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Interferon-γ and NF-κB mediate nitric oxide production by mesenchymal stromal cells

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Abstract

Mesenchymal stromal cells (MSCs) have been shown to have an immunosuppressive effect. Previously, we demonstrated that nitric oxide (NO) is one of the immunomodulatory mediators of MSCs. We herein show that primary mouse bone marrow MSCs and three cell lines that mimic MSCs suppress both differentiation and proliferation in Th1 condition, whereas the suppression in Th2 condition is mild. NO production is inversely correlated with T cell proliferation in Th1 and Th2 conditions. NO is highly induced in Th1 and minimally induced in Th2. Moreover, an inhibitor of NO synthase restores both proliferation and interferon-γ (IFN-γ) production in Th1 condition. Furthermore, an anti-IFN-γ antibody strongly inhibits NO production and an inhibitor of NF-κB reduces the level of induction of inducible NO synthase (iNOS) in MSCs. Taken together, our results suggest that NO plays a significant role in the modification of Th1 and Th2 differentiation by MSCs, and that both IFN-γ and NF-κB are critical for NO production by MSCs.

Keywords: Mesenchymal stem cells; Nitric oxide production; Interferon-γ; NF-κB; Th1 differentiation; Th2 differentiation; Immunosuppression; 10T1/2; iNOS

Although many reports indicate that mesenchymal stromal cells (MSCs) suppress T cell proliferation, the molecular mechanisms involved are poorly understood [1–6]. Human MSCs were reported to suppress Th1 differentiation and augment Th2 differentiation [4]. In this study, we investigated whether mouse bone-marrow-derived MSCs have the same effect on Th1 and Th2 differentiation while were also trying to identify the underlying molecular mechanisms of these effects.

Naïve helper T cells primarily differentiate into either Th1 or Th2 cells. Th1 cells produce IFN-γ and interleukin-2 (IL-2) while Th2 cells produce IL-4, IL-5, and IL-13. In addition, it appears that a disruption of the balance between Th1 and Th2 differentiation is associated with the development of immune diseases [7,8].

Previously, two sub-lines of the parental C3H10T1/2 (10T1/2) cell line were established by treatment of that line with 5-azacytidine. These were characterized as a preadipocyte cell line and a myoblast cell line, and were designated A54 and M1601, respectively [9]. A54 and M1601 differentiate into adipocyte and myotube under defined conditions [9]. Other studies suggest that 10T1/2 cells have the same T cell suppressive effect as primary MSCs [10]. In addition, the 10T1/2 cell line has been used as a model of MSCs [11–14]. In the present study, these three cell lines were used as MSC-like cells and demonstrated that their phenotype is similar to that of primary MSCs.

Nitric oxide (NO) is known to suppress T cell proliferation [15–18]. Previously, we demonstrated that NO is one of the major mediators of T cell suppression by mouse MSCs [19]. It is possible that NO also plays a critical role

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Then, cells were stained in FACS buffer (PBS supplemented with 10% FBS) with appropriate concentrations of antibodies for 30 min on ice, washed with FACS buffer, and analyzed with a BD LSR cytometer (BD Biosciences), and the data were analyzed using CELLQUEST software (BD Biosciences).

[^{3}H]Thymidine incorporation, immunoblot analysis, detection of inducible NO synthase (iNOS) expression. We performed these assays as described previously [19].

Results

Effects of MSCs on Th1 differentiation

Using primary MSCs and three MSC-like cell lines, the effects of co-cultivation of MSCs on Th1 and Th2 differentiation were examined. Fig. 1A, left panel illustrates that primary MSCs and the A54 cell line strongly suppressed proliferation in Th1 condition. The 10T1/2 and M1601 cells also showed modest suppression. Consistent with this, [^{3}H]thymidine incorporation demonstrated strong suppression of T cell proliferation in the presence of primary MSCs and the A54 cell line and modest suppression in the presence of the 10T1/2 and M1601 cell lines (Supplementary Fig. 1A).

Simultaneously, the production of IFN-γ in the presence of MSCs was examined. In addition to the suppression of T cell proliferation, IFN-γ production is also strongly suppressed in the presence of primary MSCs and the A54 cell line (Fig. 1A, right panel). This suppression was not due to the suppressed number of T cells because the suppression of IFN-γ was also observed with an ELISA, in which an equal number of differentiated cells were used (Supplementary Fig. 1C).

Effects of MSCs on Th2 differentiation

In contrast to Th1 differentiation, a modest suppression of Th2 cell proliferation was observed by a flow cytometric analysis (Fig. 1B, left panel). Specifically, primary MSCs and the A54 cell line showed significantly less suppression compared to the suppression in Th1 condition (Fig. 1B, left panel vs. Fig. 1A, left panel). Consistent with this, [^{3}H]thymidine incorporation assay demonstrated a less suppression of T cell proliferation in Th2 as compared to Th1 in the presence of primary MSCs and A54 cell line (Supplementary Fig. 1B vs. 1A). IL-4 production in the presence of MSCs was suppressed in Th2 condition, as measured by both flow cytometric analysis (Fig. 1B, right panel) and ELISA, in which the same number of differentiated cells were used (Supplementary Fig. 1D).

Nitric oxide production in Th1/Th2 differentiation

Since nitric oxide (NO) is one of the primary mediators of T cell suppression by MSCs [19], it is possible that NO is also involved in suppression of proliferation in Th1 condition. We found a reverse correlation between NO production and T cell proliferation in Th1/Th2 conditions (Fig. 2A and B), where NO production was highly induced in the presence of MSCs in Th1 but it was induced only minimally in Th2. In particular, primary MSCs and the A54 preadipocyte cell line, which induce strong T cell suppression in Th1 (Fig. 1A), produce high levels of NO in Th1 condition (Fig. 2A). These results suggest that NO also plays a major role in the preferential suppression of Th1 proliferation by MSCs.

Inhibition of NO synthase restores proliferation and differentiation of Th1 cells

To confirm the role of NO, a specific NO synthase inhibitor, NG-monomethyl-L-arginine (L-NMMA) was used. The presence of this inhibitor restored the T cell proliferation and IFN-γ production in Th1 condition (Fig. 3A and B), demonstrating that NO is a mediator of suppression of both T cell growth and IFN-γ production in Th1 condition. However, L-NMMA does not completely restore the T cell proliferation by primary MSCs, suggesting that there are other factors involving in this suppression, as demonstrated in the previous study [19].

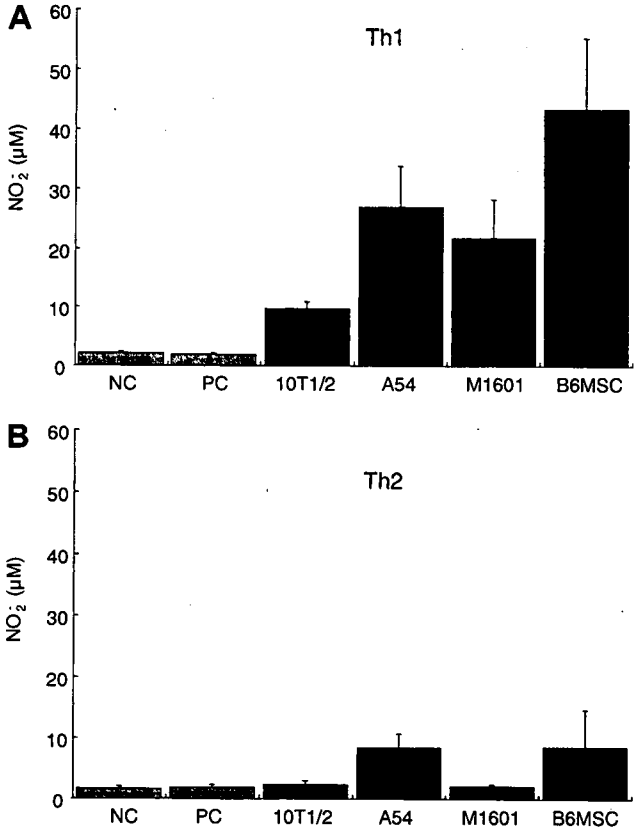

Fig. 2. NO production in Th1 and Th2 conditions in the presence of MSCs. (A) NO production in Th1 condition in the presence of MSCs. (B) NO production in Th2 condition in the presence of MSCs. Splenic $CD4^+$ T cells (1×10^6) were stimulated in either Th1 or Th2 condition in the presence of 10T1/2, A54, M1601, and primary MSCs (1×10^5) for 48 h. The concentration of NO_2 in supernatants were determined by the Greiss assay. NC, negative control; $CD4^+$ T cells alone; PC, positive control; $CD4^+$ T cells with mitogen and without MSCs.

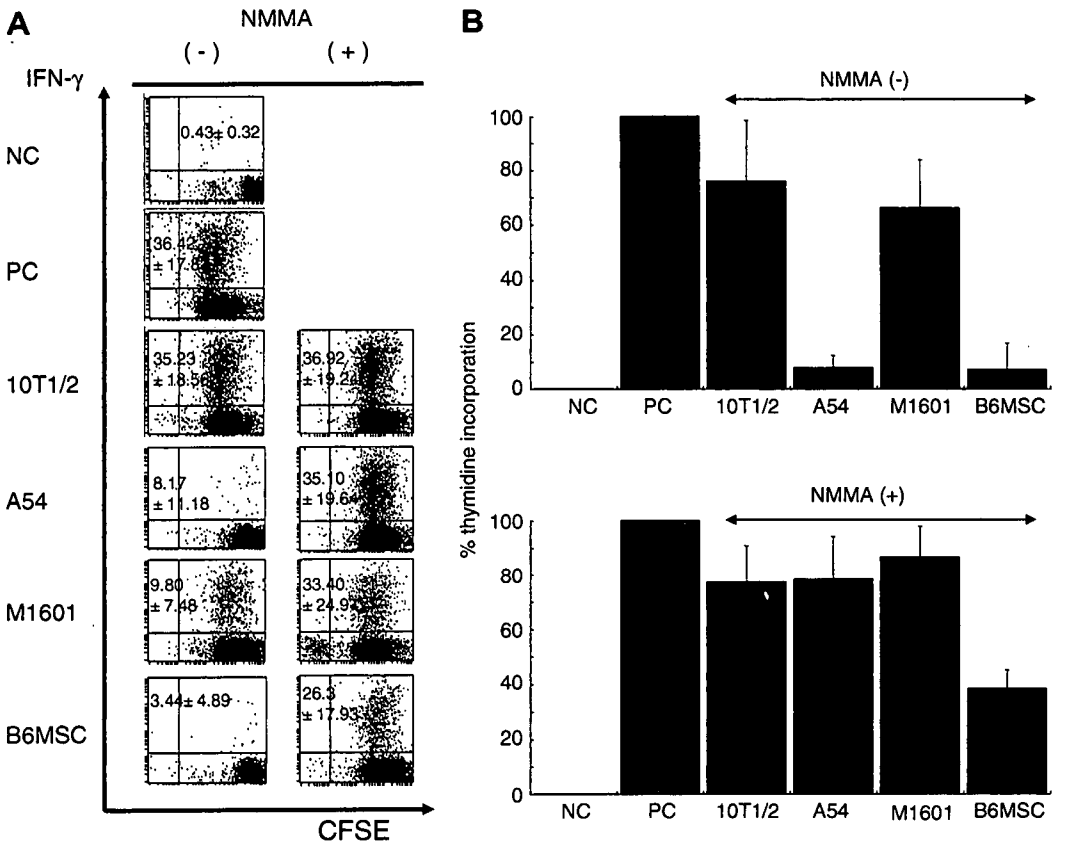

Fig. 3. A specific NO synthase inhibitor, L-NMMA, restores T cell proliferation and IFN-γ production in Th1 condition. (A) A simultaneous flow cytometric analysis of T cell differentiation and proliferation in Th1 condition as described in Fig. 1. $CD4^+$ T cells (1×10^6) were stimulated in Th1 condition in the presence of 10T1/2, A54, M1601, and primary MSCs (1×10^5) with or without 1 mM L-NMMA for 48 h. The numbers indicated are mean ± SD of the percentage of cells in upper right quadrant. (B) [^{3}H]Thymidine incorporation assay in the presence or absence of L-NMMA. $CD4^+$ T cells (1×10^5) were stimulated in Th1 condition in the presence of 10T1/2, A54, M1601, and primary MSCs (1×10^4) with or without 1 mM L-NMMA for 48 h. [^{3}H]Thymidine was pulsed for the last 6 h. 10T1/2, A54, M1601, and primary MSCs were pre-irradiated with 30 Gy to prevent [^{3}H]thymidine incorporation into MSCs. The percentage of proliferation is indicated compared to positive control, which is no MSCs. Shown are means ± SD from three independent experiments. NC, negative control; $CD4^+$ T cells alone; PC, positive control; $CD4^+$ T cells with mitogen and without MSCs.

