A mouse bone marrow stromal cell line, TBR-B, shows inducible expression of smooth muscle-specific genes

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Abstract We established an in vitro culture system which mimicked the differentiation pathway of smooth muscle cell, using TBR-B, a bone marrow stromal cell line derived from transgenic mice harboring temperature-sensitive SV40 large T-antigen gene. TBR-B cells have the potential to express smooth muscle-specific genes including h1-calponin, h-caldesmon, SM22α and α-actin, only after cultured in the differentiation medium for 2 weeks. The differentiation state of TBR-B was well controlled by using different culture medium. Using this cell line, we also found that ascorbic acid is a potent factor inducing the expression of h1-calponin and α-actin. TBR-B cells will serve as a useful tool for elucidating the regulatory mechanisms of smooth muscle-specific gene expression, and for identifying compounds that regulate the differentiation state of vascular smooth muscle cells.

Key words: Bone marrow stroma; Smooth muscle cell; Differentiation; Ascorbic acid

1. Introduction

Phenotypic modulation of vascular smooth muscle cells (SMCs) from a differentiated to a dedifferentiated state plays an important role in the pathology of atherosclerosis [1] and restenosis after coronary angioplasty [2]. It will be important to elucidate the molecular mechanisms of SMC differentiation, and to identify any factors or chemical compounds that control the differentiation state of SMC for the therapy of atherosclerosis.

For the study of the molecular mechanisms of differentiation, culture systems that mimic the differentiation process of particular cell lineages are useful. For instance, pluripotent 10T1/2 cells that can differentiate into skeletal myoblasts by the treatment with 5-azacytidine were used as a useful tool for the isolation of *MyoD*, one of the master regulators of skeletal muscle differentiation [3]. SMC-like differentiation in culture systems was also reported in the embryonal carcinoma cell line P19 treated with retinoic acid [4], co-culture of 10T1/2 cells with endothelial cells [5], and embryonic stem cells treated with either retinoic acid or dibutylyl-cAMP [6]. However, so far neither the understanding of molecular mechanisms of SMC differentiation nor the finding of useful compounds for the control of SMC differentiation were achieved. To accomplish the goal, we need to perform further analyses, including the establishment of new useful cell lines.

Our studies focused on bone marrow stromal cells. These cells are derived from multipotential stem cells and are progenitors for mesenchymal cell types, including fibroblasts, myoblasts, adipocytes, chondroblasts, osteoblasts, reticular cells and SMCs in vitro [7–12]. Differentiation of bone marrow stromal cells to several of these cell types was also observed in vivo [13,14]. For example, transplanted marrow stromal cells differentiated into osteoid, bone trabeculae, and connecting tissue lining cells of microvasculature [13]. Also, stromal cells differentiated into skeletal muscle or vascular endothelial cells after migration through the blood from the bone marrow into damaged muscle or vessels [14]. These data suggest that bone marrow stromal cells have intrinsic mechanisms to differentiate into mesenchymal cell lineages, and the compounds which induce the differentiation of these cells into SMC might be effective for the prevention of phenotypic modulation of SMCs in vivo.

We established more than 60 bone marrow stromal cell lines (TBRs) from transgenic mice harboring the temperature-sensitive SV40 large T-antigen gene [15]. These lines were classified into three cell types based on their morphology. These are endothelial-like cells, preadipocytes and fibroblasts. Some of these lines were able to differentiate into mesenchymal lineages such as adipocytes, osteogenic cells and skeletal myoblast, when cultured in the differentiation medium [15].

In this study, we demonstrated that one of these cell lines, TBR-B, showed the inducible expression of smooth muscle-specific genes such as α-SM actin, SM22α, h-caldesmon and h1-calponin [16,17]. We also found that ascorbic acid induced the expression of smooth muscle-specific gene in TBR-B cells. These results suggest that TBR-B cells can be used as a good model to study the molecular mechanisms and also served for the screening of the compounds which control the expression of smooth muscle-specific genes.

