ANIMAL CELLS AND SYSTEMS
2018, VOL. 22, NO. 3, 157–164
https://doi.org/10.1080/19768354.2018.1453546

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GM1 Induced the inflammatory response related to the Raf-1/MEK1/2/ERK1/2 pathway in co-culture of pig mesenchymal stem cells with RAW264.7

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ABSTRACT

Pig-human xenotransplantation can trigger cell-mediated immune responses. We explored the role of gangliosides in inflammation related to immune rejection in xenotransplantation. Co-culture of xenogeneic cells (pig-MSCs and RAW264.7) was used to emulate xenotransplantation conditions. MTT assay results indicated that cell viability was significantly decreased in pADMSCs co-cultured with RAW264.7 cells. GM1 and GM3 were highly expressed in pADMSCs co-cultured with RAW264.7 cells. pADMSCs co-cultured with RAW264.7 cells strongly expressed pro-inflammatory proteins such as COX-2, iNOS, p50, p65, plκBα, and TNF-α. GM1-knockdown pADMSCs co-cultured with RAW 264.7 cells did not show significantly altered cell viability, but pro-inflammatory proteins were markedly inhibited. Co-culture of pADMSCs with RAW264.7 cells induced significant phosphorylation (p) of JNK1/2 and pERK1/2. However, pERK1/2 and pJNK1/2 were decreased and MEK1/2 and Raf1 were suppressed in GM1-knockdown pADMSCs co-cultured with RAW264.7 cells. Thus, the Raf-1/MEK1/2/ERK1/2 and JNK1/2 pathways were significantly upregulated in response to increases of GM1 in co-cultured xenogeneic cells. However, the inflammatory response was suppressed in co-culture of GM1-knockdown pADMSCs with RAW264.7 cells via down-regulation of the Raf-1/MEK1/2/ERK1/2 and JNK1/2 pathways. Therefore, the ganglioside GM1 appears to play a major role in the inflammatory response in xenotransplantation via the Raf-1/MEK1/2/ERK1/2 and JNK1/2 pathways.

ARTICLE HISTORY
Received 29 November 2017
Accepted 1 March 2018

KEYWORDS
GM1; xenotransplantation; inflammation; MAPK; pig-mesenchymal stem cells

Introduction

Gangliosides are complex glycosphingolipids containing one or more sialic acids, and are a main component of cell membranes (Hakomori 1990). Some studies have reported that gangliosides are developmentally controlled in a cell type–specific manner (Yu 1994; Yamamoto et al. 1996; Yu et al. 1988). Additionally, expression of gangliosides is related to the biological processes of stem cells *in vitro* (Kwak et al. 2006).

Mesenchymal stem cells (MSCs) are multipotent cells (Pittenger et al. 1999) that can differentiate into several lineages, including adipocytes, neuron-like cells, osteoblasts, hepatocytes, and myoblasts (Ferrari et al. 1998; Pittenger et al. 1999; Sanchez-Ramos et al. 2000; Hong et al. 2005; Sato et al. 2005; Ryu et al. 2009). Several studies have reported that gangliosides are essential factors in differentiation and proliferation of MSCs (Sanchez-Ramos et al. 2000; Bergante et al. 2014).

Although xenotransplantation has vast clinical potential, it is limited by the problem of immune responses against xenogeneic tissue (Wright et al. 2016). Additionally, xenotransplanted cells, including vascularized organ xenografts, show loss of function within a short time of transplantation in dissonant species combinations. Previous studies reported that gangliosides are related to the inflammatory responses induced in co-culture of xenogeneic cells, such as pig endothelial cells (PAECs) and human leukocytes (Cho et al. 2012). The inflammatory responses were associated with the mitogen-activated protein kinase (MAPK) family (Yin et al. 2016).

The MAPK family of proteins regulates the cell death and the proliferation (Lee et al. 2002; Tarallo and Sordino 2004). The MAPK family consists of two major subgroups: the c-Jun N-terminal stress-activated protein kinase 1/2 (JNK 1/2) subgroup and the

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extracellular regulated kinase 1/2 (ERK1/2) subgroup (Jung et al. 2004). ERK 1/2, which is activated in inflammatory responses, is related to cell proliferation (Kyriakis and Avruch 2012; Marques-Fernandez et al. 2013).

