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Icariin-mediated modulation of cell cycle and p53 during cardiomyocyte differentiation in embryonic stem cells

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Abstract

The aim of this study was to investigate the possible inducible effects and to clarify the modulation by icariin of cell cycle and p53 expression in the differentiation of embryonic stem cells into cardiomyocytes in vitro. Embryonic stem cells were cultivated as embryoid bodies in hanging drops and induced to differentiate into cardiomyocytes by icariin at 10^{-7} M. Cardiomyocytes were characterized by the expression of sarcomeric proteins, α-actinin and cardiac troponin T, by immunocytochemistry. Flow cytometry revealed that 10^{-7} M icariin treatment for 48 h significantly induced the accumulation of cells in G0/G1 and reduced the proportion of cells in S phase. A marked increase in apoptosis rate was observed 48 h after icariin treatment. Icariin resulted in significantly increased expressions of p53 mRNA and protein, as determined by reverse transcription-polymerase chain reaction and Western blot analysis. During day 7+0 and 7+9 cardiac developmental stage, 10^{-7} M icariin increased the level of p53 mRNA, but caused a parallel decrease in the level of p53 protein. In conclusion, icariin at 10^{-7} M facilitated the directional differentiation of embryonic stem cells into cardiomyocytes. Results showed p53 to be an important regulator in the differentiation in embryonic stem cells treated with 10^{-7} M icariin, controlling or adjusting the balance between differentiated cells and cells undergoing apoptosis.

Keywords: Embryonic stem cells; Directional differentiation; Embryoid body; Cardiomyocyte; Icariin; Sarcomere; Cell cycle; Apoptosis; p53

1. Introduction

Embryonic growth and development entail differentiation, growth, apoptosis and morphogenesis in a highly coordinated and controlled manner. A vast spectrum of growth regulatory gene products is involved in this complex processing of signals, and many are tissue and cell-type specific. Embryonic stem cells are pluripotent cells derived from the inner cell mass of the pre-implantation blastocyst (Evans and Kaufman, 1981). These cells can be induced to differentiate into multiple cell types, including to form spontaneously beating cardiomyocytes, under certain conditions in vitro (Doestschman et al., 1985; Bradley, 1990; Robbins et al., 1992). During differentiation in vitro, cardiac-specific genes as well as proteins, receptors, and ion channels are expressed in a developmentally controlled manner, which closely recapitulates the developmental pattern of early cardiogenesis (Hescheler et al., 1997). Embryonic stem cells proliferate very actively, the S phase of their mitotic cycle is largely predominant, and the G1 phase is not longer than 1.5 h (Ludmila et al., 2002). The control of the embryonic stem cell mitotic cycle displays unusual features, and regulation of the cell cycle is critical in maintaining the transition toward differentiation (Rohwedel et al., 1996; Ludmila et al., 2002). Apoptotic signals are necessary to trigger embryonic stem cell differentiation (Kanaga et al., 1997; Sarkar and Sharma, 2002). The tumor suppressor p53 regulates cellular proliferation, differentiation, and apoptosis (Gottlieb and Oren, 1996; Ko and Prives, 1996). The processes by which embryonic stem cells differentiate into cardiomyocytes involve molecular biological activities and signal transduction, and provide the opportunity for exploring the effects

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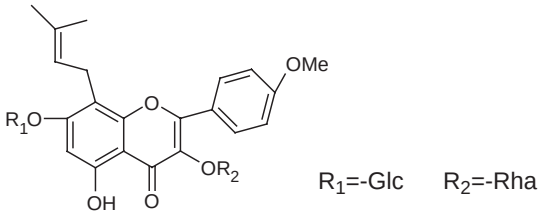


Fig. 1. Molecule structure of icariin.

of cardioactive drugs on differentiation and for detecting specific or unique targets of drug action. Furthermore, drugs interfering with the differentiation of embryonic stem cells into cardiomyocytes offer the chance to develop new treatment strategies for cardiac repair and cardiac tissue regeneration (Hassink et al., 2004).

Up to now, there have been few reports on the interfering effects of drugs on the directional differentiation of embryonic stem cells in vitro. Icariin (Fig. 1) is a constituent of *Epimedium*, a traditional Chinese herbal medicine. It possesses many kinds of biological actions, improving cardiovascular function, hormone regulation, immunological function modulation, and anti-tumor activity (He et al., 1995). Our former work has shown that icariin can be metabolized to icaritin and desmethylicaritin by human intestinal bacteria in vitro (Liu and Lou, 2004). Furthermore, icaritin and desmethylicaritin, but not icariin, exert estrogen-like activity in the modified MCF-7 cell proliferation assay (Wang and Lou, 2004). It has been reported that icariin induces the differentiation of HL-60 cells (Zhao et al., 1997a,b). In addition, icariin is a major active component of the tablets of "Xin-shen-ning" used for the treatment of heart disease (Zhang et al., 2000). However, the pharmacological effect and mechanism of action of icariin on the cardiovascular system are not yet known.

In this paper, the specific properties of embryonic stem cells and classic methods of cultures were used to confirm the inducible effects of icariin on the differentiation of embryonic stem cells into cardiomyocytes in vitro. The cardiomyocytes derived from embryonic stem cells were identified by immunocytochemistry. The possible mechanisms of the inducible effects of icariin on the modulation of cell cycle and p53 expression in the early differentiation phase were investigated by flow cytometry, reverse transcription-polymerase chain reaction (RT-PCR) and Western blot analysis.

2. Materials and methods

2.1. Embryonic stem cell culture and icariin treatment

The permanent embryonic stem cell line D3 (American Type Culture Collection, CRL-1934) (Doestschman et al., 1985) was cultivated in undifferentiated state on primary cultures of mouse embryonic fibroblasts in Dulbecco's modified Eagle's minimal essential medium (DMEM, Gibco BRL, Life Technologies, Germany), supplemented with 10% fetal calf serum (Gibco), 0.1 mM β-mercaptoethanol (Sigma), non-essential amino acids (Hyclone, stock solution diluted 1:100) and 10^6 units/l recombinant mouse leukemia inhibitory factor (Chemicon).

Cultures of differentiating embryonic stem cells were established by the formation of embryoid bodies in hanging drop cultures (Metzger et al., 1995; Scholz et al., 1999) with differentiation medium, which consisted of DMEM, 20% fetal calf serum, 0.1 mM β-mercaptoethanol, non-essential amino acids, without recombinant mouse leukemia inhibitory factor. Drops (30 μl) containing about 600 embryonic stem cells were placed on the lids of Petri dishes (Falcon) filled with D-Hanks solution, and then cultivated in hanging drops for 3 days and another 2 days in Petri dishes. On day 5, embryoid bodies were plated separately onto gelatin-coated 24-well culture plates in the above-mentioned differentiation medium. At this time, icariin (Drug Biology Product Examination Bureau, China, Batch number 0737-200011, purity: 99%) was added to the medium in concentrations of 10^{-7}, 10^{-8}, 10^{-9} M, according to preliminary test results. Embryonic stem cells treated with 10^{-8} M retinoic acid (Sigma) or with 0.1% dimethyl sulfoxide (DMSO) solvent were used as positive or negative controls.

