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Chemokine network during adipogenesis in 3T3-L1 cells

Differential response between growth and proinflammatory factor in preadipocytes vs. adipocytes

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Obesity is recognized as a low-grade chronic inflammatory state which involves a chemokine network contributing to a variety of diseases. As a first step toward understanding the roles of the obesity-driven chemokine network, we used a 3T3-L1 cell differentiation model to identify the chemokine profiles elicited during adipogenesis and how this profile is modified by epidermal growth factor (EGF) and tumor necrosis factor-α (TNF) as a growth and proinflammatory factor, respectively. The chemokine network was monitored using PCR arrays and qRT-PCR while main signaling pathways of EGF and TNF were measured using immunoblotting. The dominant chemokines in preadipocytes were CCL5, CCL8, CXCL1, and CXCL16, and in adipocytes CCL6 and CXCL13. The following chemokines were found in both preadipocytes and adipocytes: CCL2, CCL7, CCL25, CCL27, CXCL5, CXCL12, and CX3CL1. Among chemokine receptors, CXCR7 was specific for preadipocytes and CXCR2 for adipocytes. These findings indicate the development of a CXCL12–CXCR7 axis in preadipocytes and a CXCL5–CXCR2 axis in adipocytes. In addition to induction of CCL2 and CCL7 in both preadipocytes and adipocytes, EGF enhanced specifically CXCL1 and CXCL5 in adipocytes, indicating the potentiation of CXCR2-mediated pathway in adipocytes. TNF induced CCL2, CCL7, and CXCL1 in preadipocytes but had no response in adipocytes. EGFR downstream activation was dominant in adipocytes whereas NFκB activation was dominant in preadipocytes. Taken together, the adipocyte-driven chemokine network in the 3T3-L1 cell differentiation model involves CXCR2-mediated signaling which appears more potentiated to growth factors like EGF than proinflammatory factors like TNF.

Introduction

Obesity represents an increased risk factor in various diseases such as heart disease, type 2 diabetes, and certain types of cancer.[1,2] This close relationship between obesity and certain diseases is based on the fact that obesity preserves a low-grade chronic inflammatory state.[3,4] Inflammatory links between obesity and metabolic diseases are well-known mechanisms for the recruitment of immune cells into adipose tissue.[5,6] Orchestrating the recruitment of immune cells includes members of the chemokine network as a driving force.[7] Chemokines are a family of chemoattractant cytokines and consist of four subfamily groups including C (XCL1–2), CC (CCL1–28), CXC (CXCL1–17), and CX3C (CX3CL1). The main function of chemokines is to regulate leukocyte trafficking by their interaction with specific seven-transmembrane-spanning G protein-coupled receptors that are involved in development, inflammation and cancer.[8,9]

There is increasing evidence that chemokines play a pivotal role in obesity-associated diseases. Adipose tissue of obese patients elevates monocyte chemotaxis (involving CCL2, 3, 5, 7, 8, and 11 and receptors CCR1, 2, 3, and 5) and increases macrophage infiltration.[10] The CCL2/CCR2 pathway is also likely involved in obesity-related metabolic disease.[11,12] CXCL14 was found to be elevated in white adipocyte tissue of obese mice and to attenuate insulin-stimulated glucose uptake in cultured myocytes.[13] Adipose tissue-derived CXCL5 promoted insulin resistance in muscle.[14] In another study, the lack of CXCR2 in hematopoietic cells was sufficient to protect the development of insulin resistance.[15]

Interestingly, conditioned media from adipocytes stimulated production of tumor necrosis factor-α (TNF) in spleen cells, indicating the functional role of adipocytes as immune regulatory cells.[16] Obesity promoted liver inflammation and tumorigenesis; both processes involved enhancement of TNF expression.[17] TNF is particularly well known as a positive regulator for proinflammatory chemokines through NFκB signaling.[18,19] In addition, epidermal growth factor (EGF) is closely related to obesity. EGF shows biphasic effects on adipocytes: it inhibited differentiation of preadipocytes but promoted adipogenesis in differentiated cells.[20] EGF was reportedly increased in childhood

