2004).
4. **Bossolasco, P., Corti, S., Strazzer, S., Borsotti, C., Del, B. R., Fortunato, F., Salani, S., Quirici, N., Bertolini, F., Gobbi, A., Deliliers, G. L., Pietro, C. G., and Soligo, D.:** Skeletal muscle differentiation potential of human adult bone marow cells. Exp. Cell Res., **295**, 66–78 (2004).
5. **Mackay, A. M., Beck, S. C., Murphy, J. M., Barry, F. P., Chichester, C. O., and Pittenger, M. F.:** Chondrogenic differentiation of cultured human mesenchymal stem cells from bone marrow. Tissue Eng., **4**, 415–428 (1998).
6. **Wagner, W., Wein, F., Seckinger, A., Frankhauser, M., Wirkner, U., Krause, U., Blake, J., Schwager, C., Eckstein, V., Ansorge, W., and Ho, A. D.:** Comparative characteristics of mesenchymal stem cells from human bone marrow, adipose tissue, and umbilical cord blood. Exp. Hematol., **33**, 1402–1416 (2005).
7. **Pittenger, M. F., Mackay, A. M., Beck, S. C., Jaiswal, R. K., Douglas, R., Mosca, J. D., Moorman, M. A., Simonetti, D. W., Craig, S., and Marshak, D. R.:** Multilineage potential of adult human mesenchymal stem cells. Science, **284**, 143–147 (1999).
8. **Young, R. G., Butler, D. L., Weber, W., Caplan, A. I., Gordon, S. L., and Fink, D. J.:** Use of mesenchymal stem cells in a collagen matrix for achilles tendon repair. J. Orthop. Res., **16**, 406–413 (1998).
9. **Denk, P. O. and Knorr, M.:** Serum-free cultivation of human Tenon's capsule fibroblasts. Curr. Eye Res., **18**, 130 (1999).
10. **Fermor, B., Urban, J., Murray, D., Pocock, A., Lim, E., and Francis, M.:** Proliferation and collagen synthesis of human anterior cruciate ligament cells *in vitro*: effects of ascorbate-2-phosphate, dexamethasone, and oxygen tension. Cell Biol. Int., **22**, 635–640 (1998).
11. **Iyer, V. R., Eisen, M. B., Ross, D. T., Schuler, G., Moore, T., and Lee, J. C.:** The transcriptional program in the response of human fibroblasts to serum. Science, **283**, 83–87 (1999).
12. **Martin, I., Shastri, V. P., Padera, R. F., Yang, J., Mackay, A. J., and Langer, R.:** Selective differentiation of mammalian bone marrow stromal cells cultured on three-dimensional polymer foams. J. Biomed. Mater. Res., **55**, 229–235 (2001).
13. **Marui, T., Niyibizi, C., Georgescu, H. I., Cao, M., Kavalkovich, K. W., and Levine, R. E.:** Effect of growth factors on matrix synthesis by ligament fibroblasts. J. Orthop. Res., **15**, 18–23 (1997).
14. **Hosokawa, A., Takaoka, T., and Katsuta, H.:** Non-essential amino acid requirements of mammalian cells in tissue culture. Jpn. J. Exp. Med., **41**, 273–290 (1971).
15. **Eagle, H.:** Nutrition needs of mammalian cells in tissue culture. Science, **122**, 501–514 (1955).
16. **Eagle, H.:** The specific amino acid requirements of a human carcinoma cell (strain HeLa) in tissue culture. J. Exp. Med., **102**, 37–48 (1955).
17. **Eagle, H.:** The specific amino acid requirements of a mammalian cell (strain L) in tissue culture. J. Biol. Chem., **214**, 839–852 (1955).
18. **Geesin, J. C., Hendricks, L. J., Gordon, J. S., and Berg, R. A.:** Modulation of collagen synthesis by growth factors: the role of ascorbate-stimulated lipid peroxidation. Arch. Biochem. Biophys., **289**, 6–11 (1991).
19. **Chojkier, M., Houglum, K., Solis-Herruzog, J., and Brennerl, D. A.:** Stimulation of collagen gene expression by ascorbic acid in cultured human fibroblasts. A role for lipid peroxidation? J. Biol. Chem., **5**, 16957–16962 (1989).
20. **Chan, D., Lamande, S. R., Cole, W. G., and Bateman, J. F.:** Regulation of procollagen synthesis and processing during ascorbate-induced extracellular matrix accumulation *in vitro*. Biochem. J., **269**, 175–181 (1990).
