Negative Regulation of Dermal Fibroblasts by Enlarged Adipocytes through Release of Free Fatty Acids
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Subcutaneous adipose tissue lies just beneath the dermal layer, but the interaction between the two types of tissue remains obscure. Recently, we reported that obesity is associated with decreased dermal elasticity. To investigate the mechanism of the adipose tissue/dermal interaction, fibroblasts were cocultured with small or enlarged adipocytes, using a membrane insert to prevent direct contact. Enlarged adipocytes reduced 3T3-L1 fibroblast proliferation and gene expression of collagen (I)-α1 (col (I)-α1) and elastin while increasing gene expression of matrix metalloproteinase 13 (MMP13). In contrast, small adipocytes had no such effects. These results indicate that factors secreted by enlarged adipocytes influence dermal condition. As enlarged adipocytes are known to release free fatty acids (FFAs), the effects of these acids on 3T3-L1 fibroblasts were examined. Palmitic acid decreased fibroblast proliferation, reduced gene expressions of col (I)-α1 and elastin, and increased MMP13. Similar effects were observed in human dermal fibroblasts. The influence of palmitic acid on fibroblasts was inhibited by eicosapentaenoic acid (EPA), an inhibitor of Toll-like receptors (TLRs). Furthermore, EPA inhibited the effects of enlarged adipocytes on fibroblasts in the coculture system. These data indicate that enlarged adipocytes negatively control the function of dermal fibroblasts through the activation of TLRs by secreted FFAs.

INTRODUCTION
Obesity is a significant risk factor for various metabolic diseases, including hypertension, cardiovascular disease, and type 2 diabetes. Furthermore, it is also related to the development of pressure ulcers (Knudsen and Gallagher, 2003) and sagging of the face (Ezure et al., 2009), which are associated with impaired dermal conditions such as reduced synthesis and degradation of extracellular matrix proteins, resulting in delayed wound healing and loss of skin elasticity. We have reported that increase in the subcutaneous adipose layer decreases dermal elasticity in mice (Ezure and Amano, 2010a) and humans (Ezure and Amano, 2010b). However, it is still unclear how the subcutaneous adipose layer might affect the dermal layer.

Adipose tissue is composed of lipid-filled cells termed “adipocytes.” Until recently, adipocytes were considered only as inert fat-storing cells, but newer studies have demonstrated that adipocytes play dynamic roles in the highly regulated processes of uptake, storage, and release of fatty acids, and also secrete various bioactive compounds, including steroids, hormone precursors, and cytokines (Matsuzawa, 2006; Klein et al., 2007). Therefore it seems reasonable to consider that subcutaneous adipocytes may influence the dermis via endocrine action.

In obesity, the subcutaneous adipose layer increases and adipocytes become enlarged. Suganami et al. (2005) reported that enlarged adipocytes secrete free fatty acids (FFAs) such as palmitic acid, which acts on macrophages and aggravates inflammatory changes in obesity. However, the interaction between adipocytes and fibroblasts is not well understood. Furthermore, it is unclear whether normal (small) and enlarged adipocytes have different effects on fibroblast function.

In this study we examined the effects on fibroblasts of small and enlarged adipocytes by using a Transwell coculture system in which adipocytes and fibroblasts were not in direct contact. We identified a factor released by enlarged adipocytes that appears to negatively control fibroblast function.

