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Research Article

Effect of Angiotensin II on Cardiomyocyte Differentiation from Mouse Embryonic Stem Cells

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ABSTRACT

Background: Angiotensin II (Ang II), the main member of the renin arginine system (RAS), is an essential link in the pathophysiology of cardiovascular disease. Ang II is a vasoactive hormone that binds to its receptors and affects numerous parts of the body such as the blood vessels, nerves, adrenal glands, and kidneys. Embryonic stem cells (ESCs) have the capability to differentiate into a variety of cell lineages. We have sought to investigate the possible role of Ang II as an effective factor on differentiation ability of mouse ESCs (mESCs) into cardiomyocytes.

Materials and Methods: mESCs were cultivated for 6 days in suspension and treated with Ang II, oxytocin (Oxt) as the positive control, and losartan (Los) as an Ang II receptor antagonist. We divided the mESCs into seven groups: Ang II (10 nM), Ang II (1 μM), Ang II (10 μM), Los (100 μM), Ang II (10 μM) + Oxt (100 nM), OT (100 nM), and Ang II (10 μM) + Los (10 μM). Untreated ESCs comprised the normal control group. At 13 days after plating embryoid bodies (EB) in tissue culture dishes, we monitored for contraction and beating frequency in the groups. We also determined expressions of cardiac specific proteins, α actinin and troponin I, in mESC-derived cardiomyocytes.

Results: Ang II at all doses effectively induced cardiac differentiation of mESCs compared to the control group. Ang II + Oxt had a stronger effect on differentiation. Immunocytochemistry analysis confirmed the presence of cardiac specific proteins, anti-cardiac troponin I and anti-sarcomeric α actinin, in the differentiated mESCs.

Conclusion: Our results showed that Ang II, like Oxt, at the maximum doses used in this study promoted the differentiation of mESCs into cardiomyocytes.

Keywords: Angiotensin, Mouse embryonic stem cell, Cardiac differentiation, Cardiomyocytes, Oxytocin

INTRODUCTION

Embryonic stem cell (ESC) derived cardiomyocytes have the capability to serve as highly effective models for in vitro studies that intend to investigate cardiac progenitor cells, their differentiation, molecular signals associated with actions related to the onset of excitability as well as those related to excitation–contraction coupling, drug discovery, and cell therapy (1, 2). Mouse ESC (mESC) differentiation into cardiomyocytes initially involves the formation of embryoid bodies (EBs) from cultivation of ESCs. EBs are three-dimensional aggregates that contain differentiating tissues from all germ layers. When plated, they give rise to spontaneously beating cardiomyocytes that are part of an aggregate that contains a mixed population of cells (2, 3).

Angiotensin II (Ang II), a pivotal member of the renin arginine system (RAS), is a circulatory hormone that plays an essential part in the regulation of both fluid homeostasis and blood pressure. Elevated levels of renin and circulating Ang II cause a variety of pathological states - hypertension, congestive heart failure, and nitrate tolerance (4). Induction of vascular smooth muscle cell growth, vasoconstriction (5,6) stimulation of proto-oncogene expression (7-9), modulation of myocardial hypertrophy (10-13) fibrosis, and ventricular remodeling after myocardial infarction (4) are examples of the impact that Ang II has on the cardiovascular system via RAS.

Ang II types 1 and 2 cell surface receptors (AT1R and ATR2) mediate Ang II. It is well known that AT1 is responsible for the majority of Ang II actions (14). Cui et al observed expression of angiotensin type 1 receptor (AT1R) mESC derived cardiomyocytes. They reported that AT1R signaling had a primary role in cardiac differentiation of mESCs (15). Members of the TGF-β superfamily have substantial roles in cardiac development during embryogenesis as well as in various cardiac pathologies. It has been reported that TGF-β1 efficiently stimulated in vitro differentiation of explants and stem cells into cardiomyocytes as well as bone marrow derived cells. Several studies have shown that TGF-β1 mRNA and protein are readily up regulated by Ang II in cardiac fibroblasts, myofibroblasts, and myocytes (16-19).

Lee et al. reported that ouabain induced cardiac differentiation and maturation of mESC-derived cardiomyocytes by activation of Erk1/2 and more mature SR for calcium handling (20). Lagerqvist et al. assessed the effects of a number of different factors such as Ang II on beating frequency of mES derived cardiac cells. Their results showed that Ang II significantly increased the beating frequency of mESC-derived cardiomyocytes (21). Hence, Ang II might be as an effector molecule of RAS in cooperating with TGF-β to effectively elevate cardiac differentiation of mESCs. We conducted this study to investigate the probable role and effective dose of Ang II in cardiac differentiation of mESCs.