However, the expression and role of gangliosides in inflammatory responses is unclear, and has not been investigated using xenogeneic co-culture of pig MSCs with cells from other species. In this study, we investigated the role of gangliosides in inflammatory activation using co-culture of pig adipose-derived mesenchymal stem cells (pADMSCs) with RAW 264.7 macrophages.

Materials and methods

Culture of pADMSCs and RAW264.7 cells

pADMSCs were provided by the Korea Research Institute of Bioscience and Biotechnology (KRIBB). The cells were cultured in pre-warmed Dulbecco's Eagle Medium (DMEM) containing 10 ng/ml basic fibroblast growth factor (bGFGF; R&D Systems, Minneapolis, USA), 10% fetal bovine serum (FBS), and 1% (v/v) penicillin/streptomycin (P/S) solution and incubated in a humidified 5% CO_2 atmosphere at 37°C. RAW 264.7 cells were maintained in DMEM supplemented with 10% FBS and 1% P/S at 37°C in a humidified 5% CO_2 incubator.

Co-culture of mini-pig adipose-derived mesenchymal stem cells (pADMSCs) with RAW 264.7 mouse macrophages

pADMSCs were co-cultured with RAW 264.7 cells through seeding of pADMSCs (5×10^4 cells/dish) and RAW 264.7 cells (1×10^5 cells/dish). LPS (from *Escherichia coli* 0111: B4, Sigma) was administered at 10 μM to co-cultured cells and RAW 264.7 cells only.

Cell viability

Cell proliferation was determined by MTT assay 24 h after initiating culture of pADMSCs and RAW 264.7 cells. pADMSCs co-cultured with RAW 264.7 cells were transferred into 96-well plates at 1×10^4 cells/well and treated with LPS at 10 μM and GM1 synthase siRNA (10 nM), respectively. MTT solution (Sigma) was added to each well and incubated for 4 h and the absorbance was measured at 590 nm using a spectrophotometer.

Ganglioside extraction and purification

Lee *et al*. have described the methods used to extract and purify gangliosides. Briefly, cells were homogenized in distilled water at 48°C to extract total lipids, which were re-suspended in chloroform/methanol (1:1, v/v), lyophilized using N_2 gas, and subsequently dissolved in chloroform/methanol/H_2O (15:30:4, v/v/v). The column was washed with H_2O to remove non-hydrophobic lipids. Finally, the gangliosides were eluted with methanol, dried at 30°C under N_2 for 3 h, and stored at −80°C until analysis.

High-performance thin-layer chromatography

High-performance thin-layer chromatography (HPTLC) analysis of the gangliosides was conducted using a 10 × 10 cm TLC 5651 plate (Merck, Darmstadt, Germany). The purified gangliosides (600 μg protein/lane) were loaded onto TLC 5651 plates that were subsequently developed in chloroform/methanol/0.25% $CaCl_2 \cdot H_2O$ (50:40:10, v/v/v). The gangliosides were then stained with resorcinol, after which the density of the ganglioside bands was quantified by HPTLC densitometry (ImageJ, NIH). Purified mixed gangliosides (GM3, GM2, GM1, GD3, GD1a, and GD1b) (Matreya LLC, Pleasant Gap, PA, USA) were used as standards.

Design and selection of allele-specific siRNAs

GM1 and GM3 synthase-specific siRNAs and a control siRNA were synthesized by Bioneer Inc. (Daejeon, Korea). The primers for GM1 were: F, 5′-ATCGC-GAGTGTTGCTCTTCGT-3′ and R, 5′-GAGCAACACTGG-CACCTGCA-3′. The primers for GM3 were: F, 5′-ATCGGCTAACCTGGACCT-3′ and R, 5′-TACCGTTACCG-CAATTCCF-3′. All sequences were confirmed by capillary sequencing. Transfection of siRNAs or DNA vectors was performed using Lipofectamine 3000 reagent (Invitrogen, Carlsbad, USA) according to the manufacturer's recommendations. The GM1 and GM3 synthase specificity of the siRNAs was determined using HPTLC and western blot analysis to compare the activities of GM1 and GM3.