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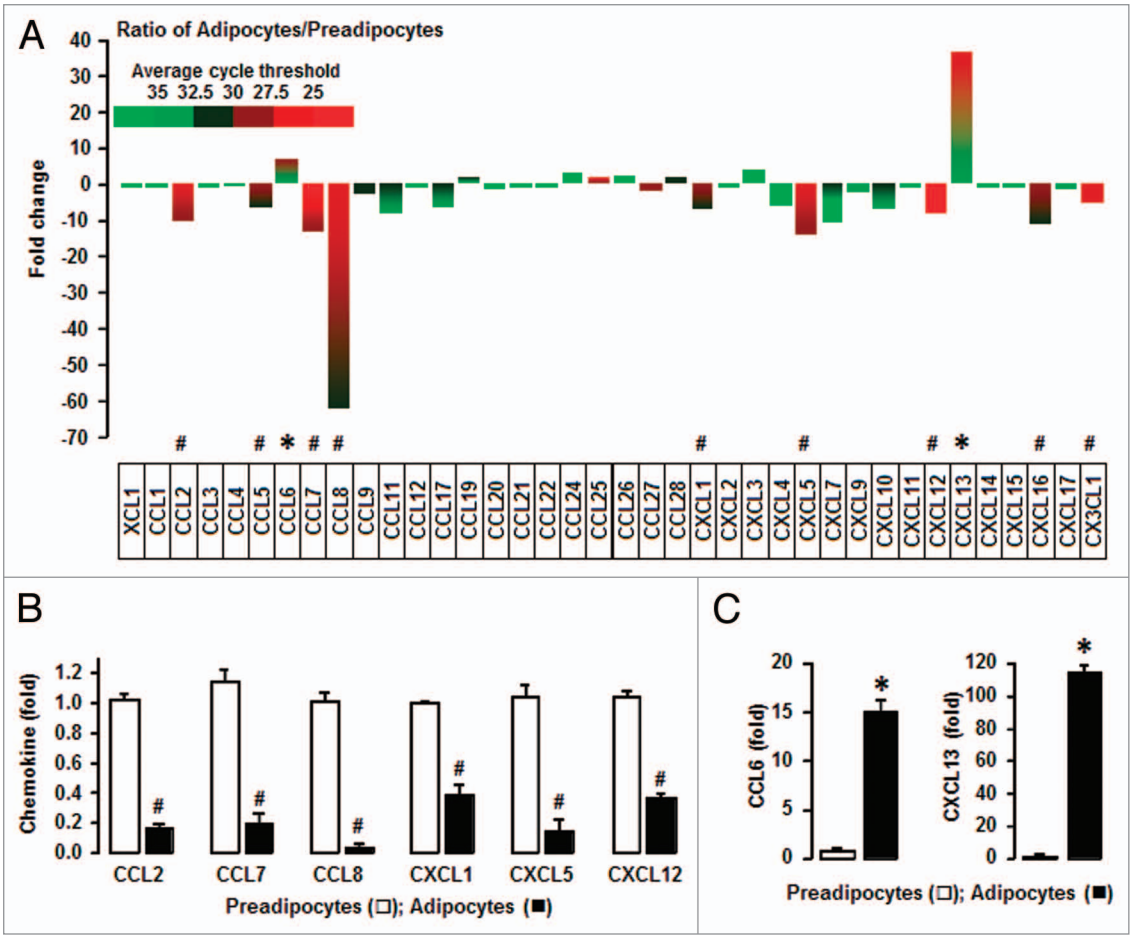

Figure 1. Adipocytes have a predominant increase in CXCL13 and a decrease in CCL8 compared with preadipocytes. (**A**) Comparison of chemokine ligands in preadipocytes and adipocytes. After isolating total RNA from nondifferentiated (preadipocytes) and differentiated cells (adipocytes), a PCR array was performed using a customized PCR array plate containing complementary sequences for mouse chemokine genes. Different colors indicate the average cycle threshold with expressions that ranged from >35 to <25. Chemokines with a >2-fold increase (*) or decrease (#) were recognized as the major differences between preadipocytes and adipocytes by excluding lowly expressed chemokines with a >30 cycle threshold. (**B**) Confirmation of preadipocyte-dominant chemokines by qRT-PCR. (**C**) Confirmation of adipocyte-dominant chemokines by qRT-PCR. After isolating total RNA, qRT-PCR was performed using primers for CCL2, CCL6–8, CXCL1, CXCL5, CXCL12, and CXCL13. Fold changes were calculated as a relative value after setting the first sample of preadipocytes as a control group (1.0). * and # indicate a significant increase or decrease ($P \leq 0.05$), respectively (Student *t* test). Experiments were performed in triplicate and all data are shown as mean ± SEM.

