Adenovirus-mediated RAR-β over-expression enhances ATRA-induced neuronal differentiation of rat mesenchymal stem cells

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Abstract

Introduction: The retinoic acid (RA) signaling pathway plays important roles in neural development. All-trans retinoic acid (ATRA) activates the RA signal by regulating RAR-β in mesenchymal stem cell (MSC)-derived neuron cells. Here, we try to investigate whether RAR-β over-expression can affect neuronal differentiation of MSCs.

Material and methods: The RAR-β gene was constructed into adenovirus Ad-RAR-β by using the AdEasy system. The MSCs were infected with Ad-RAR-β. Real time-polymerase chain reaction (RT-PCR), Western blot and immunofluorescence were performed to detect the expression and localization of RAR-β. The MSCs were treated with 1 µmol/l ATRA and modified neuronal induction medium (MNMM). Soma size and axon length of induced neuron cells were measured. Neural specific markers were detected by RT-PCR, western blot and immunofluorescence to evaluate neuronal differentiation.

Results: The 1300 bp fragment of RAR-β gene was confirmed to be correctly cloned in the adenovirus vector. Cloudiness amplification of Ad-RAR-β was observed in HEK293 cells during package. After 48 h of Ad-RAR-β infection, about 70% of MSCs were RFP-positive. RAR-β expression was increased by about 1988-fold and located in the nucleus. RAR-β over-expression did not affect neuronal differentiation efficiency; however, soma size of induced neuron cells enlarged from 716.25 ±95.96 µm² to 1160.12 ±352.65 µm² and axon length from 64.17 ±11.88 µm to 83.98 ±13.69 µm. Neural markers other than nestin – NSE, MAP-2, Tau, and Tuj1 – were increased by 4- to 11-fold in RAR-β over-expressed neuron cells with ATRA/MNMM induction compared with the Ad-null control group.

Conclusions: Our results have demonstrated that adenovirus-mediated RAR-β over-expression could facilitate neuron cell types of MSCs in vitro, indicating that the RAR-β-activated RA signal might be a vital factor in neuronal differentiation.

Key words: mesenchymal stem cells, all-trans retinoic acid, retinoic acid receptor β, neuronal differentiation.

Introduction

Perinatal hypoxic-ischemic encephalopathy (HIE) is an important disease of brain injury without a good treatment [1]. Neural stem cell transplantation in a brain damage animal model has shown its potential therapeutic applications in neuronal disease [2, 3]. Mesenchymal stem cells (MSCs)
are adult stem cells with characteristics of easy isolation, self-renewal, multi-potential and low immunogenicity, which have been widely used in tissue engineering to regenerate different cells/tissues and repair damaged tissues [4, 5]. Many reports have indicated that MSCs can differentiate into neuron cells under appropriate cellular conditions in vitro and recover learning ability and spatial memory after being transplanted into hypoxic-ischemic brain damage (HIBD) rats [6, 7]. However, compared to the high differentiation efficiency of neuron cells derived from MSCs in vitro, differentiation efficacy of MSCs in vitro and its role of functional replacement were relatively lower [6, 8]. Therefore, it is definitely required to explore a more reliable method to induce specific neuron cell types.

The retinoic acid signaling pathway can regulate gene expression and modulate a wide variety of biological processes including cell proliferation, differentiation, apoptosis and immuno-regulation [9]. We have reported that vitamin A level in vivo may affect the differentiation into mature and functional neuron cells from MSCs [7]. All-trans retinoic acid (ATRA), an active form of vitamin A, broadly participates in embryonic nervous system development and nerve cell differentiation [10]. In our previous study, ATRA treatment activated the RA signaling pathway by up-regulating retinoic acid receptor β (RAR-β) expression and subsequently promoted neuronal differentiation of MSCs [11]. Thus, we hypothesize that over-expression of RAR-β might activate RA signaling and further improve ATRA-induced neuronal differentiation of MSCs. Here, we induced RAR-β over-expression by using an adenovirus vector and investigated the effects of RAR-β on neuronal differentiation of rat MSCs.