2. Materials and methods

2.1. Cell lines and culture

Cells were cultured in the maintenance medium (RITC80-7 medium (Iwaki Glass Co., Ltd., Tokyo, Japan) supplemented with 2% fetal calf serum (Hyclone Laboratories, Logan, UT, USA), 10 μg/ml bovine transferrin (Canadian Bioclinical Ltd., Canada), 1 μg/ml insulin (Canadian Bioclinical Ltd., Canada) and 10 ng/ml recombinant EGF (Wakunaga Pharmaceutical Industrial Co., Ltd., Tokyo, Japan)) at 33°C in a humidified atmosphere of 5% CO_2 in air [16]. Cells were passaged every 1 week with trypsin treatment. To promote differentiation, a subconfluent culture of TBR-B was maintained in the differentiation medium (α-MEM (Gibco BRL, Gaithersburg, MD, USA)

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supplemented with 10% fetal calf serum and 50 μM 2-mercaptoethanol) for 2 weeks at 33°C in a humidified atmosphere of 5% CO_2 in air [15].

2.2. Western blot analysis

Cells were harvested with a rubber policeman and were centrifuged at 1000×*g* for 5 min. The cell pellet was suspended in RIPA buffer (20 mM Tris–HCl, pH 7.4, 0.1% sodium dodecyl sulfate (SDS), 1% Triton X-100, 1% sodium deoxycholate, 0.5 mM PMSF and 10 μg/ml leupeptin), and the mixture was centrifuged at 3000×*g* for 5 min. The supernatant was used for SDS–polyacrylamide gel electrophoresis. Samples were run on a 12.5% polyacrylamide gel, using the buffer system of Laemmli [18], and then transferred to the PVDF membrane. Transfer was performed at the current of 2 mA/cm^2 for 1 h. After transfer, the PVDF membrane was blocked with 5% (w/v) skim milk in phosphate-buffered saline (PBS) at room temperature for 30 min. The membranes were reacted either with monoclonal anti-SM α-actin antibody (Sigma, St. Louis, MO, USA), monoclonal anti-caldesmon antibody (Sigma, St. Louis, MO, USA) or polyclonal anti-h1-calponin antibody, kindly provided by Dr. K. Takahashi (Center of Adult Diseases, Osaka, Japan).

2.3. Immunocytochemistry

10 000 cells were grown on each well of 8-well glass chamber slides (Nunc, Naperville, IL, USA) in the maintenance medium. Two days later, the medium was changed either to the differentiation medium or the maintenance medium, and the cells were further cultured for 2 weeks. Then, the cells were washed in PBS, fixed in 2% paraformaldehyde for 5 min at room temperature and permeabilized in 0.5% Triton X-100. The chamber slides were incubated with monoclonal anti-SM α-actin antibody for 1 h at 37°C, washed in PBS, incubated with FluoroLink Cy2-labeled anti-mouse IgG (H&L) (Amersham, Arlington Heights, IL, USA) for 1 h at 37°C, washed and mounted.

2.4. Semi-quantitative reverse transcription (RT)-PCR

Total RNA was isolated from cells harvested from confluent culture in 35 mm dishes using RNeasy total RNA purification kit (Qiagen, Germany). 1 μg of total RNA was reverse-transcribed by Superscript II reverse transcriptase (Gibco BRL) using Oligo(dT) primer (Gibco BRL), and PCR was performed with this cDNA as a template. The sense and antisense primers had the following sequences, respectively: GAPDH, 5′-TTCCAGTATGACTCCACTCA-3′ and 5′-ATCACGCCACAGCTTTCCAG-3′; h1-calponin, 5′-TAACCGAGGTCCTGCCTACG-3′ and 5′-TGTGGGTGGGCTCACTCAGC-3′; SM22α, 5′-ATCAAGCTTCGCTACTCTCCTTCCAGTCCACAAACGACCA-3′ and 5′-ATCGGATCCCTTCCCTTTCTAACTGATGATCTG-3′; α-actin, 5′-GGCATCCACGAAACCACCTA-3′ and 5′-CACGAGTAACAAATCAAAGC-3′. For detection of GAPDH, SM22α and α-actin, the following PCR parameters were used: 94°C for 4 min and then 18 cycles of 45 s at 95°C, 1 min at 60°C and 2 min at 72°C. For the detection of h1-calponin, 24 cycles were performed. The amplified products were electrophoresed on a 1.8% agarose gel, stained with ethidium bromide. In the PCR conditions used, amplified products correspond to the amount of templates in the starting solution.

