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Cancer Letters 261 (2008) 253–262
CANCER Letters
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Adipocyte culture medium stimulates production of macrophage inhibitory cytokine 1 in MDA-MB-231 cells

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Received 14 August 2007; received in revised form 7 November 2007; accepted 19 November 2007

Abstract

Obesity is one of the potential risk factors in causing breast cancer. As a result, adipose tissue surrounding breast ductal cells may play an important role in the breast cancer development or progression. To identify the genes that are regulated by factors secreted from adipocytes in breast cancer cells, MDA-MB-231 cells were treated with the culture medium of adipocytes. Most of induced genes were related to immune function and wound healing, which share a common gene expression signature with cancer progression. In present study macrophage inhibitory cytokine 1 (MIC-1) gene was studied among the induced genes. It was found that both MIC-1 mRNA and protein were dramatically increased by the culture medium of adipocytes. Furthermore, proteinase K-treated adipocyte culture supernatants also induced MIC-1 expression. These findings indicate that proteins are not major MIC-1 inducing factors in adipocyte culture medium. Consequently, we examined the effect of free fatty acids such as palmitate and oleate on MIC-1 induction and found that palmitate markedly induced MIC-1 gene expression, whereas oleate did not. Adipocyte culture medium- and palmitate-induced MIC-1 gene expression was mediated by the activation of p38 MAPK, but not by the activation of JNK, ERK, and NF-κB pathway. In addition, adipocyte-CM-induced MIC-1 also increased invasiveness of MDA-MB-231 cells.

Keywords: Adipocytes; MIC-1; Breast cancer; MDA-MB-231

1. Introduction

Adipose depot has been regarded primarily as an organ for energy storage. However, after the discovery of leptin, many researchers have focused their attention on the endocrine function of adipose tissue as a source of secreted bioactive proteins. Now these secreted bioactive proteins are named as adipokines. The adipose tissue produces and releases a variety of adipokines including adiponectin, leptin, and resistin, as well as cytokines and chemokines [1]. Adipokines have been found to play an important role in regulation of glucose and lipid

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0304-3835/$ - see front matter
doi:10.1016/j.canlet.2007.11.020

metabolism as well as in maintenance of immune and vascular system homeostasis. Changes of adipokine levels caused by obesity are associated with development and progression of various diseases including type II diabetes, cardiovascular, hypertension, and cancers [2–8]. For example, levels of adiponectin are decreased in obese patients, whereas leptin levels are increased compared to normal control. In the case of breast cancer, decreased serum levels of adiponectin are associated with increased breast cancer risks [9] and increased leptin levels promote breast tumor cell proliferation [5].

Crosstalk between mammary epithelium and stroma is very important for normal mammary gland structure and function. Disruption of the crosstalk can both induce and promote breast cancer. Adipocytes are the most abundant stromal cell type in breast tissues that influence the mechanisms regulating breast cancer development and progression [10]. There have been studies demonstrating the critical interaction between the tumor and adipocytes as stromal cells. Adipocyte-secreted factor, collagen VI, promotes mammary tumor progression through induction of anti-apoptotic transcriptional programs and proto-oncogene stabilization [11,12], whereas adiponectin, an adipocyte-derived factor, suppresses breast cancer cell proliferation and metastasis [13]. Together, these reports indicate that adipocyte-derived factors could stimulate or suppress the tumor cell progression depending on the microenvironment contexts.

In the present study, we examined gene expression profiling changes in adipocyte culture medium (adipocyte-CM)-treated breast cancer cells to understand signal effects from adipocytes to breast cancer cells. It was found that immune system and wound healing-related genes were induced. Among these genes induced by adipocyte-CM, MIC-1 was studied for the reason that factors responsible for the increased MIC-1 expression in cancer are not known yet and we previously revealed that MIC-1 increased the migration and invasion of gastric cancer cell line SNU-216.

2. Materials and methods

2.1. Cell culture and reagents

MDA-MB-231 human breast cancer cells and OP9 mouse preadipocytes were obtained from the American Type Culture Collection (ATCC). MDA-MB-231 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). The OP9 cells were cultured in α-MEM supplemented with 20% FBS, and all cells were maintained at 37 °C and 5% CO_2 atmosphere. Oleic acid (O7501) and palmitic acid (P9767) were obtained from Sigma Chemical Company.