Material and methods

Isolation of rat mesenchymal stem cells

The animal study and all experiments were approved by the Ethical Committees of the Institute of Zoology and the Children’s Hospital of Chongqing Medical University. Four-week old Sprague-Dawley rats were obtained from the Experimental Animal Centre of Chongqing Medical University. As in the previously reported method [12], rats were sacrificed by decapitation, then the tibias and femurs were isolated with clearance of connective and soft tissues. MSCs were harvested by flushing bone marrow and grown in Dulbecco’s Modified Eagle Medium/Nutrient Mixture F12 (DMEM/F12, Hyclone, USA) supplemented with 10% fetal bovine serum (FBS, Hyclone, USA), 100 units/ml penicillin, and 100 µg/ml streptomycin at 37°C in 5% CO₂. Cells were passaged at a confluency of 90%. Three to 5 passages of MSCs were used for in vitro experiments.

Construction of recombinant adenovirus vector containing rat RAR-β gene

Total RNA was isolated from PC12 cells and transcribed into cDNA. The coding region of rat RAR-β was specifically amplified from the cDNA template of PC12 cells. The adenovirus was constructed using the AdEasy system [13]. Briefly, the PCR product was digested by two endonucleases BamHI and HindIII. The product was directional cloned into the pAdTrace vector to construct the pAdTrace-RAR-β plasmid. The correct recombinant pAdTrace-RAR-β was linearized by PmeI, following co-transformation with the backbone vector pAdEasy-1 in Escherichia coli BJ5183 to obtain the recombinant adenovirus vector pAd-RAR-β. After being linearized by PstI, the homologous recombinant pAd-RAR-β was liposome (Lipofectamine™ 2000, Invitrogen, USA) transfected into the HEK293 cell line to package the recombinant adenovirus vector pAd-RAR-β. Hightiter adenovirus was obtained by repeated infection in HEK293 cells.

Adenovirus-mediated over-expression of RAR-β in mesenchymal stem cells

The MSCs were seeded in 6-well culture plates at a confluence of 50-60%, and then adenovirus at multiplicity of infection (MOI) of 100 was added to the medium. After 48 h of infection, red fluorescent protein (RFP) was observed under a microscope (TE2000-S, Nikon, Japan). Cells (1 × 10⁵) were collected and suspended in 1 ml of 1% BSA PBS buffer. The ratio of RFP-positive to total cells was acquired by flow cytometry using a FACScanto™ II system and Cell Quest Pro software (BD Biosciences). The RT-PCR, western blot and immunofluorescence were performed to detect the expression of RAR-β. An empty adenovirus (Ad-null) was used as an adenovirus control.

ATRA-induced neuronal differentiation

The MSCs were cultured in DMEM/F12 medium for 24 h. Then DMEM/F12 medium containing 1 µmol/l ATRA (Sigma, USA, resolved in 100% ethanol at 10 mmol/ml storage concentration) was used to incubate with MSCs for 24 h. Afterward, DMEM/F12 medium was removed, cells were washed with D-Hank’s solution twice and induced with modified neuronal induction media (MNM) which was composed of DMEM/F12/100 units/ml penicillin, and 100 µg/ml streptomycin/1.6% DMSO/160 µmol/l H4A/20 mmol/l KCl/1.6 mmol/l valproic acidβ/8 µmol/l forskolin/0.8 µmol/l hydrocortisone/4 µg/ml insulin (all from Sigma, USA). Experimental MSCs were treated with ATRA/MNM induction, Ad-null induction and ATRA/MNM induction, respectively. In two adenovirus groups, MSCs were infected by adenovirus for 48 h before neuronal induction. The MSCs without any treatment were used as a negative control.
Quantitative analysis of neuronal differentiation

Treated MSCs were stained for neuron related markers, nestin, neuron-specific enolase (NSE) and Tau. Images of ten non-overlapping fields of view were captured under a microscope (TE2000-S, Nikon, Japan). Positive stained cells with protruding soma, axons and dendrites were counted as induced neuron cells to calculate induction efficacy. The ImageJ program was used to measure the soma size and axon length. Three independent experiments were performed and two replicates were set in each experiment.