In the experiment, day 1 referred to the day of dissociation of embryonic stem cells from mouse embryonic fibroblasts, and the initiation of differentiation by the formation of embryoid bodies. Differentiation cultures were monitored every day with light microscopy to record the morphology of the spontaneously beating embryoid bodies, the day when contraction started, and the duration of contraction from day 7. According to the percentage of beating embryoid bodies, the inducible effect of icariin on the differentiation of embryonic stem cells into cardiomyocytes was evaluated by means of a time–effect relationship curve. The rhythmically beating embryoid bodies were considered to be spontaneously beating cardiomyocytes, which was defined as a marker of successful differentiation (Metzger et al., 1995; Scholz et al., 1999).

2.2. Preparation of single cardiomyocytes and immunocytochemistry

Single cardiomyocytes were isolated from embryoid bodies by a modified procedure of Isenberg and Klockner, as described by (Maltsev et al., 1993, 1994). For immunostaining, cells were rinsed two times with phosphate-buffered saline solution (PBS) and fixed with cold acetone for 10 min. After treatment with goat serum for 30 min, specimens were incubated at 4 °C overnight together with the primary antibody, monoclonal anti-sarcomeric α-actinin (clone number EA-53, 1:200 dilution, Sigma) or monoclonal anti-troponin T (clone number JLT-12, 1:100 dilution, Sigma). After being washed with PBS three times, the specimens were incubated with the fluorescent antibody fluorescein isothiocyanate isomer I-conjugated $F(ab)_2$ frag-

ment of affinity-purified goat anti-mouse IgG (1:1000 dilution, Rockland) for 1.5 h 37 °C. Specimens were rinsed in PBS, mounted in 90% glycerol in PBS, coverslipped and examined using a fluorescence microscope (Leica DMIL, German).

For the semi-quantitative assessment of sarcomeric proteins, immunolabelled cardiomyocytes were counted and the number of positive-staining cardiomyocytes was estimated (Guan et al., 1999).

2.3. Quantification of apoptosis by direct counting of acridine orange/ethidium bromide-stained cells

For detecting apoptosis based on a change in morphology, 5×10^4 embryonic stem cells were grown on 24-well culture plates without mouse embryonic fibroblast feeders. Cells were incubated for 48 h with 10^{-7} M icariin, 10^{-8} M retinoic acid, and then stained with acridine orange and ethidium bromide for 5 min. Subsequently, visualization of the apoptotic morphology was accomplished by fluorescent microscopy using appropriate filters. Ethidium bromide was used to stain dead cells, emitting a red-orange fluorescence with a 488-nm laser excitation. At the same excitation wavelength, acridine orange emits a yellow-green fluorescence when bound to DNA (Cotter and Martin, 1996).

2.4. Analysis of cell cycle and apoptosis by flow cytometry

Exponentially growing embryonic stem cells were cultured without mouse embryonic fibroblast feeders and leukemia inhibitory factor, and then treated with 10^{-7}, 10^{-8}, 10^{-9} M icariin or 10^{-8} M retinoic acid. After 48 h, the cells were harvested and 1×10^6 cells were placed into a polypropylene tube and centrifuged. Then the supernatant was removed and 1 ml 4 °C 70% ethanol was added dropwise to the cell pellet with vortexing. The cells were kept at 4 °C until DNA was stained. Fixed cells were treated with RNase A in PBS for 1 h, followed by staining with 50 μg/ml propidium iodide in PBS. Flow cytometric analysis of cell cycle distribution and apoptosis was performed with a BD FACSCalibur with a 488-nm (blue) argon (Becton Dickinson, San Jose, CA). Data acquisition was performed with CellQuest 3.1 software and data were analyzed with ModFit LT 3.0 software (Variety Software House, Inc., Topsham, ME).

2.5. Detection of p53 mRNA expression by semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis

Embryonic stem cells were grown in the absence of fibroblast layers and leukemia inhibitory factor at a density of 5×10^5 and treated with 10^{-7}, 10^{-8}, 10^{-9} M icariin, 10^{-8} M retinoic acid or solvent for 24 h or 48 h, respectively. Total RNA was isolated from the above-mentioned samples and cardiomyocytes derived from embryoid bodies ($n=20$) induced by 10^{-7} M icariin, 10^{-8} M retinoic acid or solvent on day 7+0, day 7+5, day 7+9, using the Trizol reagent (Gibco, BRL) in accordance with the manufacturer's instructions. After extraction, mRNA was precipitated by recommended procedures and dissolved in 0.1% diethylpyrocarbonate solution. To synthesize first strand cDNA, 7 μl total RNA was incubated with 0.5 μg of oligo (dT) 6 primer (Sangon, China) and 5 μl deionized water at 65 °C for 15 min. Reverse transcription reactions of 20 μl were performed with 200 units of M-MuLV reverse transcriptase (Gibco BRL), 4 μl of 5× reaction buffer (250 mM Tris–HCl; pH 8.3 at 25 °C, 375 mM KCl, 15 mM MgCl2, 50 mM dithiothreitol) and 1 mM deoxynucleoside triphosphate (dNTP) mixture for 1 h at 42 °C. Polymerase chain reactions of 50 μl contained 1 μl of the RT reaction product, 5 μl of 10× PCR buffer (100 mM Tris–HCl; pH 8.3 at 25 °C, 500 mM KCl, 15 mM $MgCl_2$), 25 units Taq polymerase (Sangon, China), 1 μl of 10 mM dNTP mixture, and 30 pmol of each primer. The sense and antisense primers for β-actin and p53 were chosen by Primer 3.0 program, these were:

p53 (5′-AGTGGCGGTCCACTTACGAT-3′,5′-AGCTGTGAGGGCAAAATGGG-3′; 106 bp), the housekeeping gene β-actin was used as an internal standard: (5′-TGACGGGGTCACCCACACTGTGCCCATCTA-3′, 5′-CTAGAAGCATTTGCGGTGGACGATGGAGGG-3′; 660 bp).

For the semi-quantitative determination of the p53 mRNA level, the products of the reverse transcription reactions were denatured for 3 min at 94 °C, followed by 35 cycles (p53), and 30 cycles (β-actin) of amplification in the reaction with Ampli Taq DNA polymerase: 45 s denaturation at 94 °C, 40 s annealing at 56.1 °C (p53) or 55 °C (β-actin) and 45 s elongation at 72 °C. The PCR products were analyzed by 1.5% agarose gel electrophoresis, visualized with ethidium bromide staining, and then quantified using a bio-imaging analyzer (Bio-Rad, USA). The density of the products was quantitated using Quantity One version 4.2.2 software (Bio-Rad, USA).