obesity.[21] It was involved in inducing obesity in ovariectomized mice,[22] while genetically engineered obese mice were found to have a reduced production of EGF.[23] In spite of the controversial results related to EGF expression in animal models and humans, EGF is also a well-known positive regulator for proinflammatory chemokines through Akt and Erk activation.[24] These facts suggest that TNF, acting as a proinflammatory factor, or EGF as a growth factor, alters an obesity-associated chemokine profile, probably resulting in the modulation of the adipocyte's microenvironment.

Because of limited reports linking obesity and chemokine network, we attempted to identify, as a first step, the signatures of specific chemokines in preadipocytes and adipocytes, using the 3T3-L1 cell differentiation model. We also compared the differential response of the chemokine network to a growth factor (EGF) and a proinflammatory factor (TNF) in these cells.

Results

Adipocytes enhance dominantly CXCL13 when compared with preadipocytes

We used the 3T3-L1 cell differentialtion model to prepare nondifferentiated (preadipocytes) and differentiated cells (adipocytes), and performed customized PCR arrays containing genes that encode mouse chemokines and chemokine receptors. The present study used the nomenclature of chemokines approved by the IUIS/WHO Subcommittee on Chemokine Nomenclature (2002). The mRNA levels of a panel of 43 chemokines and 19 chemokine receptors were evaluated. Based on a web-based PCR Array Data Analysis protocol provided by SABiosciences (Qiagen), the absent, low, and high expression levels of chemokines were defined as >35, 30–35, and <30 average threshold cycles, respectively.

Table 1. Primers used in qRT-PCR analysis

Chemokines	Accession number	Primers (sense/antisense)	CDC no.
CCL2	BC145869	5-GCTCAGCCAG ATGCAGTTA-3 5-CTGCTGGTGA TCCTCTTGTA G-3	67–171
CCL6	BC002073	5-GGCTGGCCTC ATACAAGAAA-3 5-GATCTGTGTG GCATAGGAGA AG-3	60–175
CCL7	BC061126	5-AAGAAGGGCA TGGAAGTCTG-3 5-TCAAGGCTTT GGAGTTGGG-3	199–295
CCL8	BC117101	5-ACCTGCTGCT TTCATGTACT AA-3 5-ACAGAGAGAC ATACCCTGCT-3	91–220
CXCL1	BC132502	5-GCTGGGATTC ACCTCAAGAA-3 5-TGGCTATGAC TTCGGTTTGG-3	118–200
CXCL5	BC024392	5-GCTGCGTTGT GTTTGCTTAA C-3 5-TAGCTATGAC TTCCACCGTA GG-3	150–260
CXCL12	BC006640	5-GGTTCTTCGA GAGCCACATC-3 5-TCTTCAGCCG TGCAACAA-3	98–194
CXCL13	BC012965	5-ATTCAAGTTA CGCCCCCTG-3 5-TTGGCACGAG GATTCACAC-3	148–242
CXCR2	BC051677	5-TGTCGTCCTT GTCTTCCTG-3 5-GGCCTTGTCA ATGTCATCG-3	762–882
CXCR7	BC015254	5-GGCACCTCCA GCTATAAGAA G-3 5-GTATCAGGCA GGGACACAAA-3	451–540
β-actin	BC138611	5-CTCCCTGGAG AAGAGCTATG A-3 5-CCAAGAAGGA AGGCTGGAAA-3	702–803

CDS, coding DNA sequence.

CCL2, CCL7, CCL25, CCL27, CXCL5, CXCL12, and CX3CL1 were highly expressed in both preadipocytes and adipocytes (**Fig. 1A**). CCL5, CCL8, CXCL1, and CXCL16 were dominant chemokines in preadipocytes whereas CCL6 and CXCL13 were dominant in adipocytes. Adipogenesis from preadipocytes to adipocytes resulted in downregulation of CCL2, 5, 7, and 8, CXCL1, 5, 12, and 16 and CX3CL1. We selected CCL2, CCL7, CCL8, CXCL1, CXCL5, and CXCL12 as highly downregulated chemokines and confirmed their downregulation during adipogenesis using qRT-PCR with specific primers (**Fig. 1B**; **Table 1**). In addition, adipocyte-driven chemokines CCL6 and CXCL13 were confirmed using qRT-PCR (**Fig. 1C**). Notably, CXCL13 was primarily expressed in adipocytes as compared with preadipocytes (**Fig. 1A and C**).