IFN-γ and a factor that activates NF-κB are crucial for NO production from MSCs in the absence of T cells

To determine what inhibits the production of NO in Th2 condition, the two differentiation factors that mediate the Th2 pathway, anti-IFN-γ antibody and IL-4, were investigated. It is possible one or both of these factors is responsible for the minimum production of NO in Th2 condition. As shown in Fig. 4A, anti-IFN-γ antibody clearly inhibits the production of NO, whereas suppression by IL-4 was less evident. These results suggest that IFN-γ is a key regulator of NO production by MSCs.

Interestingly, cell supernatant collected from activated but not non-activated T cells had the ability to induce NO by MSCs (Supplementary Fig. 2A). What signal(s) are required for NO production by MSCs? As shown in Fig. 4A, IFN-γ is critical for NO production; however, in a T cell-free environment, IFN-γ alone does not induce production of NO from primary MSCs (Fig. 4B, both panels). IFN-γ in combination with LPS, but not IL-2, stimulates NO secretion from primary MSCs (Fig. 4B, left panel and data not shown), suggesting that both the IFN-γ and the signal from Toll-like receptor-4 (TLR4) are required for NO induction by MSCs. Could other TLR ligands substitute for LPS? The addition of flagellin induced NO production in combination with IFN-γ (Fig. 4B, left panel). While, synthetic double strand RNA, poly(I:C), and CpG-oligonucleotide did not induce NO (data not shown). Flagellin is a protein component of bacteria known to induce NO production from macrophages via TLR5 in the presence of either a TLR4 or IFN-γ signal [21,22]. In addition to these factors, IL-1β and TNF-α induce NO when provided in combination with IFN-γ (Fig. 4B, right panel and Supplementary Fig. 2B). As NF-κB is a downstream target of the signaling cascades activated by LPS, flagellin, IL-1β, and TNF-α, we hypothesized that activation of NF-κB is required for NO induction by MSCs. As shown in Fig. 4C, Bay 11-7085 [20], a specific inhibitor of NF-κB suppressed production of inducible NO synthase (iNOS) in MSCs, thus suggesting that NF-κB is involved in NO production by MSCs as well as IFN-γ.

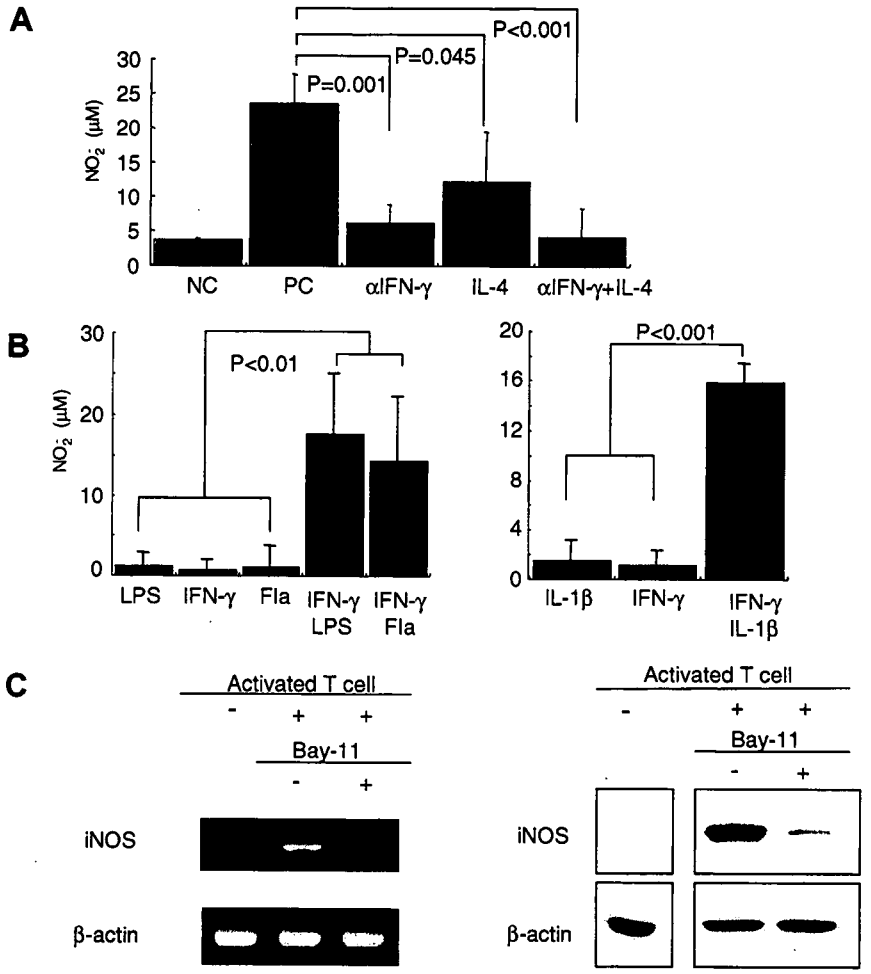

Fig. 4. IFN-γ and NF-κB are involved in NO production. (A) Anti-IFN-γ antibody inhibits NO production. $CD4^+$ T cells (1×10^6) were stimulated with anti-CD3/CD28 beads in the presence of primary MSCs (1×10^5) without any differentiation factors. A neutralizing antibody against IFN-γ (10 μg/ml), IL-4 (100 ng/ml), and a combination of both were added. NC, negative control; $CD4^+$ T cells alone; PC, positive control; $CD4^+$ T cells with mitogen. (B) LPS, flagellin, TNF-α, or IL-1β in a combination with IFN-γ induces NO production from primary MSCs in a T cell-free system. The primary MSCs (1×10^5) were treated with the indicated stimuli without T cells for 48 h and NO concentrations of supernatants were determined by the Greiss assay. (C) A NF-κB inhibitor, Bay11-7085, suppresses iNOS induction in MSCs. Unfractionated splenocytes (1×10^7) were stimulated with anti-CD3/CD28 beads for 48 h and put onto primary MSCs (1×10^6). After co-cultivation for 6 h, total RNA was harvested for RT-PCR and after 30 h, cell lysates were harvested for immunoblot analysis. In advance of co-cultivation, primary MSCs were pre-treated for 30 min with the irreversible NF-κB inhibitor, Bay11-7085 (5 μM), and washed twice with PBS. Just before harvesting, T cells were washed out extensively with PBS.

Discussion

This study demonstrates that mouse primary MSCs strongly suppress T cell proliferation and differentiation in Th1 condition, while the suppression in Th2 condition is weak. Moreover, it provided the first evidence, that NO production can elucidate the differential suppression in Th1/Th2 differentiation. We found a higher NO production in Th1 in comparison to Th2. Furthermore, L-NMMA, a specific inhibitor of NO synthases, restores not only T cell proliferation but also IFN-γ production in Th1 condition. In addition, the treatment with an anti-IFN-γ antibody resulted in a low level of NO production, suggesting that IFN-γ is critical for NO production. While the combination of IFN-γ and a NF-κB activating factor induces NO in a T cell-free system, and moreover, an inhibitor of NF-κB diminishes the induction of iNOS in MSCs, suggesting that NF-κB is also crucial for NO production by MSCs.

The suppression of IFN-γ production may be due to the suppression of cell proliferation, since differentiation is dependent upon cell division [23]. In fact, Fig. 1A, right panel shows that only proliferating cells can produce IFN-γ. However, MSCs were shown to suppress IFN-γ production, regardless of cell division [2,19].

Primary human MSCs induced a fivefold increase in IL-4 synthesis in Th2 condition [4], however, in this study, primary mouse MSCs in the Th2 pathway suppressed production of IL-4 as measured by intracellular staining and ELISA. The discrepancy may be due to species-specific difference. MSC-like cell lines also showed suppression in IL-4 production.

Our results demonstrate that LPS, flagellin, TNF-α, and IL-1β can induce NO from MSCs in a combination with

IFN-γ. These ligands are reported to induce NO from monocytes or macrophages in the presence of IFN-γ [24–28]. Monocytes and macrophages have been assumed to be prominent sources of NO. This implies, MSCs have several features in common with monocytes and macrophages. However, a combination of IFN-γ with IL-2 [27], flagellin alone [21], LPS alone [29], and IFN-γ alone [27], which induce NO from macrophages, do not induce NO from MSCs under the conditions we tested here, suggesting that MSCs also have features that make them different from monocytes and macrophages.

What is the factor required for NO induction in combination with IFN-γ? Cell supernatant from activated T cells induces NO, thus suggesting that activated T cells produce the NO inducer. Neither a soluble TNF-α receptor, a naturally occurring decoy receptor, nor a neutralizing antibody against IL-1β diminished NO production (data not shown). Our results do not define the factor from T cells but they do suggest that the factor may be a ligand that activates NF-κB in MSCs, such as LPS, flagellin, TNF-α or IL-1β.

This study demonstrate that NO is responsible for the preferential suppression of T cell proliferation in Th1 condition by MSCs, and that IFN-γ and NF-κB are involved in NO production in primary MSCs. This appears to be the first study to elucidate the molecular mechanism for preferential Th1 suppression by MSCs. Therefore, these results are considered to enhance the understanding and investigation in this field.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2007.02.054.

