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Collagen synthesis is required for ascorbic acid-enhanced differentiation of mouse embryonic stem cells into cardiomyocytes

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Abstract

Ascorbic acid has been reported to promote the differentiation of embryonic stem (ES) cells into cardiomyocytes; however, the specific functions of ascorbic acid have not been defined. A stable form of ascorbic acid, namely, L-ascorbic acid 2-phosphate (A2-P), significantly enhanced cardiac differentiation; this was assessed by spontaneous beating of cardiomyocytes and expression of cardiac-specific markers obtained from mouse ES cells. This effect of ascorbic acid was observed only when A2-P was present during the early phase of differentiation. Treatment with two types of collagen synthesis inhibitors, L-2-azetidine carboxylic acid and *cis*-4-hydroxy-D-proline, significantly inhibited the A2-P-enhanced cardiac differentiation, whereas treatment with the antioxidant *N*-acetyl cysteine showed no effect. These findings demonstrated that ascorbic acid enhances differentiation of ES cells into cardiomyocytes through collagen synthesis and suggest its potential in the modification of cardiac differentiation of ES cells.

Keywords: Ascorbic acid; Differentiation; Extracellular matrix; Myocardium; Stem cell

The generation of various differentiated cells from pluripotent embryonic stem (ES) cells, which is a renewable resource, provides insights for studying not only the mechanism of early development in vitro but also cell transplantation therapy. Among many specialized cells in adults, the cardiomyocytes are terminally differentiated and have only limited regenerative capacity after injuries such as myocardial infarctions and heart failure [1]. Thus, the transplantation of functional cardiomyocytes into the damaged myocardium would have therapeutic potential. Recent studies have demonstrated that human ES cells can differentiate into cardiomyocytes in vitro with structural and functional properties [2–6]; this suggests that the property can be used in a new therapeutic approach and will have considerable potential in treating cardiovascular diseases. However, at present, a specific protocol for the generation of cardiomyocytes from ES cells has not been established.

Since many years, ascorbic acid has been used as an antiscorbutic agent for the integrity of connective tissue [7]. It has been shown to possess several important properties. It serves as an antioxidant and as an essential cofactor in mammalian enzymatic reactions, such as synthesis of collagen, carnitine, and norepinephrine [8]. Recently, ascorbic acid has been reported to enhance the differentiation of ES cells into cardiomyocytes [9]; however, the mechanism underlying this enhancing effect remains unclear. To explore the specific functions of ascorbic acid during cardiac differentiation of ES cells, we focused on the role of collagen synthesis by ascorbic acid because the extracellular matrix (ECM) could influence the cardiac differentiation of ES cells [10]. In the present study, we examined the effect of L-ascorbic acid 2-phosphate (A2-P)—a stable form of ascorbic acid [11]—on the differentiation of mouse ES cells into cardiomyocytes and demonstrated

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that A2-P treatment enhanced cardiac differentiation through collagen synthesis.

Materials and methods

Cell culture and reagents. The ES cell line ST-1 was used in the present study; this cell line was originally established from the blastocyst of a BALB/c mouse strain, and it was transmitted through germline in the chimeric mice. ES cells were grown on mitomycin C-inactivated feeder layer of primary cultures of mouse embryonic fibroblasts to maintain them in an undifferentiated state in Dulbecco's modified Eagle's medium (DMEM: Invitrogen, Carlsbad, CA) containing 20% fetal bovine serum (FBS: Hyclone, Logan, UT), 1 mM sodium pyruvate (Invitrogen), 1 mM nonessential amino acid (NEAA: Invitrogen), 100 μM 2-mercaptoethanol (Sigma, St. Louis, MO), and 10^3 U/mL leukemia inhibitory factor (LIF: Chemicon, Temecula, CA) [12].

A2-P was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). L-2-Azetidine carboxylic acid (AzC), *cis*-4-hydroxy-D-proline (CIS), and *N*-acetyl-L-cysteine (NAC) were purchased from Sigma. The remaining reagents were obtained from Sigma unless otherwise stated.

