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Macrophage inflammatory protein-related protein-2, a novel CC chemokine, can regulate preadipocyte migration and adipocyte differentiation

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Abstract **Adipocytes not only store energy, but also secrete biologically active molecules called adipocytokines, which play a pivotal role in adipocyte-related pathological processes such as diabetes and cardiovascular disease. Recent studies have shown that preadipocyte/adipocyte expresses chemokines (e.g. monocyte chemoattractant protein-1, macrophage inflammatory protein-1 alpha) which alter adipocyte function, indicating the involvement of chemokines in adipocyte-related pathologies. The current study investigated the potential of macrophage inflammatory protein-related protein-2 (MRP-2), a novel CC chemokine, to modulate preadipocyte trafficking and adipocyte differentiation. MRP-2 and its receptors were highly expressed in preadipocytes and differentiated adipocytes as well as in the mouse fat pad. Chemotaxis assays revealed that MRP-2 was a specific chemotactic regulator in preadipocyte migration. The levels of MRP-2 expression in adipose tissue were enhanced in obese mice compared to lean mice. MRP-2 secretion by preadipocytes was suppressed during differentiation. MRP-2 suppressed the expression of adipocyte differentiation markers such as adipocyte fatty acid-binding protein and glycerol-3 phosphate dehydrogenase. Taken together, our data suggest that MRP-2 plays a role in the regulation of preadipocyte migration and adipocyte differentiation during adipose tissue development. MRP-2 may be another adipocytokine, which can be involved in the adipocyte-related pathological process.**

Key words: CC chemokine; Macrophage inflammatory protein-related protein-2; Chemotaxis; Preadipocytes; Adipocyte; Adipogenesis; Obesity; Inflammation

1. Introduction

Obesity is characterized by abnormal adipose tissue development that has been clearly shown to increase health risks such as diabetes and cardiovascular diseases. In obesity, body fat mass increases through both the enlargement of existing fat cells (hypertrophy) and the increase in adipocyte number (hyperplasia). Preadipocyte migration into fat cell clusters is an indispensable process in the initial stage of adipose tissue development [1], indicating that uncontrolled migration of preadipocytes may result in adipocyte hyperplasia and fat mass increase in obesity. However, the molecules regulating this process have not been identified. Adipocytes not only store energy, but also secrete biologically active molecules such as tumor necrosis factor-α (TNFα), interferon-γ (IFNγ), interleukin-1 and -6, transforming growth factor-β, leptin, and plasminogen activator inhibitor type 1 [2,3]. Those molecules, which are called adipocytokines [4], modulate adipose tissue metabolism and accelerate the adipocyte-related pathological process. For example, TNFα inhibited insulin action in adipocytes and thus caused obesity-related insulin resistance [5]. Plasminogen activator inhibitor type 1 directly regulated preadipocyte migration and angiogenesis, and thus was implicated in adipose tissue expansion in obesity [6]. It is now apparent that those adipocytokines act as contributing factors to adipocyte-related inflammatory diseases such as diabetes and atherosclerosis [7,8].

Chemokines, a superfamily of structurally related small (most being 8–14 kDa) chemotactic cytokines, are known as inflammatory mediators due to their ability to cause leukocyte trafficking to sites of inflammation [9]. Besides the chemotactic properties, chemokines also have broader biological functions such as growth-regulatory and angiogenic properties during the development of the immune system [10,11]. Interestingly, recent studies have shown that human adipocytes constitutively express CC chemokines such as monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein-1α (MIP-1α) [12]. Those chemokines alter lipid accumulation and leptin secretion by adipocytes [12], indicating the involvement of chemokines in regulation of adipocyte function. However, whether chemokines exhibit chemotactic properties for the recruitment of non-immune cells such as preadipocytes have not been reported.