21. **Sodek, J., Feng, J., Yen, E. H. K., and Melcher, A. H.:** Effect of ascorbic acid on the protein synthesis and collagen hydroxylation in continuous flow organ cultures of adult mouse periodontal tissue. Calcif. Tissue Int., **34**, 408–415 (1982).
22. **Harris, J. R.:** Ascorbic acid, cell proliferation, and cell differentiation in culture, p. 83–102. *In* Brigelius-Flohe, R. and Flohe, L. (ed.), Subcellular biochemistry, vol. 25. Ascorbic acid: biochemistry and biomedical cell biology. Plenum Press, New York (1996).
23. **Alcain, F. J., and Buron, M. I.:** Ascorbate on cell growth and differentiation. J. Bioenerg. Biomembr., **26**, 393–398 (1994).
24. **Savini, I., Catani, M.V., Rossi, A., Duranti, G., Melino, G., and Avigliano, L.:** Characterization of keratinocyte differen-

tiation induced by ascorbic acid: protein kinase C involvement and vitamin C homeostasis. J. Invest. Dermatol., **118**, 372–379 (2002).
25. **Arakawa, E., Hasegawa, K., Irie, J., Ide, S., Ushiki, J., Yamaguchi, K., Oda, S., and Matsuda, Y.:** L-Ascorbic acid stimulates expression of smooth muscle-specific markers in smooth muscle cells both *in vitro* and *in vivo*. J. Cardiovasc. Pharmacol., **42**, 9745–9751 (2003).
26. **Wanga, Y., Singha, A., Xua, P., Pindrusa, M. A., Blasiolia, D. J., and Kaplan, D. L.:** Expansion and osteogenic differentiation of bone marrow-derived mesenchymal stem cells on a vitamin C functionalized polymer. Biomaterials, **27**, 3265–3273 (2006).
27. **Takamizawa, S., Maehata, Y., Imai, K., Senoo, H., Sato, S., and Hata, R.:** Effects of ascorbic acid and ascorbic acid 2-phosphate, a long-acting vitamin C derivative, on the proliferation and differentiation of human osteoblast-like cells. Cell Biol. Int., **28**, 255–265 (2004).
28. **Sato, H., Takahashi, M., Ise, H., Yamada, A., Hirose, S., Tagawa, Y., Morimoto, H., Izawa, A., and Ikeda, U.:** Collagen synthesis is required for ascorbic acid-enhanced differentiation of mouse embryonic stem cells into cardiomyocytes. Biochem. Biophys. Res. Commun., **342**, 107–112 (2006).
29. **Lin, T. M., Tsai, J. L., Lin, S. D., Lai, C. S., and Chang, C. C.:** Accelerated growth and prolonged lifespan of adipose tissue-derived human mesenchymal stem cells in a medium using reduced calcium and antioxidants. Stem Cells Dev., **14**, 92–102 (2005).
30. **Tsuneto, M., Yamazaki, H., Yoshino, M., Yamada, T., and Hayashi, S.:** Ascorbic acid promotes osteoclastogenesis from embryonic stem cells. Biochem. Biophys. Res. Commun., **335**, 1239–1246 (2005).
31. **Ishikawa, S., Iwasaki, K., Komaki, M., and Ishikawa, I.:** Role of ascorbic acid in periodontal ligament cell differentiation. J. Periodontol., **75**, 709–716 (2004).
32. **Yamamoto, I., Muto, N., Murakami, K., and Akiyama, J.:** Collagen synthesis in human skin fibroblasts is stimulated by a stable form of ascorbate, 2-O-alpha-D-glucopyranosyl-L-ascorbic acid. J. Nutr., **122**, 871–877 (1992).
33. **Hata, R., Sunada, H., Arai, K., Sato, T., Ninomiya, Y., Nagai, Y., and Senoo, H.:** Regulation of collagen metabolism and cell growth by epidermal growth factor and ascorbate in cultured human skin fibroblasts. Eur. J. Biochem., **173**, 261–267 (1988).
34. **Chepda, T., Cadau, M., Girin, P. H., Frey, J., and Chamson, A.:** Monitoring of ascorbate at a constant rate in cell culture: effect on cell growth. In Vitro Cell Dev. Biol. Anim., **37**, 26–30 (2001).
35. **Wendt, M. D., Soparkar, C. N., Louie, K., Basinger, S. F., and Gross, R. L.:** Ascorbate stimulates type I and type III collagen in human Tenon's fibroblasts. J. Glaucoma., **6**, 402–407 (1997).