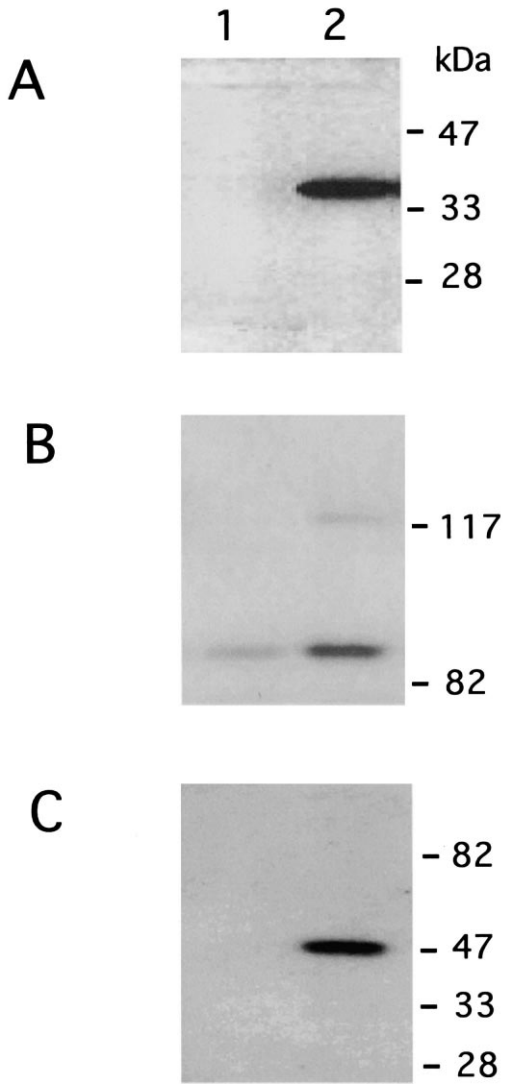

Fig. 1. Effects of culture condition on protein levels of h1-calponin, h-caldesmon and α-actin in the TBR-B cell line. Cell extracts were prepared from TBR-B cultured in the maintenance medium (lane 1) or in the differentiation medium (lane 2) at 33°C for 14 days. Proteins were resolved on an SDS–polyacrylamide gel and analyzed by Western blotting with anti-h1-calponin antibody (A), anti-caldesmon antibody (B) or anti-α-actin antibody (C).

3. Results and discussion

3.1. Expression of smooth muscle-specific markers is induced in TBR-B

We previously established culture conditions, in which TBR cell lines were either maintained pluripotent or differentiated into mesenchymal cell lineages [15]. Among those cell lines, we picked up morphologically fibroblast-like TBR cell lines and examined their abilities to express smooth muscle-specific genes during the 2 week culture period in the maintenance or the differentiation medium, at either permissive (33°C) or non-permissive (37°C) temperature. Among the 24 TBR cell lines cultured in the differentiation medium, four cell lines, designated TBR-B, 10-1, 91 and 332, expressed the smooth muscle-specific proteins, h1-calponin and α-actin (Table 1). From them, we chose the TBR-B cell for further study as it showed the clear dependence of the marker expression on the culture condition (Table 1 and Fig. 1).

We found that during the culture in the differentiation medium, some portion of TBR-B cells were differentiated into multinucleated myotubes and adipocytes [15]. We, therefore, performed immunostaining with the anti-smooth muscle α-actin antibody to determine the portion of SMC-like cells present after 2 weeks of culture in the differentiation medium. As a result, we found that about 20% of the cells cultured in the differentiation medium expressed α-actin, while few cells grown in the maintenance medium were positively stained (Fig. 2).

Although TBR-B cells grown in the differentiation medium expressed h-caldesmon and SM22α (Figs. 1 and 3), they did not express smooth muscle myosin heavy chain-1, a strict marker for SMC, in this culture condition (data not shown).