2.6. Determination of p53 protein by Western blot

Embryonic stem cells were grown in the absence of fibroblast layers and leukemia inhibitory factor at a density of 5×10^5, then were treated with 10^{-7}, 10^{-8}, 10^{-9} M icariin, 10^{-8} M retinoic acid or solvent for 24 h or 48 h, respectively. Protein was isolated from the above-mentioned samples and cardiomyocytes derived from the embryoid bodies ($n=20$) induced by 10^{-7} M icariin, 10^{-8} M retinoic acid or solvent on day 7+0, day 7+5, day 7+9. Cells were washed with PBS, collected in RIPA buffer (containing 0.2% Triton X-100, 5 mM EDTA, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin) and lysed 30 min on ice. Samples were clarified by centrifugation for 30 min at

13,000 × *g* at 4 °C. An aliquot of 40 μg of the supernatant protein from each sample was heated with 4× sodium dodecyl sulfate (SDS) sample buffer at 95 °C for 5 min, and separated electrophoretically on a 12% SDS–polyacrylamide gel. Subsequently, proteins were transferred onto 0.45-μm pore size nitrocellulose membranes for 90 min and blocked overnight. Nitrocellulose membranes were then exposed to p53 primary antibody (Santa Cruz Biotechnology, USA) for 1 h in blocking buffer at 1:2000 dilution or actin primary antibody (Santa Cruz Biotechnology, USA) for 2 h in blocking buffer at 1:2000 dilution. Then the membranes were incubated with peroxidase-conjugated affinipure goat anti-rabbit IgG at 1:10,000 dilution for 0.5 h or peroxidase-conjugated affinipure rabbit anti-goat IgG at 1:10,000 dilution for 1.0 h, respectively. The proteins were visualized autoradiographically with an enhanced chemiluminescent substrate (ECL, Pierce, USA), and scanned using a bio-imaging analyzer (Bio-Rad, USA). The density of the products was quantitated using Quantity One version 4.2.2 software (Bio-Rad, USA).

2.7. Statistical analysis

All data are expressed as means ± standard deviation (S.D.). At least three independent experiments were done. Statistical significance was evaluated by one-way analysis of variance between groups with SPSS 10.0 for WINDOWS software. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Inducible effect of icariin on the directional differentiation of embryonic stem cells into cardiomyocytes

The embryonic stem cells grew in aggregates with a clear boundary and appeared ovoid or nodule (Fig. 2A). Fig. 2B shows the shape of embryonic stem cells without feeder layers. The attached culture was established by plating a single, day 5 embryoid body formed by hanging drop culture onto a 24-well plate and allowing continued cellular proliferation and differentiation (Fig. 2C: 2 days after plating). Within this multicellular arrangement in embryoid body outgrowths, cardiomyocytes appeared as spontaneously contracting round cell clusters. Each embryoid body contained one or more beating areas (Fig. 2D). An increase in size, strength of contraction and beat frequency was observed during further differentiation.

Embryonic stem cells were induced into rhythmically beating embryoid bodies by icariin in a concentration- and time-dependent manner. The effect of icariin at 10^{-7} M on the differentiation of embryonic stem cells into cardiomyocytes was marked in comparison with control. The percentage of differentiation cultures containing contracting embryoid bodies ($n = 175$) with 10^{-7} M icariin reached a peak level of 84% on day 7+9. Longer culture times (from day 7+12) resulted in cell death and a decrease in the percentage of beating embryoid bodies to 19% at terminal

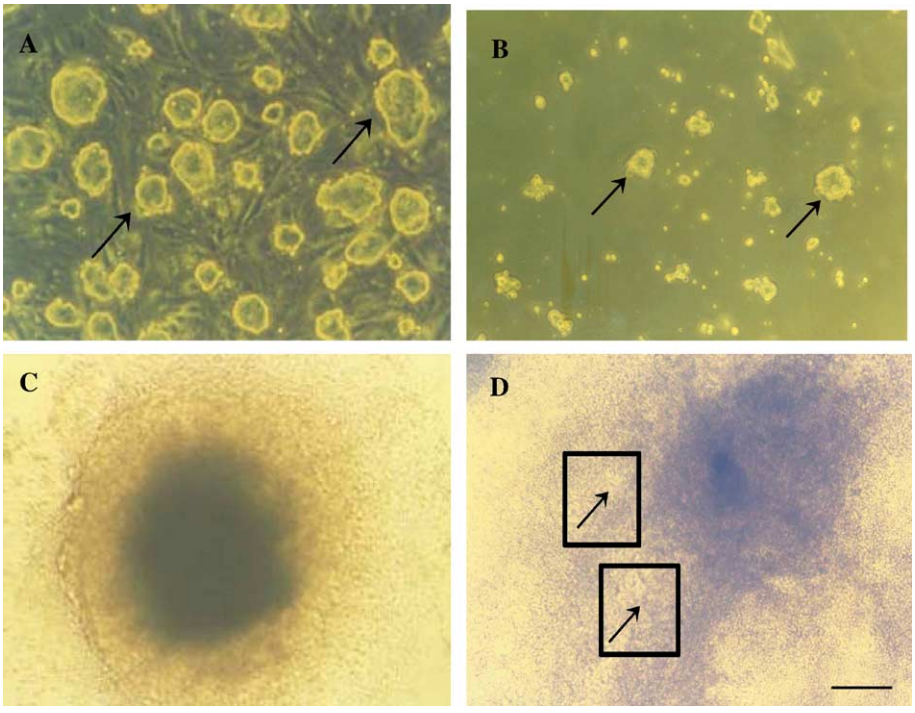


Fig. 2. Cell apparent morphological changes during the course of differentiation. (A) Colonies of embryonic stem cells on a feeder layer of murine embryonic fibroblasts (arrows: colonies of embryonic stem cells). (B) Colonies of embryonic stem cells without a feeder layer of murine embryonic fibroblasts. (C) Embryoid body within 2 days after plating onto a gelatinous layer. (D) Synchronously contracting, functional syncytium of cardiac clusters derived from an embryoid body treated with 10^{-7} M icariin on day 7+9 (rectangular frames: contracting areas ; arrows: contraction foci). Bar = 100 μm.

