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Increased Smooth Muscle Actin Expression from Bone Marrow Stromal Cells under Retinoic Acid Treatment: An Attempt for Autologous Blood Vessel Tissue Engineering

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SUMMARY Vascular replacement in vital organs is sometimes necessary for human life for example because of atherosclerosis. Blood vessel tissue engineering is applied for autologous transplantations to avoid graft rejections. Stem cells are used for blood vessel tissue engineering because they are the origin of smooth muscle cells, endothelial cells and fibroblasts. This paper shows that bone marrow stromal cells (BMSCs) can be induced to differentiate into the early stage of smooth muscle cells by using 0.01μM retinoic acid. The differentiation of BMSCs to smooth muscle cells was detected by the expression of smooth muscle alpha actin (SM α-actin), the earliest smooth muscle cell marker. The SM α-actin marker expression was demonstrated using indirect immunofluorescence technique and Western blot analysis. The induction of BMSC to form early stages of smooth muscle cells in this study is appropriate for blood vessel tissue engineering because the early stage smooth muscle cells may be stimulated to develop vascular walls with endothelial cells using a co-culture system.

There has been increasing evidence that adult stem cells have a great potential for autologous transplantations.[1, 2] In addition, the use of adult stem cells is practical since they can be obtained from various human tissues and organs. The peripheral blood, the umbilical cord blood and the bone marrow can provide a considerable number of stem cells for this purpose. The bone marrow consists of various kinds of stem cells including hematopoietic stem cells (HSCs), endothelial progenitor cells (EPCs), and bone marrow stromal cells (BMSCs).[1,3-5] The BMSCs differ from EPCs and HSCs by showing negative for the CD34 marker and by their adherence properties.[4,5] The BMSCs which can differentiate into various cell types are interesting for tissue engineering.[6] Some studies have shown that BMSCs can express SM α-actin under *in vitro* conditions both in medium containing serum as well as in serum-free medium.[7,8,9] The expression level of SM α-actin increased under treatment with TGF-β1 (transforming

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growth factor-β1) and they could perform functions during *in vitro* assays.[9]

The association between the development of the cardiovascular system and vitamin A has been recognized in mammals; Vitamin A deficiency (VAD) produces a variety of aortic arch malformations.[10,11] Retinoic acid (RA), a metabolite of vitamin A was reported to be a differentiating factor for smooth muscle cells in P19, a mouse embryonal carcinoma cell line and in mouse embryonic stem (ES) cells.[12,13] Moreover, RA is known to play a biological role in both cell proliferation and in maintenance of differentiated phenotypes in smooth muscle cells.[14] The role of RA for BMSCs as adult stem cells for tissue engineering has not yet been studied. We have investigated the possible effects of RA on BMSC in this study. The study has been designed to compare the development patterns between RA and TGF-β1 stimulation. The early development stage of smooth muscle cells was our target since in that early stage smooth muscle cells may have a better biological adaptation for application in blood vessel tissue engineering.

MATERIALS AND METHODS

The experimental protocols used in this study were approved by the Ethics Committee of Mahidol University. Informed consent was obtained from individual studied cases under the therapeutic treatment protocol.

Preparation of BMSCs

After obtaining informed consent, bone marrow was aspirated from the iliac crest of normal donors and added into Dulbecco's modified Eagle's medium (DMEM, Gibco) with heparin. An aliquot of 10 ml bone marrow in medium was washed with two-volume PBS and centrifuged at 400 x *g* for 5 minutes. The upper layer of the platelet-rich fraction was removed by aspiration and the bottom layer was applied on a Histopaque -1077 solution (Sigma). The cells were spun at 600 x *g* for 15 minutes and the upper layer of the mononuclear cell fraction was clearly separated from the bottom layer of granulocytes and red blood cells. The mononuclear cells were then resuspended in serum free medium and cultured in DMEM supplemented with 10% fetal bovine serum (FBS, HyClone), penicillin (Sigma), and streptomycin (Gibco) in tissue flasks (Corning Inc.). After 24-48 hours, the adhered cells were maintained in a growth medium whereas the floating cells were removed. The confluent cells were subcultured using 0.05% trypsin-EDTA (Sigma) and expanded in a humidified incubator at 37°C with 5%CO_2. The 3rd-5th passages of BMSCs were used in this study.