RESULTS
Enlarged adipocytes decrease fibroblast proliferation and gene expression of col (I)-α1 and elastin, and increase gene expression of MMP13
At 8 days after induction of adipogenesis, 3T3-L1 cells were differentiated into lipid-droplet-containing mature adipocytes (Figure 1a). During further culture (up to 24 days), they became enlarged and stored large lipid droplets, as
21 as compared with day 8 (Supplementary Figure S1b online). These properties of adipocytes in our system are consistent with the in vivo features of adipocyte enlargement; therefore we defined day-8 adipocytes as small (mature) adipocytes and day-21 adipocytes as enlarged adipocytes, in accordance with a previous report (Suganami et al., 2005). We found no difference in the viability of adipocytes at days 21 and 8 in the lactate dehydrogenase release test. Moreover, the expression of apoptosis-related genes, such as BCL2-associated X protein, caspase 3, caspase 8, and caspase 9, was not increased at day 21 (data not shown). To clarify the influence of enlarged or small adipocytes on fibroblasts, 3T3-L1 fibroblasts were cocultured with these adipocytes using the Transwell system. At 2 days after the start of coculture, enlarged adipocytes (day 21) significantly decreased fibroblast proliferation, whereas small adipocytes (day 8) had no effect (Figure 1b). Furthermore, gene expressions of collagen (I)-α1 (coll (I)−α1) and elastin in fibroblasts were significantly decreased by coculture with 21-day-cultured enlarged adipocytes but not by coculture with 8-day-cultured small adipocytes (Figure 1c and d). Meanwhile, the gene expression of matrix metalloproteinase 13 (MMP13), or collagenase 3, was significantly increased by coculture with enlarged adipocytes (Figure 1c), whereas small adipocytes again had no effect. Gene expressions of laminin-β1 and fibulin 5 were not affected by either small or enlarged adipocytes (data not shown). As fibroblasts and adipocytes were not in direct contact in this coculture system, some factor(s) secreted from enlarged adipocytes might be involved in modulating the function of dermal fibroblasts.

**Palmitic acid decreases cell proliferation and gene expression of col (I)-α1 and elastin, and increases gene expression of MMP13 in mouse and human fibroblasts.**

Given that enlarged adipocytes are known to secrete FFAs such as palmitic acid, oleic acid, and stearic acid (Suganami et al., 2005; Liu et al., 2010), and to induce inflammation by activating macrophages during the progression of metabolic syndrome, we examined the effect of FFAs on mouse fibroblasts at the same concentration as reported previously (Suganami et al., 2005; Liu et al., 2010). Palmitic and oleic acids significantly decreased 3T3-L1 fibroblast proliferation, whereas stearic acid had no effect (Figure 2). At this concentration, no cytotoxicity was detected by lactate dehydrogenase release assay (data not shown). Palmitic acid significantly decreased gene expression of both col (I)-α1 and elastin, and significantly increased MMP13 gene expression (Figure 3), although gene expressions of laminin-β1 and fibulin 5 were unaffected (data not shown). Both oleic and stearic acids had no effect on gene expression (data not shown). Similar results were obtained with human dermal fibroblasts (Figure 4), although the expression of MMP1 was examined in human cells instead of MMP13.

**The TLR inhibitor EPA reduces the effects of palmitic acid and enlarged adipocytes on fibroblast function.**

As palmitic acid is known to act via Toll-like receptors (TLRs) in adipocytes (Schaeffler et al., 2009), we examined the effect of a TLR inhibitor, eicosapentaenoic acid (EPA; Lee et al.,
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2003). As shown in Figure 5, EPA significantly inhibited the palmitic-acid-induced decreases of cell proliferation and gene expression of col (I)-α1 and elastin, as well as the increase of MMP13 gene expression in 3T3-L1 fibroblasts. These data suggest that the effects of palmitic acid on fibroblasts are mediated via TLRs. This was confirmed by the induction of NF-κB nuclear translocation, a downstream signal of TLRs, by palmitic acid (Supplementary Figure S2 online). This was also seen in human dermal fibroblasts (data not shown). Therefore, we next aimed to confirm the involvement of palmitic acid in the effect of enlarged adipocytes on fibroblasts in the coculture system. EPA partially but significantly inhibited the decrease of cell proliferation and gene expression of col (I)-α1 and elastin (Figure 6) in the 3T3-L1 fibroblasts. The increase of MMP13 gene expression was partly, but not significantly, inhibited by EPA. These results suggest that the palmitic acid released by enlarged adipocytes is at least partly involved in the negative regulation of fibroblasts by enlarged adipocytes.