2.2. Preparation of adipocyte culture medium

The OP9 preadipocyte cells were maintained in α-MEM supplemented with 20% FBS. To prepare preadipocyte culture medium (preadipocyte-CM), OP9 preadipocytes were plated in 100-mm plates and cultured for 2–3 days. The culture medium of preadipocytes was removed at 80% confluences, washed two times with PBS, and fresh DMEM supplemented with 10% FBS added and incubated for 1 day. After incubation, culture medium was centrifuged to remove cells. To prepare adipocyte-CM, the differentiation was induced by the addition of α-MEM supplemented with 0.5 mM of isobutylmethylxanthine, 2 μM of dexamethasone, 1.7 μM of insulin, 1 μM of rosiglitazone, and 20% FBS. The induction medium was removed 1 day after incubation. The medium was changed every 2 days with α-MEM supplemented with 20% FBS. At the time of the experiments more than 95% of OP9 cells were filled with multiple lipid droplets. The culture medium of differentiated adipocytes was removed, washed two times with PBS, and fresh DMEM supplemented with 10% FBS added and incubated for 1 day. After incubation, culture medium was centrifuged to remove adipocytes. For the preparation of boiled adipocyte-CM, adipocyte-CM was boiled at 95 °C for 10 min and for the preparation of proteinase K-treated adipocyte-CM, 100 μg/ml of proteinase K was added and incubated at 55 °C for 2 h and then boiled for 10 min.

2.3. Microarray analysis

MDA-MB-231 cells were treated with preadipocyte-CM or adipocyte-CM for 3 h and total RNA was isolated from each MDA-MB-231 cells using an RNeasy Mini Kit (Qiagen, Valencia, CA, USA). Microarray analysis was conducted in Seolin Bioscience (Seoul, Korea) using the Affymetrix Human U133 plus 2.0 chip.

2.4. RT-PCR analysis

We performed RT-PCR to verify whether expression level of MIC-1 gene is increased by adipocyte-CM treatment. Total RNA from preadipocyte-CM- or adipocyte-CM-treated MDA-MB-231 cells was prepared using RNAzolB (Tel Test, Inc., Friendswood, TX, USA), and 3 μg of total RNA was reverse transcribed using M-MuLV reverse transcriptase (Promega, Madison, WI, USA) at 37 °C for 1 h. PCR for the amplification of

MIC-1 was then performed using a primer pair (human MIC-1: forward 5′-CGGAATTCATGGCGCGCAACGGG-3′, reverse 5′-CCCTCGAGTATGCAGTGGCAGTC-3′). PCR was performed with amplification cycles of 30 s of denaturation at 94 °C, 30 s of annealing at 55 °C, and 30 s of extension at 72 °C. β-Actin was used as the control. The intensity of bands was measured using DNR Bio-imaging system (Kiryat Anavim, Israel).

2.5. Western blot analysis

Total cell lysates were prepared 24 h after treatment of adipocyte-CM, separated on a 12% SDS–polyacrylamide gel, and then transferred to a PVDF membrane (Millipore, Marlborough, MA, USA). The membrane was blocked with 5% skim milk in TBS (20 mM Tris–HCl, pH 7.5, 137 mM NaCl) for 30 min, and then incubated with phospho-p38 (Cell Signaling Technology Inc., Beverly, MA, USA) or MIC-1 antibodies (Upstate Biotechnology, Lake Palcid, NY, USA) for overnight. After washing three times with TBST (0.05% Tween 20, 20 mM Tris–HCl, pH 7.5, 137 mM NaCl) for 15 min, the membrane was incubated with the anti-rabbit IgG secondary antibody conjugated with horse-raddish peroxidase. The signal was detected using the Amersham ECL system (Amersham Pharmacia Biotech, Arlington Heights, IL, USA).

2.6. Treatment of FFA solution

FFA/BSA complex was prepared as reported previously [14]. Oleate or palmitate was dissolved in 0.1 M sodium hydroxide at 72 °C for 30 min to prepare 100 mM FFA stock solutions and 10% FFA-free BSA solutions were prepared in DMEM. To prepare 5 mM FFA/10% BSA stock solutions, 0.4 ml of 100 mM FFA stock solution was added dropwise to 7.6 ml of 10% FFA-free BSA solution with shaking and then mixed by vortexing for 10 s followed by 10 additional minutes of incubation at 55 °C. For the analysis of signaling pathway in palmitate-mediated MIC-1 induction, the various inhibitors (SP600125, 20 μM; PD98059, 20 μM; SB203580, 20 μM; pathenolide, 1.5 μM) were treated 1 h before 0.5 mM of palmitate treatment.

2.7. Invasion assays

The ability of cells to invade through Matrigel-coated filters was determined using a modified 24-well transwell (Corning Costar, Cambridge, MA, USA; 8-μm pore size) as described previously. The upper surface of the insert membranes was coated with a uniform layer of dried basement membrane matrix solution. The cells were seeded at a density of 5×10^4/ml in DMEM in the upper compartment of transwell. To determine the effect of MIC-1, recombinant MIC-1 (40 ng/ml) were added to the upper compartment of transwell and incubated for 24 h at 37 °C in 5% CO_2. Finally, the invaded cells were dissociated from the membrane using cell dissociation solution (Sigma–Aldrich Co., MI, USA) and subsequently detected with Calcein-AM solution using a fluorescence plate reader at 520 ± 10 nm.