Treatment of BMSCs for SMC differentiation

The SMC differentiation from embryonic and adult stem cells has been described elsewhere.[9,13] In this study, the treatment protocol was modified as follows. The BMSCs were resuspended in serum free medium, DMEM supplemented with 2% FBS (Hyclone), insulin-transferrin-selenium (ITS, Gibco) and penicillin-streptomycin. The results of three independent experiments were compared. Each experiment contained 5 x 10^5 bone marrow cells. The experiments included the control group (cells in serum free medium), TGF-β1 (2 ng/ml) treated cells (R & D), and retinoic acid (RA, Sigma) treated cells (concentrations of 0.01 μM and 0.1 μM respectively). The cells were continuously cultured for 5 days.

Identification of the smooth muscle cell marker

Immunofluorescence labeling

Cells were fixed in cold methanol on a glass coverslip and washed with phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA). The fixed cells were subsequently incubated for 60 minutes with primary antibodies (smooth muscle α-actin, DAKO, clone 1A4) and washed three times. The cells were then stained with a Rhodamine-labeled secondary antibody, washed, and mounted for observation with a Nikon fluorescent microscope. The cell morphology was followed up using Act-1 software (Nikon).

Western blot analysis

The SM α-actin, an early marker of SMC was measured by using Western blotting technique. Briefly, cells were lysed in lysis buffer containing 50 mM Tris, 150 mM NaCl, 10mM EDTA, 0.1% SDS, 1% Triton X-100, 1% deoxycholate, and aprotinin 25 μg/ml. The protein concentration was determined

using Lowry's method.[15] Ten micrograms of protein were denatured by boiling, reduced in 4X LDS (lithium dodecyl sulfate) sample buffer (Invitrogen), followed by electrophoresis in 12% SDS-polyacrylamide gel. The protein was transferred to a PVDF membrane (Pall Corporation) and immunoblotted by using monoclonal antibodies to SM α-actin (DAKO, clone 1A4). Secondary anti-mouse antibodies conjugated to horseradish peroxidase (Invitrogen) were used for detection using Westernbreeze technology (Invitrogen) and X-ray film exposure (Kodak). Films were analyzed using a multipolar densitometer and reported as percentage of control BMSCs depleted of treatments.

RESULTS

Morphology and cell arrangement of BMSCs under treatment

Fig. 1 shows cell organization in TGF-β1and RA treated BMSCs as compared to non treated cells. Treated cells had a greater tendency to form clusters. The clusters connected to each other by spindle shaped cells. It was noted that the TGF-β1 treated BMSCs in the cluster had taped ends with a broad shape spreading their cytoplasm onto the bottom of the culture plate whereas RA treated cells were more intact joining together in a cluster pattern. On day 5, the TGF-β1 treated cells had a greater cell density than the RA treated cells.

Under normal control conditions, bone marrow stromal cells (BMSCs) in our culture mainly appeared elongated with taped ends. The cells were randomly arranged. Spindle shaped cells were observed throughout the experiment (Fig. 2A). Under TGF-β1 treatment, BMSCs maintained a similar morphology to the controls up to 24 hours and then became flat within 48 hours (Fig. 2B). Cells with a ribbon-like shape with cytoplasmic fibers were noted as well. This has been observed throughout the culture period. In the RA 0.01μM treated BMSCs, cell