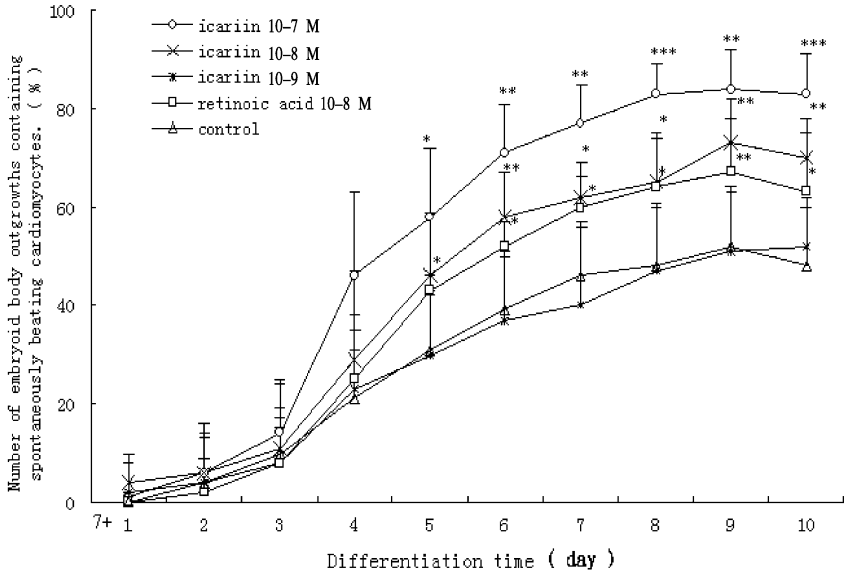


Fig. 3. Percentage of beating embryoid bodies. Embryoid bodies were treated with 10^{-7}, 10^{-8}, 10^{-9} M icariin, positive control 10^{-8} M retinoic acid or negative control. Embryoid bodies were plated on day 5 and the number of beating embryoid bodies was evaluated during cultivation from day 7+1 to day 7+11. $n=6$ independent experiments. Results are means±standard deviation (S.D.). *$P<0.05$, **$P<0.01$, ***$P<0.001$ vs. control. ○: embryoid bodies ($n=175$) treated with 10^{-7} M icariin; ×: embryoid bodies ($n=96$) treated with 10^{-8} M icariin; *: embryoid bodies ($n=90$) treated with 10^{-9} M icariin; □: embryoid bodies ($n=186$) treated with 10^{-8} M retinoic acid ; △: embryoid bodies ($n=200$) treated with control medium.

stage (data not shown). In the control only about 50% of the beating embryoid bodies ($n=200$) were found on day 7+9. The potential of embryoid bodies treated with 10^{-7} M or 10^{-8} M icariin to undergo cardiac differentiation was significantly enhanced in comparison with those in control cultures over a period from day 7+5 ($P<0.05$). Treatment of embryoid bodies ($n=186$) with 10^{-8} M retinoic acid also resulted in a remarkable increase in the number of embryoid bodies with spontaneously beating cardiomyocytes from day 7+5 ($P<0.05$) (Fig. 3).

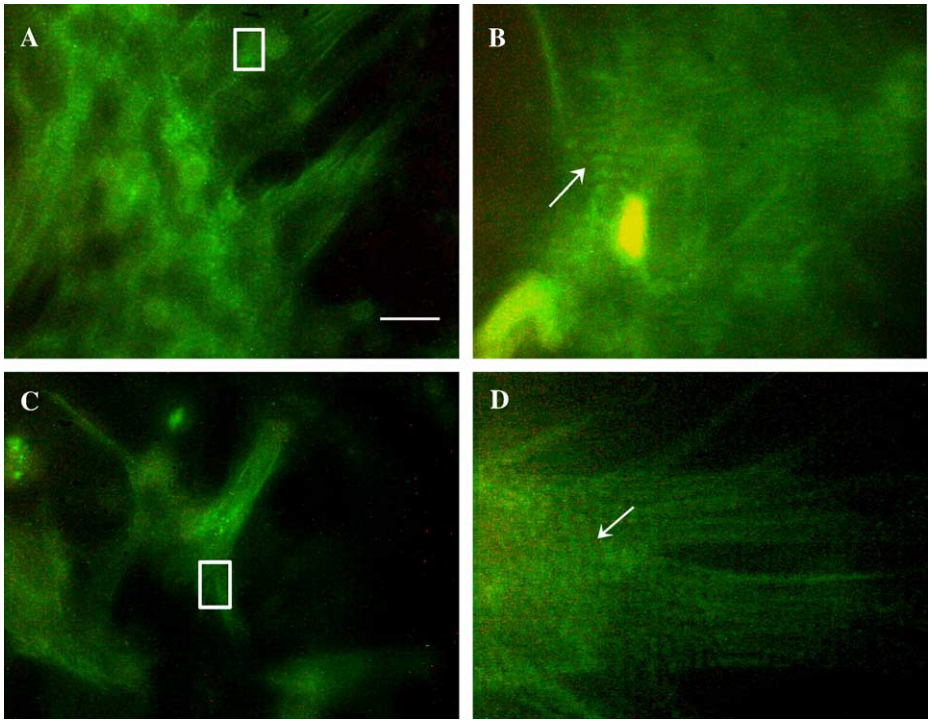


Fig. 4. Positive immunostaining of dispersed cardiomyocytes from beating embryoid bodies treated with 10^{-7} M icariin on day 7+11. The cardiomyocytes were identified by the expression of cardiac-specific sarcomeres. (A) Positive staining with anti-sarcomeric α-actinin mAb. The rectangular frame indicates the part magnified in panel B. (B) Higher magnification of sarcomeric α-actinin staining (arrow). (C) Positive staining with anti-cardiac-troponin T mAb. The rectangular frame indicates the part magnified in panel D. (D) Higher magnification of sarcomeric troponin T staining (arrow). Bar=25 μm (A, C), 10 μm (B, D).

Table 1
Evaluation of the positive expression of sarcomeric α-actinin and cardiac troponin T in embryonic stem cell-derived cardiomyocytes on day 7+11

	Control	Retinoic acid (10^{-8} M)	Icariin (10^{-7} M)	Icariin (10^{-8} M)	Icariin (10^{-9} M)
α-Actinin	46.7±2.3	61.3±5.4[a]	62.1±6.9[a]	55.6±2.9[a]	47.8±3.1
Troponin T	6.0±1.4	15.7±0.5[b]	16.5±4.9[a]	12.3±4.1	6.1±2.1

Shown are percentages of cardiomyocytes exhibiting positive staining. $n=3$ independent experiment. Results are means±standard deviation (S.D.).
[a] $P<0.05$ vs. control.
[b] $P<0.001$. vs. control.

3.2. Effect of icariin on expression of cardiac-specific proteins during differentiation of embryonic stem cells into cardiomyocytes

To test whether the observed cell types induced by icariin expressed tissue-specific proteins, indirect immunofluorescence was performed. The differentiated beating cardiac cells stained positively with anti-α-actinin mAb and anti-troponin T mAb (Guan et al., 1999). Icariin at 10^{-7} M accelerated the differentiation of the embryonic stem cells into cardiomyocytes expressing cardiac-specific proteins for sarcomeric structures. Fig. 4 shows the representative staining results for the cardiac-specific sarcomeric α-actinin and troponin T proteins. A semi-quantitative assessment further verified these structural findings (Table 1): On day 7+11, only about 47% of the cells stained positively for sarcomeric α-actinin in the control, while about 62% of the cardiomyocytes treated with 10^{-7} M icariin or 10^{-8} M retinoic acid stained for α-actinin. The proportion of cardiomyocytes induced by 10^{-7} M icariin or 10^{-8} M retinoic acid that contained positive troponin T-protein-labelled sarcomeres was significantly increased by about 16% in contrast with the case of 6% in the control.