Induction of cardiac differentiation of ES cells. The ES cells were spontaneously differentiated into beating cardiomyocytes as described previously [12,13]. In brief, the cells were dissociated with 0.25% trypsin, 1% chicken serum (Invitrogen), and 1 mM EDTA in phosphate-buffered saline (PBS). The cells were resuspended in Iscove's modified Dulbecco's medium (IMDM: Invitrogen) containing 20% FBS, 1 mM sodium pyruvate, 1 mM NEAA, and 100 μM 2-mercaptoethanol without LIF, and a hanging drop was prepared at a concentration of 1000 cells per 50 μL drop. The hanging drop was cultured in an atmosphere of 5% CO_2 at 37 °C for 5 days. An individual 5-day-old embryoid body (EB) was cultured in each well of a 48-well culture plate. The emergence frequency of contracting cells in the EB outgrowths that indicate cardiac differentiation was monitored daily. The percentage of beating EBs, beating area (mm^2), and percentage of beating area were assessed using NIH image software (Bethesda, MD).

Cell viability assay. Cell viability was assessed using CytoTox nonradioactive cytotoxicity assay kit (Promega Corporation, Madison, WI) according to the manufacturer's instructions.

Collagen assay. Collagen content was measured using a colorimetric method described by Leon and Rojkind [14] by means of a collagen stain kit (Collagen Research Center, Tokyo, Japan). In brief, the cells were incubated for 30 min at room temperature with a saturated solution containing a green dye that stains noncollagenous proteins and a sirius red dye that specifically stains collagen. The presence of collagen (red) and noncollagenous protein (green) was observed using a fluorescence microscope (IX-70, Olympus, Tokyo, Japan). The amount of collagen was calculated using a formula described previously [14].

Immunocytochemical staining. Immunocytochemistry for the detection of cardiac troponin I (cTnI) was carried out as described previously [15]. The cells were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) and incubated with the primary antibody against cTnI (diluted 1:1000; Biogenesis Ltd., England, UK) for 2 h at room temperature. Cy3-labeled goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) was used as the secondary antibody. Irrelevant mouse IgG (Vector Laboratories Inc., CA) was used as a negative control. The cells were incubated with 4′, 6-diamidino-2-phenylindole (DAPI: Wako Pure Chemical Industries Ltd.) for nucleic acid staining and observed using a fluorescence microscope (IX-70, Olympus).

Reverse transcription-polymerase chain reaction. Reverse transcription-polymerase chain reaction (RT-PCR) analysis was performed as described previously [16]. In brief, total RNA from the outgrowths of the EBs was extracted using Isogen (Wako Pure Chemical Industries) according to the manufacturer's instructions. Single stranded complementary DNA was synthesized from 1 μg total RNA by using SuperScript I First-strand Synthesis System (Invitrogen). The PCR was performed using a *Taq* PCR kit (Qiagen, Hilden, Germany). The following primers specific for mouse cardiac genes (oligonucleotide sequences are given in parentheses in the order of antisense and sense primer followed by the annealing temperature, cycles used for PCR, and length of the amplified fragment) were used: Nkx2.5 (5′-CAGTGGAGCTGGACAAAGCC and 5′-TAGC GACGGTTCTGGAACCA; 58 °C; 30 cycles; 647 bp), GATA binding protein 4 (GATA-4, 5′-TGAAGAGATGCGCCCCATCAA and 5′-AG CTGTCCCCACAAGGCTAT; 60 °C; 25 cycles; 1704 bp), atrial myosin light chain (MLC-2A, 5′-CAGACCTGAAGGAGACCT and 5′-GTCA GCGTAAACAGTTGC; 58 °C; 30 cycles; 196 bp), ventricular myosin light chain (MLC-2V, 5′-GCCAAGAAGCGGATAGAAGG and 5′-CT GTGGTTCAGGGCTCAGTC; 60 °C; 25 cycles; 83 bp), α-myosin heavy chain (α-MHC, 5′-CTGCTGGAGAGGTTATTCCTCG and 5′-GGAAG AGTGAGCGGCGCATCAAGG; 60 °C; 25 cycles; 302 bp), glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 5′-TTCAACGGCACAGT CAAGG and 5′-CATGGACTGTGGTCATGAG; 60 °C; 30 cycles; 297 bp).