Adipocytes specifically increase CXCR2 when compared with preadipocytes

We then compared chemokine receptors in preadipocytes and adipocytes. Almost all of chemokine receptors were absent or were seen in trace amounts in both preadipocytes and adipocytes (**Fig. 2A**). However, CXCR7 was predominantly expressed in preadipocytes and CXCR2 was highly induced in adipocytes. Based on our qRT-PCR results, adipocytes had a significantly increased CXCR2 level when compared with preadipocytes (**Fig. 2B**). On the other hand, preadipocytes expressed higher CXCR7 levels than adipocytes (**Fig. 2C**). These facts indicate the potentiation of CXCR7-mediated signaling in preadipocytes and CXCR2-mediated signaling in adipocytes.

Adipocytes are more responsive to EGF than TNF when compared with preadipocytes

We selected EGF as a growth factor and determined the effects of EGF on the chemokine network in preadipocytes and adipocytes. Preadipocytes induced CCL2 and CCL7 in response to EGF (**Fig. 3A**). In addition to CCL2 and CCL7, EGF significantly enhanced CXCL1 and CXCL5 in adipocytes (**Fig. 3B**). Next we selected TNF as an inflammatory factor and identified TNF-responsive chemokines in preadipocytes and adipocytes. Preadipocytes induced CCL2, CCL7, and CXCL1 in response to TNF (**Fig. 4A**). Interestingly, it had no or less effect on the chemokine network in adipocytes (**Fig. 4B**). In addition, we defined the effects of EGF and TNF on chemokine receptors in preadipocytes and adipocytes. Unlike the chemokine ligands, chemokine receptors were less responsive to EGF and TNF in both preadipocytes and adipocytes (**Fig. S1**). We next confirmed the effects of EGF and TNF on CCL2, CCL7, CXCL1, and CXCL5 in preadipocytes and adipocytes using qRT-PCR. Although EGF significantly induced CCL2 and CCL7 in preadipocytes, the effect of TNF on CCL2 and CCL7 was significantly greater. TNF also increased CXCL1 and CXCL5 in preadipocytes (**Fig. 5A**). In adipocytes, EGF significantly enhanced CCL2, CCL7, CXCL2, and CXCL5 levels while TNF had no effect on these chemokines (**Fig. 5B**). These facts indicate a dominant effect of TNF on chemokines in preadipocytes and EGF in adipocytes. The difference in dominant chemokines may be attributed from differential response of EGF or TNF in preadipocytes vs. adipocytes.