Western blot analysis

pADMSCs and RAW 264.7 cells were homogenized in RIPA buffer (Sigma), and then centrifuged at 13,000 rpm for 20 min at 4°C. Proteins (30 μg/lane) were separated on a 10% SDS polyacrylamide gel and then transferred to a nitrocellulose membrane (Hybond ECL; Amersham Pharmacia Biotech, Piscataway, NJ). The blots were blocked for 2 h with 5% bovine serum albumin (BSA) in Tris-buffered saline, and the membrane was incubated for 16 h with the following primary antibodies: BCl-2, Caspase-8, Caspase-9, Caspase-3, and β-actin (1:500; Santa Cruz Biotechnology, Santa Cruz,

USA). The blot was then incubated with the corresponding horseradish peroxidase-conjugated secondary antibodies, such as anti-mouse and anti-rabbit (Santa Cruz Biotechnology), and proteins were visualized using the ECL system (Pierce, Rockford, USA).

Statistical analysis

All data are presented as mean (SD). Multi-group associations were analyzed using one-way ANOVA and two-way ANOVA, followed by Tukey's and Bonferroni post-hoc pairwise comparisons. A p-value < 0.05 was considered statistically significant. All statistical analyses were executed using GraphPad Prism (Ver. 5.00; GraphPad Software Inc., La Jolla, USA).

Results

Cell viability and ganglioside expression patterns in pADMSCs, RAW 264.7 cells, and pADMSCs co-cultured with RAW264.7 cells

The co-culture was designed to emulate the conditions of xenograft. The cell culture groups consisted of pADMSCs only, RAW264.7 cells plus LPS, and co-culture of pADMSCs with RAW264.7 cells. Figure 1 shows cell viability as determined by MTT assays. Cell viability was significantly decreased when pADMSCs were co-cultured with RAW264.7 cells (Figure 1(A)). However, cell viability was similar to control in LPS only group (Figure 1(A)). In addition, we examined the ganglioside expression profile in pADMSCs only, RAW264.7 cells only, and pADMSCs co-cultured with RAW264.7 cells. GM2 and GD3 were weakly expressed in pADMSCs only and RAW264.7 cells only (Figure 1(B)). However, GM1 and GM3 were highly expressed in co-culture of pADMSCs with RAW264.7 cells (Figure 1(B)).

Expression of pro-inflammatory factors in pADMSCs co-cultured with RAW264.7 cells

We investigated expression levels of pro-inflammatory factors including COX-2, iNOS, p50, p65, pIκBα, and TNF-α. Pro-inflammatory factors were very weakly expressed in pADMSCs and RAW264.7 cells (Figure 2). Conversely, RAW264.7 cells plus LPS, the positive control, showed significantly increased expression of pro-inflammatory factors (Figure 2, $p < 0.001$). Pro-inflammatory proteins, such as COX-2, iNOS, p50, p65, pIκBα, and TNF-α, were strongly expressed in pADMSCs co-cultured with RAW264.7 cells, compared with pADMSCs only and RAW264.7 cells only (Figure 2, $p < 0.001$).

Cell viability in pADMSCs co-cultured with RAW 264.7 cells with knockdown of GM1 and GM3 synthase using siRNA

We investigated the effects of GM1 and GM3 knockdown in pADMSCs using GM1 and GM3 synthase siRNA. Figure 3(A) shows the results of GM1 and GM3 knockdown in co-culture of pADMSCs with RAW264.7 cells (Figure 3(A)). Cell viability significantly decreased in pADMSC co-cultured with RAW264.7 cells as a positive control (Figure 3(B)). However, cell viability was significantly higher in pADMSCs (GM1 synthase knockdown) co-cultured with RAW264.7 cells than in pADMSCs co-cultured with RAW264.7 cells (Figure 3(B)).