2.8. Measurement of free fatty acids (FFAs)

According to the manufacturer's information, the FFAs released from mature adipocytes were measured using Cultured Human Adipocyte Lipolysis Assay Kit (Zen Bio, Research Triangle Park, NC, USA). Briefly, preadipocytes were differentiated in 96-well plates. On day 6 of differentiation, culture medium was removed from each well and adipocytes were washed three times with 200 μl of wash buffer. After the washing step, 75 μl of assay buffer was added and incubated at 37 °C for 3 h. At the end of incubation, 30 μl of assay buffer was mixed with 50 μl of FFA Reagent A, and then 100 μl of FFA Reagent B was added after incubation of 10 min. After additional 10 min, the absorbance was measured at 540 nm.

3. Results

3.1. Adipocyte-CM increases genes associated with immune system and wound healing

Stromal cells have significant impact on both tumor cell suppression and progression. In the breast tissue, stromal cells consist of mesenchymal fibroblasts, myoepithelial cells, macrophages, and adipocytes. Although it has been known that fibroblasts play an important role in the metastasis of tumor cells by secreting MMPs, little is known about the role of adipocytes, which are one of most abundant stromal cell types in breast tissues. To examine the change in gene expression profile of adipocyte-CM-treated MDA-MB-231 cells, adipocyte-CM was prepared as follows. Mouse bone marrow-derived cell line OP9 cells were differentiated into adipocytes using differentiation cocktail including 0.5 μM isobutylmethylxanthine, 2 μM dexamethasone, and 1.7 μM insulin. The lipid droplets inside of cells were observed 6 days after the induction of differentiation. One day before the time of experiments, the culture medium was changed with fresh DMEM supplemented with 10% FBS and the differentiated adipocytes were cultured to obtain adipocyte-CM for an additional day.

In order to find early responsive genes that regulated by adipocyte-CM, MDA-MB-231 cells were treated with the prepared adipocyte-CM or preadipocyte-CM for 3 h. Total RNA was isolated from adipocyte-CM- or preadipocyte-CM-treated MDA-MB-231 cells and changes in the expression profile of genes were analyzed using microarray analysis. Interestingly, immune system and wound

Table 1
List of upregulated genes by adipocyte-CM

Gene name	Accession No.	Fold increase	Classification
Interleukin 1, alpha (IL-1α)	M15329	109.1	Response to wound healing
Inhibin, beta A (INHBA)	M13436	62.68	Response to wound healing
Chemokine (C–C motif) ligand 20 (CCL20)	NM_004591	52.36	Inflammatory response
Epstein–Barr virus induced gene 3 (EBI3)	NM_005755	39.12	Immune response
Tumor necrosis factor (TNF)	NM_000594	28.64	Immune response
Colony stimulating factor 3 (CSF3)	NM_000759	27.67	Immune response
Complement component 2 (C2)	BC029781	27.47	Immune response
Chemokine (C–X–C motif) ligand 3 (CXCL3)	NM_002090	26.54	Tumor angiogenesis
C-type lectin superfamily 2, member D (CLEC2D)	AF285089	15.03	Immune response
Selectin E (SELE)	NM_000450	12.64	Response to wound healing
Matrix metalloproteinase 10 (MMP10)	NM_002425	11.71	Response to wound healing
Pregnancy-associated plasma protein A, pappalysin 1 (PAPPA)	BF107618	10.78	Metallopeptidase activity
Guanylate binding protein 5 (GBP5)	BG271923	10.48	Immune response
Interleukin 1, beta (IL-1β)	NM_000576	10.06	Immune response
B-factor (BF)	NM_001710	9.65	Immune response
Regenerating islet-derived 3 alpha (REG3A)	NM_002580	9.13	Acute-phase response
Macrophage inhibitory cytokine 1 (MIC-1)	AF003934	9.06	Growth factor activity
Interleukin 24 (IL-24)	NM_006850	8.88	Immune response
Chemokine (C–X–C motif) ligand 2 (CXCL2)	M57731	8.22	Proliferation and growth
Matrix metalloproteinase 1 (MMP1)	NM_002421	7.46	Response to wounding
Tumor necrosis factor superfamily, member 18 (TNFSF18)	NM_005092	6.96	Inflammatory responses

healing-associated gene expressions that play important roles in breast cancer cell progression were increased in MDA-MB-231 cells treated with adipocyte-CM (Table 1).