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Nitric oxide plays a critical role in suppression of T-cell proliferation by mesenchymal stem cells

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The molecular mechanisms by which mesenchymal stem cells (MSCs) suppress T-cell proliferation are poorly understood, and whether a soluble factor plays a major role remains controversial. Here we demonstrate that the T-cell–receptor complex is not a target for the suppression, suggesting that downstream signals mediate the suppression. We found that Stat5 phosphorylation in T cells is suppressed in the presence of MSCs and that nitric oxide (NO) is involved in the suppression of Stat5 phosphorylation and T-cell proliferation. The induction of inducible NO synthase (NOS) was readily detected in MSCs but not T cells, and a specific inhibitor of NOS reversed the suppression of Stat5 phosphorylation and T-cell proliferation. This production of NO in the presence of MSCs was mediated by CD4 or CD8 T cells but not by CD19 B cells. Furthermore, inhibitors of prostaglandin synthase or NOS restored the proliferation of T cells, whereas an inhibitor of indoleamine 2,3-dioxygenase and a transforming growth factor–β–neutralizing antibody had no effect. Finally, MSCs from inducible NOS$^{-/-}$ mice had a reduced ability to suppress T-cell proliferation. Taken together, these results suggest that NO produced by MSCs is one of the major mediators of T-cell suppression by MSCs. (Blood. 2007;109:228-234)

Introduction

Because mesenchymal stem cells (MSCs) differentiate into osteocytes, chondrocytes, myotubes, and adipocytes,[1-3] they are expected to become a source of cells for regenerative therapy. Also, MSCs support hematopoietic stem cell engraftment[4-9] and modulate immunologic responses by unknown mechanisms.[9-14] Here, we investigated the molecular mechanisms by which MSCs suppress T-cell proliferation.

Transforming growth factor–β (TGF-β), hepatocyte growth factor, indoleamine 2,3-dioxygenase (IDO), and prostaglandin E2 (PGE_2) have been reported to mediate T-cell suppression by MSCs.[13-15] Specifically, neutralizing antibodies against TGF-β or hepatocyte growth factor,[13] an inhibitor of IDO,[14] or an inhibitor of prostaglandin production reverse the inhibition of T-cell proliferation by MSCs.[15] In addition, some reports have shown that a soluble factor is the major mediator of suppression,[13-17] whereas some reports have demonstrated that T-cell–MSC contact is required for this suppression.[12-14,16,17] In the current study, we sought to resolve these conflicting results by using a mouse bone marrow–derived MSC system.

One candidate soluble factor for T-cell suppression is nitric oxide (NO) because it is known to inhibit T-cell proliferation.[18-25] NO is produced by NO synthases (NOSs), of which there are 3 subtypes: inducible NOS (iNOS), endothelial NOS, and neuronal NOS. Like MSCs, it has been known that macrophages suppress T-cell proliferation. This suppression was reported to be mediated by NO inhibition of Stat5 phosphorylation.[18,19] Also, MSCs were reported to produce NO when they differentiate into chondrocytes.[26] We therefore investigated whether MSCs can produce NO and whether NO is involved in their ability to suppress T-cell proliferation.

Materials and methods

Materials

N-nitro-L-arginine methyl ester (L-NAME), indomethacin, and concanavalin A (Con A) were purchased from Wako (Osaka, Japan). Con A was used at 5 μg/mL. Indomethacin was used at 5 μM. Phorbol 12-myristate 13-acetate (PMA) and ionomycin were from Sigma (St Louis, MO) and were used at concentrations of 50 ng/mL and 1 μg/mL, respectively. Antimouse CD3/CD28 beads (Dynal Biotech ASA, Oslo, Norway) were used at 10 μL per 10^6 cells. The transwell system with 1-μm pores for 12-well dishes was from BD Falcon (Franklin Lakes, NJ). Monoclonal antibodies for CD4, CD8, CD11b, CD25, CD29, CD44, CD45, CD69, Sca-1, B220, Gr-1, and interferon-γ (IFN-γ) were from BD Pharmingen (San Diego, CA). An inhibitor of IDO, 1-methyl-DL-tryptophan (1-MT), was purchased from Sigma. An antibody for TGF-β was purchased from Peprotech (Rocky Hill, NJ). Lipopolysaccharide was from Sigma.

MSCs

MSCs were obtained from wild-type or iNOS$^{-/-}$ C57BL/6 mice. Bone marrow cells were harvested from femurs and tibias by a standard flushing method[1] and then cultivated in a plastic dish in Iscove modified Dulbecco medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal calf serum (Sigma), 2 mM L-glutamine, 0.1 mg/mL streptomycin, and 100 U/mL penicillin G (Invitrogen) or in MF medium (Toyobo, Tokyo, Japan).

All primary MSCs were characterized at least once by flow cytometry and an in vitro differentiation assay. All MSCs were positive for CD29, CD44, and Sca-1, negative for CD11b, Gr-1, and CD45, and able to

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differentiate into adipocytes and osteoblasts (Figure S1, available at the *Blood* website; see the Supplemental Figures link at the top of the online article). We used at least 2 independently isolated batches of MSCs. These cells can be propagated for a long time and retain their surface phenotype and capacity to differentiate for at least 4 months.

Flow cytometric analysis

Cells were incubated with Fc block (BD Pharmingen) to inhibit nonspecific binding of antibodies to Fc receptors. Next, cells were stained in FACS buffer (phosphate-buffered saline [PBS] supplemented with 10% fetal bovine serum) with antibodies for 30 minutes on ice, washed with FACS buffer, and analyzed on a BD LSR flow cytometer (Becton Dickinson, Franklin Lakes, CA). Collected data were analyzed with CELLQUEST software (Becton Dickinson).

Enzyme-linked immunosorbent assay (ELISA)

ELISA kits for mouse IFN-γ and mouse interleukin-2 (IL-2; BD Pharmingen) were used according to the manufacturer's instructions.

Selection of CD4+, CD8+, and CD19+ cells

$CD4^+$, $CD8^+$, and $CD19^+$ cells were selected using mouse CD4, CD8, and CD19 MACS beads and an autoMACS system (Miltenyi Biotech, Auburn, CA). The purity of the cells as determined by flow cytometry with antibodies against CD4, CD8, and B220 was more than 80%.

Thymidine incorporation

Splenocytes were grown in 96-well plates containing RPMI 1640 (Invitrogen) supplemented with 10% fetal calf serum (Sigma), 2 mM L-glutamine (Invitrogen), 50 μM 2-mercaptoethanol (Sigma), 0.1 mg/mL streptomycin, and 100 U/mL penicillin G (Invitrogen). During the cultivation, the cells were pulsed for the last 8 hours of culture with 1 mCi (3.7×10^7 Bq) of [^{3}H]-thymidine (Amersham Biosciences, Piscataway, NJ). After 48 hours of growth, cells were harvested with a Packard FilterMate harvester (Perkin Elmer Life Sciences, Boston, MA), transferred to a UniFilter plate (Perkin Elmer Life Sciences), and analyzed using a TopCount microplate scintillation counter (Perkin Elmer Life Sciences). In the coculture system, MSCs were γ-irradiated (30 Gy) prior to cultivation to prevent thymidine incorporation. When using the transwell system (BD Falcon), one tenth of the cells were harvested and counted because the system includes 12-well culture dishes, which contain 10-fold more cells than the wells of a 96-well plate.

Western blot analysis

Polyclonal antibodies to phosphorylated Stat5 (Cell Signaling Technology, Danvers, MA), Stat5 (Santa Cruz Biotechnology, Santa Cruz, CA), cyclin D2 (Cell Signaling Technology), and Kip1 (Cell Signaling Technology) were used for Western blotting. Cells were lysed on ice for 15 minutes with a buffer consisting of 50 mM Tris (pH 7.5), 150 mM NaCl, 0.5% NP40, Complete proteinase inhibitor cocktail (Roche Diagnostics, Mannheim, Germany), and 1 mM Na_3VO_4. Lysates were centrifuged at 13 000*g* for 15 minutes, and supernatants were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred from the gel to a PVDF membrane (Invitrogen), and Western blotting was performed using enhanced chemiluminescence reagents (Pierce, Rockford, IL) to visualize the immunoreactive proteins.

Assay for NO production

NO is quickly converted to NO_2 and NO_3 in culture medium. Because of the presence of NO_3 in RPMI medium, we measured NO_2 production using a Griess reagent kit (Wako).

Detection of iNOS expression

Total RNA was prepared using an RNeasy kit (Qiagen, Valencia, CA) and 1 μg was reverse-transcribed using a First Strand Synthesis Kit (Invitrogen), and one tenth of the product was subjected to PCR using the following primers: for iNOS, 5′-GAGATTGGAGTTCGAGACTTC-3′ and 5′-TGGCTAGTGCTTCAGACTTC-3′; and for β-actin, 5′-CCATCATGAAGTGTGACGTTG-3′ and 5′-GTCCGCCTAGAAGCACTTGCG-3′.

Western blotting was performed using a polyclonal antibody for iNOS (BD Transduction Laboratories, Lexington, KY) or a monoclonal antibody for β-actin (Sigma) to confirm equal loading. Immunofluorescence was carried out using the polyclonal iNOS antibody (BD Transduction Laboratories) followed by Alexa Fluor 488 goat anti–rabbit IgG (Molecular Probes, Eugene, OR). To distinguish splenocytes from MSCs, cells were simultaneously stained with phycoerythrin-conjugated anti-CD45 monoclonal antibody (BD Pharmingen). Cells were fixed with ProLong Gold antifade reagent (Molecular Probes) and were visualized by confocal microscopy (Nikon, Tokyo, Japan), and images were analyzed with the accompanying confocal image analysis software (Bio-Rad, Hercules, CA).

Intracellular staining

Intracellular staining was performed using a BD Cytofix/Cytoperm kit (BD Pharmingen) according to the manufacturer's instructions.

Mice

Wild-type mice (C57BL/6) were purchased from Clea Japan (Tokyo). The $iNOS^{-/-}$ mice (C57BL/6 background) were purchased from Jackson Laboratory (Bar Harbor, ME).

Cell lines

RAW264.7 mouse macrophage cells were a generous gift from Dr Matsuura (Jichi Medical University, Tochigi, Japan). HeLa human cervical carcinoma cells were used as negative control for iNOS expression.

Statistical analysis

We used the Student *t* test for statistical analysis. Differences were considered statistically significant at *P* values less than .05.

Results

Characteristics of T-cell suppression by MSCs

Although many reports[9-17,27] have shown that MSCs suppress T-cell proliferation, the molecular mechanisms and the signaling molecules inhibited by MSCs have not been defined. We therefore investigated the status of activated T cells in the presence of MSCs. The expression of activation markers and the production of IL-2 and IFN-γ were evaluated by flow cytometry, ELISA, and intracellular staining. The expression of the activation markers CD25 and CD69 on CD4 or CD8 T cells was not changed by the presence of MSCs (Figure 1A). In addition, MSCs suppressed the production of IFN-γ but not IL-2 (Figure 1B, upper panel). Also, IFN-γ production was diminished after 24 hours in the presence of MSCs (Figure 1B, lower panel). These findings are in agreement with previous results,[27] but they do not explain the strong suppression of T-cell proliferation by MSCs; for example, thymidine incorporation by T cells is reduced more than 10-fold in the presence of MSCs (data not shown).

We next induced T-cell proliferation using a combination of PMA and ionomycin, which act downstream of the T-cell–receptor complex by activating protein kinase C and inducing Ca^{2+} influx, respectively. This proliferation was suppressed by MSCs (Figure 1C), suggesting that the T-cell receptor complex is not a target for the suppression and that MSCs influence signals downstream of protein kinase C and Ca^{2+} influx. As demonstrated in Figure 1C, the proliferation of both purified CD4 and CD8 T cells as well as unfractionated splenocytes was suppressed by MSCs.