Macrophage inflammatory protein-related protein-2 (MRP-2), a new member of the CC chemokine family, is a potent chemoattractant for monocytes, lymphocytes, neutrophils, and eosinophils [13]. Our previous studies have shown that the levels of MRP-2 are enhanced in mice fed high fat diets as well as in human atherosclerosis patients [14,15], indicating

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Abbreviations: MRP-2, macrophage inflammatory protein-related protein-2; TNFα, tumor necrosis factor alpha; IFNγ, interferon gamma; MCP-1, monocyte chemoattractant protein-1; MIP-1α, macrophage inflammatory protein-1 alpha; CCR, chemokine receptor

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MRP-2 could be associated with the obesity-related pathological process. The current study investigated the potential of MRP-2 to induce preadipocyte migration and to modulate adipocyte function. Here, we present for the first time, the evidence that MRP-2 is a potent chemoattractant for preadipocyte, and the expression levels of MRP-2 in adipose tissue is elevated in obese mice. In addition, we show that MRP-2 is associated with regulation of adipocyte differentiation. The CC chemokine MRP-2 may be a critical factor in the regulation of preadipocyte recruitment and adipocyte differentiation during adipose tissue development.

2. Materials and methods

2.1. Cell culture

3T3-L1 murine preadipocytes (ATCC) were cultured in a basal medium consisting of Dulbecco's modified Eagle's medium (DMEM) supplemented with 200 μM ascorbic acid, 10% calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in a 5% CO_2 atmosphere. Two days after reaching confluence (day 0), the cells were incubated in a differentiation medium containing the inducing mixture (0.25 μM dexamethasone, 10 μg/ml insulin, and 0.5 mM 1-methyl-3-isobutylxanthine as in the basal medium. After 40 h, the cell culture medium was changed to a maturation medium containing 5 μg/ml insulin with the basal medium, and fresh maturation medium was changed every 2 days for 7 days [16].

2.2. Preparation of adipose tissue sample

To examine the MRP-2, chemokine receptor (CCR)-1 and CCR-3 mRNA or protein expression in the fat pad, epididymal adipose tissue samples were isolated from Balb/c female mice. Obese (db/db obese, 8 weeks, male, Japan CLEA, Tokyo) and lean mice (C57BL, 8 weeks, male, Japan CLEA, Tokyo) were used to examine the expression of MRP-2, aP2, and leptin mRNA. The care and treatment of experimental animals conformed to the NIH guidelines for the ethical treatment of laboratory animals.

2.3. Chemotaxis assay

Cell migration was assessed in a multiwell microchemotaxis chamber (Neuroprobe, USA). Briefly, 3T3-L1 preadipocytes were suspended in the DMEM medium at a concentration of 5×10^5 cells/ml and 50 μl of the cell suspension was placed into the upper well of a 96-well chamber separated by a gelatin-coated 8-μm polycarbonate filter from a lower well containing MRP-2. A medium that does not contain additional MRP-2 served as the untreated control. In order to assess MRP-2 or the receptor-specific chemotaxis, anti-MRP-2 (50 μg/ml, R&D Systems, MN, USA), anti-CCR1 (50 μg/ml, R&D Systems, MN, USA), or anti-CCR3 (50 μg/ml, R&D Systems, MN, USA) antibody was added to the culture medium for 30 min to neutralize MRP-2 or CCR1/CCR3. Normal rat IgG or mouse IgG was used as the negative control. The medium from untreated cells was used to determine basal migration. After incubation for 16 h at 37°C, non-migrated cells were removed by scraping. The cells that migrated across the filter were fixed and stained with Diff-Quik (International Reagents Corp., Japan). Stained cells were counted in three randomly chosen high-power fields (×400) under a light microscope. Results were expressed as means ± S.E.M. from triplicate samples.

2.4. Oil red-O staining

Adipocyte differentiation was monitored by staining intracellular lipid with oil red-O. Briefly, cells were washed gently with PBS, fixed in 10% formalin solution for 1 h, and washed in water. Cells were treated with 60% isopropanol for 1 min, and then stained for 2 h at room temperature with 0.18% oil red-O in 60% isopropanol. After washing in 60% aqueous isopropanol, the cells were visualized.