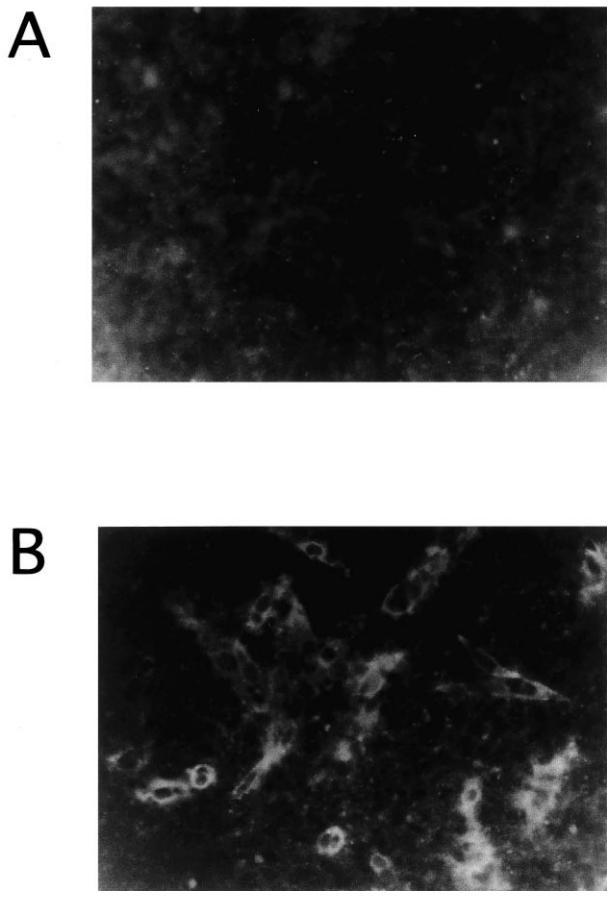

Fig. 2. Immunostaining of TBR-B cell line with antibody to α-actin. TBR-B cells were grown in the maintenance medium (A) or in the differentiation medium (B) at 33°C for 14 days, fixed and stained first with monoclonal antibody to α-actin and then with a Cy2-labeled secondary antibody as described in Section 2. (100×).

This suggests that the cells represent primitive SMCs seen at an early stage in the smooth muscle development [19].

We also examined the mRNA levels of smooth muscle-specific genes in TBR-B by semi-quantitative RT-PCR analysis as described in Section 2. Interestingly, h1-calponin, SM22α and α-actin mRNAs were induced after 2 days of culture in the differentiation medium, while the induction of h1-calponin and α-actin protein expression required at least 10 days in the same culture condition (data not shown). Since mRNA expression of h1-calponin and α-actin was detected at an earlier stage than protein expression, there might exist more than two regulatory mechanisms for the expression of smooth muscle-specific genes, including transcriptional and translational regulations. We are now trying to identify the regulatory factors for the expression of smooth muscle-specific markers by using the TBR-B cell line.

Table 1
Expression of h1-calponin and α-actin in TBR cell lines

TBR lines	33°C		37°C	
	MM	DM	MM	DM
B	–	A/C	–	A/C
10-1	A/C	A/C	A/C	A/C
91	–	C	–	C
332	–	A	–	A/C

TBR cells were cultured for 2 weeks in the maintenance medium (MM) or in the differentiation medium (DM) at 33°C or 37°C, respectively. Expression of α-actin (A), h1-calponin (C) was detected as described in Section 2.

3.2. *Ascorbic acid is an inducing factor for smooth muscle-specific markers*

In order to examine whether TBR-B cells can be used for screening compounds inducing SMC differentiation, we further investigated the conditions of marker expression in detail. The expression of h1-calponin and α-actin was induced in TBR-B cells cultured in the differentiation medium at both permissive and non-permissive temperature (Table 1), suggesting that the induction depended not on the activity of temperature-sensitive SV40 large T-antigen, but on the culture medium. Thus we searched for the ingredients of the medium that affect the expression of smooth muscle-specific genes. Change of serum concentration or the addition of insulin, transferrin or EGF did not affect the expression (data not shown). In contrast, α-MEM, which is the base of the differentiation medium, induced the expression of h1-calponin and α-actin in TBR-B cells, while RITC80-7, which is the base of the maintenance medium, did not (data not shown).

L-Ascorbic acid was suspected to be an inducing factor of the differentiation because it is included only in α-MEM at 0.3 mM but not in RITC80-7. As shown in Fig. 4, after 2 weeks in the maintenance medium containing 0.3 mM L-ascorbic acid, TBR-B cells expressed h1-calponin and α-ac-