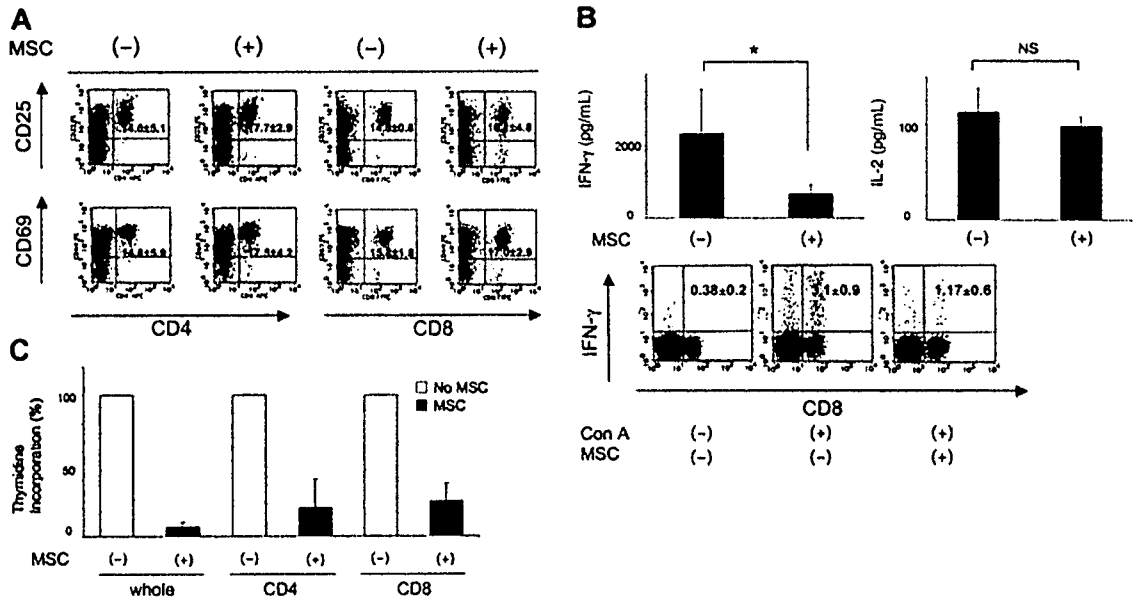

Figure 1. Status of activated T cells in the presence of MSCs. (A) Expression of the T-cell activation markers CD25 and CD69 on CD4 or CD8 cells 24 hours after stimulation of splenocytes (1×10^6 cells) in a 12-well dish with anti-CD3/CD28 beads (10 μL) in the presence or absence of 1×10^5 MSCs. The numbers in the top right quadrants indicate the percentage ± the standard deviation (SD). (B) Top panel, cytokine production in the same condition as in panel A. Concentrations of IL-2 and IFN-γ were determined at 48 hours by ELISA. The values are the means ± SD from 3 independent experiments. Bottom panel, intracellular staining of IFN-γ at 24 hours. GolgiStop (monensin) was used for the last 8 hours. The values are the mean percentages of CD8/IFN-γ–positive cells ± SD from 3 independent experiments. (C) MSCs suppress the induction of $CD4^+$ and $CD8^+$ T-cell proliferation by PMA and ionomycin. Splenocytes (1×10^5), $CD4^+$ cells (1×10^5), or $CD8^+$ cells (1×10^5) were stimulated in the presence or absence of irradiated MSCs (1×10^4) in the wells of a 96-well plate. The incorporation of [^{3}H]-thymidine is shown relative to that in the absence of MSCs. The values are the means ± SD from 3 independent experiments. *$P < .05$. NS indicates $P > .05$.

Stat5 phosphorylation is inhibited by MSCs

Although T cells from Stat5ab$^{-/-}$ mice do not proliferate upon stimulation with anti-CD3, they up-regulate CD25.[28] Because this phenotype is similar to the status of activated T cells in the presence of MSCs (Figure 1A), we hypothesized that they suppress Stat5 phosphorylation. Indeed, as shown in Figure 2, Stat5 phosphorylation was diminished in activated T cells in the presence of MSCs despite equivalent IL-2 production (Figure 1B). The reported changes in cell-cycle–related proteins, including the down-regulation of cyclin D2 and up-regulation of p27 Kipl in the presence of MSCs,[27] were observed less consistently; in most cases, we found that MSCs up-regulated cyclin D2 and down-regulated p27 Kipl in activated splenocytes compared with freshly isolated splenocytes (data not shown).

Dose dependency and time course of NO production

Macrophages have been reported to suppress T-cell proliferation[19-23] due to the production of NO and its inhibition of Stat5 phosphorylation.[18,19] This prompted us to examine the production of NO in our mouse MSC system. We found that MSCs caused a significant and dose-dependent production of NO (Figure 3A). NO could be first detected approximately 12 hours after the activation of T cells in the presence of MSCs. In the transwell system, in which the T cells were separated from the MSCs by a 1-μm-pore membrane, NO production was initially detected approximately 24 hours after the activation of T cells (Figure 3B).

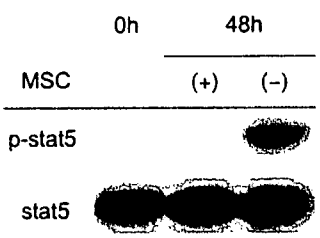

Figure 2. Inhibition of Stat5 phosphorylation in the presence of MSCs. Western blot analysis of Stat5 phosphorylation. Splenocytes (2×10^6) were activated with anti-CD3/CD28 beads in the presence or absence of 1×10^5 MSCs. After 48 hours, splenocytes were collected, lysed, and analyzed by Western blotting. Each lane contains 20 μg protein. Western blotting with anti-Stat5 is shown as a loading control. Shown are representative results from more than 5 experiments.

T-cell suppression and NO

Significant amounts of NO were not produced by MSCs cocultured with T cells in the absence of Con A or by Con A–treated MSCs or T cells (Figure 4A). In the presence of a direct interaction between T cells and MSCs, there was a high level of NO production accompanied by a strong suppression of T-cell proliferation (Figure 4A-B). In contrast, both NO production and T-cell suppression were reduced in a transwell system (Figure 4A-B). We further examined whether such a difference is observed using the RAW264.7 macrophage cell line, a well-characterized producer of NO. As with MSCs, T-cell suppression and NO production were inhibited in the transwell system using the RAW264.7 cells (Figure 4B, right side, and data not shown), suggesting that the difference reflects common aspects of T-cell suppression by NO.

T cells but not B cells induce NO

We next asked which cell type causes the NO production. We found that purified $CD4^+$ and $CD8^+$ T cells induce similar degrees of T-cell suppression as unfractionated splenocytes (Figure 1C). Therefore, it is not surprising that they also produce NO in the presence of MSCs (Figure 4C). Although MSCs suppress B-cell proliferation (~50%; data not shown), purified $CD19^+$ B cells did not appear to induce NO production in the presence of MSCs, suggesting that the mechanisms of B-cell and T-cell suppression are different.

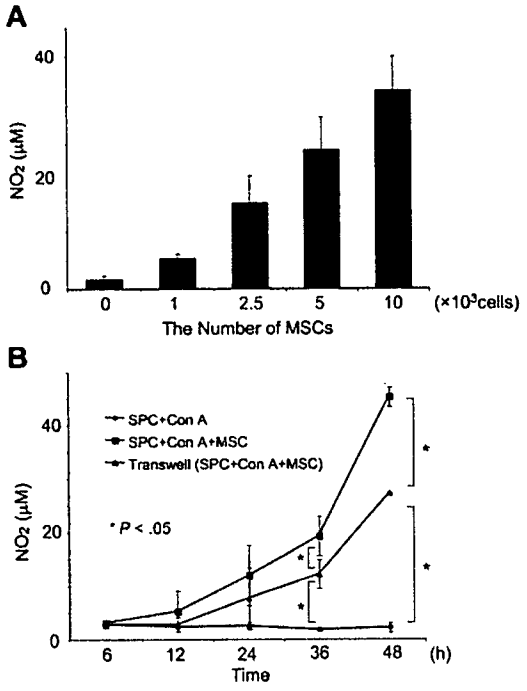

Figure 3. NO production in the presence of MSCs. (A) Dose-dependent effect of MSCs on NO production. Splenocytes (1×10^6) were activated with Con A (5 μg/mL) in the presence of the indicated number of MSCs for 48 hours in a 12-well dish. The concentrations of NO were determined by Griess assay. (B) Time course of NO production. MSCs (1×10^5/well) were treated as in panel A for the indicated amount of time. "Transwell" indicates experiments performed in 12-well dishes in which the T cells were separated from MSCs by a 1-μm-pore membrane. Values represent the means ± SD from 3 independent experiments. *$P < .05$.

MSC–T-cell interaction and NO production

There are 2 possible explanations for the difference in NO production in the presence and absence of the transwell system. First, it is possible that there is a difference in the time course of NO production in the 2 systems. In the transwell system, a significant level of NO was typically detected after 24 hours, whereas NO production was detected after 12 to 18 hours in the presence of a direct interaction. Thus, the amount of NO produced in the transwell system was always lower than that in the presence of a direct interaction (Figure 3B). These findings suggest that a direct interaction is critical for the early and efficient production of NO as well as for the strong suppression of T-cell proliferation. A second possible explanation for the different results obtained in the transwell and direct interaction systems is that, because NO is highly unstable, in the transwell system it can lose its activity before it influences T cells.

MSCs are a producer of NO

If this second explanation is correct, MSCs should be the main producer of NO. Therefore, we examined whether MSCs can produce NO. It is known that there are 3 NO synthases (iNOS, endothelial NOS, and neuronal NOS), and only one of these, iNOS, can be induced by cell stimulation.[29] Therefore, we suspected that iNOS is induced in either T cells or MSCs. Reverse transcriptase–polymerase chain reaction (RT-PCR) (Figure 5A), Western blot analysis (Figure 5B), and immunofluorescence (Figure 5C) detected the induction of iNOS in MSCs cocultured with activated splenocytes but not in MSCs alone, splenocytes alone, or HeLa cells. The immunofluorescence studies showed that iNOS was exclusively expressed by large adherent $CD45^-$ cells, which correspond to MSCs (Figure 5C). In addition, iNOS appeared to be expressed throughout the cytoplasm as previously found in Kupffer cells and hepatocytes (Figure 5C).[30]

Specific inhibitor of NOS restores T-cell proliferation and Stat5 phosphorylation

Next, we investigated the effects of L-NAME, a specific inhibitor of NOS. As expected, L-NAME dose-dependently inhibited the production of NO by MSCs in the presence of activated T cells (Figure S2). Importantly, L-NAME restored T-cell proliferation (Figure 6A, left panel). The effect of L-NAME was dose dependent and more efficient when lower numbers of MSCs were used (Figure 6A, right panel). Using 2.5×10^3 MSCs, 1 mM L-NAME resulted in up to an approximately 80% recovery compared with the positive control (Figure 6A, left panel), suggesting that NO is one of the most important factors for T-cell suppression under the stringent conditions of our assays. On the other hand, even under these conditions, 100% recovery was not achieved, implying that other factors also contribute to the suppression of T-cell proliferation by MSCs.