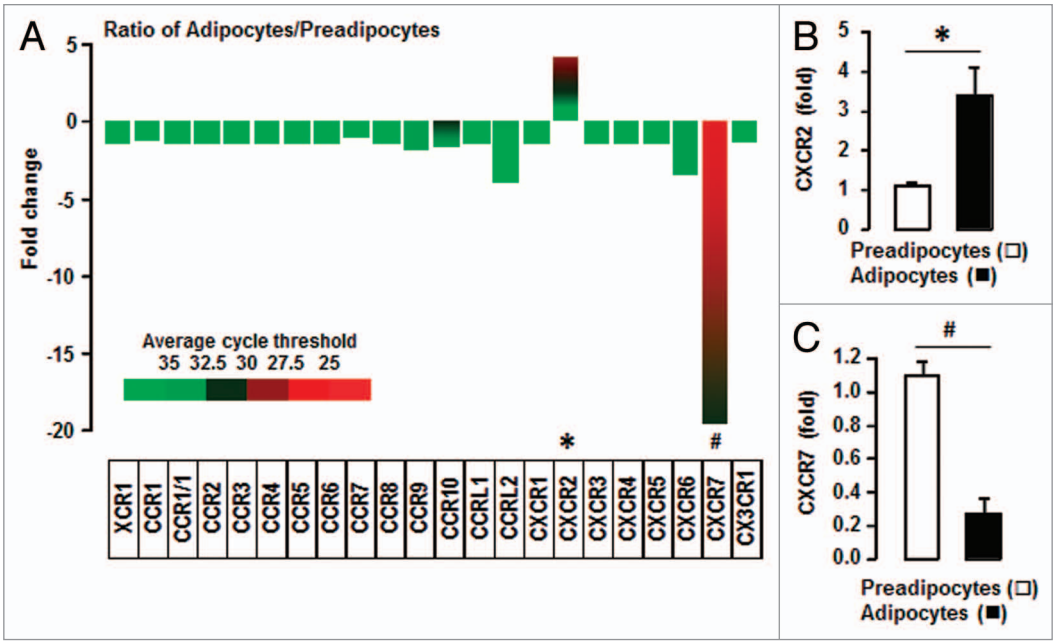

Figure 2. Adipocytes have a significantly increased expression of CXCR2 and a decreased expression of CXCR7 relative to preadipocytes. (**A**) Comparison of chemokine receptors in preadipocytes and adipocytes. After isolating total RNA from preadipocytes and adipocytes, a PCR array was performed using a customized PCR array plate containing complementary sequences for mouse chemokine receptor genes. (**B**) Confirmation of increased CXCR2 mRNA in adipocytes by qRT-PCR. (**C**) Confirmation of decreased CXCR7 mRNA in adipocytes of the by qRT-PCR. After isolating total RNA, qRT-PCR was performed using primers for CXCR2 and CXCR7. Fold changes were calculated as a relative value after setting the first sample of preadipocytes as a control group (1.0). * and # indicate significant increase or decrease ($P \leq 0.05$), respectively (Student *t* test). Experiments were performed in triplicate and all data are shown as mean ± SEM.

We then compared EGF- or TNF-mediated signaling pathways in preadipocytes and adipocytes, including Akt, Erk, and IκB. Akt activation was greater in adipocytes whereas Erk was more responsive in preadipocytes (**Fig. 5C and D**). EGF clearly activated Akt and Erk in adipocytes whereas it activated Erk in preadipocytes, but had only a slight effect on Akt and IκB (**Fig. 5C**). On the other hand, TNF clearly activated IκB (and gradually Akt and Erk) in preadipocytes whereas it had no or less effect in adipocytes (**Fig. 5D**). These differential signaling pathways support the dominant effect of TNF on chemokines in preadipocytes and the dominant effect of EGF in adipocytes.

Discussion

The primary findings of this study are that the adipocyte-driven chemokine network has a CXCL5–CXCR2 axis and that EGF-induced CXCL1 and CXCL5 may potentiate the CXCR2-mediated signaling, indicating an alteration in the adipocyte microenvironment. Although adipogenesis downregulated CXCL5, adipocytes still express CXCL5 in quite low levels as compared with preadipocytes. Other authors also demonstrated using 3T3-L1 cells that CXCL5 was downregulated during adipogenesis.[14] The CXCL5 promoter contains several NFκB binding sites and TNF was found to induce CXCL5 via NFκB activation in human embryonic 293 cells.[25] Thus a lower NFκB activation to TNF in adipocytes may be associated with downregulation of CXCL5. Interestingly, a decrease in the weight of epididymal white adipose tissue following castration resulted to upregulation of CXCL5 levels.[26] On the other hand, obese subjects have a higher serum CXCL5 level than lean subjects.[14,27] The source of this CXCL5 is most likely macrophages in white adipose tissue.[14,26] This finding suggests that macrophages in adipose tissues contribute to the enhanced CXCL5 levels in the obese group despite downregulation of CXCL5 during adipogenesis. Consistent with our results, human adipocytes highly express CXCR2 compared with preadipocytes.[28]

Other reports indicate the significance of CXCR2-mediated signaling in obesity. $CXCR2^{-/-}$ mice are protected against obesity-induced insulin resistance.[14] Even the lack of CXCR2 in hematopoietic cells is sufficient to protect adipose macrophage recruitment and the development of insulin resistance in diet-induced obese mice.[15] These facts indicate that CXCR2-mediated signaling is involved in obesity-related diseases such as diabetes and some types of cancer. In comparison to preadipocytes, EGF further induced CXCL1 and CXCL5 in adipocytes, thereby probably potentiating the CXCR2-mediated signaling that was diminished due to downregulation of the CXCR2 ligands, CXCL1 and CXCL5, during adipogenesis. Although CXCL1 and CXCL5 are NFκB-activated chemokines,[18,25] EGF increased CXCL1 mRNA in ovarian cancer cells[24] and CXCL5 mRNA in human umbilical vein endothelial cells and the ileum.[29,30] EGFR-transactivated Akt signaling was involved in CXCR2-driven ovarian cancer progression by upregulating proinflammatory chemokines CXCL1/2.[31] Interestingly, Erk activation was not involved in upregulating the proinflammatory chemokines.[31] Therefore, higher Akt activation to EGF