2.5. RT-PCR analysis

Total RNA was extracted from 1×10^7 cells or from a 10 mg tissue using a Tri-Reagent (MRC). Total RNA (0.5 μg) was reverse-transcribed into cDNA according to the manufacturer's instructions. For semiquantitative analysis, the linearity of amplification of MRP-2, MIP-1α, adipocyte fatty acid-binding protein (aP2), glycerol-3 phosphate dehydrogenase (GPDH), leptin, CCR-1, CCR-3 and β-actin cDNAs was established in preliminary experiments. 20 cycles for aP2, leptin, 30 cycles for GPDH, MRP-2 and 40 cycles each for CCR-1, CCR-3, MIP-1a amplification were found to be the optimum conditions for obtaining a satisfactory profile. The following sets of primers were used in PCR amplification: MRP-2 (GenBank accession number U15209): forward, 5′-TGCCTGTCCTATAACTCACGGATT-3′, reverse, 5′-AGCAAATGTTATTGTTTGTAGGTCCG-3′; MIP-1α (X53372): forward, 5′-ACCAAGTCTTCTCAGCGCCAT-3′, reverse, 5′-AAAATAGTCAACGATGAATTGGCG-3′; adipocyte fatty acid-binding protein (aP2) (K02109): forward, 5′-AAGACAGCTCCTCCTCGAAGGTT-3′, reverse, 5′-TGACCAAATCCCCATTTACGC-3′; GPDH (J02655): forward, 5′-AGACACCCAACTTTCGCATCAC-3′, reverse, 5′-CAATGGACTTTCCAGTTCGAGC-3′; leptin (M96827): forward, 5′-AGCACTTGGTTCGCTATCGCT-3′, reverse, 5′-TTCCCCACGTAGAGCGTTAGG-3′; CCR-1 (NM001295): forward, 5′-AGTGGGAGTTCACTCACCGTACC-3′, reverse, 5′-AGTAATAGCAAATATCAGACGCACGG; CCR-3 (AF026535): forward, 5′-ACAATTGACAGATACCTGGCTATCGT-3′, reverse, 5′-AAAAAGGAGAACCAGGTTGTACGG-3′; β-actin (X03672): forward, 5′-ATGAAGATCCTGACCGAGCGT-3′, reverse, 5′-AACGCAGCTCAGTAACAGTCCG-3′. Amplification products obtained by PCR were electrophoretically separated on a 2% agarose gel. SYBR green-stained bands for the target gene and β-actin were photographed. The intensity of the bands was densitometrically measured with a NIH image analyzer. All gene signals were normalized to mRNA levels of the housekeeping gene, β-actin, and expressed as a relative ratio.

2.6. Western blot analysis

Adipose tissue samples were prepared and their protein content was determined. Equal amounts of protein (100 μg/lane) were loaded and electrophoresed on a 17% SDS–polyacrylamide gel. After transferring the separated proteins to nitrocellulose membrane, the membrane was incubated overnight in a blocking buffer (2% non-fat dry milk, 10 mM Tris (pH 7.5), 100 mM NaCl and 0.1% Tween 20) and was treated with a goat polyclonal MRP-2 antibody (R&D System, MN, USA) for 2 h. After washing, the membrane was incubated with a horseradish peroxidase (HRP)-conjugated anti-goat IgG antibody. The immunoreactive protein was detected with a chemiluminescent system (ECL kit, Amersham, UK). After exposure to an X-ray film, band density was calculated from optical density with an image analyzer.

2.7. Measurement of MRP-2

Immunoplates (Nunc, Denmark) were coated with purified MRP-2 (2 μg/ml, R&D Systems) in PBS (100 μl/well) overnight at 4°C. After blocking, 100 μl of the test sample was added to each well. A biotinylated polyclonal anti-MRP-2 antibody (100 μg/ml) was added (100 μl/well) after washing, and then the plates were incubated for 1 h at room temperature. After washing, HRP-labeled streptavidin was added at 100 μl/well and the plates were incubated for 30 min at room temperature. The plates were washed before adding the substrate kit (Pharmingen, San Diego, CA, USA), and the reaction was quenched by the addition of 50 μl of 0.4 M NaOH. The absorbance at 450 nm was measured using a microplate reader (Molecular Devices).

2.8. Statistical analysis

All experiments were repeated at least twice. Results were expressed as means ± S.E.M. Statistical analysis was performed using ANOVA and Duncan's multiple range test. Differences were considered to be significant at $P<0.05$.