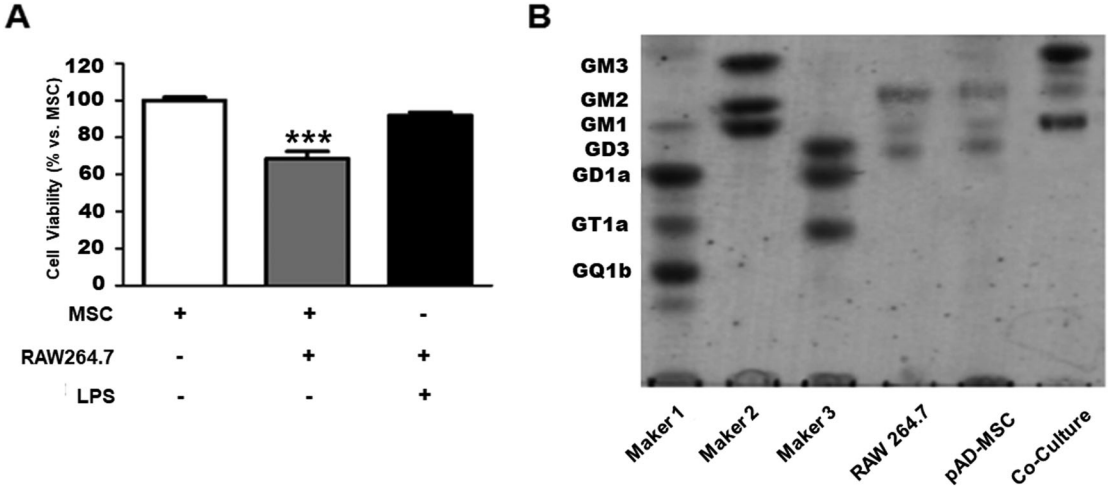

Figure 1. Ganglioside expression patterns and cell viability in GM1-knockdown pADMSCs co-cultured with RAW264.7 cells. (A) Cell viability of pADMSCs co-cultured with RAW264.7 cells. (B) Expression of gangliosides as detected by HPTLC in co-culture of GM1-knockdown pADMSCs with RAW264.7. *** $p < 0.001$ indicates a significant difference from the pADMSCs.