3.3. Quantification of apoptosis by direct counting of acridine orange/ethidium bromide-stained cells

The normal morphology of embryonic stem cells is shown in Fig. 5A: the cells were stained and had a bright green fluorescence. When treated with 10^{-7} M icariin, 10^{-8} M retinoic acid for 48 h, cells exhibited characteristic morphological change of apoptosis and emitted dense yellow-green fluorescence with condensation of chromatin (Fig. 5C,D). Only a few control embryonic stem cells showed such staining (Fig. 5B).

3.4. Analysis of cell cycle and apoptosis by flow cytometry

The cell cycle distribution of embryonic stem cells was analyzed and considered as a parameter of their differentiation state (Rohwedel et al., 1996; Ludmila et al., 2002). This offered the possibility to understand the effect of icariin on early differentiation events before a shift to the cardiomyocyte phenotype. The results of cell cycle analysis of propidium iodide-stained cells showed a smaller percentage of embryonic stem cells in G0/G1 phase compared with the percentage of differentiated cells in the control (about 30% and 40%, respectively). Icariin at 10^{-7} M significantly induced the accumulation of cells in G0/G1 (44%). Treatment

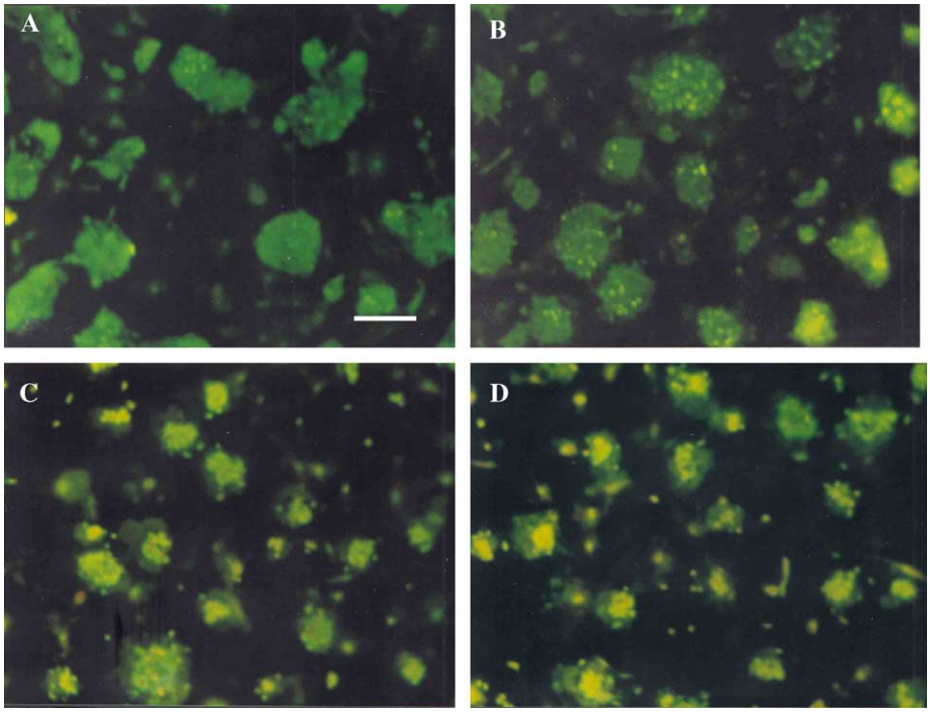


Fig. 5. Qualitative analysis of apoptosis by counting of acridine orange/ethidium bromide-stained cells. (A) embryonic stem cells, (B) spontaneously differentiated embryonic stem cells (control), (C) embryonic stem cells treated with 10^{-8} M retinoic acid for 48 h, (D) embryonic stem cells treated with 10^{-7} M icariin for 48 h. Bar=100 μm.

of embryonic stem cells with 10^{-8} M retinoic acid had a similar effect on the accumulation of cells in G1 (48%). The proportion of cells in S phase in either 10^{-7} M icariin-treated cells or 10^{-8} M retinoic acid-treated cells was strongly reduced (29% and 27% vs. 47% of S phase in embryonic stem cells) (Fig. 6).

Apoptosis plays a vital role in development, by removing unwanted cells (Uren and Vaux, 1996), which is the possible checkpoint in the transition toward differentiation of embryonic stem cells. Embryonic stem cells incubated with 10^{-7} M icariin for 48 h showed a higher proportion of apoptosis, 10% compared to 4% in spontaneously differentiated embryonic stem cells (control). Treatment with 10^{-8} M retinoic acid for 48 h also leads to apoptosis in 8% of cells (Fig. 6).

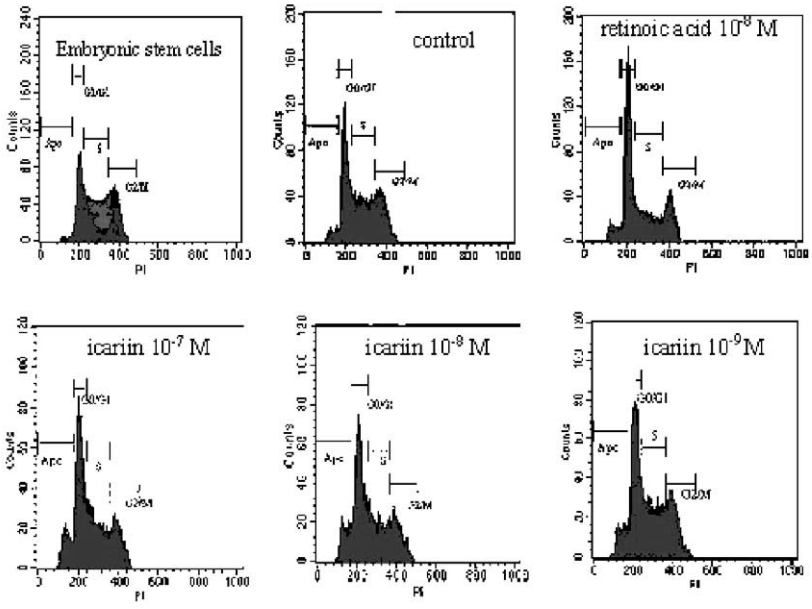
3.5. Detection of p53 gene by RT-PCR analysis

To elucidate the pattern of p53 gene expression during differentiation in embryonic stem cells, semi-quantitative RT-PCR was analyzed. Undifferentiated embryonic stem cells expressed a high level of p53. When embryonic stem cells were plated without feeder layers and leukemia inhibitory factor for 24 h, the mRNA level of p53 was dramatically decreased. However, the p53 mRNA level increased in embryonic stem cells treated with 10^{-7} M icariin for 24–48 h (Fig. 7A,B). When embryonic stem cells were treated with 10^{-8} M retinoic acid for 24–48 h, no change in p53 mRNA level was detected. The results suggested that retinoic acid-mediated apoptosis preceded the changes in p53 expression; however, p53 induction initiated icariin-induced apoptosis during embryonic stem cell differentiation.