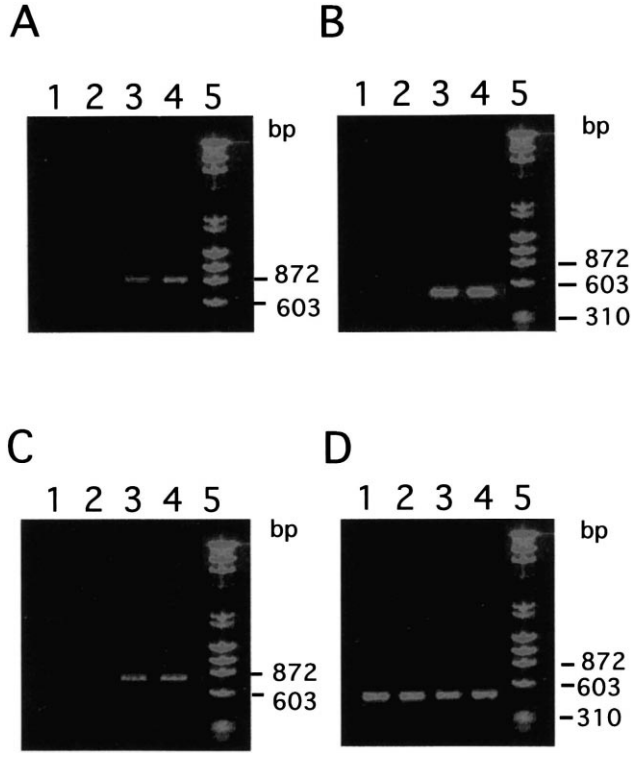

Fig. 3. RT-PCR analysis of smooth muscle-specific markers in the TBR-B cell line. Total RNA was extracted after 2 days culture in the maintenance medium (lanes 1 and 2) or in the differentiation medium (lanes 3 and 4) at 33°C. RT-PCR was performed as described in Section 2. An 841 bp band for h1-calponin (A), a 418 bp band for α-actin (B), a 662 bp band for SM22α (C) and a 455 bp band for G3PDH (D) were observed. Lane 5 shows the molecular marker, λ*Hin*dIII+ϕX174/*Hae*III digest.

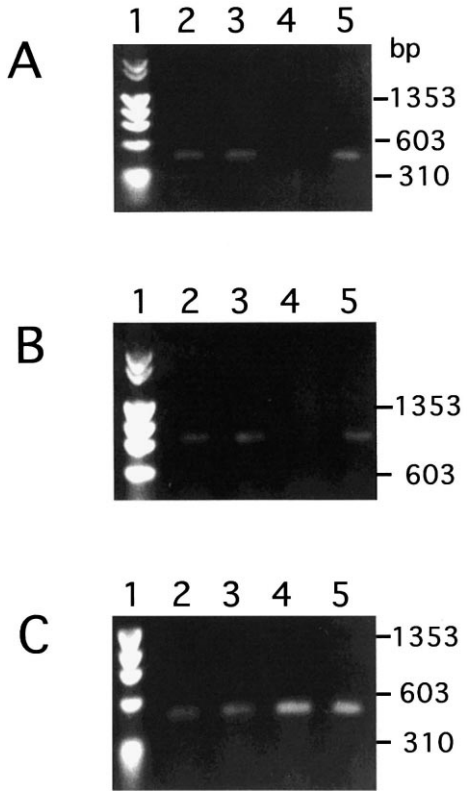

Fig. 4. L-Ascorbic acid and L-ascorbic acid 2-phosphate induced h1-calponin and α-actin expression in the TBR-B cell line. Total RNA was extracted 11 days after culture in the maintenance medium (lane 4), in the maintenance medium containing 0.3 mM L-ascorbic acid (lane 2), in the maintenance medium containing 0.3 mM L-ascorbic acid 2-phosphate (lane 3) or in the differentiation medium (lane 5). Media were changed every 2 days. RT-PCR was performed as described in Section 2. A 418 bp band for α-actin (A), an 841 bp band for h1-calponin (B) and a 455 bp band for G3PDH (C) were observed. Lane 1 shows the molecular marker, λ*Hin*dIII+φX174/*Hae*III digest.

tin mRNAs. L-Ascorbic acid 2-phosphate, a long-acting derivative of L-ascorbic acid [20], also induced the marker expression (Fig. 4). L-Ascorbic acid and L-ascorbic acid 2-phosphate were reported to be required for the differentiation of mesenchymal derived tissues such as skeletal muscle, cartilage and bone [21–23]. We here for the first time demonstrated that L-ascorbic acid also has the potential to induce smooth muscle differentiation.

However, unlike the case of the culture in the differentiation medium, in which the expression of h1-calponin and α-actin proteins was induced, the expression of these proteins was not induced by adding L-ascorbic acid and L-ascorbic acid 2-phosphate to the maintenance medium (data not shown). Again, it may suggest that there exist at least two mechanisms regulating the expression of smooth muscle-specific genes, and that other factors in α-MEM are necessary for the expression of these proteins.

In summary, we showed that the bone marrow stromal cell line, TBR-B, has the ability of expressing the smooth muscle-specific genes, and that the phenotype of TBR-B cell line could be controlled by changing culture conditions. TBR-B cells will serve as a useful model system for the study of regulatory mechanisms of smooth muscle-specific gene expression, and for the development of the factors inducing SMC differentiation.

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