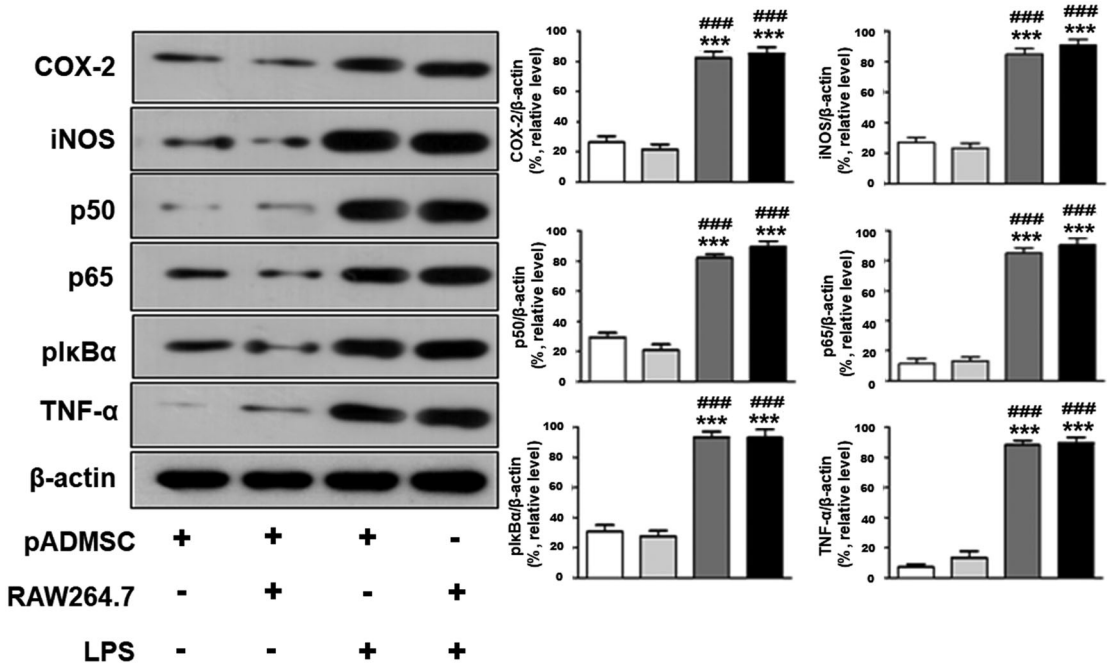

Figure 2. Increase of pro-inflammatory factors in co-culture of xenogeneic cells (pADMSCs with RAW264.7). Expression of β-actin and pro-inflammatory factors such as COX-2, iNOS, p50, p65, pIκbα, and TNF-α was detected by western blotting. *** $p < 0.001$ indicates a significant difference from pADMSCs. ### $p < 0.001$ indicates a significant difference from RAW264.7 cells.

Inhibition of expression of pro-inflammatory factors by knockdown of GM1 and GM3 synthase using siRNA

We examined inhibition of expression of pro-inflammatory factors, including COX-2, iNOS, p50, p65, pIκBα, and TNF-α. The pro-inflammatory factors were significantly expressed in pADMSCs co-cultured with RAW264.7 cells and RAW264.7 cells plus LPS (Figure 4). In contrast, pro-inflammatory factors such as COX-2, iNOS, p50, p65, pIκBα, and TNF-α were markedly

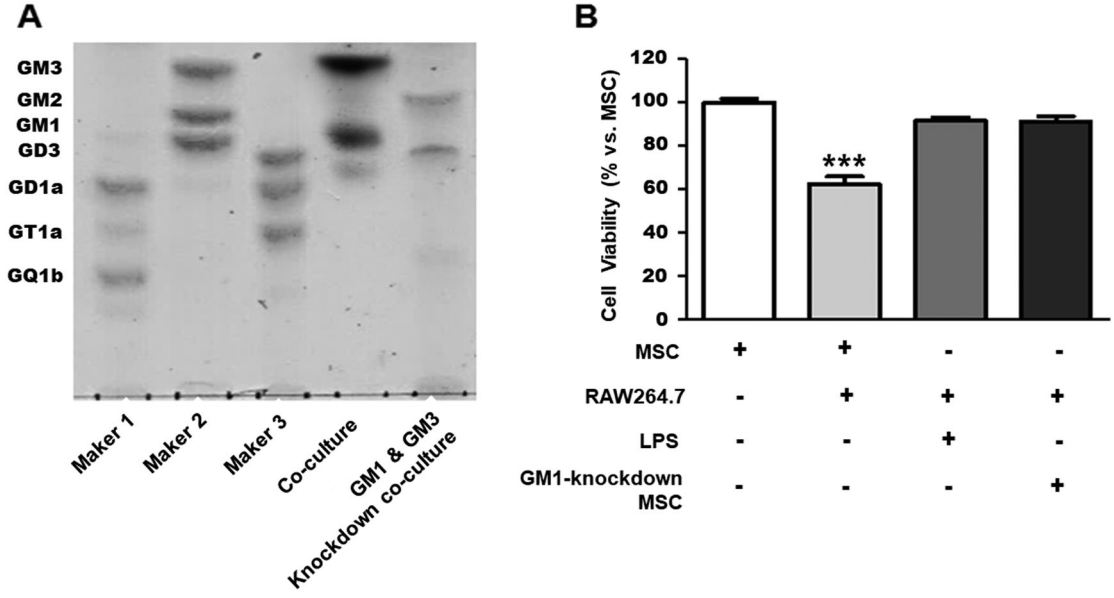

Figure 3. Expression of the ganglioside GM1 and cell viability in GM1-knockdown pADMSCs co-cultured with RAW264.7 cells. (A) Knockdown of GM1 and GM3 was detected by HPTLC in co-culture of GM1-knockdown pADMSCs with RAW264.7 cells. (B) Cell viability of GM1-knockdown pADMSCs co-cultured with RAW264.7 cells. *** $p < 0.001$ indicates a significant difference from pADMSCs co-cultured with RAW264.7 cells.