Statistical analysis. Data are expressed as means ± SD. For comparisons between multiple groups, we determined the significance of difference between the group means by one-way analysis of variance (ANOVA). All analyses were performed using the StatView software (Abacus Concepts, Inc., Berkeley, CA). The differences with values of $p < 0.05$ were considered to be statistically significant.

Results

Effect of A2-P on cardiac differentiation

ST-1 cells are pluripotent ES cells with a typical morphology. The initiation of cardiac differentiation was indicated by EB formation. The EBs adhered to the plates and continued to proliferate and then differentiated into beating cardiomyocytes. We first examined the effect of A2-P on the differentiation of ES cells into cardiomyocytes. On day 3 of the A2-P (1–100 μM) treatment, the percentage of EBs containing beating areas showed a significant increase in a dose-dependent manner (Fig. 1A). On days 5 and 7, this percentage in the cells treated with 100 μM A2-P remained significantly higher than that in the untreated cells. We further assessed other parameters (beating area and percentage of beating area) of cardiac differentiation and showed that these parameters also significantly increased by the A2-P treatment on day 7 (Figs. 1B and C).

We next assessed the period during which ascorbic acid is required for differentiation. A2-P treatment during the entire differentiation period (production of EBs and their culture after plating) led to a significant increase in cardiac differentiation compared to the control EBs, whereas A2-P treatment during the culture after plating showed no effect on cardiac differentiation (Fig. 1D). Furthermore, the level of cardiac differentiation in the cells treated with A2-P during the production of EBs was almost equivalent to that in the cells treated during the entire differentiation period. These findings indicated that ascorbic acid affected the early phase of cardiac differentiation of ES cells.

Effects of collagen synthesis inhibitors and an antioxidant

Since the favorable effects of ascorbic acid are attributed to its antioxidative and collagen synthesis properties [8], we investigated the mechanism by which ascorbic acid-enhanced cardiac differentiation of ES cells by using the