RNA isolation and RT-PCR analysis

As previously described [14], extraction of total RNA was carried out using an RNA extraction kit (Genemega Inc, USA) according to the manufacturer’s instructions. Then, 10 µg of total RNA was reverse transcribed into cDNA with random primer and Superscript II reverse transcriptase (TAKARA, Japan). The Bio-Rad protocol for realtime PCR amplification was performed as follows: 94°C x 20", 58°C x 20", 70°C x 20" and reading plate for 40 cycles. Primary data of Ct value were taken to calculate fold change by normalizing each cDNA to GAPDH.

Western blot analysis

Western blot analysis and quantification were performed as previously described [14]. Briefly, treated MSCs from T25 culture flasks were collected and lysed with RAPI buffer containing PMSF. Approximately 20 µg of total proteins per lane were loaded onto an 8-10% SDS/PAGE gel (Beyotime, China). Electrophoretic separation was performed at an applied voltage of 120 V for 1.5-4 h in SDS running buffer. Then, proteins were transferred to an Immobilon-P membrane (Millipore, Billerica, USA). After approximately 20 µg of total proteins per lane were loaded onto an 8-10% SDS/PAGE gel (Beyotime, China). Electrophoretic separation was performed at an applied voltage of 120 V for 1.5-4 h in SDS running buffer. Then, proteins were transferred to an Immobilon-P membrane (Millipore, Billerica, USA). After transferring, the membrane was incubated with anti-NSE (Abcam, USA), anti-neuron-specific class III β-tubulin (Tuj1) (Abcam, USA) or anti-β-actin (Santa Cruz Biotechnology, USA) primary antibody at 4°C overnight respectively, followed by incubation with an appropriate secondary antibody conjugated with horseradish peroxidase (Santa Cruz Biotechnology, USA) at room temperature for 1 h. The presence of the proteins was detected and quantified by using Luminata Crescendo Western HRP Substrate (Millipore, Billerica, USA) with the Syngene GBox Image Station.

Immunofluorescence staining

Immunofluorescence staining was carried out using previous methods [14]. First, treated MSCs were fixed with methanol at −20°C for 15 min and blocked with 5% bovine serum albumin at room temperature for 1 h, followed by incubation with NSE, nestin or Tau primary antibody (all from Abcam, USA) at 4°C overnight. After washing twice, DyLight 488 conjugated secondary antibody (Jackson ImmunoResearch Laboratories, Inc, USA) was added to incubate cells at room temperature for 30 min. DAPI (Sigma, USA) was used to stain nuclei. The presence of the proteins was examined under a reverse fluorescence microscope (TE2000-S, Nikon, Japan). Untreated MSCs stained by non-specific IgG were used as negative controls.

Statistical analysis

All data were analyzed using the SPSS 15.0 software package and presented as mean ± standard deviation (SD). A two-tailed Student’s t-test assuming equal variances was performed to measure significant differences between two samples. A p < 0.05 was considered statistically significant.

Results

Construction of recombinant adenovirus vector containing rat RAR-β gene

An approximately 1300 bp fragment of the full-length RAR-β gene was PCR amplified from the recombinant adenoviral plasmid pAd-RAR-β, and a specific 4500 bp fragment was digested by Pac I.
restriction enzyme from the pAd-RAR-β plasmid and compared with pAdEasy-1 bone vector (Figure 1 A). The target genes were confirmed to be correctly cloned in the adenovirus vector by gene sequencing and matched to the RAR-β sequence in GenBank. The pAdTrace-TOX vector contains an RFP code region driven by the CMV promoter. Thus, we can check RFP to measure the transfection and infection efficiency. At 24 h post Pac I linearized pAd-RAR-β plasmid transfection, more than 10% of HEK293 cells were RFP positive. Amplification of Ad-RAR-β exhibited cloudiness in the HEK293 cell line at day 10 during package (Figure 1 B). This result demonstrated that the recombinant adenovirus vector containing the rat RAR-β gene was successfully constructed.