3. Results

3.1. Expression of MRP-2 and the receptors in fat cells and fat tissue

The expression of MRP-2 and its receptors at the mRNA level in 3T3-L1 preadipocytes, differentiated adipocytes, and fat pad was investigated. RT-PCR and Western blot analysis revealed that MRP-2 mRNA/protein is expressed in both the epididymal fat pad and the 3T3-L1 cells (Fig. 1A,B). Our data revealed, for the first time, that fat cells constitutively expressed MRP-2. Interestingly, the expression of MRP-2 un-

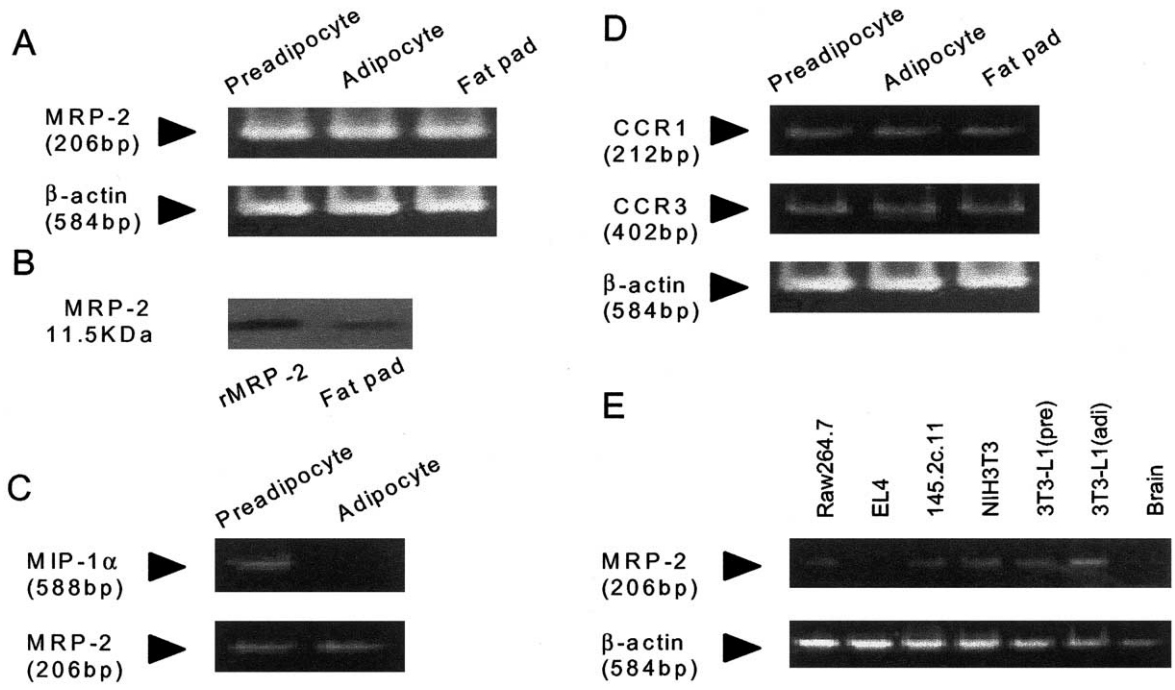

Fig. 1. Expression of MRP-2 and its receptor at mRNA or protein level in murine 3T3-L1 preadipocytes, differentiated adipocytes, and mouse fat tissue. Total RNA was extracted from 3T3-L1 preadipocytes, differentiated adipocytes, and mouse epididymal adipose tissue. A: MRP-2 mRNA expression by RT-PCR analysis. B: MRP-2 protein expression by Western blot analysis. Recombinant MRP-2 served as the positive control. C: MIP-1α mRNA expression by RT-PCR analysis D: CCR1 and CCR3 mRNA detected by RT-PCR. (E) MRP-2 mRNA expression in various cell lines. Data presented is a representative of three independent experiments.

changed in differentiated adipocyte (Fig. 1C), whereas the expression of MIP-1α, a family of CC chemokine, was down-regulated, as previously reported [10]. The expression of CCR1 and CCR3 mRNA was also observed in the 3T3-L1 preadipocytes, adipocytes, and fat pad (Fig. 1D). MRP-2 was detected in macrophage (RAW264.7), fibroblast (NIH3T3), and B lymphocyte hybridoma (145.2c.11), but not in T lymphoma cell line (EL4). MRP-2 mRNA is widely expressed in most organs (e.g. liver, lung, thymus, heart) except brain [17]. Consistent with the previous report, MRP-2 was not detected in the brain.