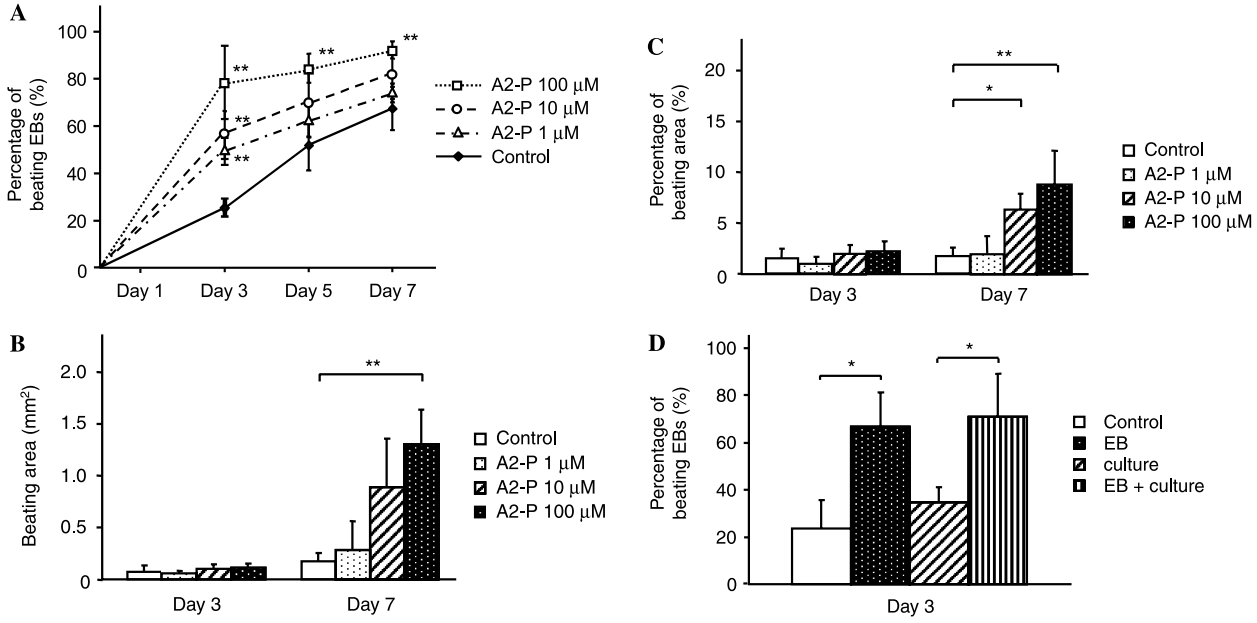

Fig. 1. Effect of A2-P on cardiac differentiation. The cardiac differentiation of mouse ES cells in the presence or absence of A2-P at the indicated concentrations. The emergence frequency of contracting cells was monitored daily. Percentage of beating EBs (A), beating area (B), and percentage of beating area (C) were assessed. (D) A2-P (100 μM) was added to the culture media during entire differentiation period (EB + culture), production of EB (EB), or during the culture after plating (culture). Data are expressed as means ± SD ($n = 3$–4). $^{*}p < 0.05$ and $^{**}p < 0.01$.

antioxidant NAC and two types of collagen synthesis inhibitors, namely, AzC and CIS [11]. As shown in Fig. 2A, NAC (100 and 1000 μM) treatment could not mimic the effect of A2-P on cardiac differentiation. In contrast, AzC (100 and 300 μM) treatment significantly inhibited A2-P-enhanced cardiac differentiation during days 3–10 (Figs. 2B and C). In addition, AzC treatment inhibited cardiac differentiation even in A2-P-untreated cells. Another collagen synthesis inhibitor CIS (100 and 300 μM) also inhibited A2-P-enhanced cardiac differentiation (Fig. 2D). Cell viability assessed by lactate dehydrogenase (LDH) release showed that NAC, AzC, and CIS had no cytotoxic effects at these concentrations (Fig. 2E). Similar results were obtained using trypan blue exclusion (data not shown).

Collagen synthesis and cardiac marker expression

To examine whether AzC actually inhibits collagen synthesis in ES cells, collagen was stained and quantified. As shown in Fig. 3, A2-P treatment significantly increased collagen production, and this increased production was significantly inhibited by AzC (100 and 300 μM) treatment.

We further confirmed the expression of various cardiac markers in the A2-P-treated cells by RT-PCR and immunostaining. The RT-PCR analysis revealed that A2-P increased the mRNA expression of Nkx2.5, GATA-4, MLC-2V, MLC-2A, and α-MHC (Fig. 4A), and this expression was inhibited by AzC (100 and 300 μM) treatment. Similar to the mRNA expression pattern, immunostaining showed that A2-P increased the number of cTnI-expressing cells, and this increase was inhibited by AzC treatment (Fig. 4B).

Discussion

The major findings of this study are (1) A2-P, a long-acting ascorbic acid, significantly enhanced cardiac differentiation of ES cells, and this effect of A2-P was observed when A2-P was added during the early phase of EB culture; (2) treatment with the antioxidant NAC failed to mimic the effect of ascorbic acid on cardiac differentiation; (3) two distinct inhibitors of collagen synthesis, i.e., AzC and CIS, significantly inhibited A2-P-enhanced cardiac differentiation. The findings obtained from this study provide a new insight into the mechanism of cardiac differentiation of ES cells and the generation of cardiomyocytes for future clinical treatment.