DISCUSSION

In this study we used a coculture system to investigate the influence of enlarged adipocytes on fibroblasts. We found that enlarged adipocytes decreased cell proliferation and col (I)-α1 and elastin gene expression, and increased MMP13 gene expression. Palmitic acid, which is known to be produced by enlarged adipocytes, showed similar effects. Furthermore, EPA, which is a ligand of TLRs and is known to compete with palmitic acid (Lee et al., 2003), inhibited these effects of both palmitic acid and enlarged adipocytes in coculture. Therefore, our results indicate that enlarged adipocytes negatively control the function of dermal fibroblasts at least partly through the activation of TLRs by secreted palmitic acid.

Obesity is a significant risk factor for various metabolic diseases, and also aggravates skin ulcers (Knudsen and Gallagher, 2003) and sagging of the face (Ezure et al., 2009). We have reported that increase of the subcutaneous adipose layer is negatively correlated to dermal elasticity (Ezure and Amano, 2010b). Furthermore, we have previously reported that adiponectin, which is secreted mainly from the small adipocytes found in healthy subjects (not subjects with obesity), increases hyaluronic acid gene expression in dermal fibroblasts (Ezure and Amano, 2007), and this is expected to improve dermal condition. Therefore, it has been presumed that the status of the subcutaneous adipose layer has an

Figure 2. Effect of FFAs on fibroblast proliferation. 3T3-L1 fibroblasts were cultured alone, and FFAs (known to be produced by enlarged adipocytes) were added at the indicated concentration. After 2 days, cell abundance was measured by means of Alamar blue assay. (a) Palmitic and (b) oleic acids significantly decreased fibroblast abundance, (c) but the effect of stearic acid was not significant. Results in the form of percentage (%) of control (Cont.; without fatty acid) are expressed as means ± SEM of three wells in each group. Statistical significance of differences was determined by analysis of variance, followed by Fisher’s protected least significant difference as a multiple-comparison test. **P<0.01 and ***P<0.001 compared with non-cocultured cells. FFA, free fatty acid; NS, not significant.

Figure 3. Palmitic acid decreased col (I)-α1 and elastin gene expression, and increased MMP13 gene expression. 3T3-L1 fibroblasts were cultured alone, and palmitic acid was added at the indicated concentration. After 2 days, relative gene expression levels were measured by real-time PCR. Results in the form of relative value compared with the control (Cont.; without palmitic acid) are expressed as means ± SEM of three wells in each group. (a) Col (I)-α1 and (b) elastin mRNA abundance was significantly decreased by palmitic acid. Meanwhile, (c) MMP13 mRNA abundance was significantly increased by palmitic acid. Statistical significance of differences was determined by analysis of variance followed by Fisher’s protected least significant difference as a multiple-comparison test. *P<0.05 and ***P<0.001 as compared with non-cocultured cells. col (I)-α1, collagen (I)-α1; MMP13, matrix metalloproteinase 13; NS, not significant.
Local influence of secreted FFAs has also been reported in the mainly bound to serum proteins in the blood, and therefore adipocytes in other tissues, such as omentum, are likely to control fibroblast function in the dermis. FFAs secreted from enlarged adipocytes in obese subjects may negatively show cytotoxicity. Therefore, palmitic acid released acid is an effective modulator of fibroblast function, without (Suganami et al., 2005). In this study, we found that palmitic acid inhibited fibroblast proliferation and gene expression of collagen (I)-α1 and elastin, and increased MMP13 gene expression; these changes were markedly inhibited by EPA, which is an inhibitor of the TLRs pathway (Lee et al., 2003). We also observed the induction by palmitic acid of NF-κB nuclear translocation, which is a downstream signal of TLRs. Palmitic acid activates the TLRs/NF-κB pathway (Lee et al., 2001); NF-κB activation of nuclear translocation is known to inhibit gene expression of collagen (I)-α1 (Rippe et al., 1999) and elastin (Kuang et al., 2002), and to increase gene expression of MMP13 (Mengshol et al., 2000). FFA-TRL signaling is also reported in macrophages in obesity (Suganami et al., 2009); hence, these results suggest that the negative control of fibroblast function by palmitic acid may be mediated by activation of the TLR signaling pathway.