3.2. Chemotaxis of 3T3-L1 preadipocytes by MRP-2

In order to determine whether MRP-2 can activate 3T3-L1 preadipocytes that express CCR1 and CCR3, specific receptors of the CC chemokine MRP-2, we examined whether

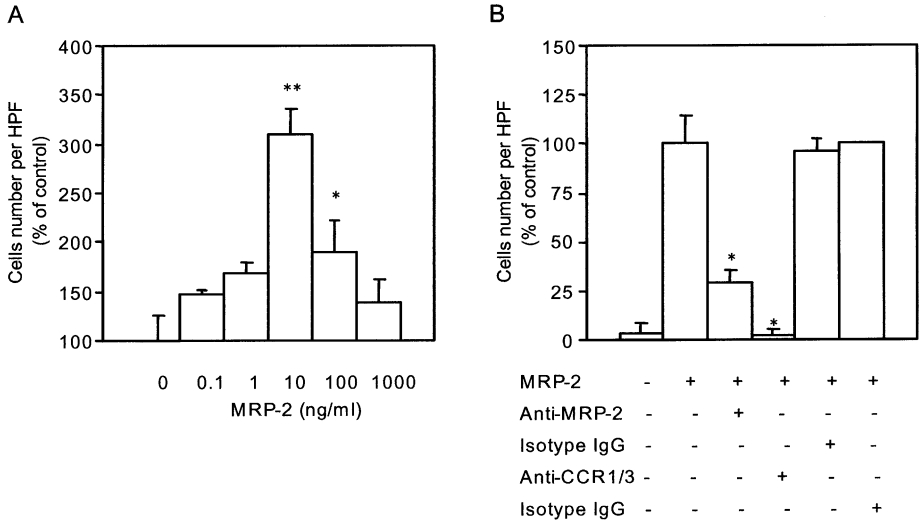

Fig. 2. Chemotaxis of 3T3-L1 preadipocytes by MRP-2. A: 3T3-L1 preadipocytes were exposed to MRP-2 (0.1–1000 ng/ml) or a vehicle for 16 h and cells migration was determined as described in Section 2. A medium that does not contain MRP-2 served as the untreated control. Migration was expressed as percentage of the untreated control. B: Preincubation with a neutralizing anti-MRP-2 or anti-CCR-1/CCR-3 antibody resulted in significant inhibition of chemotaxis, whereas preincubation with non-immune isotypes of mouse IgG or rat IgG had no effect. Migration was expressed as percentage of the MRP-2 (10 ng/ml)-treated control. Values are means ± S.E.M. from three independent experiments. $*P < 0.05$, $**P < 0.01$, significantly different from control.