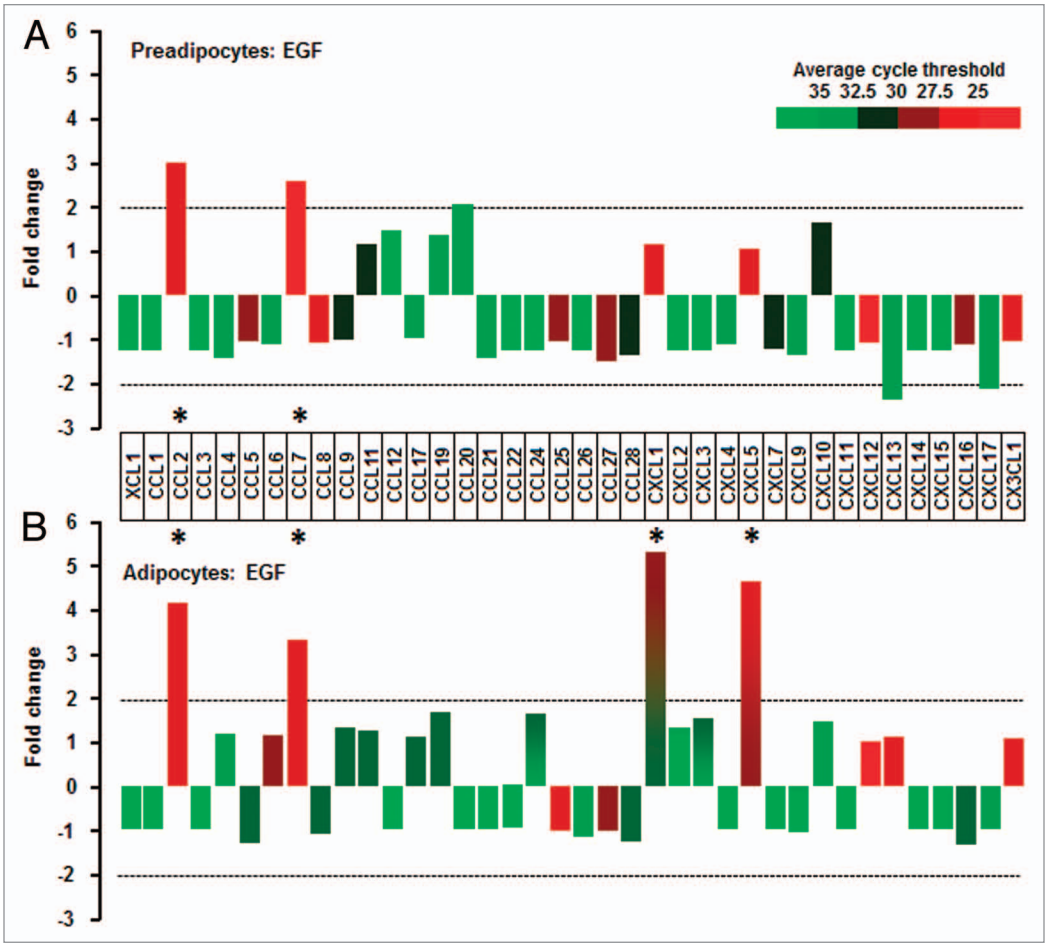

Figure 3. EGF-responsive chemokines in preadipocytes and adipocytes. (**A**) In preadipocytes, EGF significantly increases CCL2 and CCL7. (**B**) In adipocytes, EGF resulted in significant increase in not only CCL2 and CCL7 but CXCL1 and CXCL5. Cells were treated with EGF (10 ng/ml) for 1 h. After isolating total RNA from preadipocytes and adipocytes, a chemokine PCR array was performed. Different colors indicate the average cycle threshold with expression ranges from >35 to <25. Chemokines with a >2-fold increase (*) were recognized as the major effects of EGF by excluding lowly expressed chemokines with >30 cycle threshold.

in adipocytes may contribute to EGF-induced CXCL1 and CXCL5, rather than that seen in preadipocytes.