During the differentiation of embryonic stem cells into cardiomyocytes, the expression of p53 mRNA was also examined. The results showed that the level of mRNA for p53 was increased by 10^{-7} M icariin or 10^{-8} M retinoic acid during early cardiac development between 7+0 and 7+9 days (Fig. 7C).

3.6. Expression of p53 protein by Western blot

The expression of p53 protein was investigated in undifferentiated embryonic stem cells and after differentiation was induced by 10^{-7}, 10^{-8}, 10^{-9} M icariin, 10^{-8} M retinoic acid or solvent for 24–48 h. Undifferentiated embryonic stem cells expressed a high level of p53 protein



cell cycle phase	G0/G1 (%)	S (%)	G2/M (%)	apoptosis(%)
embryonic stem cells	30.8±2.2	46.6±6.4	21.8±6.3	1.6±0.3
control	39.7±1.3[b]	33.5±4.9[a]	23.6±6.3	3.6±1.2[a]
retinoic acid 10^{-8} M	48.3±0.6[ce]	26.7±0.4[b]	16.8±1.0	8.5±1.5[bd]
icariin 10^{-7} M	43.9±1.2[bd]	28.7±0.2[b]	17.4±1.4	10.1±2.5[bd]
icariin 10^{-8} M	43.7±3.3[b]	27.2±2.6[b]	20.1±1.6	9.7±2.2[bd]
icariin 10^{-9} M	42.4±2.5[b]	31.0±1.1	18.5±1.1	8.4±1.3[bd]

Fig. 6. Cell cycle analysis of icariin treated embryonic stem cells by FCM. The table shows the percentage of G0/G1, S, G2/M phase and apoptotic cells under each treatment condition. $n=3$ independent experiments. Results are means±standard deviation (S.D.). [a]$P<0.05$, [b]$P<0.01$, [c]$P<0.001$ vs. embryonic stem cells group, [d]$P<0.05$, [e]$P<0.001$ vs. control.

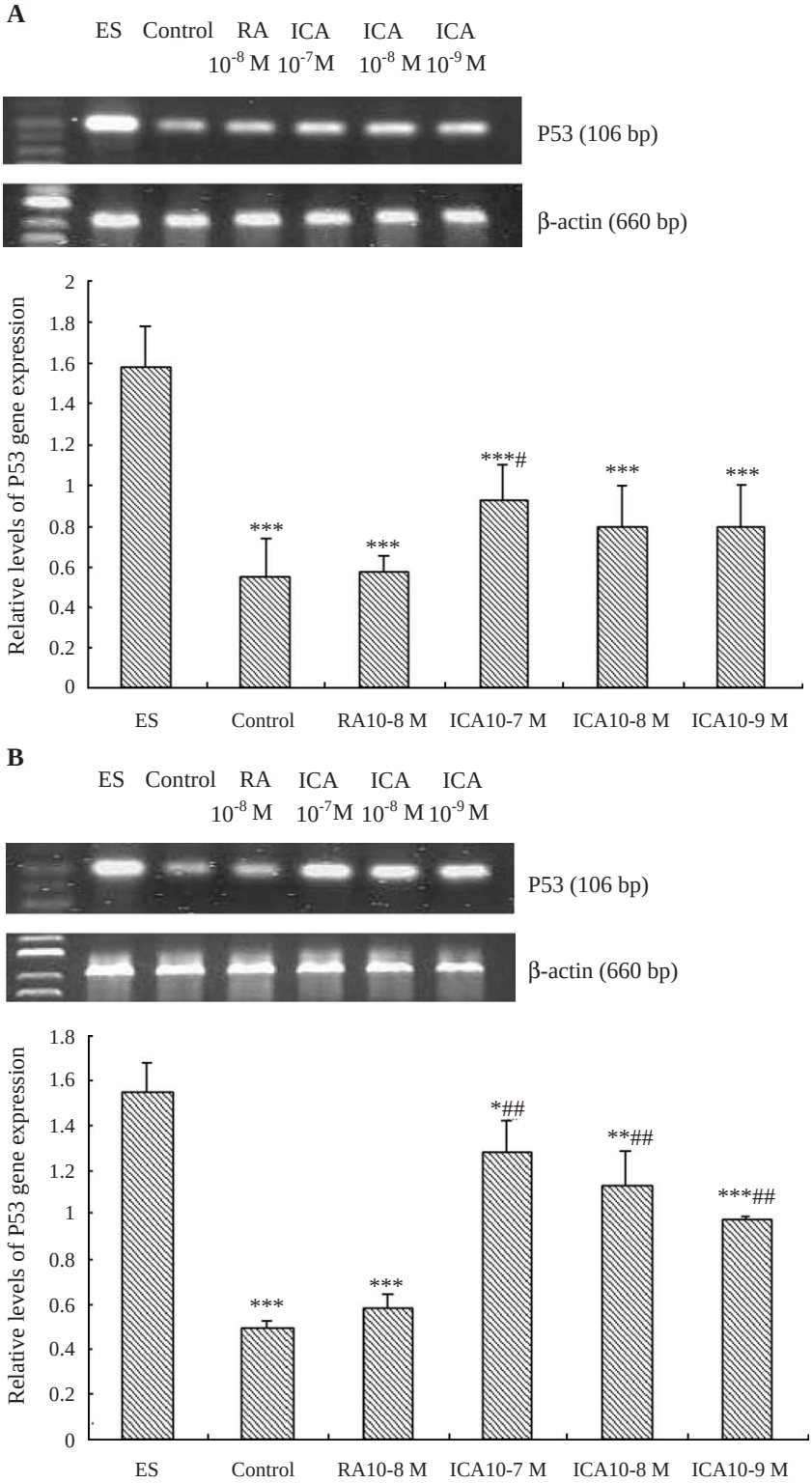


Fig. 7. (A) Level of p53 mRNA in embryonic stem cells treated with 10^{-7}, 10^{-8}, 10^{-9} M icariin (ICA), 10^{-8} M retinoic acid (RA) for 24 h analyzed by semi-quantitative RT-PCR. (B) Level of p53 mRNA in embryonic stem cells treated with 10^{-7}, 10^{-8}, 10^{-9} M icariin, 10^{-8} M retinoic acid for 48 h. (C) Level of p53 mRNA in embryoid bodies treated with 10^{-7} M icariin, 10^{-8} M retinoic acid on day 7+0, day 7+5, day 7+9. The ethidium bromide-stained gels of PCR products were analyzed by computer-assisted densitometry and the data were plotted for p53 genes in relation to β-actin gene expression. Similar data were obtained in three independent RT-PCR experiments. $^{*}P<0.05$, $^{**}P<0.01$ vs. embryonic stem cells group (A,B), $^{\#}P<0.05$, $^{\#\#}P<0.01$ vs. control (A,B). $^{*}P<0.05$ vs. control (C).