3.2. Adipoctye-CM increases MIC-1 expression in MDA-MB-231 cells

In the present study, we focused on MIC-1 because we previously showed that MIC-1 increased the migration and invasion of gastric cancer cell line SNU-216. Firstly, we confirmed that microarray results were reproducible. When MDA-MB-231 cells were treated with adipocyte-CM for 3 h, the expression of MIC-1 mRNA was markedly increased compared with preadipocyte-CM treatment (Fig. 1A). To further confirm this result, MDA-MB-231 cells were treated with the preadipocyte-CM or adipocyte-CM and the culture supernatants were subjected to Western blot analysis using anti-human-MIC-1 antibody. The secreted MIC-1 protein from MDA-MB-231 cells was greatly increased in the adipocyte-CM-treated culture supernatants (Fig. 1B). Next, the time kinetics of MIC-1 mRNA induction by adipocyte-CM was examined. The expression of MIC-1 mRNA was increased as early as 2 h and sustained until 24 h (Fig. 1C). In order to know whether the adipocyte-CM-mediated MIC-1 mRNA induction is sustained in the absence of adipocyte-CM, the adipocyte-CM was changed with fresh DMEM supplemented with 10% FBS 6 h after the treatment of adipocyte-CM. The adipocyte-CM-induced MIC-1 expression started to decrease at 3 h and returned to the basal level at 6 h, indicating that continuous presence of adipocyte-CM is necessary for MIC-1 mRNA induction.

3.3. Adipocyte-derived palmitate increases MIC-1 mRNA expression in MDA-MB-231 cells

It is well known that adipocytes secrete adipokines including leptin, resistin, and adiponectin as well as FFAs. To verify which factors are responsible for the induction of MIC-1 mRNA expression, proteinase K was added into adipocyte-CM to remove protein components from the adipocyte-CM. The proteinase K-treated adipocyte-CM was still able to induce MIC-1 mRNA expression in MDA-MB-231 cells (Fig. 2A). To determine whether protein components in the proteinase K-treated adipocyte-CM were efficiently removed, the proteinase K-treated adipocyte-CM was subjected to Western blot analysis using anti-mouse adiponectin antibody because adiponectin is abundantly secreted in adipocytes. The adiponectin was completely removed in the proteinase K-treated culture supernatants (Fig. 2B). In addition, TNF-α and IL-6, which are factors known to increase MIC-1 mRNA expression, failed to increase MIC-1 mRNA expression (data not shown). This finding shows that protein components in adipocyte-CM are not the factors responsible for the induction of MIC-1 mRNA expression.

We therefore speculated that FFAs secreted from adipocytes may be able to increase MIC-1 mRNA expression. Oleate and palmitate are major FFAs that are secreted from adipocytes [15,16]. Thus, MDA-MB-231