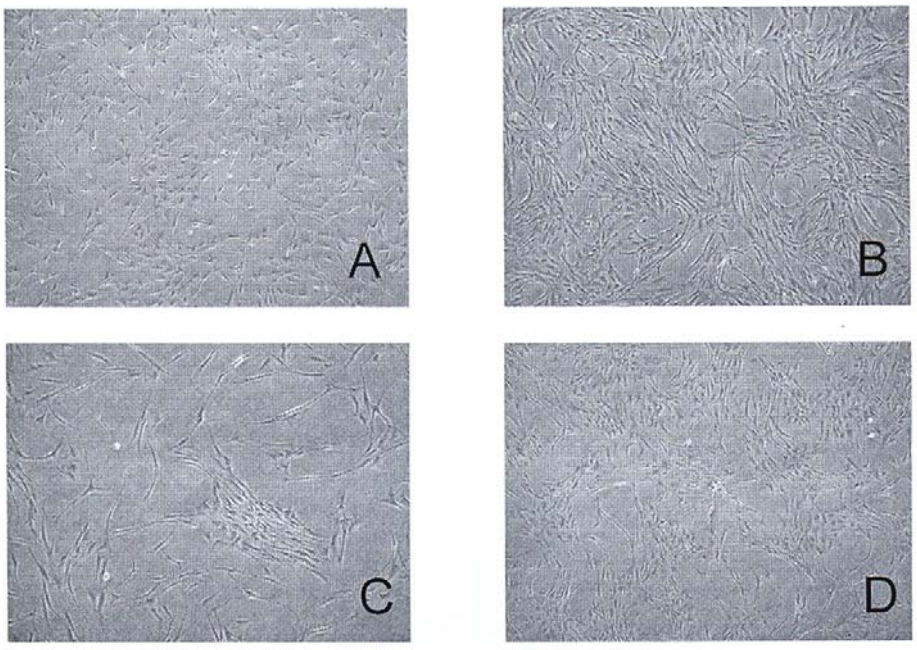

Fig. 1 Patterns of cell arrangement of BMSCs under various treatment conditions. On day 5, the cultured cells arranged themselves in different patterns: (A) Untreated cells in ITS medium (control condition) showed randomly dispersed cells with no distinct orientation. (B) TGF-β1 treatment. High cell density with cell orientation in whorl-like fashion. (C) 0.01 μM and (D) 0.1 μM retinoic acid treatment. The RA treated cells arranged themselves into multi-clusters. Each cluster was joined together by spindle shaped cells. A lesser degree of cell density was noted as compared to TGF-β1 treatment.

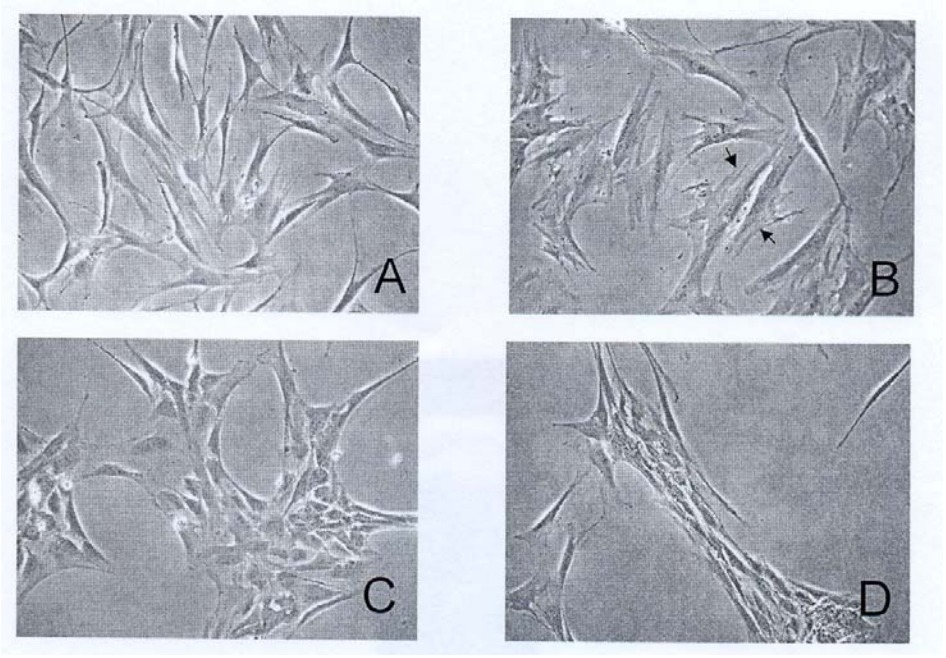

Fig. 2 Phase-contrast micrographs of BMSCs during treatment: (A) Control BMSCs cultured in serum free medium (2% FBS, ITS) (original magnification, x 200), (B) a more rectangular shape of BMSCs was observed after treatment with TGF-β1 within 48 hours and some cells had stress fibers in the cytoplasm (arrows). The retinoic acid treated cells showed characteristics of cell clumping with cell orientation (C, D) (original magnification, x 200).

orientation was shown by the presence of cell clusters. The cell clusters were joining by spindle shaped cells within 48 hours (Fig. 2C). The same pattern was shown in 0.1μM RA treated BMSCs (Fig. 2D).

