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Impact of high-/middle-molecular-weight adiponectin on the synthesis and regulation of extracellular matrix in dermal fibroblasts

Hideki Nakasone[a], Kiriko Terasako-Saito[a], Rie Yamazaki[a], Miki Sato[a], Yukie Tanaka[a], Kana Sakamoto[a], Masakazu Kurita[b], Ryoko Yamasaki[a], Hidenori Wada[a], Yuko Ishihara[a], Koji Kawamura[a], Tomohito Machishima[a], Masahiro Ashizawa[a], Shun-ichi Kimura[a], Misato Kikuchi[a], Aki Tanihara[a], Junya Kanda[a], Shinichi Kako[a], Junji Nishida[a], Shigeki Yamada[c], and Yoshinobu Kanda[a]

[a]*Division of Hematology, Saitama Medical Center, Jichi Medical University, Saitama, Japan;* [b]*Department of Plastic Surgery, Kyorin University School of Medicine, Tokyo, Japan;* [c]*Department of Pathology, Saitama Medical Center, Jichi Medical University, Saitama, Japan*

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Adiponectin has been shown to play a critical role in immunity. Recently, we reported that the adiponectin levels after allogeneic stem cell transplantation were higher in recipients with chronic graft-versus-host disease (cGVHD). However, the effects of adiponectin on extracellular matrix (ECM) and regulatory factors in dermal fibroblasts remain unclear. We compared the messenger RNA (mRNA) levels of collagen type1 (COL1A), fibronectin 1 (FN1), matrix metalloproteinase (MMP)1, MMP3, tissue inhibitor of metalloproteinase (TIMP)1, TIMP3, transforming growth factor-β (TGF-β), and TGF-β receptor 2 (TGF-βR2) in human normal dermal fibroblasts cultured with and without adiponectin, and we assessed the degree of synthesis of ECMs by immunofluorescent microscopy. Furthermore, we also assessed these mRNA levels after blocking of TGF-βR2. Adiponectin induced higher mRNA levels of FN1, MMP1, MMP3, TIMP1, TIMP3, and TGF-βR2 in a dose-dependent manner, but did not significantly affect COL1A or TGF-β. In addition, adiponectin was shown to upregulate FN1, MMPs, and TIMPs after blocking of TGF-βR2. Immunofluorescent microscopy revealed that adiponectin promoted a greater synthesis of ECMs than in the control in vitro. The finding that adiponectin upregulated ECM-associated factors might mean that high levels of adiponectin could modulate dermal fibrosis was observed in recipients with cGVHD. Further basic investigation is warranted to elucidate whether the adiponectin-pathway could be a target for the treatment of sclerotic cGVHD. © **2014 ISEH - Society for Hematology and Stem Cells. Published by Elsevier Inc.**

Allogeneic stem cell transplantation (SCT) is an important curative treatment for hematologic diseases. However, SCT is associated with many adverse complications, including graft-versus-host disease (GVHD). GVHD is thought to be the result of alloreactive and autoreactive interactions among donor T and B cells, host antigen-presenting cells, and host tissues [1–3]. In particular, chronic GVHD (cGVHD) significantly impairs the recipient's quality of life [4,5]. The detailed mechanism of cGVHD has not been elucidated, although previous reports have investigated various biomarkers for cGVHD [6]. Almost all these biomarkers were shown to be inflammatory cytokines, such as tumor necrosis factor α, soluble interleukin-2 receptor, and soluble B cell activation factor, which are associated with the activation or inhibition of immune cells, including T and B cells [2,7–9]. However, few reports have focused on other endocrine substances in the view of pathophysiology of cGVHD.

Recently, it has been revealed that adiponectin, an adipokine that is secreted by adipose tissues, plays an important role in immunity and inflammation [10–15]. Adiponectin is thought to exist in a globular isoform, trimers, and middle-/high-molecular-weight (MMW/HMW-) multimers. AdipoR1, AdipoR2, and T-cadherin have been identified as specific receptors for each, respectively [16]. The functions of adiponectin are diverse and may depend on the target organ and its isoforms [16,17].