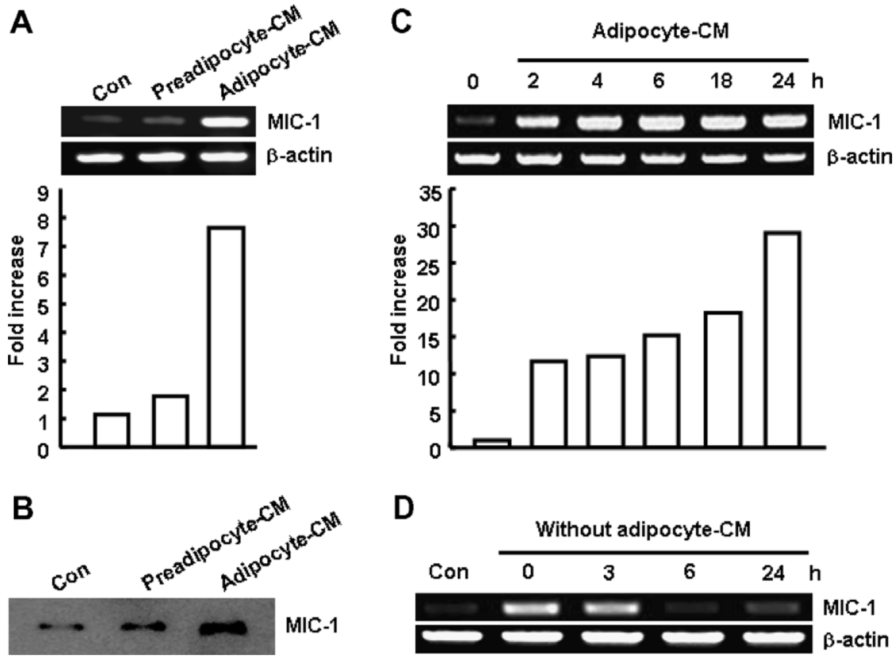

Fig. 1. MIC-1 mRNA and protein expression are increased by adipocyte-CM but not preadipocyte-CM in MDA-MB-231 cells. (A) MDA-MB-231 cells were incubated with preadipocyte-CM or adipoctye-CM for 3 h. The total RNA was isolated and subjected to RT-PCR analysis using MIC-1 primers. The intensity of bands was measured using DNR Bio-imaging system. (B) MDA-MB-231 cells were incubated with preadipocyte-CM or adipoctye-CM for 24 h. MIC-1 protein level in the culture medium was determined using Western blot analysis. (C) MDA-MB-231 cells were treated with adipocyte-CM. At the indicated time points, cells were harvested and total RNA was isolated. Then RT-PCR analysis was performed using MIC-1 primer. The intensity of bands was measured using DNR Bio-imaging system. (D) MDA-MB-231 cells were treated with adipocyte-CM for 24 h and total RNA was isolated at the indicated time points after removal of the adipocyte-CM, and then subjected to RT-PCR analysis.

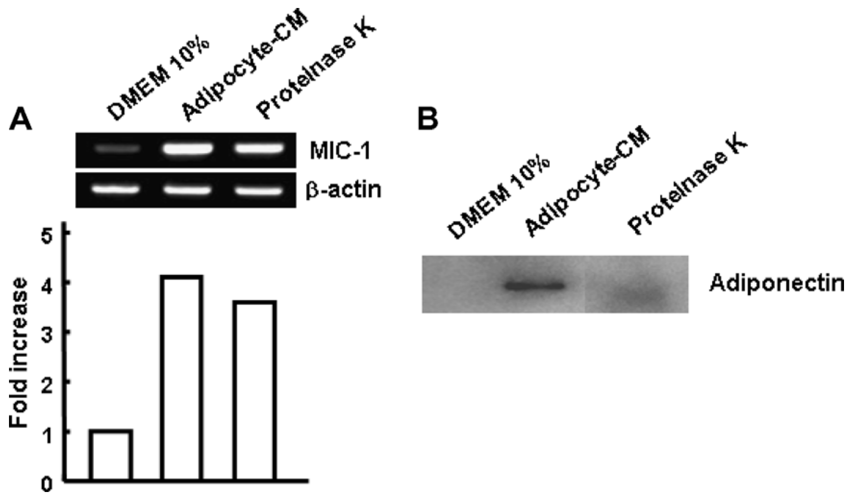

Fig. 2. MIC-1 induction is not mediated by protein factors secreted from adipocytes. (A) MDA-MB-231 cells were treated with adipocyte-CM or proteinase K-treated adipocyte-CM for 6 h. The total RNA was isolated and subjected to RT-PCR analysis using MIC-1 primers. The intensity of bands was measured using DNR Bio-imaging system. (B) To determine efficiency of proteinase K treatment, adipocyte-CM and proteinase K-treated adipocyte-CM were subjected to Western blot analysis using anti-adiponectin antibody.

cells were treated with oleate and palmitate to examine the induction of MIC-1 mRNA expression. Palmitate greatly induced MIC-1 mRNA expression at a concentration of more than 0.5 mM, whereas oleate failed to induce

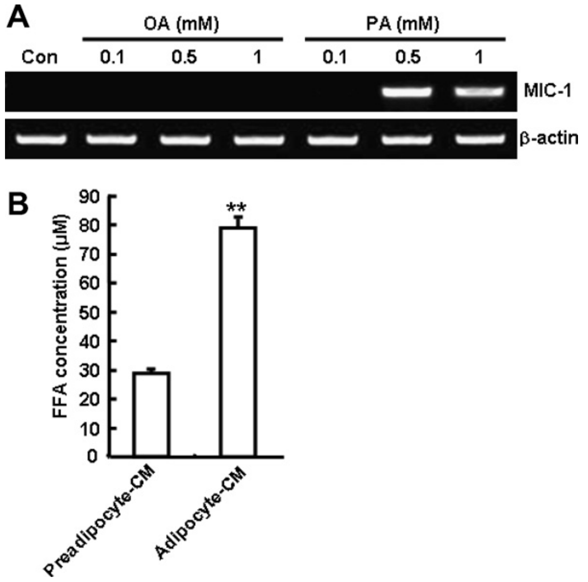

Fig. 3. Palmitate increases MIC-1 gene expression in MDA-MB-231 cells. (A) MDA-MB-231 cells were incubated with 10% BSA as a control, oleate, and palmitate for 6 h. The total RNA was isolated and subjected to RT-PCR analysis using MIC-1 primers. (B) The FFAs released from mature adipocytes were measured using Cultured Human Adipocyte Lipolysis Assay Kit. The experiment was performed in triplicates. Data represent means ± SEM. $^{**}P < 0.01$ versus preadipocyte-CM.