Immunofluorescence detection of treated BMSCs

Using the indirect immunofluorescence technique, BMSCs induced with TGF-β1 and 0.01 μM RA (Fig. 3C-D, E-F) showed a strongly positive reaction with SM α-actin, the early marker of SMCs. The strong staining indicated that the BMSCs have been induced to differentiate into SMCs under the treating protocol. Since the BMSCs themselves were weakly positive for SM α-actin (Fig. 3A, B), further confirmation using western blot analysis was done in this study.

Western blot analysis of BMSCs

The western blot of the SM α-actin expression (Fig. 4) was analyzed by a densitometer and summarized as percentage of control cells. The TGF-β1 treated BMSCs on day 3 and day 5 showed increased SM α-actin expression (Mean ± SE, 149 ± 26% and 216 ± 86%, respectively) as compared to the control BMSCs (100%). Regarding the different dosages of RA treatment, 0.01 μM RA could increase SM α-actin expression at a steady level during day 3 (129 ± 29%) to day 5 (125 ± 9%) whereas, 0.1 μM RA showed an unchanged SM α-actin expression similar to the control.

DISCUSSION

The BMSCs can express smooth muscle characteristics after a long term marrow culture by their own.[7] These BMSCs may be the progeny of mesenchymal cells which can follow the smooth muscle differentiation pathway.[16] Many factors have been reported to be involved in smooth muscle cell differentiation of different cell types. TGF-β1 has been reported to be an inducer of smooth muscle differentiation for different cell types such as bone marrow stem cells[9] and 10T1/2 cell lines.[17] However, TGF-β had no such inducing effect on fetal liver

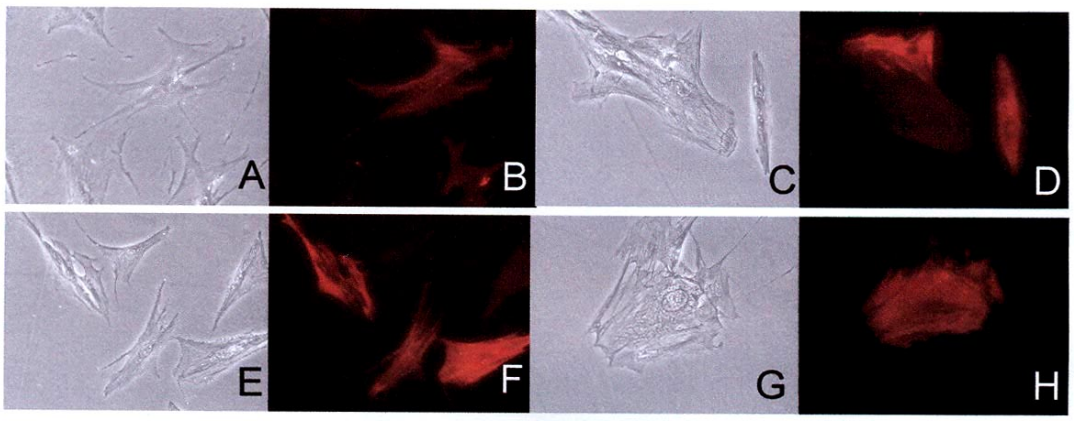

Fig. 3 Expression of SM α-actin in BMSC in the control culture (A, B), as well as in TGF-β1 (C, D), 0.01 μM RA (E, F) and 0.1 μM RA (G, H) treated cells. Both TGF-β1 and 0.01 μM RA treated cells gave a much stronger reaction than the control cells.