L-NAME restored not only T-cell proliferation but also Stat5 phosphorylation (Figure 6B), indicating that NO inhibits Stat5 phosphorylation. Because Stat5 is required for T-cell division,[28] we suspect that NO first inhibits Stat5 phosphorylation, which then results in arrest of the cell cycle.

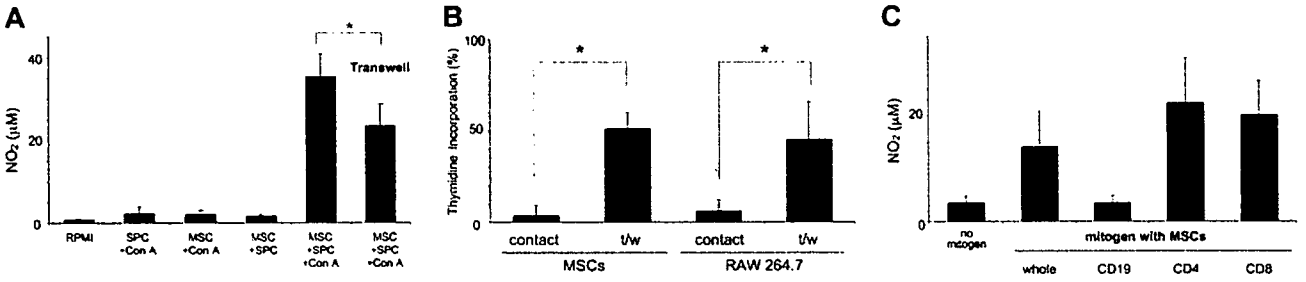

Figure 4. Relationship between NO production and T-cell suppression. (A) Production of NO. Splenocytes (1×10^6) were incubated with or without Con A in the presence or absence of MSCs for 48 hours. "Transwell" indicates experiments performed in 12-well dishes in which the T cells were separated from MSCs by a 1-μm-pore membrane. The values are the means ± SD from 3 independent experiments. *$P < .05$. (B) T-cell proliferation in the presence or absence of the transwell system. Splenocytes (1×10^6) were activated with Con A in the presence or absence of 1×10^5 MSCs or RAW264.7 cells for 48 hours with or without the transwell. The incorporation of [^{3}H]-thymidine is shown relative to that in the absence of MSCs. The values are the means ± SD from 3 independent experiments. *$P < .05$. (C) T cells but not B cells induce NO production. Purified CD4 or CD8 T cells (1×10^5; ~80% purity) induce NO in the presence of MSCs (1×10^4), whereas purified CD19 B cells (1×10^5; ~95% purity) do not induce significant NO production. The mitogen for T cells was Con A, and for B cells, it was lipopolysaccharide (1 μg/mL). The values are the means ± SD from 3 independent experiments. *$P < .05$.

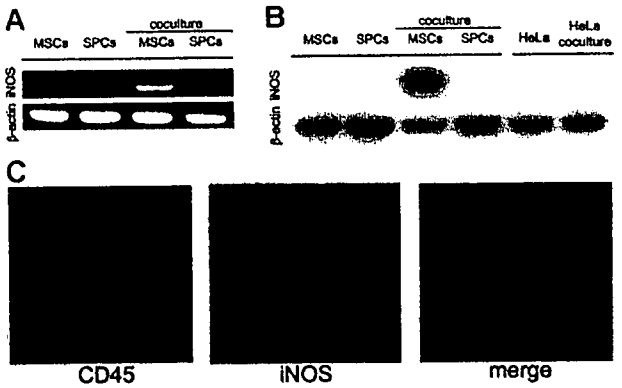

Figure 5. Induction of iNOS in MSCs. Total RNA and cell lysates were collected from MSCs alone, splenocytes alone, MSCs cocultured with activated T cells, or activated splenocytes cocultured with MSCs. MSCs were harvested just after washing out activated T cells with PBS. (A) RT-PCR analysis of iNOS mRNA. β-actin is shown as a control. (B) Western blot analysis of iNOS protein. Each lane contains 20 μg protein. β-actin is shown as a loading control. HeLa cells were used as negative control because the antibody also reacts with human iNOS protein. (C) Immunofluorescence of iNOS protein. Left panel, confocal immunofluorescent image of CD45 protein. Middle panel, confocal immunofluorescent image of iNOS protein. Right panel, merged confocal immunofluorescent images of CD45 protein and iNOS protein. Splenocytes (1×10^6) were activated with Con A in the presence of 1×10^5 MSCs for 48 hours. Images were visualized using a Nikon Eclipse TE300 microscope (Nikon, Tokyo, Japan) equipped with a 100×/1.40 numerical aperture oil objective lens, Nikon CFI Plan APO (Nikon). Images were acquired using Lasersharp software version 2.1 (Bio-Rad).

Other candidates as mediators of suppression by MSCs

Because TGF-β, IDO, and PGE_2 were reported as mediators of T-cell suppression by MSCs,[13-15] we further compared the effects of L-NAME with inhibitors of each mediator. Indomethacin (inhibitor of PGE_2 production) but not 1-MT (inhibitor of IDO) or an anti–TGF-β–neutralizing antibody restored T-cell proliferation as effectively as L-NAME (Figure 7A); however, the effects of L-NAME and indomethacin were not additive, suggesting that the NO and PGE_2 share signaling pathways leading to T-cell suppression (Figure 7A).

MSCs from iNOS$^{-/-}$ mice have reduced activity in T-cell suppression

Finally, we used MSCs from iNOS$^{-/-}$ mice to confirm that NO is produced by MSCs and that NO suppresses T-cell proliferation. MSCs from iNOS$^{-/-}$ mice were less effective than MSCs from wild-type mice at suppressing T-cell proliferation, suggesting that NO produced by MSCs is a major mediator of this effect (Figure 7B, left panel). We also confirmed that MSCs from iNOS$^{-/-}$ mice do not produce NO even in the presence of activated T cells (Figure 7B, right panel).

Discussion

Here, we demonstrate for the first time that the production of NO is involved in the suppression of T cells by MSCs. We also showed that NO inhibits Stat5 phosphorylation. Although NO was already known to suppress T-cell proliferation, NO has not been previously reported to mediate T-cell suppression by MSCs.[29] Our hypothesis that NO is produced by MSCs and that it suppresses T-cell proliferation in part through Stat5 inhibition was supported by the following facts: (1) NO was readily detected in the medium in the presence of MSCs; (2) L-NAME restored T-cell proliferation as well as Stat5 phosphorylation; and (3) MSCs from iNOS$^{-/-}$ mice had markedly reduced abilities to suppress T-cell proliferation. This hypothesis was further confirmed by the finding that iNOS was detected only in MSCs.

Compared with experiments in which cells were in direct contact, experiments performed in transwells showed a lag in NO production, suggesting that T-cell–MSC contact is critical for the early and efficient production of NO and, thus, T-cell suppression. Whether a soluble factor is a main mediator of T-cell suppression by MSCs has been controversial because results from transwell systems have been inconsistent.[12,14,16-17] Our finding that the transwell reduces but does not abolish T-cell suppression (Figure 4B) may help explain these conflicting reports. Although we could not define the mechanism by which NO production is suppressed in the transwell system, the amount of NO production appears to correspond with the extent of T-cell suppression.

Under stringent conditions, in which a lower number of MSCs was used, the restoration of T-cell proliferation by L-NAME reached up to approximately 80%, suggesting that NO is one of the major mediators; however, 100% restoration was never attained, suggesting that other factor(s) contribute to the suppression. Because TGF-β, IDO, and PGE_2 have been considered as possible mediators of T-cell suppression by MSCs,[13-15] we examined the effect of specific inhibitors of each. We found that indomethacin (inhibitor of PGE_2 production) restores T-cell proliferation as previously reported[15] but that neither 1-MT (inhibitor of IDO) nor the TGF-β antibody had an effect. Also, the effects of indomethacin

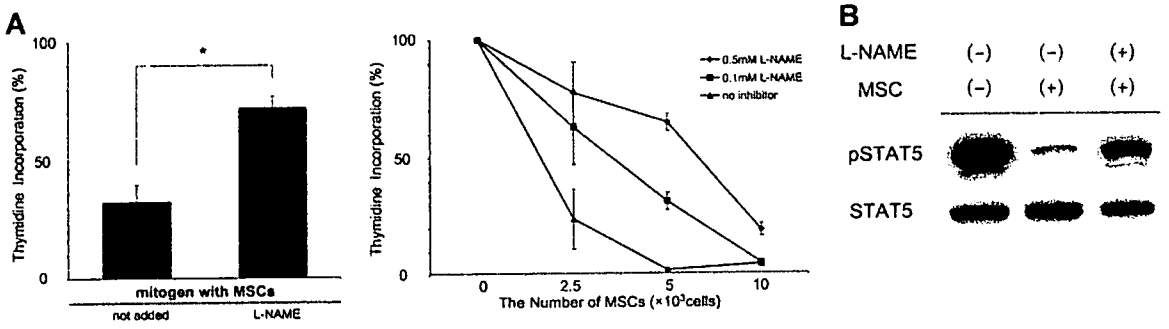

Figure 6. A specific inhibitor of NOS restores T-cell proliferation and Stat5 phosphorylation. (A) Effect of L-NAME on thymidine incorporation. Top panel, splenocytes (1×10^5) were activated with Con A in the presence or absence of 2.5×10^3 irradiated MSCs and in the presence or absence of 1 mM L-NAME. The incorporation of [^{3}H]-thymidine is shown relative to that in the absence of MSCs. The values are the means ± SD from 3 independent experiments. *$P < .05$. Bottom panel, dose-dependent restoration of T-cell proliferation by L-NAME. Splenocytes (1×10^5) were activated with Con A in the presence of the indicated number of irradiated MSCs for 48 hours. The concentrations of L-NAME are shown. Shown is a typical result of 3 independent experiments. (B) L-NAME restores Stat5 phosphorylation. Splenocytes (2×10^6) were activated with anti-CD3/CD28 beads in the presence or absence of 0.5×10^5 to 1×10^5 MSCs for 48 hours and in the presence or absence of 1 mM L-NAME. Western blotting for phosphorylated and total Stat5 was performed as described in Figure 2. Shown is a representative result from 5 independent experiments.