MIC-1 mRNA expression (Fig. 3A). These results suggest that palmitate in the adipocyte-CM is a MIC-1 expression inducing factor. To examine level of FFAs in adipocyte-CM, FFAs concentration in culture medium of differentiated OP9 cells was determined. The concentration of FFAs 3 h after the culture of differentiated OP9 cells was 80 μM (Fig. 3B). Therefore, accumulated concentration of FFAs in adipocyte-CM cultured for 24 h will be more than 0.5 mM. This result indicates that level of FFAs in adipocyte-CM is enough to stimulate MIC-1 production.

3.4. Adipocyte-CM increases MIC-1 expression via p38 MAPK signaling pathway in MDA-MB-231 cells

In order to determine which signaling pathway is involved in adipocyte-CM-induced MIC-1 gene expression, MDA-MB-231 cells were treated with various signal blockers in the presence of adipocyte-CM and subsequently RT-PCR analysis was performed. NF-κB inhibitor, pathenolide; JNK inhibitor, SP600125; ERK inhibitor, PD98059, did not affect MIC-1 gene expression induced by adipocyte-CM treatment. However, p38 MAPK inhibitor, SB203580, completely inhibited the adipocyte-CM-induced MIC-1 mRNA expression (Fig. 4A). If palmitate in the adipocyte-CM is the major component inducing MIC-1 gene expression, palmitate-induced MIC-1 gene induction will be also suppressed by the treatment of SB203580. As expected, SB203580 markedly inhibited palmitate-induced MIC-1 gene expression (Fig. 4B). To further confirm this result, we examined whether palmitate is able to induce the phosphorylation of p38 MAPK. Palmitate increased the phosphorylation of p38 MAPK as early as 10 min. These findings show that MIC-1 gene induction by the adipocyte-CM is occurred by palmitate-mediated p38 MAPK activation.

3.5. MIC-1 increases invasiveness of MDA-MB-231 cells

In order to verify the impact of adipocyte-CM treatment on the invasiveness of MDA-MB-231 cells, firstly, MDA-MD-231 cells were treated with NIH3T3-CM, preadipocyte-CM, and adipocyte-CM in upper chambers of transwell for 24 h and then invasiveness was measured. MDA-MB-231 cells treated only with adipocyte-CM in the upper chamber showed significant increase in the invasiveness compared with NIH3T3-CM and preadipocyte-CM (Fig. 5A). We previously showed that MIC-1 increases the invasiveness of gastric cancer cells [17], therefore, it is probable that adipocyte-CM-induced MIC-1 would increase the invasiveness of MDA-MB-213 cells. To test this possibility, we examined whether recombinant MIC-1 is also able to increase the invasiveness of MDA-MB-231 cells. Recombinant MIC-1 significantly enhanced the invasiveness of MDA-MB-231 cells as the adipocyte-CM treatment did (Fig. 5B), indicating that adipocyte-CM-induced MIC-1 is responsible factor to enhance the invasiveness of MDA-MB-231 cells.

4. Discussion

Stromal–epithelial interactions in the mammary gland are required for normal development [18]. Generally, normal stroma can efficiently inhibit the expression of neoplasm characteristics [19,20]. However, disruptions in stromal–epithelium interactions enhance cancer development and progression [19,21]. Adipocytes are the most abundant stromal cells in breast tissues, but little is known about the role of the adipocytes in the breast cancer cells. There are only several reports describing adipocyte-breast cancer cell interactions. One report showed that irradiation of gland-free mammary fat pad of mice causes malignant progression of transplanted normal mammary cells [22], indicating that adipocytes normally prevent malignant progression of normal mammary cells. Meanwhile, other group showed that adipocyte-secreted factors promote proliferation of breast cancer cells including MCF-7 and MDA-MB-231 and stimulate angiogenesis *in vitro* [11,23]. In the present study, we