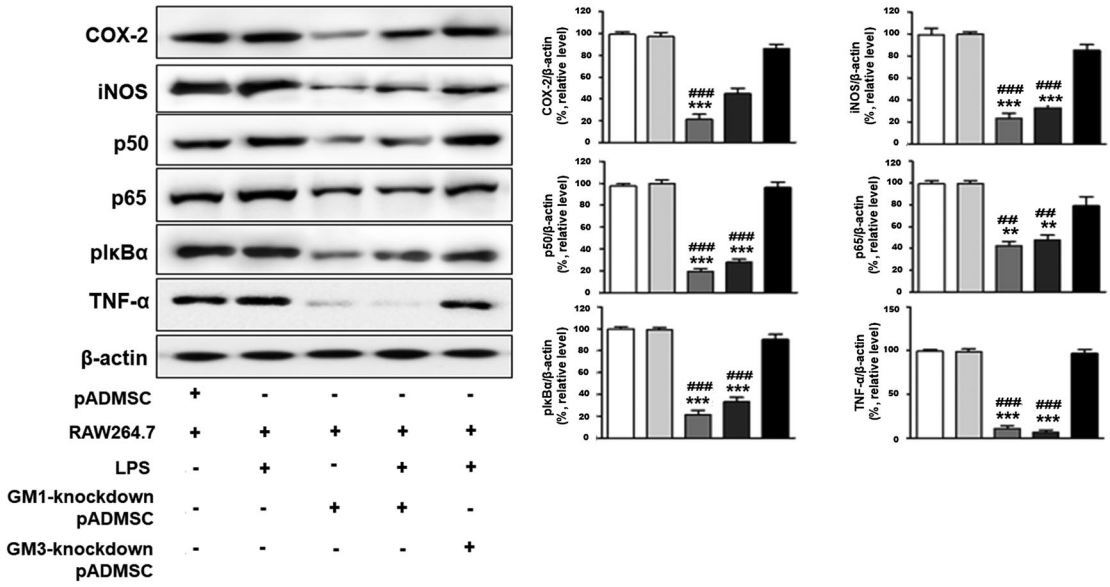

Figure 4. Inhibition of pro-inflammatory factors in co-culture of GM1-knockdown pADMSCs with RAW264.7 cells. Expression of β-actin and pro-inflammatory factors such as COX-2, iNOS, p50, p65, pIκbα, and TNF-α was detected by western blotting. *** $p < 0.001$ indicates a significant difference from co-culture of pADMSCs with RAW264.7. ### $p < 0.001$ indicates a significant difference from RAW264.7 cells treated with LPS.

inhibited in GM1-knockdown pADMSCs co-cultured with RAW 264.7 cells (Figure 4). Moreover, pro-inflammatory factors were meanly inhibited in GM1-knockdown pADMSCs co-cultured with RAW264.7 cells plus LPS (Figure 4). However, expression of pro-inflammatory factors was significantly increased in GM3-knockdown pADMSCs.co-cultured with RAW264.7 cells plus LPS.

Involvement of the MAPK pathway with GM1 in inflammation of co-cultured pADMSCs and RAW264.7 cells

We attempted to determine the role of MAPK and to elucidate its mechanism of action in co-culture of pADMSCs with RAW264.7 cells with or without knockdown of GM1. As shown in Figure 5(A), co-culture of pADMSCs with RAW264.7 cells induced significant phosphorylation (p) of pJNK1/2 and pERK1/2. However, pERK1/2 and pJNK1/2 were markedly decreased in GM1-knockdown pADMSCs co-cultured with RAW264.7 cells compared with co-culture of pADMSCs and RAW264.7 cells (Figure 5(A)). In addition, we investigated the upstream ERK1/2 signaling, including mitogen-activated protein kinase 1/2 (MEK1/2) and Raf1. MEK1/2 and Raf1 were significantly activated in co-culture of pADMSCs and RAW264.7 cells (Figure 5(B)). Moreover, activation of MEK1/2 and Raf1 was strongly increased in GM3-knockdown pADMSCs. However, in co-culture of GM1-knockdown pADMSCs with RAW264.7 cells, MEK1/2 and Raf1 were meanly decreased compared with co-culture of