**Ad-RAR-β over-expressed RAR-β in MSCs**

Ad-RAR-β was added to infect MSCs. After 48 h of infection, 71.61% of RFP-positive MSCs were observed. Meanwhile, 69.63% of RFP-positive MSCs with Ad-null infection were taken as the control. From real-time PCR and western blot results, we found that MSCs hardly expressed endogenous RAR-β, Ad-null infection did not affect the expression of RAR-β, and Ad-RAR-β adenovirus could significantly induce high expression of RAR-β (Figures 2 A, B). The immunofluorescence picture showed the nuclear localization of exogenous RAR-β over-expression (Figure 2 C). Therefore, Ad-RAR-β could effectively infect MSCs and increase the mRNA and protein expression of RAR-β.

**RAR-β over-expression improved ATRA-induced neuronal differentiation of MSCs**

As we previous described, ATRA treatment could improve neuronal differentiation and only stimulate expression of the RAR-β [11]. As a neuron-specific receptor, RAR-β is necessarily required in neuron development and is widespread in different neuron
tissues [15]. However, RAR-β is barely detected in MSCs. Next, we sought to determine whether over-expression of RAR-β in MSCs could activate the ATRA-induced signal pathway and facilitate neuronal differentiation in MNM induction. As shown in Table II and Figure 3 A, 24 h after ATRA/MNM induction, about 88% of MSCs became retracted and refraction-enhanced, exhibiting long axons or dendrites which were counted as induced neuronal cells. There was no significant difference of neuronal induction efficacy among the three treated groups. The soma sizes of ATRA/MNM, Ad-null/ATRA/MNM and Ad-RAR-β/ATRA/MNM treated cells were 739.65 ±41.11 µm², 716.25 ±95.96 µm² and 1160.12 ±352.65 µm², respectively. Axon length is another index to evaluate neuronal cells. Ad-null did not affect soma size of induced cells, but axon length of Ad-null/ATRA/MNM treated cells was shorter than that of ATRA/MNM treated cells. With Ad-RAR-β-mediated RAR-β over-expression, induced cells exhibited longer axons (83.98 ±13.69 µm) compared with 64.17 ±11.88 µm of axons in Ad-null groups. Next, we detected neuronal specific markers to measure neuronal cells induced from MSCs. Real-time PCR and western blot showed that MSCs hardly expressed neuronal related markers, nestin, NSE, MAP-2, Tau and Tuj1, which were significantly increased in ATRA/MNM-induced cells. No difference was seen between ATRA/MNM and Ad-null/ATRA/MNM groups. RAR-β over-expression resulted in 4 to 11-fold higher expression of NSE, MAP-2, Tau and Tuj1, but not nestin (Figure 3 B). The above results were also confirmed by immunofluorescence staining. The expression of NSE and Tau proteins was detected in cytoplasm (Figure 3 C). The green fluorescence of nestin was at similar intensity among the three induction groups. NSE and Tau protein had increased expression with Ad-RAR-β treatment (Figure 3 D). Taken together, we demonstrated that adenovirus-mediated RAR-β transfection could improve neuronal differentiation of MSCs.

Table II. RAR-β over-expression improved neuronal differentiation of MSCs

| Variable                      | ATRA + MNM | Ad-RFP + ATRA + MNM | Ad-RAR-β + ATRA + MNM |
|-------------------------------|------------|----------------------|------------------------|
| Neural differentiation efficiency [%] | 83.35 ±5.05 | 79.20 ±8.62           | 82.41 ±11.03            |
| Soma size [µm²]               | 739.65 ±41.11 | 716.25 ±95.96         | 1160.12 ±352.65*        |
| Axonal length [µm]            | 81.6 ±9.72 | 64.17 ±11.88          | 83.98 ±13.69*           |

Neuronal differentiation efficacy, soma size and axon length of induced neuron cells treated with ATRA/MNM, Ad-null/ATRA/MNM, and Ad-RAR-β/ATRA/MNM, respectively (n = 10, *p < 0.05 vs. Ad-null/ATRA/MNM group)
Zechel [24] reported differential requirement of RAR isotypes during the initial stages of neural differentiation of PCC7 cells. Endogenous expression of RAR-α and RAR-γ is detectable in MSCs, but RAR-β rarely exhibits it. Combination of ATRA pretreatment with MNM induction is a mature method to induce neuronal differentiation of MSCs. However, only RAR-β was selectively upregulated by 24 h ATRA pretreatment and had a higher increased expression in induced neuron cells compared with RAR-α and RAR-γ, indicating ATRA activation and neuronal differentiation mediated through RAR-β in MSCs [11]. RAR-β might be an important factor to activate the RA signaling pathway in developing neuronal differentiation of MSCs in vitro. So, we hypothesize that, if MSCs have a relatively high expression of RAR-β, it
might be easier to induce MSCs to differentiate into neuron cells by ATRA and MNM induction.