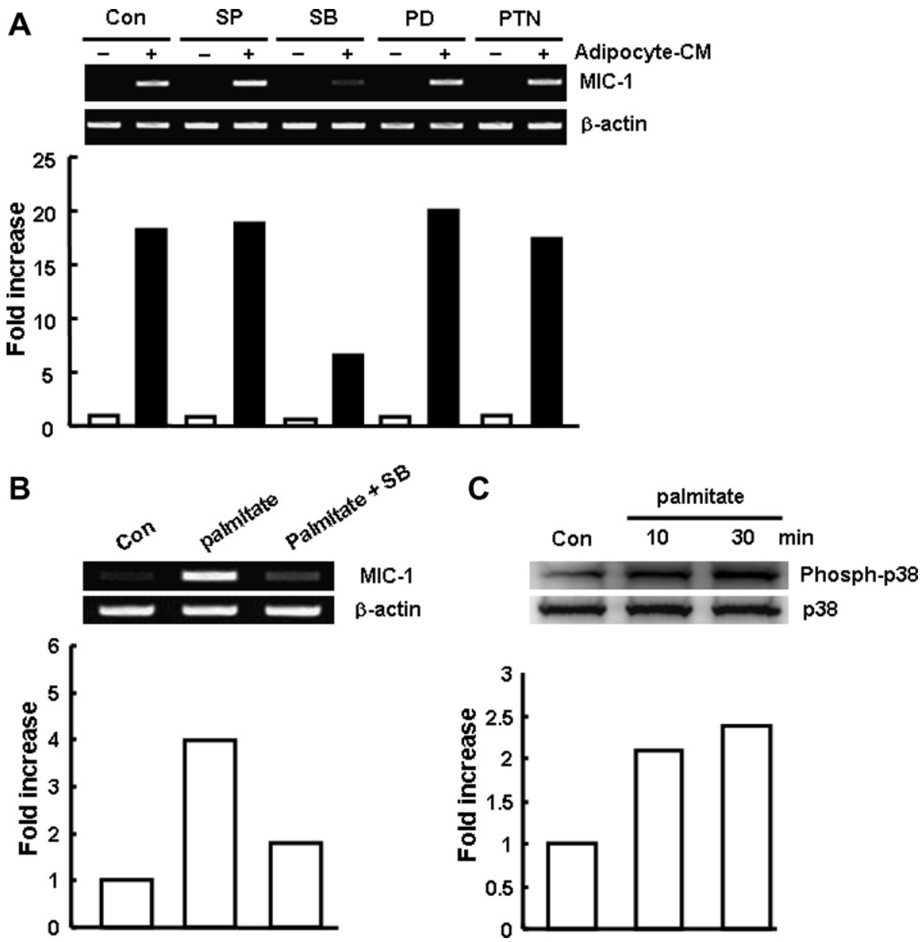

Fig. 4. Adipoctye-CM and palmitate increase MIC-1 expression in MDA-MB-231 cells through activation of p38 MAPK pathway. (A) MDA-MB-231 cells were pretreated with 20 μM of SP600125, 20 μM of PD98059, 20 μM of SB203580, and 1.5 μM of pathenolide (PTN) for 1 h and changed with adipocyte-CM and then incubated for 6 h. The total RNA was isolated and subjected to RT-PCR analysis using MIC-1 primers. The intensity of bands was measured using DNR Bio-imaging system. (B) MDA-MB-231 cells were pretreated with 20 μM of SB203580 for 1 h and changed with fresh culture medium containing 0.5 mM of palmitate. The total RNA was isolated and subjected to RT-PCR analysis using MIC-1 primers. The intensity of bands was measured using DNR Bio-imaging system. (C) MDA-MB-231 cells were treated with 0.5 mM of palmitate. At the indicated time points, cells were harvested and cell lysates were prepared. Total cell lysates were subjected to Western blot analysis using phospho-p38 and p38 antibodies. The intensity of bands was measured using DNR Bio-imaging system.

used OP9 cells as a source of adipocyte-CM to study the crosstalk between adipocytes and breast cancer cells because OP9 is bone marrow-derived cell line and breast cancer cells frequently metastasized to the bone marrow. The culture supernatants of differentiated OP9 adipocytes stimulated MDA-MB-231 cells to produce MIC-1, leading to increased invasiveness. This finding indicates that bone marrow adipocytes are able to contribute to the preparation of microenvironment for tumor metastasis, although other roles of adipocyte-CM-induced MIC-1 in MDA-MB-231 cells remain to be elucidated.

Various components including proteins and lipids in adipocyte-CM are involved in the regulation of proliferation and invasion of breast cancer cells, and these components from adipocyte-CM determine the fate of breast cancer cells such as promotion or suppression. However, in order to know the exact molecular mechanism by which adipocyte-CM affects tumor invasion, we need to understand the function of each single factor in the crosstalk between adipocyte and breast cancer cells. We therefore studied MIC-1 among the genes that were induced by adipocyte-CM because the role of MIC-1 has not been identified yet for breast cancer