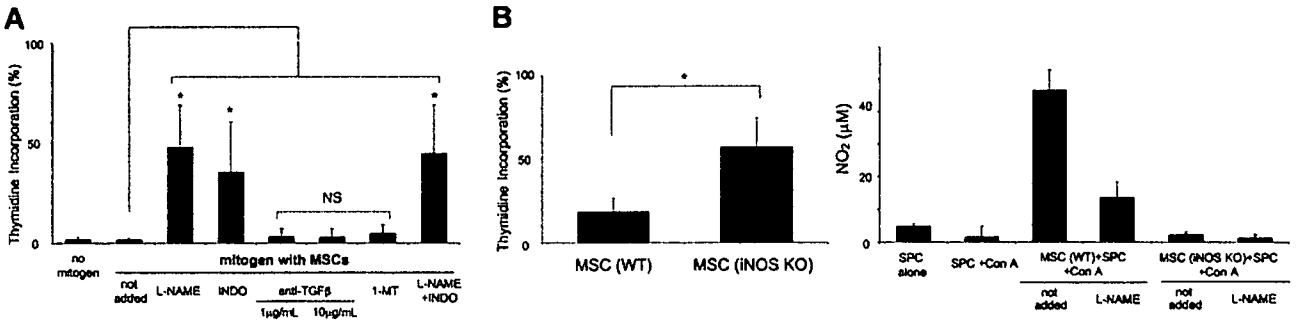

Figure 7. Effect of inhibitors and T-cell suppression by MSCs from iNOS$^{-/-}$ mice. (A) L-NAME and indomethacin (INDO) restored T-cell proliferation, but TGF-β antibody and 1-MT had no effect. Splenocytes (1×10^5) were activated with Con A in the presence or absence of irradiated MSCs (2.5×10^3), 1 mM L-NAME, 5 μM indomethacin,[14] 1 μg/mL or 10 μg/mL TGF-β antibody, and 1 mM 1-MT for 48 hours. The incorporation of [^{3}H]-thymidine is shown relative to that in the absence of MSCs. Shown is the mean ± SD of 3 independent experiments. *$P < .05$. NS indicates $P > .05$. (B) MSCs from iNOS$^{-/-}$ mice have a reduced ability to inhibit T-cell proliferation. Splenocytes (1×10^5) were activated with Con A in the presence or absence of MSCs (2.5×10^3) from either wild-type or iNOS$^{-/-}$ mice. Left panel, incorporation of [^{3}H]-thymidine relative to that in the absence of MSCs. Right panel, production of NO. The values are the means ± SD from 3 independent experiments. *$P < .05$.

were not additive with those of L-NAME. These results, combined with previous reports,[31,32] suggest that NO acts upstream of PGE_2. Furthermore, our results imply that NO may be the central mediator of T-cell proliferation.

Under our standard conditions (1:10 ratio of MSCs to splenocytes), 1 mM L-NAME restored T-cell proliferation to approximately 25%. Similarly, 5 μM indomethacin also produced an approximately 25% recovery (data not shown). These results suggest that the restoration by L-NAME or indomethacin is not specific to the more stringent conditions (1:40 ratio of MSCs to splenocytes).

Although most of the results from our mouse MSC system are consistent with previous reports,[12,15,27] we did not find a clear correlation between T-cell suppression and the up-regulation of Kip1 or the down-regulation of cyclin D2. Instead, our results suggest that the inhibition of Stat5 phosphorylation is more important for T-cell suppression, at least under the conditions of our experiments. In the conditions studied here, after coculture with MSCs, T cells could respond to a second mitogenic stimulation (data not shown), whereas they could not respond to a second stimulation in a previous report,[27] suggesting that the status of T cells in our experiments is different than that in the previous report.

Our results provide new insight into how MSCs modulate immune function. Although it is known that the NO-Stat5 pathway is important for T-cell suppression by macrophages, this is the first report demonstrating that the NO-Stat5 pathway is also critical for T-cell suppression by MSCs. The physiologic role of NO produced by MSCs is unknown, and we are currently investigating the possibility that MSCs in bone marrow protect hematopoietic stem cells from T-cell–mediated destruction by inhibiting T-cell proliferation.

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Authorship

Contribution: K.S. performed the research and analyzed data; K. Ozaki designed the research and wrote the paper; K.H. performed Western blotting; I.O. carried out experiments regarding PMA plus ionomycin; T.N. provided technical advice; A.M. and K.M. provided some reagents and analyzed data; and K. Ozawa organized the research project.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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Screening of genes responsible for differentiation of mouse mesenchymal stromal cells by DNA micro-array analysis of C3H10T1/2 and C3H10T1/2-derived cell lines

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Background

The molecular mechanisms underlying the biologic effects or differentiation of mesenchymal stromal cells (MSC) have not been clarified. Screening for genes differentially expressed at different stages is an important step in determining these molecular mechanisms.

Methods

In this study, we analyzed the gene expression profiles of C3H10T1/2 (10T1/2) cells and two sublines, A54 (pre-adipocyte) and M1601 (myoblast), as a model of MSC and downstream committed progenitors.

Results

We found up-regulated expression of delta-like-1 (Dlk), Wnt-5a and IL-1 receptor-like-1 (ST2) in 10T1/2 cells; stem cell factor (SCF) and stromal derived factor-1 (SDF-1) in A54 cells; and cardiac muscle-specific gene in M1601 cells. Overexpression of Dlk in A54 cells did not induce any effects on their differentiation into adipocytes. After differentiation into adipocytes, A54 cells reduced the expression of SCF, SDF-1 and Ang-1 as well as the ability to support the formation of a cobblestone appearance.

Discussion

The results suggest that these three lines have different gene profiles and are a useful system for analyzing the differentiation and function of MSC and progenitor cells.

Keywords

mesenchymal stromal cells, 10T1/2, DNA micro-array, expression profiles.

Introduction

Mesenchymal stromal cells (MSC) are a non-hematopoietic component of BM. These cells account for a small portion of BM and they have the capacity to differentiate into adipocytes, osteocytes and chondrocytes [1]. Because MSC can be readily isolated and expanded *in vitro*, it is expected that they can be used for regenerative therapies. Recent studies have demonstrated that MSC are capable of supporting hematopoiesis and regulating the immune response [2–5]. Clinical studies have been carried out regarding the use of MSC for suppressing GvHD in allogeneic stem cell transplantation [6,7] and for regenerative therapy [8,9]; however, the molecular mechanism underlying the biologic effects of MSC remains unclear. The identification of key molecules for the differentiation, immunosuppression and hematopoietic support of MSC is a crucial step for their clinical application.

Some investigators have used the 10T1/2 cell line, derived from C3H mouse embryo cells, as a model of mouse MSC [5,10]. In the presence of 5-azacytidine, 10T1/2 cells can be differentiated into adipocytes, osteocytes and chondrocytes [11]. We have previously

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established two sublines from 10T1/2, designated A54 (pre-adipocyte) and M1601 (myoblast) [12]. Under appropriate conditions, A54 and M1601 cells can terminally differentiate into adipocytes and myotubes, respectively, whereas the parental 10T1/2 cells remain undifferentiated under the same conditions [12].

Ang-1 is expressed on osteoblasts in the BM niche, and Tie-2, the receptor for Ang-1, is expressed on vascular endothelium and hematopoietic stem cells [13]. It has been suggested that the interaction between Ang-1 and Tie-2 is essential for cobblestone formation by hematopoietic stem cells *in vitro* [14]. Activation of Tie-2 is reported to participate in the self-renewal of hematopoietic stem cells within the BM niche [14]. Except for its expression in osteoblasts, the distribution of Ang-1 expression in the BM has not been clearly determined.

In the present study, we used 10T1/2 cells as a model of MSC and A54 and M1601 cells as committed mesenchymal progenitors, and compared their gene expression profiles by DNA micro-array analysis. We found that each cell line showed unique gene expression profiles. Furthermore, we identified several molecules that might participate in the differentiation of MSC.

Methods

Cell lines and induction of terminal differentiation

The parental 10T1/2 cell line was obtained from the Japanese Cancer Research Resources Bank (Tsukuba, Japan) and the two derivative cell lines, A54 and M1601, were established as described previously [12]. All cell lines were cultured in Iscove's modified Dulbecco's medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% FCS (Sigma, St Louis, MO, USA). Adipocyte differentiation of A54 cells was induced by incubating them for 7 days in medium supplemented with 10^{-5} M insulin (Sigma) and 10^{-6} M dexamethasone (Wako, Osaka, Japan). Myotube differentiation of M1601 cells was induced by incubating them for 7 days in medium containing 2% horse serum (GIBCO, Gland Island, NY, USA) instead of FCS.

Oligonucleotide micro-array

Total RNA was extracted from the cells using RNeasy (QIAGEN, Valencia, CA, USA) and 10 μg total RNA was subjected to cDNA synthesis using a T7-$(dT)_{24}$ primer that contained the T7 promoter sequence. After second-strand synthesis, *in vitro* transcription was performed to synthesize biotin-labeled complementary RNA (cRNA). The cRNA (15 μg) was hybridized for 16 h at 45°C onto an Affymetrix Mouse Genome U74A version-2 GeneChip (Affymetrix, CA, USA), which contains more than 6000 target genes. The hybridized chip was washed and stained with streptavidin-conjugated PE using the fluidics station. The fluorescence signal intensity was captured by a laser scanner and calculated using Microarray Suite version 5.0 (Affymetrix).

Micro-array data analysis

GeneSpring software (Silicon Genetics, Foster, CA, USA) was used for the analysis of micro-array data. To eliminate noise, only transcripts with more than a 2-fold increase in relative intensity and those indicated as 'present' by Microarray Suite version 5.0 (Affymetrix) were selected as up-regulated genes.

Quantitative real-time RT-PCR

Total RNA was extracted from the cells and cDNA was synthesized using the SuperScript® first-strand synthesis kit (Invitrogen) from 4 μg total RNA. PCR was performed with 1/30th of the original cDNA synthesis reaction (corresponding to 133 ng total RNA) using a Quantitect® SYBR Green PCR kit (QIAGEN) according to the manufacturer's instructions. The incorporation of the SYBR green dye into the PCR products was monitored with an ABI Prism 7700 sequence detection system (PE Applied Biosystems, Foster, CA, USA). A standard curve was produced in each reaction to confirm the target product was within the standard curve and to calculate the copy number of the target. To create templates for the standard curve, PCR products were directly cloned into a plasmid vector (TOPO TA Cloning® Kits for sequencing; Invitrogen). After determination of the plasmid DNA concentration, the copy number of the standard could be calculated using the following formula: DNA concentration /(plasmid length in base pairs × 660) × 6.022×10^{23}.

Quantitative RT-PCR was repeated three times using the same cDNA but at different time points. Statistical analysis was based on the Student's *t*-test. $P < 0.05$ was considered statistically significant.

Flow cytometric analysis

PE-conjugated rat anti-mouse CD90.2 MAb was from BD Pharmingen (San Diego, CA, USA). Rat anti-mouse Dlk

mAb was a gift from Dr Atsushi Miyajima (University of Tokyo, Tokyo, Japan). PE-conjugated goat anti-rat IgG was from Cederlane (Ontario, Canada). Fc block (BD Pharmingen) was used to prevent non-specific binding of Ab to Fc receptors. Cells were stained with MAb for 30 min on ice. Flow cytometric analysis was performed using a BD LSR (Becton Dickinson, San Diego, CA, USA) and the collected data were analyzed using CELLQUEST software (Becton Dickinson).

Retrovirus-mediated gene transduction

A54 cells were infected with either empty MSCV-IRES-EGFP retroviral vectors or the one encoding hDlk (MSCV-IRES-EGFP vector was a gift from Dr Akihiro Kume, Jichi Medical School, Tochigi, Japan) [15]. Retroviral vectors were transiently transfected into the BOSC23 packaging cell line using Lipofectamine® and Plus reagent (Invitrogen). Supernatants from BOSC23 were supplemented with 4 μg/mL polybrene. Transduction into A54 cells was accomplished by centrifugation at 1080 *g* for 40 min at 32°C, followed by incubation for 4 h at 37°C.