Although EPA completely inhibited the effect of palmitic acid on fibroblasts, the inhibition of fibroblast function by enlarged adipocytes was only partially inhibited by EPA. Adipocytes are known to secrete various factors in addition to palmitic acid, and the secretion of factors such as IL-6 and tumor necrosis factor-α is also known to be altered in enlarged adipocytes (Matsuzawa, 2006). Given that adiponectin increases collagen production (Ezure and Amano, 2007), the reduced secretion of adiponectin in enlarged adipocytes might be involved in the decrease of collagen gene expression in cocultures of enlarged adipocytes (Figure 1c), in addition to the effects of increased secretion of palmitic acid (Figure 3a). Thus, factor(s) other than palmitic acid may also be involved in the negative regulation of dermal fibroblasts by enlarged adipocytes, although they remain to be identified.

There is no basement membrane between the subcutaneous adipose layer and the dermis; rather, only a membrane surrounding adipocytes is present. As it is well established that

**Figure 4.** Palmitic acid influences human dermal fibroblasts in the same way as mouse 3T3-L1 fibroblasts. Instead of mouse 3T3-L1 fibroblasts, human neonatal dermal fibroblasts were used to confirm the effect of palmitic acid. Human dermal fibroblasts were cultured alone, and palmitic acid was added at the indicated concentration. After 2 days, cell abundance was measured by Alamar blue assay, and relative gene expression was measured by real-time-PCR. Results in the form of percentage (%) of control or relative value compared with the control (Cont.; without palmitic acid) are expressed as means ± SEM of three wells in each group. (a) Palmitic acid (Pal.) significantly decreased proliferation of dermal fibroblasts. Statistical significance of differences was determined by analysis of variance, followed by Fisher’s protected least significant difference as a multiple-comparison test. (b) Collagen (I)-α1 and (c) elastin mRNA abundance was significantly decreased by palmitic acid. Meanwhile, (d) MMP1 mRNA abundance was significantly increased by palmitic acid. Statistical significance of differences was determined by using Student’s t-test. *P<0.05 and ***P<0.001 as compared with non-cocultured cells. Collagen (I)-α1, collagen (I)-α1; MMP1, matrix metalloproteinase 1.
adipocyte-derived FFAs interact with macrophages, this membrane is likely to be permeable to FFAs. Therefore, it is reasonable that the subcutaneous adipose layer might influence the dermis via secreted factors such as palmitic acid.

In conclusion, enlargement of the adipose layer in skin may affect dermal function by negatively modulating fibroblast function through factors secreted by enlarged adipocytes. Palmitic acid appears to be one of regulatory molecules secreted by enlarged adipocytes to control dermal fibroblast functions such as cell proliferation and the production and degradation of extracellular matrix proteins.

MATERIALS AND METHODS

Materials

3T3-L1 fibroblasts were purchased from Lonza Walkersville (Walkersville, MD). Oleic acid, stearic acid, IBMX, and dexamethasone were purchased from Sigma (St Louis, MO). Superscript II, calf serum, and DMEM were from Gibco/BRL (Carlsbad, CA). Fetal bovine serum (FBS) was from Biowest (Nuaille, France). Oil Red-O was from Wako Pure Chemical Industries (Osaka, Japan). Cell culture flasks and dishes were from Falcon (Becton Dickinson, Franklin Lakes, NJ). DNase and RNeasy Protect Kit were purchased from Qiagen (Valencia, CA). Palmitic acid and EPA were purchased from Nakarai Tesque (Kyoto, Japan). Free fatty acid quantification kit was purchased from BioVision (Mountain View, CA). Rabbit anti-NF-kappaB monoclonal antibody and anti-rabbit IgG antibody were purchased from Cell Signaling Technology (Boston, MA).