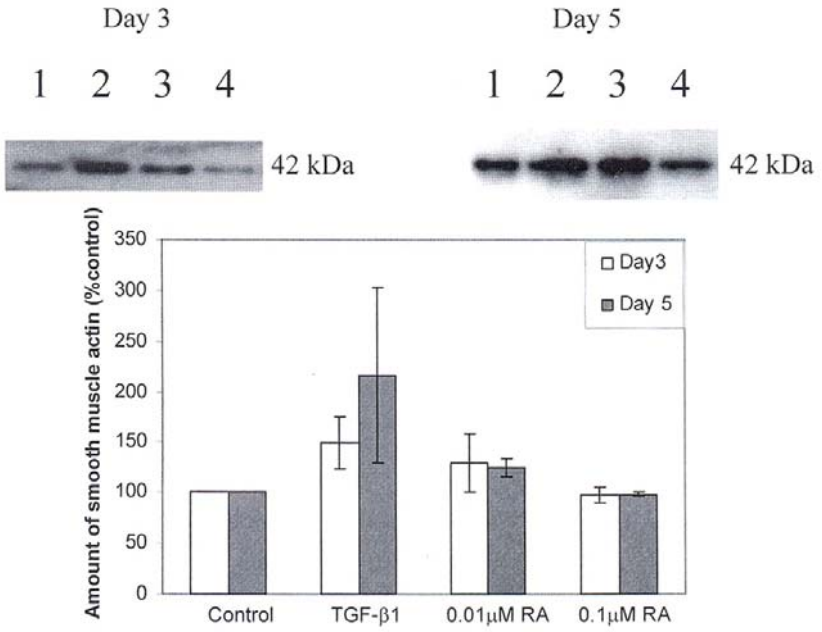

Fig. 4 Western blot analysis of SM α-actin expression on treated BMSCs. Upper figure: lane 1, control; Lane 2, TGF- β1 treatment; Lane 3, 0.01 μM RA treatment, and Lane 4, 0.1 μM RA treatment. The figure shows a comparison of treated cells for three days and five days. Each lane represents 10 μg loading of total protein. The histogram (lower figure) shows the results of the densitometry (n = 3) in this experiment as the mean ± S.E., relative to the control BMSCs (100%).

kinase-1 (flk-1) positive cells derived from embryonic stem cells.[18] Our study also demonstrated that TGF-β treated BMSCs could express an increasing amount of alpha smooth muscle actin, the early expression marker of smooth muscle cells. RA is another factor reported to be an inducer of smooth

muscles like cells in the P19 embryonic carcinoma cell line[12] and in mouse embryonic stem cells.[13] It was shown that when arterial smooth muscle cells were cultured in RA, the differentiation stage of smooth muscle cells could be maintained as shown by an increase of both SM α-actin and myosin heavy chain expression and by a lowering of proliferation and ECM (extracellular matrix) synthesis.[19] We found that BMSC cultured in an optimal concentration (0.01μM) of retinoic acid expressed SM α-actin. A higher concentration of RA (0.1 μM) unexpectedly resulted in a lower SM α-actin expression. The inhibitory effect of a high dose of RA on SM α-actin expression was previously shown in vascular smooth muscle cells.[19] Our results confirmed that the inhibitory effect of high-dose RA could also be found in stem cells in addition to differentiated smooth muscle cells from vascular origin.

RA has been shown to regulate the expression of the homeobox gene which is a powerful regulator of pattern formation of the developing embryo. Several members of the HOX clusters show a related role for the cardiovascular system and vasculogenesis process.[20] The paired-related homeobox genes MHox or Prx 1 and Prx 2 or S8 have been shown to play an important role in vasculogenesis and early vessel development.[21] Prx1 was shown to promote the expression of genes that contain the CArG element in their promoters such as c-fos.[22] The SM α-actin promoters have been shown to contain two CArG elements. A higher expression of SM α-actin can be promoted via serum responsive factor (SRF) binding to these elements.[23] RA can promote both MHox and SM α-actin expression in P19 embryonal carcinoma cells.[12] The SRF was shown to be highly expressed in novel P19-derived clonal cell lines after treatment with RA.[24] However, the mechanism how RA influences the homeobox gene of BMSCs still has to be elucidated. The cell orientation in a loop-like fashion in RA treated BMSCs in our study may indicate morphological evidence of vasculogenesis from stem cells.

In conclusion, this paper has shown that triggering BMSC differentiation may require RA at an optimal concentration. According to the microscopic investigation, the RA -induced BMSCs had an intact cell morphology with strong cell to cell interaction by forming cell clusters and spindle shaped cells. With increasing the dose of RA to 0.1 μM, the BMSCs produced a lower amount of SM α-actin although they shared a similarity in cell morphology and arrangement with the 0.01 μM RA induced BMSCs. In addition, the expression of an early marker of smooth muscle cells in 0.01 μM RA induced BMSCs may be suitable for further co-culture with endothelial cells since vascular formation requires an early stage of cells. We suggest that 0.01 μM RA induced BMSCs may be useful in blood vessel tissue engineering.

ACKNOWLEDGEMENTS

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