In addition to CXCL5, CCL2, CCL5, CCL7, CCL8, CXCL1, CXCL12, CXCL16, and CX3CL1 are decreased during adipogenesis. Because chemokines such as CCL2,[32] CCL5,[33] CXCL1,[18] CXCL16,[34] and CX3CL1[35] are regulated by NFκB, the smaller response to TNF in adipocytes may be involved in downregulating these particular chemokines. In particular, CCL2 is one of chemokines studied intensively in obesity. CCL2 was found to be highly expressed in obese subjects.[10,36,37] Consistent with our results, CCL2 levels have been shown to be higher in preadipocytes than adipocytes.[28,38,39] Also, downregulation of CXCL1 during adipogenesis has been supported in another report.[15] Obesity has been found to be associated with decreased CXCL16 levels.[40] Because CCL7[18] and CCL8[41] are induced by TNF, the downregulation of these chemokines during adipogenesis may result again, from the smaller response to TNF in adipocytes.

In comparison, the preadipocyte-driven chemokine network is the CXCL12–CXCR7 axis, based on high expression of CXCL12 and CXCR7 in these cells. Although CXCL12 binds to two specific receptors (CXCR4 and CXCR7), CXCR4 is not expressed in preadipocytes or adipocytes despite intensive attention on CXCL12–CXCR4 axis in the cancer field.[42] The downregulation of CXCL12 during adipogenesis is supported by the decrease in CXCL12 levels in diet-induced obese mice.[27]

Interestingly, NFκB and Erk activation was attenuated while Akt activation was potentiated during adipogenesis. Similarly LPS-induced NFκB and Erk activation was found to decrease as differentiation of human adipocytes increased.[43] Particularly, TNF has been reported to suppress adipocyte-specific genes such as Akt and GLUT4 in 3T3-L1 adipocytes[44] and inhibit adipocyte differentiation,[45] indicating a preadipocyte preference for TNF actions. As NFκB is important for TNF-induced lipolysis in human adipocytes,[46] many studies indicate that TNF has a clear impact in both adipocyte biology[47] and obesity.[48]

CCL6 and CXCL13 were found to be significantly increased in adipocytes. This finding will require further study, if these two chemokines are critical to adipogenesis. Thus far the roles of CCL6 and CXCL13 in obesity have not been clarified. CCL6 is a rodent-specific chemokine and plays critical roles in