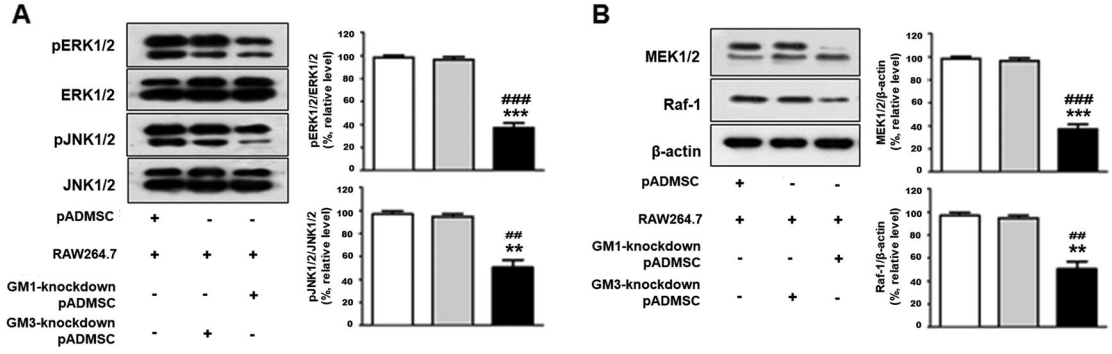

Figure 5. Suppression of Raf-1/MEK1/2ERK1/2 phosphorylation and JNK1/2 in co-culture of GM1-knockdown pADMSCs with RAW264.7. (**A**) β-actin, total ERK1/2, phosphorylated ERK1/2 (pERK1/2), total JNK1/2, and pJNK1/2, and (**B**) MEK1/2 and Raf-1 were detected by western blotting. *** $p < 0.001$ vs co-culture of pADMSCs with RAW264.7. ### $p < 0.001$ vs RAW264.7 cells treated with LPS.

pADMSCs with RAW264.7 cells plus LPS (Figure 5(B)). These results indicated that GM1 inhibited both pathways (Raf1/MEK1/ERK1/2 pathway and JNK1/2 pathway) in inflammation induced by co-culture of pADMSCs and RAW264.7 cells.

Discussion

Inflammation results from an excessive immune response, as part of the mechanism for protection against damaged tissue and foreign substances (Fitzpatrick 2001). Inflammatory mediators such as NO are produced by macrophages (Lee et al. 2016). Inducible iNOS and COX-2 are expressed following increased production of NO in LPS-stimulated macrophages (Zhao et al. 2013). iNOS is an important factor for the development of inflammation and subsequent maintenance of the inflammatory response. COX-2 is another important factor in inflammation (Lee et al. 2009). In addition, the activities of iNOS and COX-2 related to production of TNF-α play important roles in inflammatory responses (Shin et al. 2015). NF-κB is an important transcription factor associated with inflammatory responses, which induces the expression of various inflammatory factors, including iNOS, COX-2, and TNF-α (An et al. 2011; Yamada et al. 2014). In LPS-stimulated RAW264.7 macrophage cells, NF-κB is activated by the protein I-κBα (Ramaswami et al. 2012). Some reports have shown that gangliosides can induce production of cyclooxygenase-2. In this study, we observed that pro-inflammatory factors including COX-2, iNOS, p65, p50, p-IκBα, and TNF-α were significantly increased in pADMSCs co-cultured with RAW264.7 cells (Figure 2). Moreover, the increases in pro-inflammatory factors in co-culture of pADMSCs with RAW 264.7 cells were similar to the pro-inflammatory factor expression observed in LPS-stimulated RAW 264.7 cells, as a positive control (Figure 2). However, pro-inflammatory factors were significantly suppressed in GM1-knockdown pADMSCs co-cultured with macrophages (Figure 2). These results indicated that in co-culture of xenogeneic cells (pig MSCs with RAW264.7 mouse macrophages) inflammation related to the rejection of xenografts was mediated by GM1.