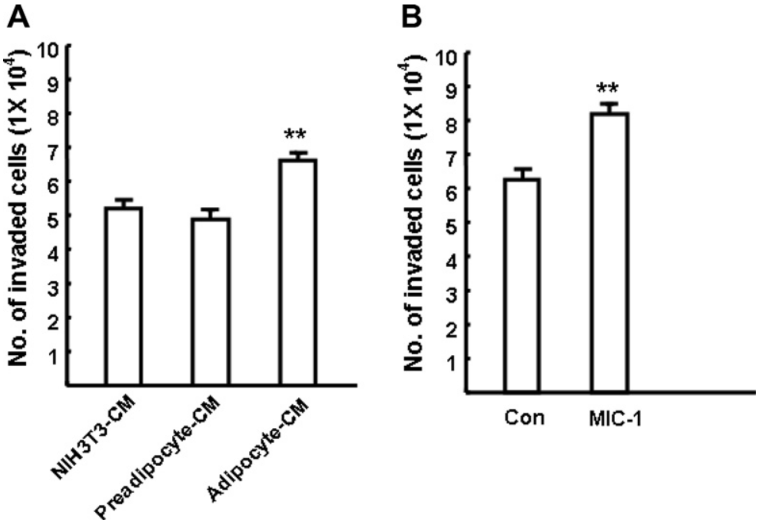

Fig. 5. Adipocyte-CM and MIC-1 increases invasiveness of MDA-MB-231 cells. (A) MDA-MB-231 cells were incubated with NIH3T3-CM, preadipocyte-CM, and adipocyte-CM in upper chamber of transwell for 24 h. After incubation for 24 h, the cells that invaded to lower chamber were disassociated with 500 μl of disassociation buffer containing Calcein-AM reagents. Invaded cells were detected using Wallac 1420 Victor3 plate reader. (B) MDA-MB-231 cells were incubated with 40 ng/ml of MIC-1 in upper chamber of transwell for 24 h. After incubation for 24 h, the cells that invaded to lower chamber were measured. The experiment was performed at the triplicates. Data represent means ± SEM. $^{**}P < 0.01$ versus control.

even though the serum concentration of MIC-1 has been known to be highly increased in breast, colorectal, and prostate cancer. On the other hand, although there has been a report that MIC-1 expression is increased by TNF-α and IL-1β administration in liver [24], MIC-1 gene induction was not observed (data not shown) when MDA-MB-231 cells were treated with TNF-α and/or IL-1β. It is the reason why proteinase K-treated adipocyte-CM still induced MIC-1 gene expression (Fig. 2).

One of the important roles of adipocytes is triglyceride synthesis for energy storage and release of FFA for the regulation of energy balance. Palmitate, a major FFA released from adipocytes [25], increased MIC-1 gene induction in MDA-MB-231 cells, whereas oleate did not (Fig. 3A). Palmitate stimulated p38 MAPK and then activated p38 MAPK, leading to an increase in the production of MIC-1, which in turn was able to increase the invasiveness of MDA-MB-231 cells as did the adipocyte-CM treatment. It has been known that palmitate inhibits protein kinase B and stimulates an intrinsic pathway of pro-apoptotic signaling in microvascular mesangial cells and MDA-MB-231 cells [26,27]. It is also known that MIC-1 overexpression in tumors also causes growth arrest and apoptosis [28,29]. In this study, we however showed that adipocyte-CM containing palmitate increased the invasiveness of MDA-MB-231 cells without inducing apoptosis. The following explanation will be the probable reason for this discrepancy of result. Besides palmitate that promotes apoptosis in MDA-MB-231 cells, adipocyte-CM also contains other tumor growth promoting factors such as previously identified collagen IV and hepatocyte growth factor [11,30]. Thus, it is not surprising that other factors including oleate, collagen IV, and hepatocyte growth factor in adipocyte-CM, are able to inhibit palmitate-induced apoptosis of MDA-MB-231 cells. In addition, the previous report showed that cotreatment of palmitate with oleate overcame palmitate-induced apoptosis of MDA-MB-231 cells [26]. It is, therefore, conceivable that palmitate-induced MIC-1 is able to increase invasiveness of tumor cells without palmitate-induced apoptosis of tumor cells.

In summary, we showed that subsets of genes associated with immune and wound healing, which regulate tumor microenvironment, were increased by the adipocyte-CM treatment. Palmitate, a lipid component in adipocyte-CM, increased MIC-1 production in MDA-MB-231 cells and the increased MIC-1 stimulated the invasion of MDA-MB-231 cells. Since adipocyte-CM contains many protein and lipid factors, study on the relative roles of each factor will contribute to elucidate the microenvironment made by adipocytes. Further studies on other functions of MIC-1 in breast cancer cells will be more helpful in understanding the crosstalk between adipocytes and breast cancer cells.

Acknowledgements

This work was supported by the SRC program of KOSEF (R11-2005-017-01002-0) and the Biogreen 21 project (20070301034031) of Rural Development Administration.

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