2. MATERIALS AND METHODS

2.1 Cell culture and differentiation

We cultivated the MUKF3 ESC line (22) in DMEM high glucose medium (Gibco) supplemented with 20% FBS, 2 mM L-glutamine (Gibco, 15039-027), 0.1 mM β-mercaptoethanol (Sigma, M7522), 1% non-essential amino acids (Sigma, M7145) and 10 ng/ml leukemia inhibitory factor (LIF, Chemicon, ESGRO). In order to produce EBs, we cultivated 5×10^4 cells/ml of ESCs in petri dishes. Each plate contained 5 ml of medium and one of the following (7 groups): Ang II (10 nM, 1 µM, or 10 µM), losartan (Los; 100 µM), Ang II (10 µM) + Los (10 µM), oxytocin (Oxt; 100 nM), and Ang II (10 µM) + Oxt (100 nM). The control group consisted on untreated cells. After 6 days, we individually plated EBs on 0.1% gelatin-coated 96-well plates (TPP, Switzerland). Day 0 was considered the first treatment day. We analyzed the cell populations from days 7–19 of the entire differentiation protocol.

2.2 Immunocytochemistry

We performed immunocytochemistry analyses to evaluate the existence of cardiac specific proteins, anti-cardiac troponin I and anti-sarcomeric α actinin. Briefly, differentiated cells were washed with PBS and treated with 4% paraformaldehyde for 20 min at room temperature, washed twice with PBS, and permeabilized in the presence of PBS that contained 0.1% triton X-100. Unspecific binding was blocked with 0.02% triton X-100 and 1% BSA for 45 min at room temperature. Cells were then incubated with primary antibodies diluted 1:50 and dissolved in block/permeabilized buffer for 45 min at room temperature or overnight at 4ºC. Goat anti-mouse antibody conjugated with Texas red (1:100) and goat anti-rabbit antibody conjugated with FITC at a 1:100 dilution were the secondary antibodies. Differentiated cells were visualized and photographed under an inverted fluorescent microscope (IX71, Olympus, Japan).

2.3 Statistical analysis

We used analysis of variance and Tukey's test to compare the percentage of beating EBs in the different groups and on different days. Significance was set at 5%. Data were analyzed with statistical software SPSS, version 23.

3. RESULTS

We observed that Ang II efficiently stimulated the production of beating EBs. Oxt and Los were the positive and negative control groups. In all experimental groups, the EBs had spontaneous beating on day 1 after plating (Figure 2). The maximum number of beating EBs was observed in the 10 µM Ang II treated group at days 7-19 of differentiation. Table shows the percent of beating EBs at different days of differentiation. Our findings showed that Ang II at the maximum dose had a stronger effect in stimulation of cardiac differentiation compared to the other experimental and control groups (Figure 1). In addition to 10 nM of Ang II, Oxt had the same effect. Ang II + Oxt effectively increased the number of beating EBs. We investigated the presence of cardiac specific proteins, anti-troponin 1 and α actinin, in the differentiated cells by immunocytochemistry analyses. The results for both markers were positive (Figure 3). We also performed immunocytochemistry analyses of the normal control samples (not shown) under the same conditions (without primary antibodies) for the mentioned markers. The differentiated cardiomyocytes had spindle, multi-faceted and spherical morphologies (Figure 3).

	Day 7	Day 10	Day 13	Day 16	Day 19
Normal control	3	8.4	11.4	14.4	17.4
Ang II (10 nM)	4	12.8	17.4	17.4	18.4
Ang II (1 µM)	6	11.6	14	17	20
Ang II (10 µM)	6	17.4	20.2	23	23
Los (100 µM)	1.2	6.4	9.2	10.4	10.4
Ang (10 µM) + Los (10 µM)	4.4	8.4	12.4	14	14
Oxt (100 nM) (positive control)	7	17.2	20	23.2	23.4
Ang II (10 µM) + Oxt (100 nM)	9	19.2	23	25.4	27.2

Table1. Percentages of beating embryoid bodies (EBs) in the experimental groups during different days of the differentiation period. Ang II (10 µM) and oxytocin (Oxt; 100 nM) had the same effect. Ang II (10 µM) + Oxt (100 nM) had a stronger impact on cardiomyocyte differentiation in the aggregated cells. Losartan (Los), a blocker of Ang II receptors, diminished the positive effects of Ang II and significantly decreased mouse embryonic stem cell (mESC) differentiation in the Ang II and Los group.