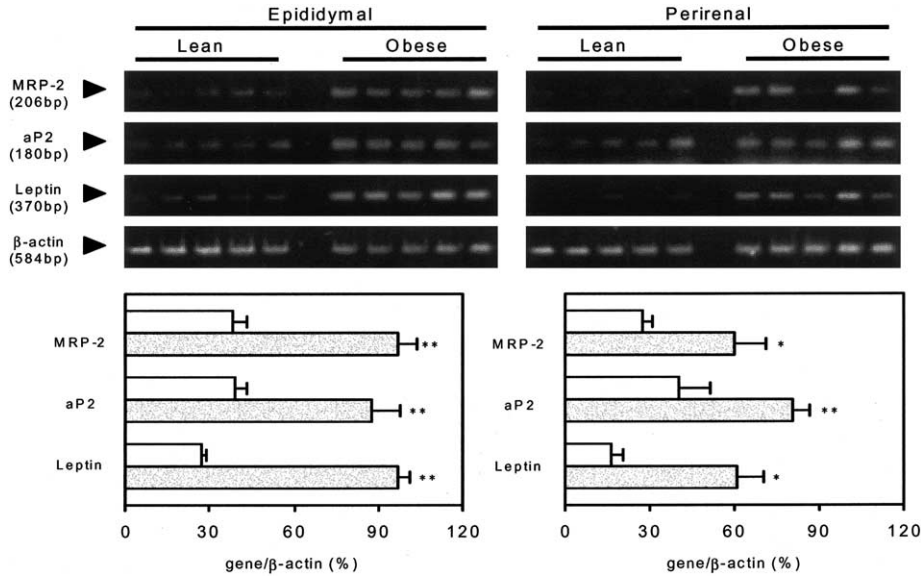

Fig. 3. Expression of MRP-2, aP2, or leptin mRNA in epididymal and perirenal adipose tissues of obese and lean mice. Epididymal and perirenal adipose tissues are isolated from obese ($N=5$) and lean mice ($N=5$), and total RNA was extracted from the adipose tissues. The expressions of MRP-2, aP2, leptine mRNA were measured by RT-PCR analysis and the intensity of the bands was densitometrically measured and normalized to mRNA levels of β-actin. Open boxes show the data for lean mice and closed boxes the data for obese mice. Values are means ± S.E.M. $*P<0.001$, $**P<0.01$, significantly different from control.

MRP-2 could induce preadipocyte migration. Using a chemotaxis assay we observed that MRP-2 is a strong chemoattractant for 3T3-L1 preadipocytes (Fig. 2A). MRP-2 (0.1–1000 nM) induced chemotactic migration in a bell-shaped response pattern with an optimal response at 10 ng/ml. This MRP-2-induced chemotactic migration was significantly ($P<0.01$) inhibited by the anti-MRP-2 or anti-CCR-1/CCR-3 antibodies (Fig. 2B), whereas the non-immune isotype of mouse IgG or rat IgG had no effect.

3.3. Expression of MRP-2 in adipose tissues of obese and lean mice

To examine whether MRP-2 expression is upregulated in obesity, we compared the levels of MRP-2 mRNA expression in epididymal and perirenal adipose tissues from obese and lean mice. The expression of MRP-2 was upregulated 2.5 fold in obese epididymal and 2.2 fold in perirenal adipose tissue compared to lean mice (Fig. 3A). The expression of leptin and aP2 mRNA in the fat tissue, which is known to be enhanced in obesity, was significantly upregulated in obese mice compared to those the expression in lean mice (Fig. 3B), as previously reported [18–20].

3.4. MRP-2 release by 3T3-L1 preadipocytes

Lipid accumulation in differentiated adipocytes was determined by oil-red O staining (Fig. 4A). The release of MRP-2

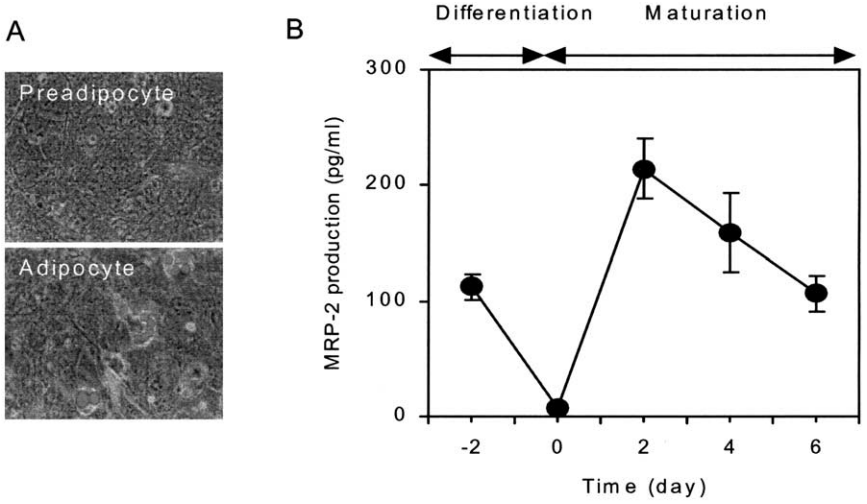

Fig. 4. MRP-2 release by 3T3-L1 preadipocytes and differentiated adipocytes. At 48 h after reaching confluence, 3T3-L1 preadipocytes were incubated in the differentiation medium containing 0.25 μM dexamethasone, 10 μg/ml insulin, and 0.5 mM 1-methyl-3-isobutylxanthine. After 40 h, the cell culture medium was changed to the maturation medium containing 5 μg/ml insulin, and the maturation medium was replaced with a fresh one every 2 days for 7 days. At day 7, lipid accumulation in adipocytes was determined by oil red-O staining. The amount of MRP-2 protein released by preadipocytes or adipocytes was measured by ELISA as described in Section 2. AL Oil red-O staining. B: MRP-2 release. Values are means ± S.E.M. from three independent experiments. $*P<0.01$, significantly different from the undifferentiated control.