Co-culture of the mouse hematopoietic stem cell fraction with 10T1/2, A54 and M1601 cells

BM cells were obtained from the femurs of 10-week-old C3H/HeN mice by flushing with PBS (Invitrogen). BM suspensions were layered over 1.087 g/mL Lympholyte-M (Cedarlane, Ontario, Canada) and centrifuged at 480 *g* for 30 min to obtain BM mononuclear cells. These cells were magnetically labeled with a cocktail of biotinylated Ab to lineage markers, including CD5, B220, CD11b, Gr-1, 7-4 and Ter119, followed by anti-biotin microbeads (Miltenyi Biotec, Auburn, CA, USA). Magnetically labeled cells were depleted with AutoMACS (Miltenyi Biotec) according to the manufacturer's instructions. Subsequently, the lineage-negative fraction was magnetically labeled with anti-Sca-1 microbeads (Miltenyi Biotec) and positively selected. Lin(−)Sca-1(+) cells (2.4×10^4) were plated on 2×10^4 10T1/2, A54 or M1601 cells in 12-well dishes and co-cultured for 6 days. Prior to co-cultivation, the 10T1/2, A54 and M1601 cells were irradiated with 30 Gy to prevent excess proliferation. Hematopoietic cobblestone formation was evaluated by phase-contrast microscopy.

Western blot analysis

Protein samples were extracted from 10T1/2, A54, M1601 and OP9 cells using an NP-40-based cell lysis buffer. Equal amounts of protein (20 μg) were fractionated by SDS–PAGE. The proteins were then electrophoretically transferred onto PVDF membranes (Invitrogen) and blotted with an Ab to mouse Ang-1 (1:10000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) or a MAb to β-actin (1:5000; Sigma), followed by horseradish peroxidase-conjugated anti-mouse or anti-rabbit Ig Ab (Amersham Biosciences, Uppsala, Sweden). Blots were visualized using West Pico chemiluminescent reagent (Pierce, Rockford, IL, USA) and exposure to X-ray film (Kodak, Rochester, NY, USA).

Results

Terminal differentiation of 10T1/2-derived cells into adipocytes or myotubes

Previously we had established two sublines, pre-adipocyte A54 and myoblast M1601 cells, from the parental mouse embryo fibroblast cell line 10T1/2 by treatment with 5-azacytidine [12]. 10T1/2, A54 and M1601 exhibit similar morphologies under maintenance conditions. A54 and M1601 cells can be differentiated into adipocytes and myotubes, respectively, under appropriate conditions,

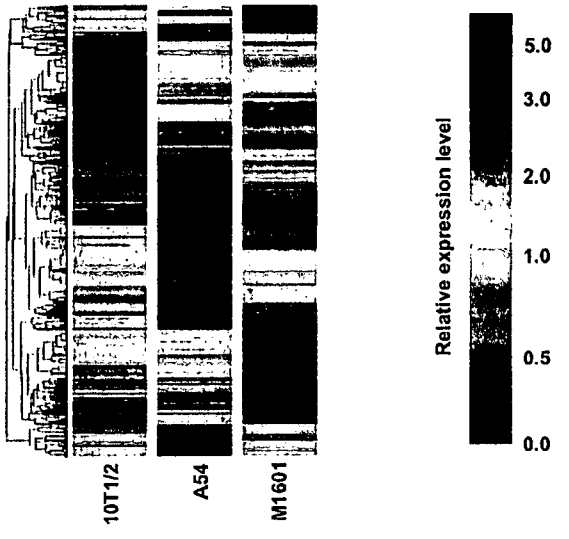

Figure 1. Gene expression profiles of 10T1/2, A54 and M1601 cell lines. Hierarchical clustering analysis of selected genes based on their expression patterns in 10T1/2 and 10T1/2-derived cell lines was performed, and the results are shown as a dendrogram. The relative signal intensity was calculated and color-coded as indicated on the right. Each row corresponds to a single gene.

Table 1. Genes up-regulated in 10T1/2 (A), A54 (B) and M1601 (C) Genes specifically up-regulated in 10T1/2, A54 and M1601 cells are listed. Functionally unknown genes have been excluded to simplify the lists. The common name.

A

Genbank ID	Common name	Description
U69137	Cdh10	cadherin 10
AF100778	Wisp2	WNT inducible signaling pathway protein 2
AW120746	Scoc	short coiled coil proten
AI845934	Ebna1bp2	EBNA1 binding protein 2
AI854020	Cd01	cysteine dioxygenase 1. cytosolic
AF059567	Cdkn2b	cylin-dependent kinase inhibitor 2B (p 15. inhibits CDK4)
X69619	Activin	inhibin beta-A
Y09257	nov	nephroblastoma overexpressed gene
Z12171	dlk	delta-like 1 homolog (Drosophila)
AF002718	IF1	ATPase inhibitor
AI839950	Fhl11	four and a half LIM domains 1
AB017697	TLP21	TATA box binding protein-like 1
AI849615	Gas5	growth arreset specific 5
AI853375	Mdm2	transformed mouse 3T3 cell double minute 2
AB021228	mt3-mmp	matrix metalloproteinase 16
D13695	ST2	interleukin 1 receptor-like 1
M28845	Krox-24	early grown response 1
X90875	FXR1	fragile X mental retardation gene 1. autosomal homolog
X90875	Wnt-5a	wingless-related MMTV integration site 5A
M89798	NCX1	solute carrier family 8 (sodium/calcium exchanger). member 1
AF004666	Cdkn2a	cyclin-dependent kinase inhibitor 2A
AF044336	Thy-1.2 (CD90.2)	thymus cell antigen 1. theta
X60367	CRBPI	Retinol binding protein 1, cellular
AF022072	Grb10	Growth factor receptor bound protein 10

B

Genbank ID	Common name	Description
U49513	Cc19	chemokine (C-C motif) ligand 9
X61800	C/ebp delta	CCAAT/enhancer binding protein (C/EBP), delta
J04596	Cxcl1	chemokine (C-X-C motif) ligand 1
X70058	CCL7;MCP-3	chemokine (C-C motif) ligand 7
M62362	mc/EBP	Mouse CAATT/enhancer binding protein gene
AF002719	SLPI	secretory leukocyte protease inhibitor
X79199	Tna	tetranectin (plasminogen binding protein
AV139913	Cxcl12	chemokine (C-X-C motif ligand 12
AF004874	Ltbp2	latent transforming growth factor beta binding protein 2
X56848	BMP-4	bone morphogenetic protein 4
AF054623	frizzled-1	frizzled homolog 1, (Drosophila)
X57413	TGF-beta2	transforming growth factor, beta 2
U27267	Cxcl5	chemokine (C-X-C motif) ligand 5
U88566	Sfrp1	secreted frizzled-related sequence protein 1
M62362	mc/EPB	CCAAT/enhancer binding protein (C/EBP), alpha
X83202	11beta-HSD1A	hydroxysteroid 11-beta dehydrogenase 1
AF011450	Col15a1	procollagen, type XV
M57647	SCF	kit ligand
U49915	adipoQ	adipocyte complement related protein
U10374	Pparg	Peroxisome proliferators-activated receptor gamma
X04480	Igf1	insulin-like growth factor 1
X66405	Col6a1	procollagen, type VI, alpha 1

Table 1 (*Continued*)

L19932	beta ig-h3	transforming growth factor, beta induced 68 kDa
U60091	TAP2-cas	transporter 2, ATP-binding cassette, sub-family B (MDR/TAP)

C

Genbank ID	Common name	Description
U21301	Mertk	c-mer proto-oncogene tyrosine kinase
L48989	Tnnt3	troponin T3, sakeletal, fast
U77943	Mylpf; Mlc2; MLC-2	myosin light chain
M25944	Car2; Call; Car-2:	carbonic anhydrase 2
M14537	Chrnb1	cholinergic receptor, nicotinic, beta polypeptide 1
Z38015	DM-PK	dystrophia myotonica kinase, B15
AF000236	Cmkor1	chemokine orphan receptor 1
AV362816	Star	steroidogenic acute regulatory protein
AV324706	Ncam1	neural cell adhesion molecule 1
AV248455	Chrng	cholinergic receptor, nicotnic, gamma polypeptide
M74753	Myh3	myosin, heavy polypeptide 3
M18879	MyoD	myogenic differentiation 1
X12973	Mylf; MLC1f; MLC3f	myosin light chain, alkali, fast skeletal muscle
D26532	Runx1	runt related transcription factor 1
X67140	Atp2a1	ATPase, Ca+ + transporting
U73620	Vegfc; VEGF-C	vascular endothelial growth factor C
D88689	Flt 1	FMS-like tyurosine kinase 1
AF041847	Crap; Alrp; CARP	cardiac responsive adriamycin protein
AFO42487	Kcnn4	potassium conductance calcium-activated channel
AF045801	Cktsf1b1	cysteine knot superfamily 1, BMP antagonist 1
M12347	alpha-actin	actin, alpha 1, skeletal muscle
Z80112	Icr-1	chemokine (C-X-C motif) receptor 4

whereas parental 10T1/2 cells maintain the same morphologic phenotype under the same conditions (see the Methods) [12].

Gene expression profiles of 10T1/2 and 10T1/2-derived cells

Using these three cell lines as a model of MSC and downstream progenitors, we performed high-throughput gene analysis to identify genes important for the differentiation of MSC. Total RNA from these three cell lines was subjected to biotinylation and hybridization with an Affymetrix GeneChip containing more than 6000 genes. Gene transcripts showing a more than 2-fold increase in relative intensity compared with the other two cell lines were selected. Each of the three cell lines (10T1/2, A54 and M1601) showed a unique gene expression profile despite morphologic similarities (Figure 1).

We identified 105 genes that were elevated in the parental 10T1/2 cells, including *activine*, *Dlk*, *Nov*, *Grb10*, *p15* and many functionally uncharacterized molecules (Table 1A). *Dlk* and *Nov* are known to be involved in the Notch signaling pathway and are reported to have the ability to inhibit MSC differentiation into adipocytes and osteoblasts [16,17]. In pre-adipocyte A54 cells, we found 201 genes that were up-regulated. These included genes known to be involved in adipocyte differentiation, such as *C/EBPα*, *C/EBPδ*, *PPAR-γ*, *PAI-I* and *Frizzled-1* (Table 1B) [18–21]. Finally, the myoblast M1601 cells showed 137 up-regulated genes, including ones related to skeletal muscle differentiation, such as *MyoD*, *MLC1F*, *α-skeletal actin*, *myosin heavy chain* and *myosin light chain* [22], as well as genes related to cardiac muscle differentiation, including *α-cardiac actin*, *cardiac troponin C* and *troponin T2* (Table 1C) [23].