Here, we tried to over-express exogenous RAR-β and determine its role in neuronal induction of MSCs. Because of stem cell derivation, a general euakaryotic expression vector is hardly transfected into MSCs by lipofection or calcium phosphate co-precipitation [25]. In this study, we successfully constructed an adenovirus vector carrying the RAR-β gene by using the replication-defective AdEasy adenovirus system. Ad-RAR-β was confirmed to infect MSCs with efficacy of more than 60%, and significantly enhance mRNA and protein expression level of RAR-β. RAR-β is classified as a nuclear receptor to regulate the transcription of adjacent genes [26]. Immunofluorescence results indicated nuclear localization of RAR-β in MSCs, suggesting that Ad-RAR-β can induce a biologically active RAR-β form.

It has been reported that ATRA dose-dependently affects target gene expression [27]. We previously detected the effect of different concentrations of ATRA on neuronal differentiation of MSCs and found that 1 µmol/l of ATRA pretreatment may induce neuron cells with the longest axon and soma diameter, and the highest expression of neuronal specific markers. Actually, ATRA at 10 µmol/l was also as good as, if not better than, ATRA at 1 µmol/l in the role of neuronal induction. However, the high cell death rate under this circumstance was a major problem; the reason might be the cytotoxicity of ATRA at high concentrations [28]. So, we considered that when RAR-β is over-expressed in MSCs, 1 µmol/l of ATRA might be easier to activate the RA signaling pathway so that it plays a more effective role. Though induction efficiency was not distinct, soma size and axon length were improved in induced neuron cells with Ad-RAR-β infection. Ad-null control was set up in following experiments since adenovirus might affect axon length of induced neuron cells. Most of the neuronal specific markers had increased expression with RAR-β over-expression. Nevertheless, we did not find a change of nestin between ATRA/MNN and Ad-RAR-β/ATRA/MNM groups. Nestin is an intermediate filament protein which is specifically expressed in neuroepithelial stem cells, and can also transiently appear in other progenitor cells [29]. Nestin only expresses in neural epithelium at an early stage of embryonic development, and stops expression after birth [30, 31]. During the ATRA/MNM-induced neuronal differentiation process, the expression of nestin increased within the first 18 h and decreased quickly. The Ad-RAR-β/ATRA/MNM-induced neuron cells crossed the peak of nestin expression, and only a low level of nestin expression was found in the mature neuron cells.

In conclusion, we demonstrated that adenovirus-mediated RAR-β over-expression in MSCs could improve ATRA-induced neuronal differentiation of MSCs in following MNM induction, providing us with a promising method for neuronal differentiation in vitro. As a neural specific receptor, RAR-β is also a critical candidate molecule for RA signal activation. Further studies on RAR-β should contribute to the biochemical mechanism of the RA signaling pathway in neuronal development and differentiation.

Acknowledgments
Yang Bi and Min Gong contributed equally to the work.

This work was supported by grants from the National Natural Science Foundation of China (30830106 and 81100309), China Postdoctoral Science Foundation (20100480667), Natural Science Foundation of Chongqing City (CSTC2010BB097), and the health bureau of Chongqing (2010-2-217).

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Appendix
Illustration of construction of recombinant adenovirus vector containing rat RAR-β gene

**pAdEasy-1 (33414 bp)**

- Initially transformed into bacteria
- Selection with Kan

**pAd-Trace-TOX (9086 bp)**

- Transformation into bacteria
- Selection with Kan

**pAd-Trace-RAR-β (10445 bp)**

- Linearized with Pmel

**pAd-RAR-β (36969 bp)**

- Linearized with PacI

- Transfection of 293 cells

- Repeated infection in 293 cells to obtain high titer adenovirus