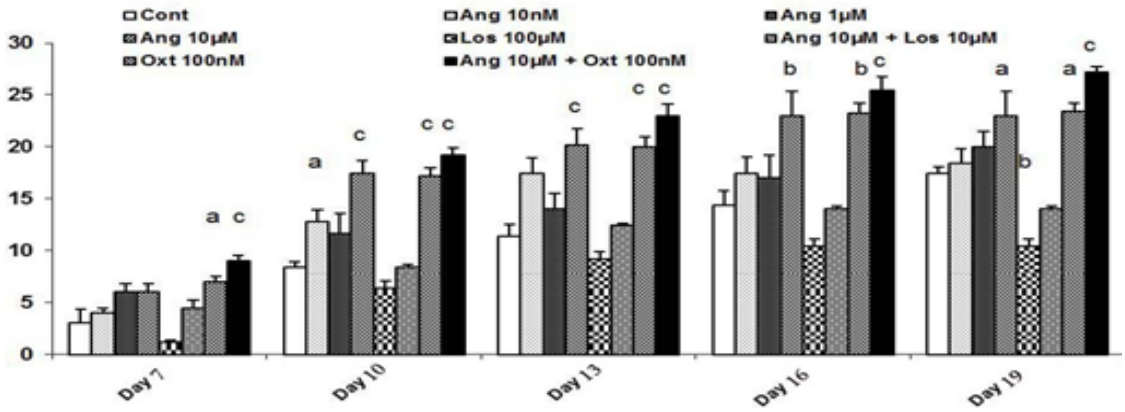

Figure 1. Differentiation efficiency was examined by calculating the percentage of beating embryoid bodies (EBs) after culture in medium treated with angiotensin II (Ang II). EBs were pre-incubated for 6 days with Ang II (10 nM), Ang II (1 µM), Ang II (10 µM), losartan (Los; 100 µM), Ang (10 µM) + oxytocin (Oxt; 100 nM), Oxt (100 nM), Ang

(10 µM) + Los (10 µM). The medium was changed every other day. Percentages of beating EBs were calculated from days 7 to 19. The significance level was set at 5% versus the control (without Ang II or Los treatment).

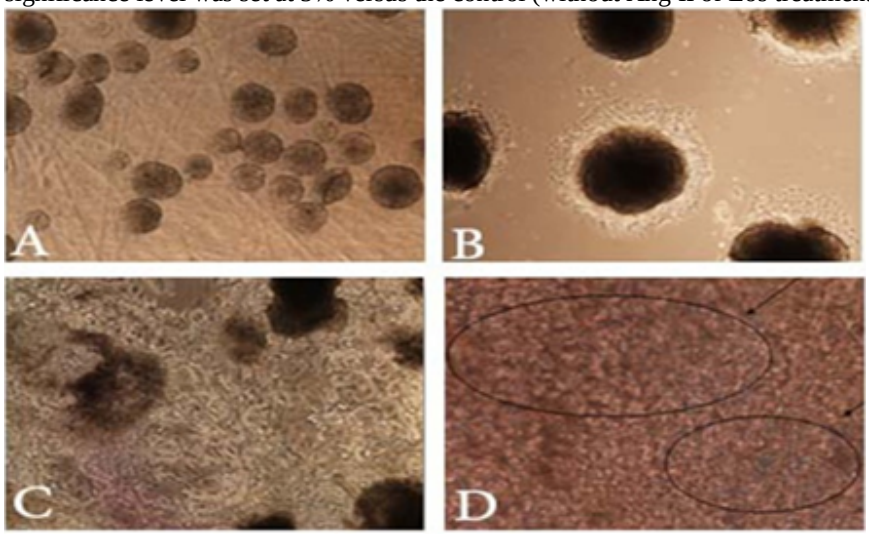

Figure 2. Effect of angiotensin II (Ang II) on cardiac differentiation of mouse embryonic stem cells (mESCs). Mukf3 mESCs were seeded at a density of 5×10^4 cells/5 cm in petri dishes to form embryoid bodies (EBs) (A) from days 0 to 6 in the presence of Ang II (10 nM), Ang II (1 µM), Ang II (10 µM), losartan (Los; 100 µM), Ang II (10 µM) + oxytocin (Oxt; 100 nM), Oxt (100 nM), Ang II (10 µM) + Los (10 µM). Untreated cells were the normal control group. Immigration and expansion of EBs were distinguishable (B and C). Expanded EBs spontaneously began to beat (D). We monitored all groups for contraction and beating frequency 13 days after EBs were plated in tissue culture dishes.