36. **Feng, J., Melcher, A. H., Brunette, D. M., and Moe, H. K.:** Determination of L-ascorbic acid levels in culture medium: concentrations in commercial media and maintenance of levels under conditions of organ culture. In Vitro, **13**, 91–99 (1977).
37. **Hata, R. and Senoo, H.:** L-Ascorbic acid 2-phosphate stimulates collagen accumulation, cell proliferation, and formation of a three-dimensional tissue like substance by skin fibroblasts. J. Cell Physiol., **138**, 8–16 (1989).
38. **Senoo, H. and Hata, R.:** Extracellular matrix regulates and L-ascorbic acid 2-phosphate further modulates morphology, proliferation, and collagen synthesis of perisinusoidal stellate cells. Biochem. Biophys. Res. Commun., **200**, 999–1006 (1994).
39. **Alcain, F. J. and Buron, M. I.:** Ascorbate on cell growth and differentiation. J. Bioenerg. Biomembr., **26**, 393–398 (1994).
40. **Duarte, T. L. and Lunec, J.:** When is an antioxidant not an antioxidant? A review of novel actions and reactions of vitamin C. Free Radic. Res., **39**, 671–686 (2005).
41. **Clement, M. V., Ramalingam, J., Long, L. H., and Halliwell, B.:** The *in vitro* cytotoxicity of ascorbate depends on the culture medium used to perform the assay and involves hydrogen peroxide. Antioxid. Redox Signal, **3**, 157–163 (2001).
42. **De Laurenzi, V., Melino, G., Savini, I., Annicchiarico-Petruzzelli, M., Finazzi-Agro, A., and Avigliano, L.:** Cell death by oxidative stress and ascorbic acid regeneration in human neuroectodermal cell lines. Eur. J. Cancer, **31A**, 463–466 (1995).
43. **Lee, W. C., Rubin, J. P., and Marra, K. G.:** Regulation of alpha-smooth muscle actin protein expression in adipose-derived stem cells. Cells Tissues Organs, **183**, 80–86 (2006).
44. **Peled, A., Zipori, D., Abramsky, O., Ovadia H., and Shezen, E.:** Expression of alpha-smooth muscle actin in murine bone marrow stromal cells. Blood, **78**, 304–309 (1991).
45. **Galmiche, M. C., Koteliansky, V. E., Briere, J., Herve P., and Charbord, P.:** Stromal cells from human long-term marrow cultures are mesenchymal cells that differentiate following a vascular smooth muscle differentiation pathway. Blood, **82**, 66–76 (1993).
46. **Kinner, B., Zaleskas, J. M., and Spector, M.:** Regulation of smooth muscle actin expression and contraction in adult human mesenchymal stem cells. Exp. Cell Res., **278**, 72–83 (2002).
47. **Bishun, N., Basu, T. K., Metcalfe, S., and Williams, D. C.:** The effect of ascorbic acid (vitamin C) on two tumor cell lines in culture. Oncology, **35**, 160–162 (1978).
48. **Bram, S., Froussard, P., Guichard, M., Jasmin, C., Augery, Y., Sinoussi-Barre, F., and Wray, W.:** Vitamin C preferential toxicity for malignant melanoma cells. Nature, **284**, 629–631 (1980).
49. **Leung, P. Y., Miyashita, K., Young, M., and Tsao, C. S.:** Cytotoxic effect of ascorbate and its derivatives on cultured malignant and nonmalignant cell lines. Anticancer Res., **13**, 475–480 (1993).
50. **Liotti, F. S., Menghini, A. R., Guerrieri, P., Talesa, V., and Bodo, M.:** Effects of ascorbic and dehydroascorbic acid on the multiplication of tumor ascites cells *in vitro*. J. Cancer Res. Clin. Oncol., **108**, 230–232 (1984).
51. **Liotti, F. S., Bodo, M., Menghini, A. R., Guerrieri, P., and Pezzetti, F.:** Antagonism between catalase and ascorbic acid in control of normal and neoplastic cell multiplication. Cancer Lett., **33**, 99–106 (1986).
52. **Malicev, E., Woyniak, G., Knezevic, M., Radosavljevic, D., and Jeras, M.:** Vitamin C induced apoptosis in human articular chondrocytes. Pflugers Arch., **440**, R46–R48 (2000).
53. **Gardiner, N. S. and Duncan, J. R.:** Enhanced prostaglandin synthesis as a mechanism for inhibition of melanoma cell growth by ascorbic acid. Prostaglandins Leukot. Essent. Fatty Acids, **34**, 119–126 (1988).