We recently reported that high levels of HMW-adiponectin were observed after SCT in recipients who suffered from cGVHD, and were associated with the severity of cGVHD [18]. However, it is still unclear whether the increase in HMW-adiponectin is a primary or secondary event, as is

Offprint requests to: Yoshinobu Kanda, M.D., Ph.D., Division of Hematology, Saitama Medical Center, Jichi Medical University, 1-847, Amanuma-cho, Omiya-ku, Saitama 330-8503, Japan; E-mail: ycanda-tky@umin.ac.jp

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the role that HMW-adiponectin plays in the pathophysiology of cGVHD. We hypothesized that HMW-adiponectin might have fibrotic or antifibrotic effects on dermal fibroblasts, because skin fibrosis is a major symptom of cGVHD. Skin and organ fibrosis are both defined as the excessive deposition and accumulation of extracellular matrix (ECM), including collagen type 1 and fibronectin [19]. This ECM is produced by dermal fibroblasts, and is known to be increased in scleroderma [20]. The ECMs produced by fibroblasts are regulated by matrix metalloproteinase (MMP), which can degrade ECMs, and by tissue inhibitor of metalloproteinase (TIMP), which can inhibit the activity of MMPs. Therefore, MMPs might improve fibrosis, whereas TIMPs might accelerate the deposition of ECMs and fibrosis [19]. Both MMPs and TIMPs are also produced by fibroblasts.

To date, there has no thorough investigation of the effects of HMW-/MMW-adiponectin on ECM, MMPs, and TIMPs in dermal fibroblasts. Therefore, we assessed the changes in the gene expression of ECMs and regulatory factors, including transforming growth factor (TGF), MMPs and TIMPs, with or without MMW-/HMW-adiponectin in normal dermal fibroblasts in vitro.

Methods

Fibroblast culture

A skin sample was obtained during plastic surgery with informed consent. Superficial dermal samples were incubated with 0.25% trypsin and 0.02% ethylenediaminetetraacetic acid (EDTA) in phosphate-buffered saline (PBS) for 16–24 hours at 4°C, and the epithelium was separated from the superficial dermal sample. Next, human fibroblasts were isolated and cultured for explant at 37°C under a humidified atmosphere of 5% CO_2 in fibroblast growth medium including Dulbecco's modified Eagle's medium with 10% fetal calf serum and 0.6 mg/mL glutamine. Approximately 3 weeks later, primary cultures were subcultured. These dermal fibroblasts derived from a normal subject were used for all experiments during 7–12 passages.

Human recombinant HMW-/MMW-oligomer-rich adiponectin was purchased commercially (BioVendor, Asheville, NC, USA). Fibroblasts were cultured in a humidified atmosphere of 5% CO_2 at 37°C until subconfluence, and harvested with 0.025% trypsin and 0.01% EDTA. At day −1, 5,000 fibroblasts/cm^2 were seeded in each well of an IWAKI 24-well plate in M106 medium (Kurabo, Osaka, Japan) containing 2% fetal bovine serum with 10 μg/mL gentamicin and 0.25 μg/mL amphotericin. After 24 hours (at day 0), each well was washed with PBS and exchanged for 0.5 mL of fresh M106 medium with or without HMW-/MMW-adiponectin.

For the time-dependent assessment, 0 or 10 μg/ml of HMW-/MMW-adiponectin was added to control and target wells, respectively. Fibroblasts were collected at 0, 24, 48, and 72 hours after the addition of adiponectin.

For the dose-dependent assessment, 0, 1, 5, 10, or 20 μg/mL of HMW-/MMW-adiponectin was added to each well. Fibroblasts were collected 3 days after the addition of adiponectin. The adiponectin level in normal subjects is considered to range between 2 and 10 μg/ml [11,16].