Ascorbic acid (vitamin C) is widely known for its role as an essential nutrient in guinea pigs and primates. The deficiency of ascorbic acid in these species leads to a pathological condition known as scurvy. Ascorbic acid also has multiple biological activities; it serves as a water-soluble antioxidant and a cofactor in the synthesis of collagen, carnitine, and norepinephrine [8]. Furthermore, recent studies have shown that ascorbic acid can regulate the differentiation of ES cells into some types of cells. Lee et al. [17] reported that ascorbic acid enhances the differentiation of ES cells into neurons. Tsuneto et al. [18] showed that ascorbic acid promotes osteoclastogenesis of ES cells via increasing the number of Flk-1-positive cells. In regard to cardiac differentiation, Takahashi et al. [9] recently screened a broad range of compounds to identify a chemical that stimulates cardiac differentiation of ES cells and found that ascorbic acid markedly increases the efficiency of cardiac differentiation. Passier et al. [19] demonstrated that the

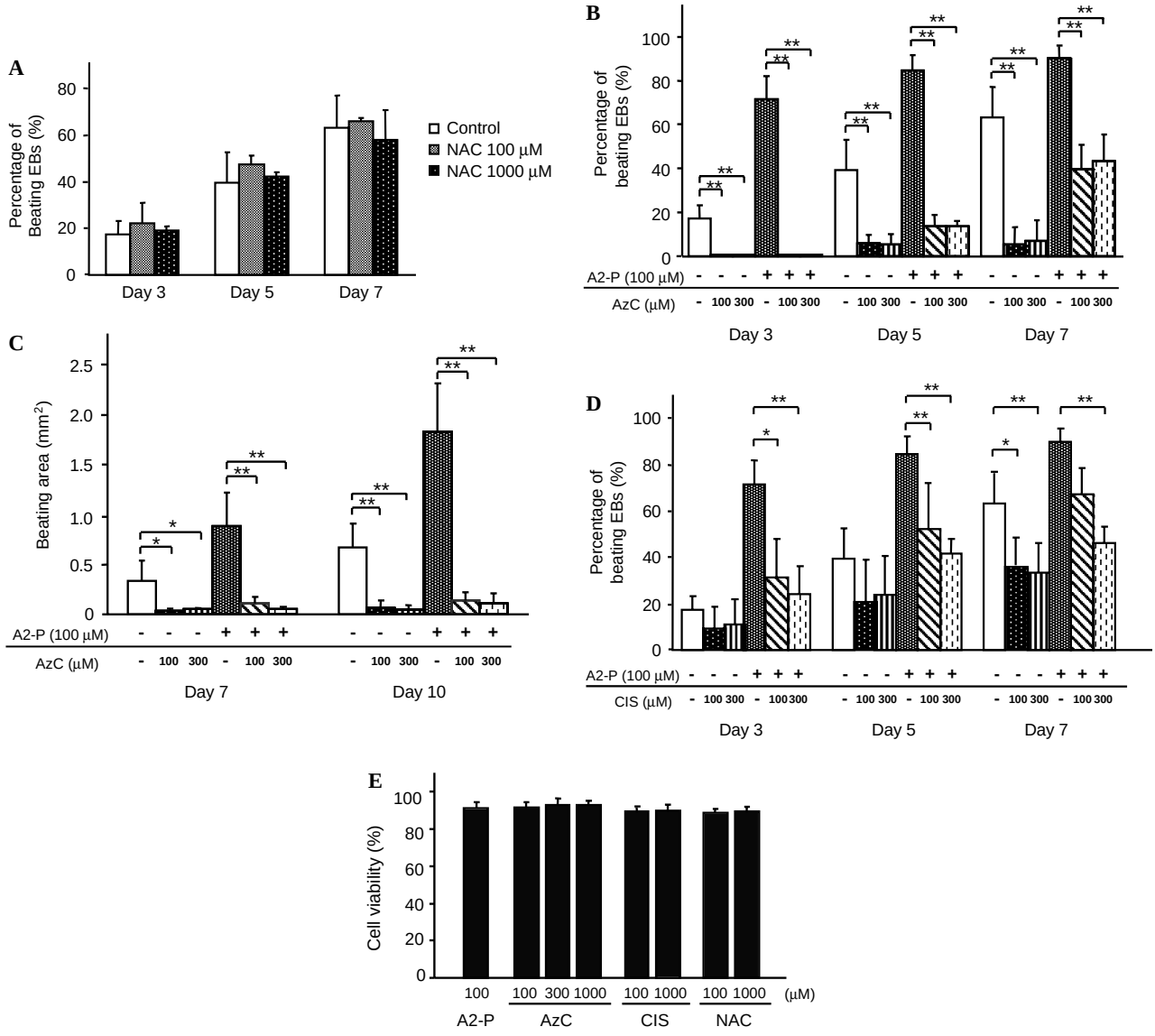

Fig. 2. Effects of antioxidants and collagen-synthesis inhibitors. A2-P (100 μM)-enhanced cardiac differentiation in the presence or absence of NAC (A), AzC (B,C), or CIS (D) at the indicated concentrations. The emergence frequency of contracting cells was monitored daily. Percentage of beating EBs (A, B, and C), beating area (C), and cell viability (E) was assessed. Data are expressed as means ± SD ($n = 3$–10). $^{*}p < 0.05$ and $^{**}p < 0.01$.

addition of ascorbic acid increased the number of beating areas in human ES cells. Consistent with these reports, we also observed that ascorbic acid significantly increased cardiac differentiation of ES cells in a dose-dependent manner. We further observed that A2-P, a long-acting form of ascorbic acid, was more effective than the usual form of ascorbic acid (data not shown), thereby suggesting the potential use of A2-P in the generation of cardiomyocytes from ES cells.