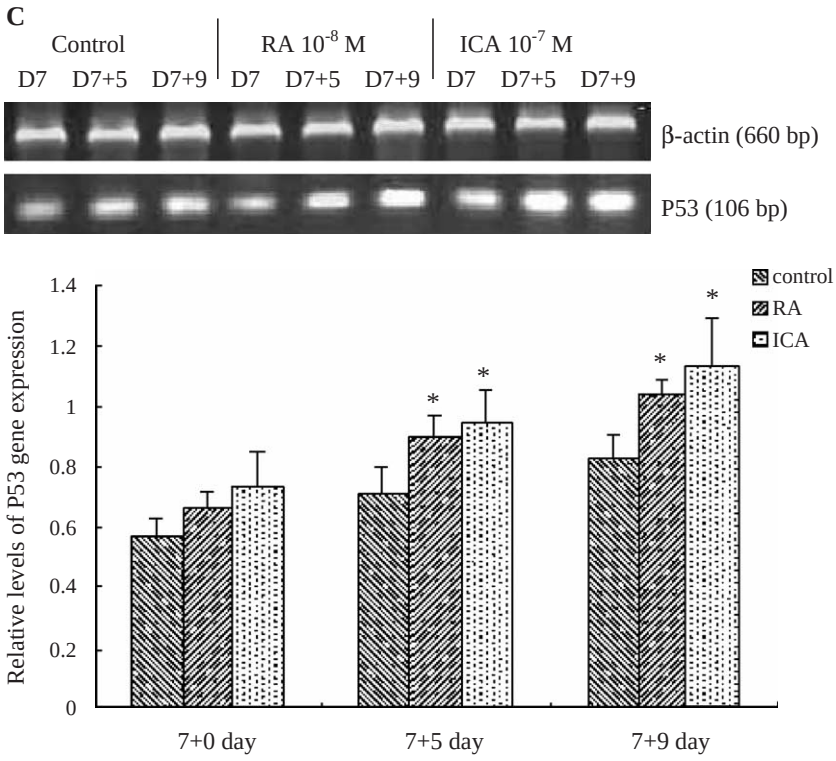


Fig. 7 (*continued*).

that declined during differentiation for 24 h in control. The level of p53 protein expression increased in icariin-treated cells after 24 and 48 h. Barely no change in the expression of p53 protein was observed after 24 and 48 h of exposure to retinoic acid (Fig. 8A). Protein expression during initial differentiation and that mediated by icariin or retinoic acid paralleled mRNA expression.

During the differentiation of embryonic stem cells into cardiomyocytes, the expression of p53 protein was also examined. Interestingly, the level of p53 protein in 10^{-7} M icariin or 10^{-8} M retinoic acid groups decreased significantly during cardiac development between 7+0 and 7+9 days (Fig. 8B). The decrease in the expression of p53 protein coincided with the increased number of cardiomyocytes.

4. Discussion

Icariin is one of the constituents of *Epimedium*, a traditional Chinese herbal medicine. Icariin improves the function of the cardiovascular system (Zhang et al., 2000), reinforces immunity (Zhao et al., 1996), and induces tumor cells to differentiate (Zhao et al., 1997a,b). Our former study focused on the metabolism of icariin by human intestinal bacteria in vitro (Liu and Lou, 2004) and the estrogenic-like activity of icariin compounds in relation to metabolism (Wang and Lou, 2004). Icariin appears to be a major active component of traditional treatments for heart disease (Zhang et al., 2000). However, the pharmacological effect and mechanism of action of icariin on the cardiovascular system are still not well known.

Embryonic stem cells are pluripotent cells and capable of contributing to normal embryogenesis. Therefore they are thought to be a good model for studying cardiomyocyte differentiation and regenerative cell therapy, and for investigating the mechanisms and specific targets of the cardioactive action of drugs during cardiogenesis. We investigated the possible inducible effects of icariin on the differentiation of embryonic stem cells into cardiomyocytes in vitro. The results verified that treatment with icariin at 10^{-7} M increased and accelerated the differentiation of embryonic stem cells into beating cardiomyocytes. To determine the partial mechanisms by which icariin is involved in embryonic stem cell differentiation, the expression of cardiac-specific proteins was monitored by immunocytochemistry, and the modulation of cell cycle and the expression of p53 were clarified by flow cytometry, RT-PCR and Western blot analysis.

It has been reported that embryonic stem cell-derived cardiomyocytes express cardiac gene products and cardiac-specific proteins in a developmentally controlled manner (Hescheler et al., 1997; Wobus and Guan, 1998; Manuilova et al., 2001). Here, the cardiomyocytes derived from embryoid bodies were characterized by the expression of cardiac-specific genes, such as α-myosin heavy chain and ventricular myosin light chain-2v (unpublished data), as well as the expression of cardiac-specific sarcomeric proteins