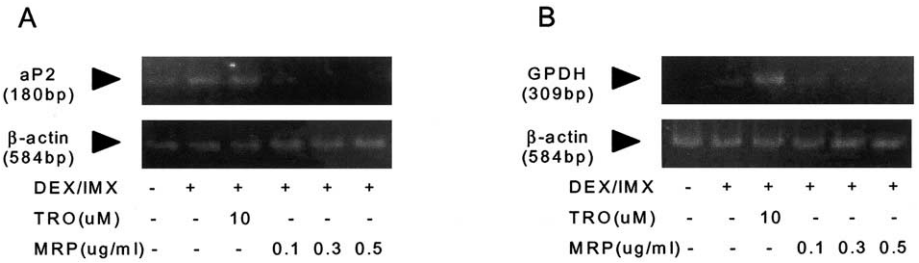

Fig. 5. Effect of MRP-2 on the levels of aP2 and GPDH mRNA expression in adipocyte. 3T3-L1 preadipocytes were incubated in the differentiation medium containing 0.25 μM dexamethasone, 10 μg/ml insulin, and 0.5 mM 1-methyl-3-isobutylxanthine. Total cellular RNA was isolated at 6 days after the induction of differentiation. The expressions of aP2 (A), GPDH (B) mRNA were measured by RT-PCR analysis. Data presented is a representative of three independent experiments.

by 3T3-L1 adipocytes before and after differentiation was detected in the culture medium. 3T3-L1 preadipocytes differentiated in the inducing mixture, then underwent maturation for 7 days. Undifferentiated 3T3-L1 cells produced MRP-2 (113 ± 11 pg/ml), the production of which was significantly ($P < 0.01$) reduced during differentiation (7 ± 0.6 pg/ml), as shown in Fig. 4B. The amount of released MRP-2 gradually increased and reached maximal levels (214 ± 26 pg/ml) at day 2 of maturation, and returned toward baseline of the undifferentiated cells at day 6 of maturation.

3.5. *Effect of MRP-2 on the expression of aP2 and GPDH mRNA*

Because MRP-2 release by preadipocytes decreased during differentiation, we examined whether MRP-2 could affect cell differentiation where exogenous mediators promoted adipogenesis. MRP-2 treatment during adipocyte differentiation resulted in suppression of adipocyte-specific differentiation markers such as message expression of aP2 and GPDH at day 6 after induction of differentiation (Fig. 5). Troglitazone, a synthetic thiazolidinedione derivative, which is known to promote adipocyte differentiation [16] resulted in upregulation of the adipocyte-specific differentiation markers.

4. Discussion

In the present study, we revealed for the first time the role of the CC chemokine MRP-2 in the recruitment of preadipocytes and adipogenesis.

The CC chemokine MRP-2 is known to be a chemotactic cytokine, recruiting leukocytes into the sites of inflammation and activating the cells [13]. However, it is not known whether that chemokine can act as a modulator to induce chemotaxis for non-immune cells and/or to alter their functions. Our data showed that MRP-2 mRNA and protein are highly expressed in murine 3T3-L1 preadipocytes, differentiated adipocytes, and mouse adipose tissue. The expression of MRP-2 in an adipocyte cell line as well as in adipose tissue of normal mice indicates that MRP-2 might modulate adipocyte function, and not be limited to the recruitment of immune cells. It has been shown previously that preadipocytes constitutively express CC chemokine (e.g. MCP-1, MIP-1α) and their receptors [12], and that expression of chemokine mRNA was decreased in differentiated adipocytes compared with preadipocytes. It is important to note, however, that mRNA steady-state levels of MRP-2 did not decrease in differentiated adipocytes compared to preadipocytes, whereas MIP-1α expression decreased in adipocytes as previously reported [12]. The results indicate that there may be a role for regulation of adipocyte function. Mouse adipose tissue and 3T3-L1 cells also expressed the receptors CCR1 and CCR3. That receptor expression in preadipocytes and adipocytes suggests that MRP-2 may function as an autocrine or a paracrine regulator of both preadipocytes and adipocytes.