Cell culture

3T3-L1 fibroblasts were cultured in flasks in DMEM containing 10% (w/v) calf serum, in a humidified atmosphere of 5% CO2 in air at 37 °C. When the cells became subconfluent, they were harvested with 0.025% trypsin and 0.01% EDTA, and plated at a density of 7,500 cells per cm2 in six-well dishes. Culture was continued for an additional 2 days after the cells had reached confluence, then adipogenesis was induced, as reported previously (Green and Kehinde, 1975; Student et al., 1980). Briefly, cells were cultured in DMEM with 10% FBS containing 150 μM 3-isobutyl-1-methylxanthine, 1 μg ml⁻¹ insulin and 0.3 μM dexamethasone at 37 °C for 2 days, then cultured in DMEM containing 10% FBS and 1 μg ml⁻¹ insulin.
insulin for an additional 2 days, and further cultured in DMEM containing 10% FBS. Lipids were stained with oil red-O (Ramirez-Zacarias et al., 1992). Cytotoxicity was evaluated in terms of lactate dehydrogenase release from the cells, measured with a colorimetric cytotoxicity kit (Roche, Mannheim, Germany) according to the manufacturer’s instructions (Decker and Lohmann-Matthes, 1988).

**Coculture of fibroblasts with adipocytes.** Mouse 3T3-L1 fibroblasts were plated in Transwells (Falcon) at a density of 7,500 cells per cm² with 10% cell serum containing DMEM. After 6 hours, the medium was changed to 0.5% FBS containing DMEM. At the same time, adipocytes were similarly changed to 0.5% FBS containing DMEM. Twelve hours later, fibroblasts were layered onto the adipocytes, and incubation in 0.5% FBS containing DMEM was continued. Two days later, the upper well containing fibroblasts was transferred to a new plate without adipocytes, and cell abundance was measured by means of Alamar blue assay (AccuMed International, Chicago, IL). Immunofluorescence staining of NF-kB was performed with NF-kB antibody according to the manufacturer’s instructions.

**Quantitative real-time PCR**

Extraction of RNA was performed using an RNaseasy Kit, followed by DNase treatment according to the manufacturer’s instructions. Then RNA was translated to complementary DNA using SuperScript II according to the manufacturer’s instructions. The 28S rRNA was quantified as an internal control, and quantification of complementary DNA for each selected gene was conducted by real-time PCR amplification using a LightCycler, Roche, Indianapolis, IN (Ezure and Amano, 2009). The reaction mixture included 1X FastStart DNA Master SYBR Green master Mix (Roche), 2–4 μM MgCl₂, 500 nM primer, and 1 μl of complementary DNA. Cycling conditions were 95°C for 10 minutes, followed by 35 cycles at 95°C for 15 seconds, at 60°C for 5 seconds, and at 72°C for 4–8 seconds. The products were continuously detected by fluorescence measurement. The cycle threshold values calculated by Lightcycler software version 3.5 were normalized to 28S rRNA for each sample. The standard curves of cycle threshold value against complementary DNA concentration gave r > 0.99 for each gene. These analyses were performed in duplicate for samples from three different cell cultures, and each experiment was performed 2–4 times. Primers and probes used in this study are shown in Supplementary Table S1 online. Amplification of the target genes was confirmed by sequence analysis of the PCR products.

**Statistical analysis**

All data were expressed as means ± SEM. Differences between groups were examined for statistical significance using Student’s t-test or analysis of variance, followed by Fisher’s protected least significant difference as a multiple comparison test, performed by Microsoft Excel. A P-value <0.05 was considered to indicate a significant difference.

**CONFLICT OF INTEREST**

The authors state no conflict of interest.

**SUPPLEMENTARY MATERIAL**

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

**REFERENCES**