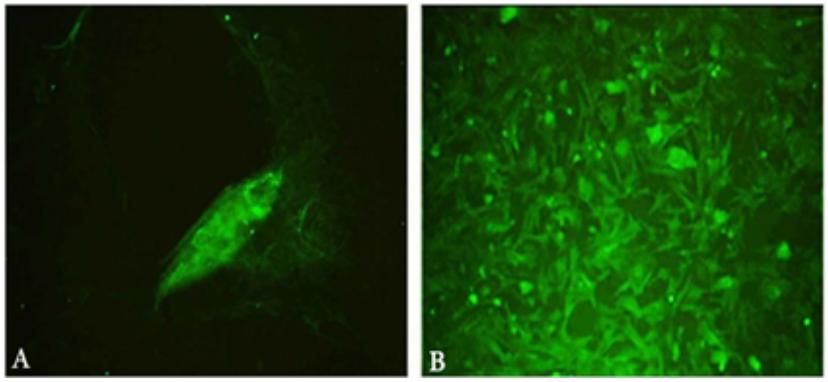

Figure 3. Immunostaining of MUKF3 embryonic cell-derived cardiomyocytes after Ang (10 uM) treatment (200x). Mouse embryonic stem cell (mESC) derived embryoid bodies (EBs) were treated from days 0 to 6 with Ang II (10 uM) and stained with an antibody against α actinin or troponin I. Immunoreactivity signals were obtained for EBs treated with Ang II (10 uM) which confirmed expression of α actinin (A) and troponin I (B) antigens.

DISCUSSION

We used mESCs to investigate the effects of Ang II on cardiac differentiation. Our results demonstrated that the maximum dose of Ang II (10 nM) efficiently stimulated ESCs to cardiac differentiation. Ang II combined with Oxt showed greater efficacy than Oxt or Ang II individually. We observed a decrease in beating in cells treated with Los, an Ang II antagonist.

Cui et al. reported that cardiomyocytes derived from mESCs expressed AT1R (15). Lager et al. investigated the effects of Ang II and other factors on the beating frequency of cardiomyocytes from mESCs. They determined that Ang II increased both the contraction amplitude and frequency of beating (21). Xing et al. evaluated cardiomyocyte differentiation of rat bone marrow MSCs with the combination of Ang

II and 5-azacytidine. In their study, Ang II was effective in cardiomyocyte differentiation (23).

Recently, Wu et al. reported that Ang II promoted cardiac differentiation via an AT1R. They observed that 1 μM of Ang II effectively stimulated cardiac differentiation in ESCs with no significant difference between Los and the normal control. However, in the current study, 10 μM of Ang II had greater effect compared to 1 μM of Ang II in cardiomyocyte differentiation. A significant difference existed between Los and the normal control. This was probably due to different exposure treatment time (24 h) for Los in their study versus 144 h in the current study, or the different mESC lines (24).

Based on our results, Ang II increased production of cardiomyocytes from ESCs whereas Los caused a decrease. Los, an AT1R inhibitor, negatively impacted cardiac differentiation of ESCs. However, the patients who received transplanted ESCs required caution in using Los. Other studies showed that in vitro differentiation of explants and stem cells into cardiomyocytes were stimulated by TGF-β1 (25, 26). It has been reported that both RAS and TGF-β1 have important roles in the development of cardiac hypertrophy and heart failure (25). Recent studies reported that Ang II and TGF-β1 as part of a signaling network, promote cardiac remodeling (13). TGF-β1 has been shown to give rise to cardiac fibroblasts growth and their phenotypic conversion to myofibroblasts. Hence it mediates Ang II-induced structural remodeling of the ventricular wall in an auto-/paracrine manner. TGF-β can be upregulated through AT1 by Ang II in cardiac myocytes and fibroblasts. Stimulation of this cytokine is absolutely required for Ang II induced cardiac hypertrophy in vivo (25).

An experimental group was treated with Oxt. Previously, the effects of Oxt on promotion of cardiac differentiation of ESCs have been reported. Ang II + Oxt showed a stronger effect on beating EBs formation which encouraged the development of a potential combination therapy for heart failure (27).

Animal models of myocardial infarction have shown limited success with stem cell transplantation in regenerating damaged myocardia (28, 29).

Most studies have focused on transplantation of undifferentiated stem cells, however obstacles must be overcome before stem cell therapies can be used in the clinic setting (27). One of the main obstacles is teratoma formation due to the multilineage differentiation tendency of undifferentiated ESCs, because only a small portion of ESCs can differentiate into a desired lineage. Therefore, the clinical efficacy of ES therapy can be affected during in vivo transplantation. It is essential to discover new, safe drugs to promote cardiac differentiation of ESCs (29-31).

In conclusion, the encouraging use of Ang II to promote cardiomyocytes from ESCs necessitates additional, more extensive studies to determine the effect of Ang II and Los on ESC-derived cardiomyocytes and their potential benefits and hazards.

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