Several studies have reported that phosphorylation of three MAPKs (ERK, JNK, and p38) occurs by NF-κB activation (Hwang et al. 2011; Li et al. 2011). Szelenyi and Uros reported that the ERK1/2 pathway is a dominant and highly responsive pathway in inflammation (Szelenyi and Urso 2012). MEK-mediated ERK activation is the most important regulatory step in inflammation (Parthasarathy and Philipp 2014). A previous study indicated that the anti-inflammatory mechanism of flavonoids was related to inhibition of ERK phosphorylation by down-regulation of the expression of iNOS and COX-2 (Han et al. 2013), but was independent of the JNK and P38 pathway (Mazier et al. 2001). In addition, another report indicated that GM1 can activate ERKs in young rats and that GM1 induces activation of ERK1/2 by the Raf-1/MEK1/2 pathway in the VSMCs pathway (Duchemin et al. 2002). Ceramide, an important component of gangliosides, is known to be related to the ERK1/2 and the JNK pathways (Maziere et al. 2001). In particular, ceramide regulates the ERK1/2 pathway via activated RAf-1 and MEKs in various cell types (Willaime et al. 2001). In this study, we investigated how the MAPK pathway is involved with inflammation in pADMSCs co-cultured with RAW264.7 macrophage cells, and we found that expression of ERK1/2 and JNK1/2 were meanly activated in pADMSCs co-cultured with RAW264.7 cells (Figure 5 (A)). Furthermore, activation of MEK1/2 and Raf-1, the up-stream pathway of ERK1/2, was strongly increased in pADMSCs co-cultured with RAW 264.7 cells (Figure 5 (B)). However, other components of the MAPK pathway, including ERK1/2, JNK1/2, MEK1/2, and Raf-1, were significantly suppressed in co-culture of pADMSCs with GM1 knockdown (Figure 5). These results indicated that ERK1/2 phosphorylation by upregulation of MEK1/2/Raf-1 was associated with macrophage inflammation mediated by an increase of the ganglioside GM1 in co-culture of xenogeneic cells (pig MSCs with RAW264.7 mouse macrophages).

Conclusion

Cell - mediated immune responses can induce by xenotransplantation of pig and human. We explored the role of gangliosides in inflammation related to immune rejection in xenotransplantation. We used the co-culture of xenogeneic cells (pig-MSCs and RAW264.7) for emulate xenotransplantation conditions. We observed the highly expression of GM1 and GM3 when pADMSCs co-cultured with RAW264.7 cells. Pro-inflammatory proteins such as COX-2, iNOS, p50, p65, pIκBα, and TNF-α strongly expressed when pADMSCs co-cultured with RAW264.7 cells. In GM1-knockdown pADMSCs co-cultured with RAW 264.7 cells, pro-inflammatory proteins were markedly inhibited. In addition, we observed the phosphorylation (p) of JNK1/2 and pERK1/2 was significant induced in co-culture of pADMSCs with RAW264.7 cells. However, pERK1/2, pJNK1/2, MEK1/2 and Raf1 were suppressed in GM1-knockdown pADMSCs co-cultured with RAW264.7 cells. Thus, GM1 increases significantly up regulated the Raf-1/MEK1/2/ERK1/2 and JNK1/2 pathways in co-cultured xenogeneic cells. However, we find that inflammatory response suppressed by down-regulation of the Raf-1/MEK1/2/ERK1/2 and JNK1/2 pathways in co-culture

of GM1-knockdown pADMSCs with RAW264.7 cells. Therefore, the ganglioside GM1 appears to play a major role in the inflammatory response in xenotransplantation via the Raf-1/MEK1/2/ERK1/2 and JNK1/2 pathways.

Disclosure statement

No potential conflict of interest was reported by the author(s).

Funding

This research was supported by Basic Science Research Program through the National Research Foundation of Korea(NRF) grant funded by the Ministry of Science, ICT & Future Planning [grant number 2017R1A4A1015594, 2016R1D1A1B03932393], and the Korea Research Institute of Bioscience and Biotechnology (KRIBB) Research Initiative Program [grant number KGM4251824], Republic of Korea.

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