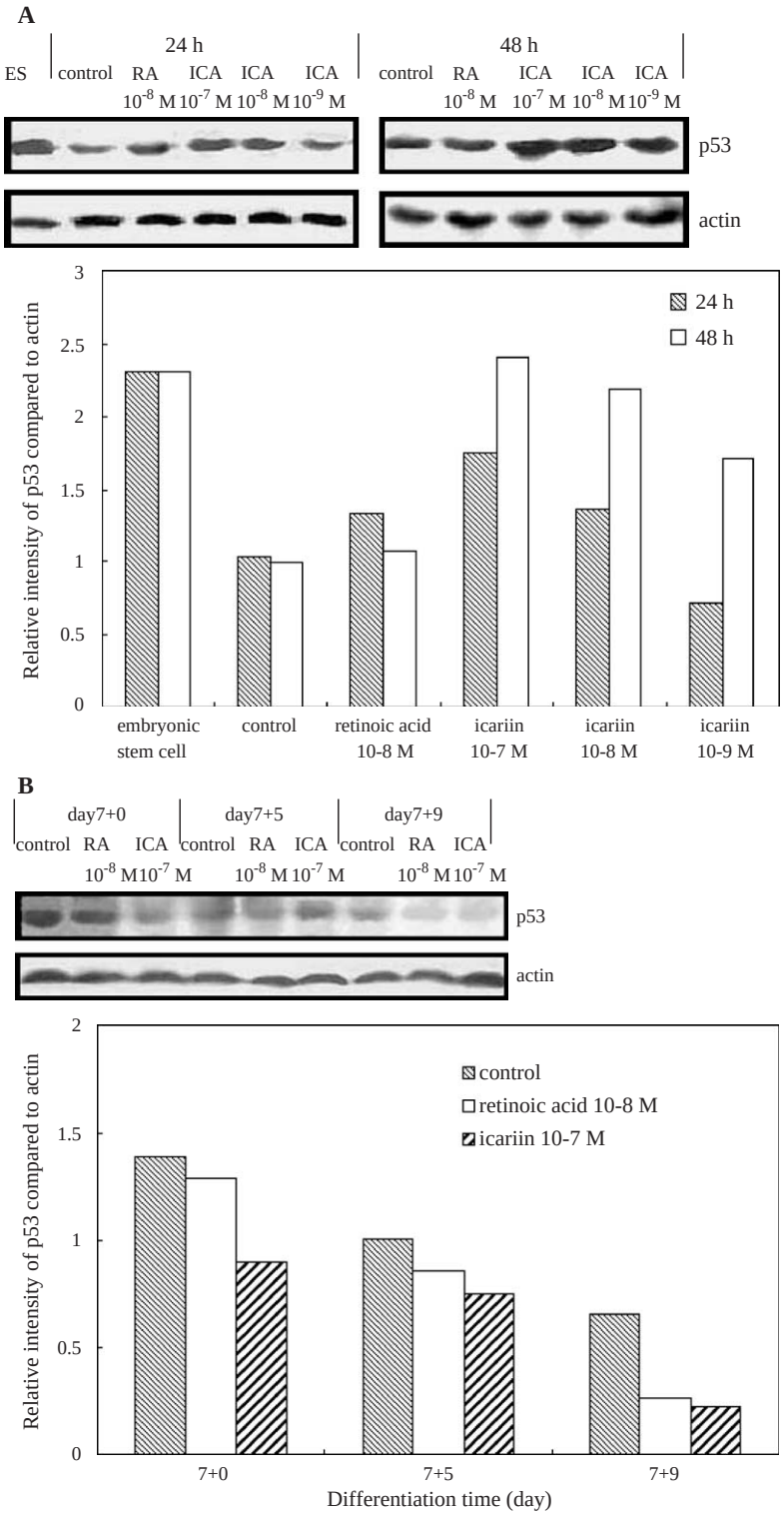


Fig. 8. (A) Level of p53 protein in embryonic stem cells treated with 10^{-7} M icariin (ICA), 10^{-8} M retinoic acid (RA) for 24 h and 48 h analyzed by Western blot. (B) Expression of p53 protein in embryoid bodies treated with 10^{-7} M icariin (ICA), 10^{-8} M retinoic acid (RA) on day 7+0, day 7+5, day 7+9 analyzed by Western blot. The products of Western blot were analyzed by computer-assisted densitometry and the data were plotted for p53 protein in relation to actin protein expression. A representative experiment is shown. An aliquot of 40 μg of the supernatant protein was separated electrophoretically and probed chemiluminescently.

such as α-actinin and cardiac troponin T. α-Actinin is known to bind to the cytoplasmic domain of β_1 integrin, which is expressed relatively early (Otey et al., 1990). Troponin T-labelled cells were characterized by their spindle- and triangular-shaped morphology, which is typical for ventricular and atrial cells in a relatively late differentiation phase (Hescheler et al., 1997; Guan et al., 1999). In addition, icariin treatment significantly increased and accelerated the expression of α-actinin and cardiac troponin T at an early differentiation stage. Thus the effects of icariin on promoting cardiac differentiation are related to acceleration of the sarcomerogenesis for α-actinin and cardiac troponin T.

A fine balance between positive and negative regulators of the cell cycle is critical in maintaining differentiation. It has been reported that embryonic stem cells have a low proportion of cells in G1 phase and a high proportion of cells in S phase (Savatier et al., 1995). The cell cycle distribution of embryonic stem cells is considered a parameter of their differentiation state (Rohwedel et al., 1996; Ludmila et al., 2002). Icariin is new biological response modifier and differentiational agent. It could induce some cells to differentiate by regulating the cell cycle (Mao et al., 2000; Di and Zhang, 2003). Our results showed that when embryonic stem cells were treated with icariin, more cells accumulated in G0/G1 phase and fewer in S phase. Furthermore, icariin induced apoptosis during differentiation before a shift to the cardiomyocyte phenotype. Thus the inducible effect of icariin is associated with cell cycle arrest and apoptosis. A key aspect of the differentiation of embryonic stem cells was the icariin-mediated modulation of the cell cycle. Furthermore, various molecules, including tumor suppressor genes and cell cycle regulators, may also be involved in the modulation of the cell cycle induced by icariin treatment.

P53 as a tumor suppressor regulates the cell cycle and differentiation. It is now known that p53 is an important mediator of cell cycle checkpoint at G1-S phase transition. In addition, p53 also induces cell apoptosis. The system of embryonic stem cell differentiation could recapitulate the in vivo differentiation process, including the occurrence of apoptosis accompanying differentiation (Kanaga et al., 1997; Sarkar and Sharma, 2002). A apoptotic signal is necessary to trigger embryonic stem cell differentiation. Apoptosis is regulated by the balance of various molecules, including tumor suppressor p53 (Morgenbesser et al., 1994). P53 plays a complex role during development, and the exact function of p53 is not yet known. Recent reports suggested that p53 levels may determine the balance between apoptosis or cell cycle arrest. High levels of p53 have been shown to induce apoptosis and low levels to cause cell cycle arrest (Chen et al., 1996). P53 has also been implicated in the regulation of cell differentiation, exerting a cell-type-specific and stage-specific role (Kanaga et al., 1997). Our studies showed that embryonic stem cells were unique in that they tolerated high levels of p53, while spontaneous differentiation resulted in a reduction in p53 levels. However, the proportion of cells undergoing apoptosis was increased early in the differentiation of embryonic stem cells treated with icariin, and was accompanied by up-regulation of p53 mRNA and protein. But apoptotic events mediated by retinoic acid occurred earlier than p53 induction. Thus p53 may play a direct role in apoptosis during the early differentiation of embryonic stem cells induced by icariin, but not by retinoic acid.

During early cardiomyocyte differentiation, both retinoic acid and icariin treatment increased p53 mRNA expression, but decreased the p53 protein level. It has been reported that p53 mRNA is readily detected in embryonic heart and that its expression decreases at birth. But p53 protein could barely be detected at any stage of heart development (Kyung et al., 1994). Differentiation results in a reduction in p53 protein levels in differentiated cells derived from embryonic stem cells, with a concomitant reduction in its functional activity and a shift in its conformational status. Low and functional inactivation of p53 protein seems to allow the differentiating cells to escape from apoptosis (Kanaga et al., 1997). Our results also suggested that a decline in p53 protein would favor the production of newly formed cardiomyocytes. The p53 protein may mediate its functions in a transcription-independent fashion (Jeremy and Michele, 1997). The activation of p53 would result in transactivation of downstream target genes, such as Mdm2, that target p53 for degradation (Haupt et al., 1997), so the decline in p53 protein may be associated with an increase in Mdm2 protein level following icariin treatment. These results provide new insights into the role of p53 as an important early regulator in the icariin-induced differentiation process, controlling the balance between cells undergoing apoptosis and differentiation.

In conclusion, embryonic stem cells can be induced by 10^{-7} M icariin to differentiate into cardiomyocytes, characterized by sarcomeric proteins for α-actinin and cardiac troponin T. The mechanisms of icariin involve regulation of the cell cycle, induction of apoptosis, as well as modulation of p53 during early differentiation and cardiac development.

Acknowledgments

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