Interestingly, it was observed that, to achieve efficient cardiac differentiation, ascorbic acid needs to be added during the early phase of differentiation and not during the late phase. This finding indicates that ascorbic acid influences the initiation of cardiac differentiation of ES cells. This finding was supported by Tsuneto et al. [18], who reported that ascorbic acid promotes stronger osteoclastogenesis in ES cells when it was added during the first 4 days than during the last 4 days. Since both cardiomyocytes and osteoclasts are derived from mesodermal cells, these findings suggest that ascorbic acid might affect the initiation of mesodermal development.

The mechanism of ascorbic acid-enhanced cardiac differentiation remains unknown. In the present study, we clearly showed that ascorbic acid-induced collagen synthesis was required for the cardiac differentiation of ES cells. This finding was supported by Baharvand et al. [10], who recently reported that ES-derived cardiomyocytes cultured on ECM secreted from fibroblasts matured more rapidly, suggesting that the matrix components are involved in cardiac differentiation and cardiomyocyte growth. Cardiomyocytes are surrounded by a basement membrane consisting of collagen type VI, laminin, fibronectin, and

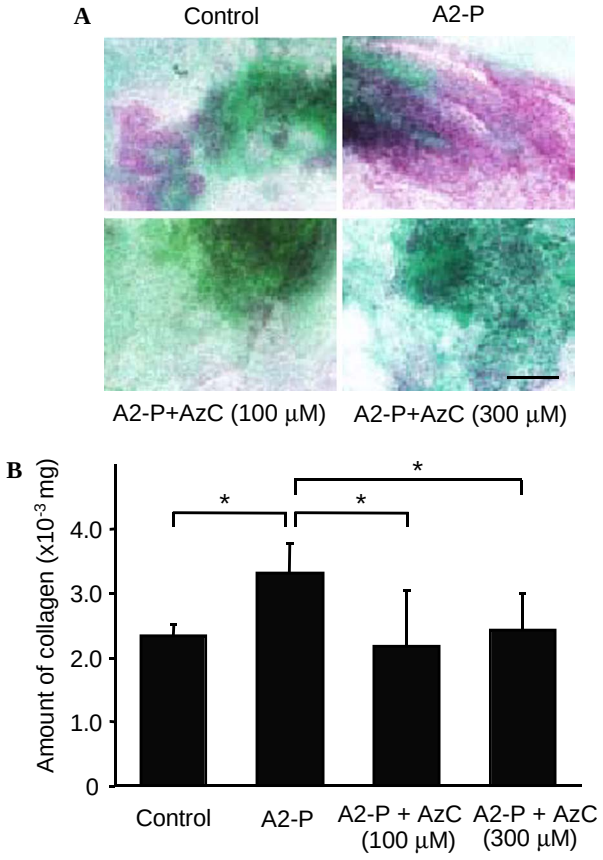

Fig. 3. Collagen synthesis. A2-P (100 μM)-enhanced cardiac differentiation in the presence or absence of AzC at the indicated concentrations. Collagen was stained (A) (collagen: red, noncollagenous proteins: green) and quantified. The bar indicates 200 μm. (B) The results of four independent experiments are shown. Data are expressed as means ± SD ($n = 3$). $^{*}p < 0.05$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