Since the recruitment of leukocytes are mediated by the chemokines and their specific receptor signaling, we thought that preadipocytes may respond to MRP-2-induced chemotaxis in a manner similar to other immunocompetent cells such as monocytes. Our data clearly revealed that 3T3-L1 preadipocytes exhibited the ability to migrate when treated with the CC chemokine MRP-2. The treatment with the anti-MRP-2 or anti-CCR-1/CCR-3 antibody blocked MRP-2-induced migration. Those results indicated that preadipocytes had the same chemotactic properties as monocytes mediated by specific receptors. Considering the fact that preadipocyte migration into fat cell clusters is a prerequisite for adipose tissue development, the effect of MRP-2 on preadipocyte migration has significant physiological relevance in adipose tissue development. It has been reported that chemokines exhibit growth regulatory and angiogenic properties [11,12,21], which can also affect tissue development. Therefore, our result could be interpreted that MRP-2 produced locally by preexisting adipocytes may play an important role in recruiting preadipocytes into adipocyte clusters.

The chemotactic properties of MRP-2 for preadipocytes led us to speculate that MRP-2 may be associated with abnormal adipose tissue development in obesity. To find the potential involvement of MRP-2 in obesity, we determined the difference of the levels of MRP-2 expression in lean and obese mice. Our data revealed that the levels of MRP-2 expression of epididymal adipose tissue were markedly enhanced in obese mice compared to those of lean mice. Consistent with other reports [18–20], upregulation of aP2 and leptin expression was observed in the adipose tissues from obese mice. Considering the chemotactic properties of MRP-2, the upregulation of MRP-2 in fat tissues may cause abnormal adipose tissue expansion in the obese mice.

Interestingly, it has been shown that preadipocytes exhibited functional features of macrophages, such as phagocytosis and anti-microbial activity, suggesting that preadipocytes play a role in the inflammatory process and in an immune response [22]. MRP-2-induced preadipocyte trafficking in our observa-

tions strongly supports the idea that preadipocytes could migrate to inflammatory sites and directly affect inflammatory processes mimicking macrophages. It has been reported that obese subjects have a higher incidence of atherosclerosis. Our previous studies demonstrated that MRP-2 expression was upregulated in atherosclerotic lesions, especially in foam cell-rich areas [14,15]. Therefore, alternatively, MRP-2 may contribute to the recruitment of preadipocytes into the atherosclerotic lesion sites.

The secretion of MRP-2 from both preadipocytes and differentiated adipocytes was detected. Interestingly, the release of MRP-2 by preadipocytes was markedly decreased when the inducing mixture was added. It has been previously shown that MRP-2 suppressed colony formation by bone marrow stem and progenitor cells, suggesting that MRP-2 is associated with cell differentiation [13]. Recent studies have indicated potential regulation of adipogenesis by a chemokine signaling pathway (e.g. MIP-1α and MCP-1) [12]. These results support the idea that MRP-2 may function as a regulatory molecule in adipocyte differentiation. When the expression of differentiation markers was measured in adipocytes, we found that MRP-2 suppressed the expression of aP2 and GPDH mRNA in mature adipocytes, indicating that MRP-2 inhibited adipocyte differentiation. Our results together with those of others suggest that MRP-2 may modulate adipogenesis. Further studies are needed to demonstrate the mechanism of MRP-2 action in adipogenesis.

In conclusion, MRP-2 and its receptors are highly expressed in adipose tissue and 3T3-L1 cells. MRP-2 functions as a chemotactic modulator in preadipocyte migration. The levels of MRP-2 expression in adipose tissue were enhanced in obese mice. MRP-2 secretion by preadipocytes is suppressed during differentiation. Moreover, MRP-2 inhibited the expression of aP2 and GPDH mRNA in 3T3-L1 adipocytes. Taken together, our data suggest that MRP-2 plays an important role in the recruitment of preadipocytes and adipocyte differentiation during adipose tissue development. MRP-2 may be another adipocytokine which is important in the adipocyte-related pathological process.

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