Real-time PCR analysis of selected genes from micro-array experiments in parental 10T1/2 cells

Next, we selected six genes, *Dlk*, *Wnt-5a*, *ST2*, *CrbpI*, *p15* and *CD90*, as candidates for further analysis in 10T1/2 cells. According to the micro-array data, these genes had a more than 3-fold higher expression in 10T1/2 than in the other two cell lines. To confirm this differential expression, we performed RT real-time PCR. As shown in Figure 2A,

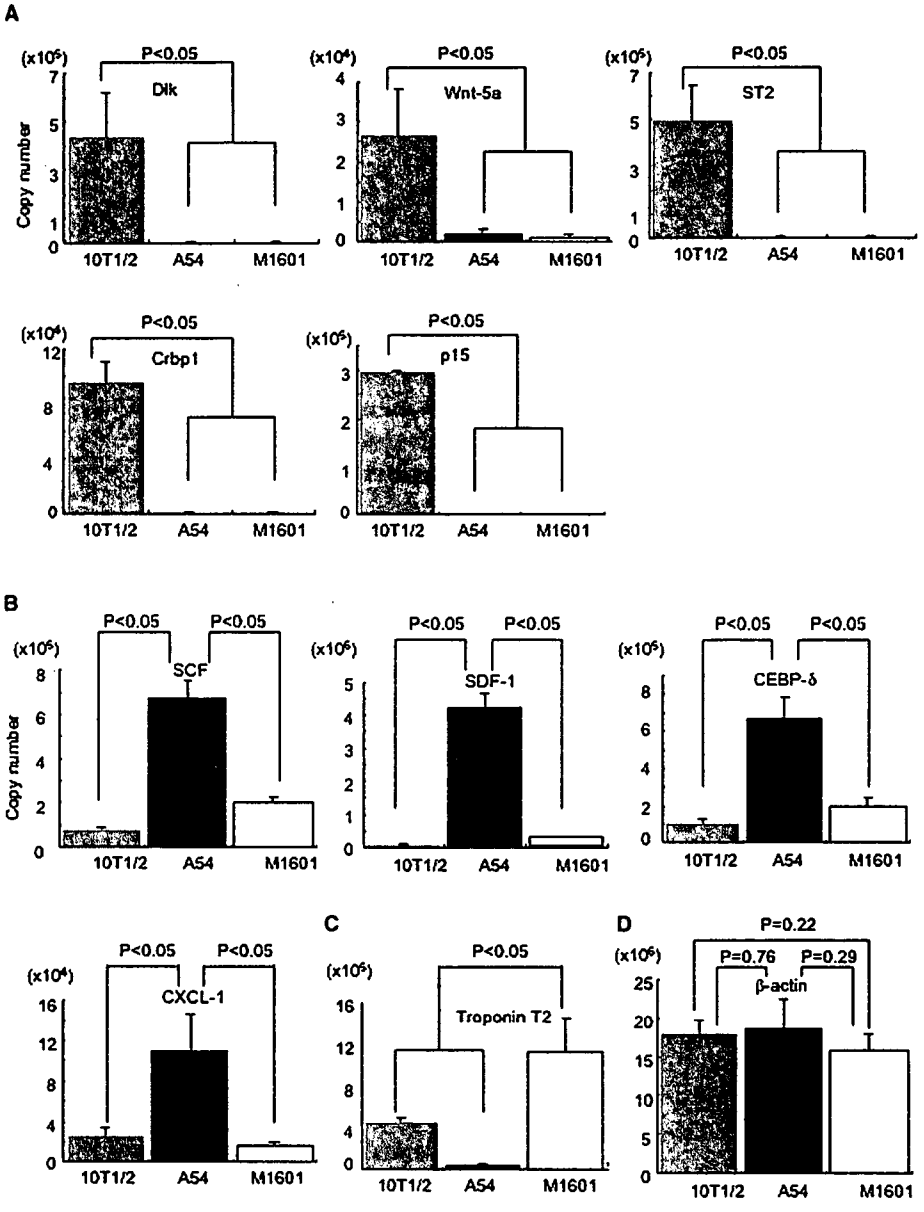

Figure 2. Up-regulated genes in 10T1/2(A), A54(B) and M1601(C) cells. Total RNA was extracted independently from the micro-array experiments and used to synthesize cDNA. The cDNA was subjected to real-time PCR with specific primers for Dlk, Wnt-5a, ST2, Crbpl and p15 (A), SCF, SDF-1 and adipogenic markers C/EBP-δ and CXCL-1 (B), cardiac troponin T2 (C) and β-actin (D). The copy number was used to compare the level of expression. The mean ± SD from three independent reactions using the same cDNA is shown.

we confirmed that the levels of transcripts for these genes were elevated in 10T1/2 cells. We also confirmed the up-regulation of CD90 in 10T1/2 cells by flow cytometry (data not shown). Although the function of CD90 in MSC is unclear, it may be useful for distinguishing immature MSC from committed progenitors because CD90 was not detected in the other two cell types. We analyzed the expression of the *Dlk* gene further, because *Dlk* is known to inhibit adipocyte and osteoblast differentiation [16]. Overexpression of human *Dlk* in A54 cells by transduction with a retroviral vector did not affect adipocyte differentiation (Figure 3). Further experiments are required to determine the function of the genes up-regulated in 10T1/2 cells.

SCF and SDF-1 are up-regulated in pre-adipocyte A54 cells

Previous studies have shown that pre-adipocytes have a greater ability to support hematopoiesis *in vitro* than other kinds of stromal cell components [24,25]. The results of

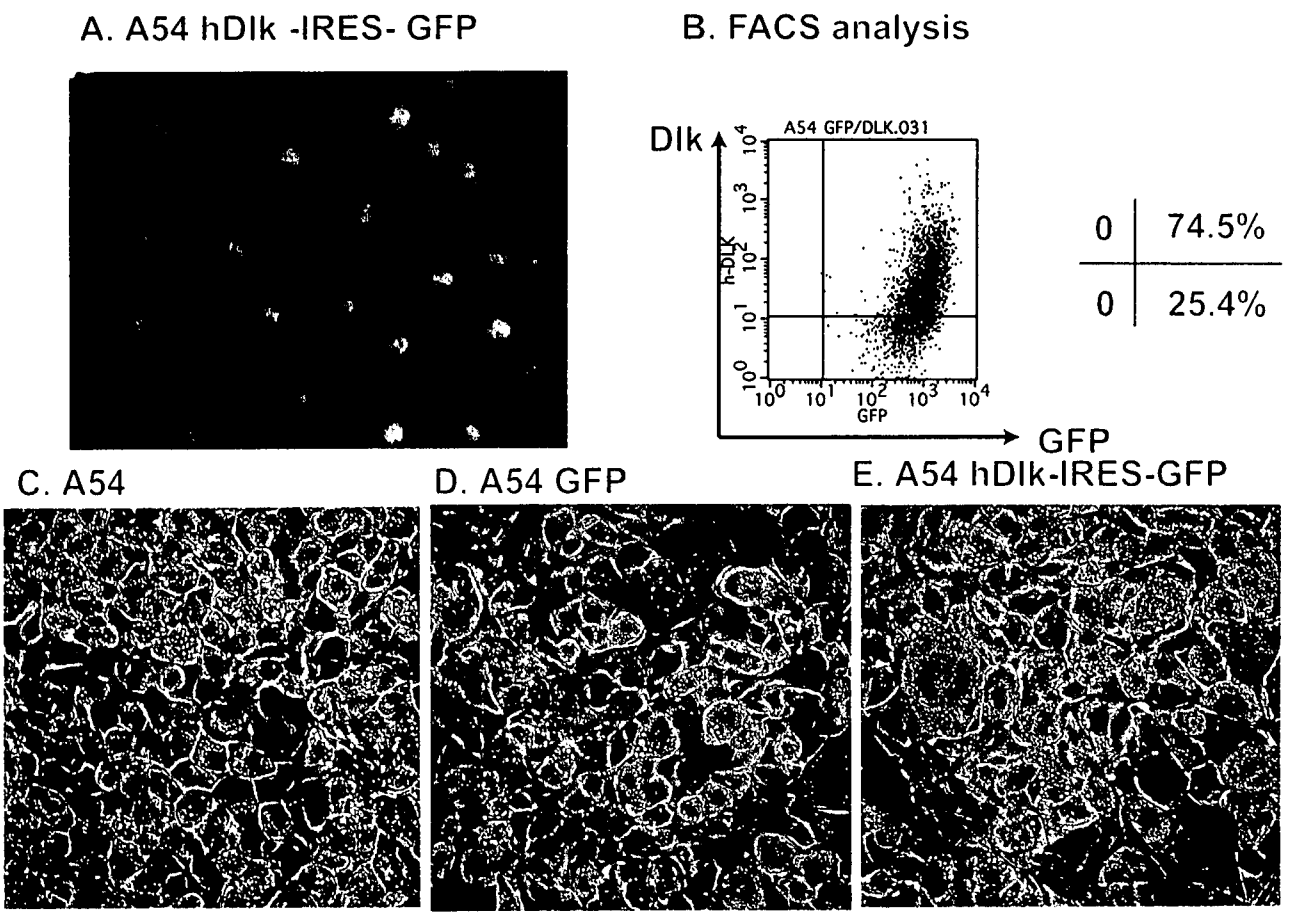

Figure 3. Enforced Dlk expression in A54 cells did not inhibit terminal differentiation into adipocyte. (A) GFP expression in A54 cells transfected with hDlk-IRES-EGFP. (B) Flow cytometric analysis for expression of hDlk in A54 cells transfected with hDlk-IRES-EGFP. (C–E) Adipocyte differentiation among parental A54 cells (C), A54 cells transfected with control IRES-EGFP (D) and A54 cells transfected with hDlk-IRES-EGFP (E).

our gene expression profiling revealed that critical cytokines for hematopoiesis, such as SCF and SDF-1, are upregulated in pre-adipocyte A54 cells. In addition, many chemokines, such as CXCL-1 and CCL-7, were also upregulated (Table 1B). We performed real-time PCR analysis of *SCF*, *SDF-1*, *CEBP-δ* and *CXCL-1*. As shown in Figure 2B, among the three cell lines the expression of these genes was highest in A54 cells. The level of Ang-1 mRNA in A54 cells was similar to that in OP9 cells (Figure 4A), which are a well-characterized BM stromal cell line. Moreover, of the three cell lines, A54 was the only one with which we could detect the expression of Ang-1 protein, and its level apparently decreased after adipocyte differentiation (Figure 4B).

The ability of the three cell lines to support formation of a cobblestone appearance

To examine the effects of these three lines on hematopoietic cells, we co-cultured them with a mouse hematopoietic stem cell fraction. The experimental protocol is shown in Figure 5A. The BM cells were depleted of lineage marker-positive cells using the Auto-MACS magnetic beads system. Thereafter Sca-1$^+$ cells were selected. The resulting Lin(−)Sca-1(+) fractions were plated on 10T1/2, A54, or M1601 cells in 12-well dishes. After 6 days of co-culture, a cobblestone appearance was observed only on the A54 cells (Figure 5B–D). These results suggest that, of the three cell lines, only A54 cells have the ability to support hematopoietic cell growth, which agrees with our previous report [12]. We did not observe hematopoietic cell colonies on the layer of the terminally differentiated A54 adipocytes (Figure 5E), suggesting that pre-adipocyte A54 cells lose the ability to support cobblestone formation after differentiation into adipocytes.

To understand the molecular mechanisms underlying this effect, we examined the levels of *SCF*, *SDF-1* and *Ang-1* expression after adipocyte differentiation by real-time