Furthermore, we compared the gene expression of ECMs, MMPs, and TIMPs under TGF-β receptor 2 (TGF-βR2)-blocked conditions using 20 μg/ml of anti-human TGF-βR2 antibody (R&D Systems, Minneapolis, MN, USA) and 20 μg/ml of HMW-/MMW-adiponectin. A dose of 10–20 μg/mL of antihuman TGF-βR2 antibody has often been used for the neutralization of TGF-pathways [21]. In the same manner as described earlier, fibroblasts were collected 3 days after cytokine administration. The targets and controls each included two or three wells.

Messenger RNA extraction, complementary DNA synthesis, and quantitative real-time reverse-transcript polymerase chain reaction

After fibroblasts were collected, messenger RNA (mRNA) extraction and complementary DNA (cDNA) synthesis were performed using an RNAspin mini RNA isolation kit (GE Healthcare, Tokyo, Japan) and SuperScript III First-Strand Synthesis SuperMix for qRT-PCR (Life Technologies, Tokyo, Japan) according to the respective manufacturer's instructions. We then performed quantitative real-time reverse-transcript polymerase chain reaction (qRT-PCR) using Taqman Universal Master Mix II (Life Technologies, Tokyo, Japan) according to the manufacturer's instructions. All specific primers and probes for targets and internal control genes were purchased from Life Technologies (Tokyo, Japan): T-cadherin (Hs00169908_m1*), fibronectin 1 (FN1) (Hs01549976_m1*), collagen type I alpha 2 (COL1A2) (Hs00164099_m1*), TIMP-1 (Hs00171558_m1*), TIMP-3 (Hs00165949_m1*), MMP-1 (Hs00899658_m1*), MMP-3 (Hs00968305_m1*), TGF-β1 (Hs99999918_m1), and TGF-βR2 (Hs00234253_m1*). β-actin (433762F) was used as an internal control. All qRT-PCR procedures were performed with a 7900HT FAST Real Time PCR system (Life Technologies, Tokyo, Japan). Relative transcripts were determined by the following formula: $2^{-(\text{CT target} - \text{CT control})}$.

Immunofluorescent microscopy

Observations of ECMs in vitro were performed by immunofluorescent microscopy as described in previous reports [22–24]. At day −1, 5,000 fibroblasts/cm^2 were seeded on cover glasses in IWAKI 35 mm dishes with M106 medium (Kurabo, Osaka, Japan) containing 2% FBS. After 24 hours (day 0), each dish was washed with PBS and exchanged for fresh M106 medium without any cytokines, with 20 ng/mL of TGF-β or 20 μg/mL of HMW-/MMW-adiponectin. At day 4, cells were washed with PBS and fixed with 3.3% formaldehyde of CellFIX (BD Biosciences, Tokyo, Japan) for 15 minutes and 0.5% triton X (Nacalai Tesque, Kyoto, Japan) for 5 minutes. After three washes with PBS, the samples were blocked with PBS containing 2% FBS for 30 minutes. After three washes with PBS, the samples were incubated with primary antibodies of anti-fibronectin IgG produced in rabbit (1:200 in PBS; F3648; Sigma, Tokyo, Japan) and anti-collagen type1 IgG produced in mouse (1:1000 in PBS; C2456; Sigma) at 4°C for 4 hours. After three washes with PBS, anti-rabbit IgG produced in chicken AlexaFluor488 (A21441; Life Technology) and anti-mouse IgG produced in donkey AlexaFluor594 (A21203; Life Technologies) were added (1:500 in PBS for each) and incubated for 30 minutes. ProLong Gold Antifade Reagent with 4′,6- diamidino-2-phenylindoldilactate (Life Technologies, Tokyo, Japan) was added to each sample after three washes with PBS. Images were obtained by laser confocal microscopy (Fluoview Systems FV500; Olympus, Tokyo, Japan).