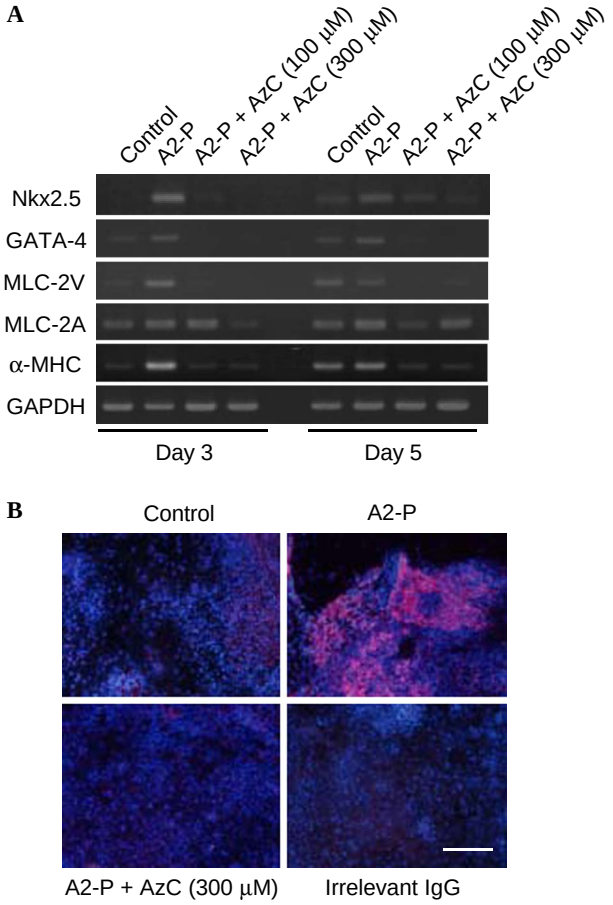

Fig. 4. Expression of cardiac marker mRNAs and proteins. A2-P (100 μM)-enhanced cardiac differentiation in the presence or absence of AzC at the indicated concentrations. (A) Total RNA was extracted and analyzed for the presence of Nkx2.5, GATA-4, MLC-2V, MLC-2A, α-MHC, and GAPDH by RT-PCR analysis. GAPDH was used as internal controls. (B) Immunocytochemical analysis for the detection of cTnI (red: cTnI expression, blue: DAPI). The results of three independent experiments are shown. The bar indicates 200 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

several proteoglycans; these are normally synthesized by the cardiomyocytes. The extracellular space in the heart is occupied by many other molecules; the predominant molecules are interstitial collagen type I and III [20]. Thus, collagen and other ECM proteins may constitute the "niche" in which cardiomyocytes exist. In fact, it has been reported that the phenotypes of cardiomyocytes are modified based on the composition and orientation of the ECM [21]. Furthermore, several lines of evidence indicate that ECM plays a role in the regulation of cardiac differentiation of ES cells. Studies on β1-integrin-deficient ES cells revealed retardation of cardiac differentiation; this was demonstrated by the delayed expression of cardiac-specific genes and action potentials [22]. Since the ECM predominantly transmits signals via the integrins, some types of ECMs such as collagen might be necessary for cardiac differentiation of ES cells. Further investigations are required for understanding the role of ECM in cardiac differentiation of ES cells.

In conclusion, we demonstrated that ascorbic acid enhances the differentiation of ES cells into cardiomyocytes through the upregulation of collagen synthesis. In particular, the early phase of cardiac differentiation was preferentially enhanced by ascorbic acid. These findings suggest the potential of ascorbic acid in the modification of cardiac differentiation of ES cells and provide new insights into the mechanisms involved in this process.

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