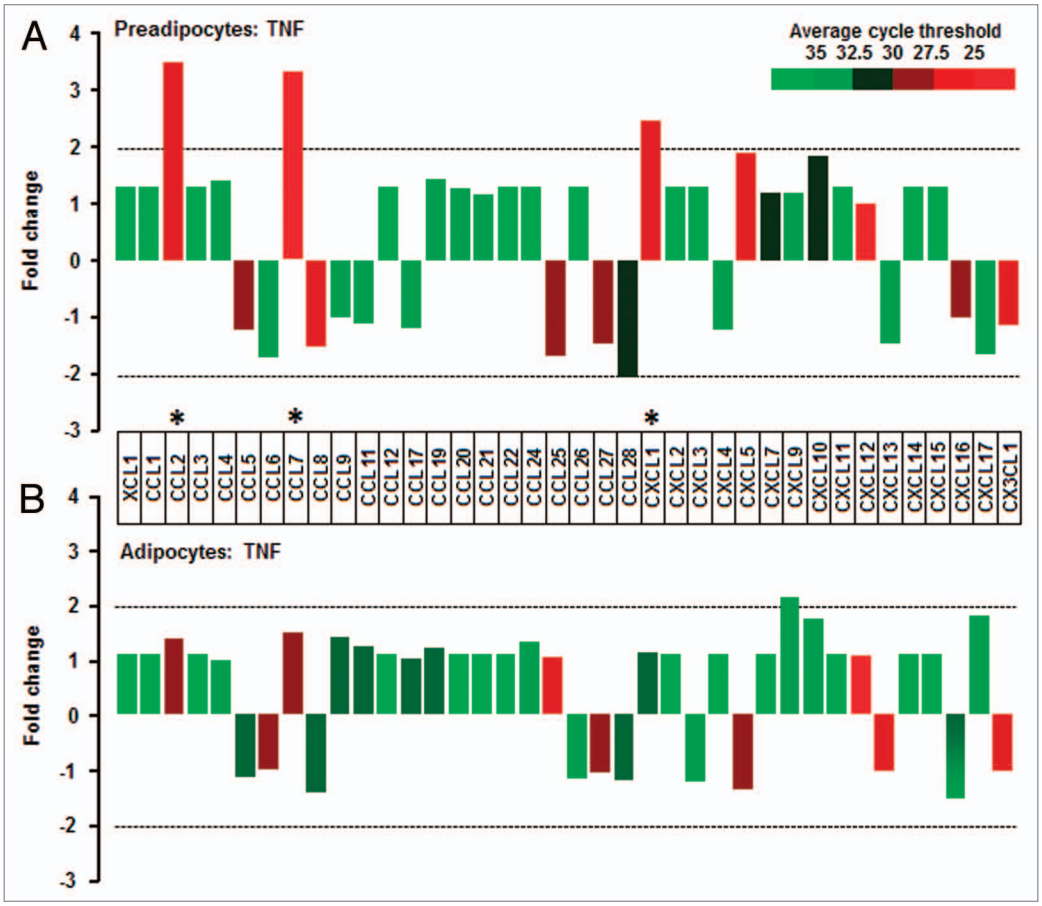

Figure 4. TNF-responsive chemokines in preadipocytes and adipocytes. (**A**) TNF resulted in a significant increase in CCL2, CCL7, and CXCL1 in preadipocytes, while (**B**) TNF had no significant effect on chemokines in adipocytes. Cells were treated with TNF (10 ng/ml) for 1 h. After isolating total RNA from preadipocytes and adipocytes, a chemokine PCR array was performed. Different colors indicate average cycle threshold with expression ranges from >35 to <25. Chemokines with a >2-fold increase (*) were recognized as the primary effects of TNF by excluding low expressed chemokines with >30 cycle threshold.

IL-13-induced lung inflammation through CCR1[49] and macrophage infiltration.[50] B cells from *ob/ob* mice have a greater propensity to migrate to the liver through a CXCL13-mediated signaling pathway.[51] These facts suggest that CCL6 and CXCL13 likely are involved in macrophage and lymphocyte infiltration in obesity, leading to chronic inflammation.

We summarize the characteristics of the chemokine network in preadipocytes and adipocytes, and describe the development of expected chemokine network for cell–cell communication in the preadipocyte and adipocyte microenvironments (**Fig. 6**). CCL2, CCL7, CCL25, CCL27, CXCL5, CXCL12, and CX3CL1 are commonly expressed in both preadipocytes and adipocytes. CXCR7 expression in preadipocytes and CXCR2 in adipocytes can drive to establish CXCL12–CXCR7 axis in preadipocytes and CXCL5–CXCR2 axis in adipocytes. Common chemokines (CCL2, CCL7, CCL25, CCL27, CXCL12, and CX3CL1), preadipocyte-driven chemokines (CCL5, CCL8, CXCL1, and CXCL16) and adipocyte-driven chemokines (CCL6 and CXCL13) can communicate with other cells containing specific receptors for these chemokines. TNF and EGF commonly induce CCL2 and CCL7. Additionally TNF induced CXCL1 in preadipocytes, and EGF enhanced CXCL1 and CXCL5 in adipocytes. Further induction of CXCR2 ligands such as CXCL1 and CXCL2 by TNF and EGF may lead to potentiation of CXCR2-mediated signaling in adipogenesis, adipocyte biology, and obesity.

This study represents the first step to clarify the role of the identified chemokines on adipogenesis for future direction. In conclusion, the CXCL1/5–CXCR2 axis is a central adipocyte-driven chemokine network and growth factors like EGF potentiate CXCR2-mediated signaling rather than proinflammatory factors like TNF, in the adipocyte microenvironment.

Materials and Methods

Reagents

Recombinant human EGF (236-EG-200) and TNF (210-TA-020) were obtained from R&D Systems. Antibodies for IκB (8219), Akt (8200), Erk (8201) and their phosphorylated forms were purchased from Cell Signaling Technology. The PCR array for customized mouse chemokines (CAMP10242) and SYBR® Green Master Mix (330503) came from SABiosciences/

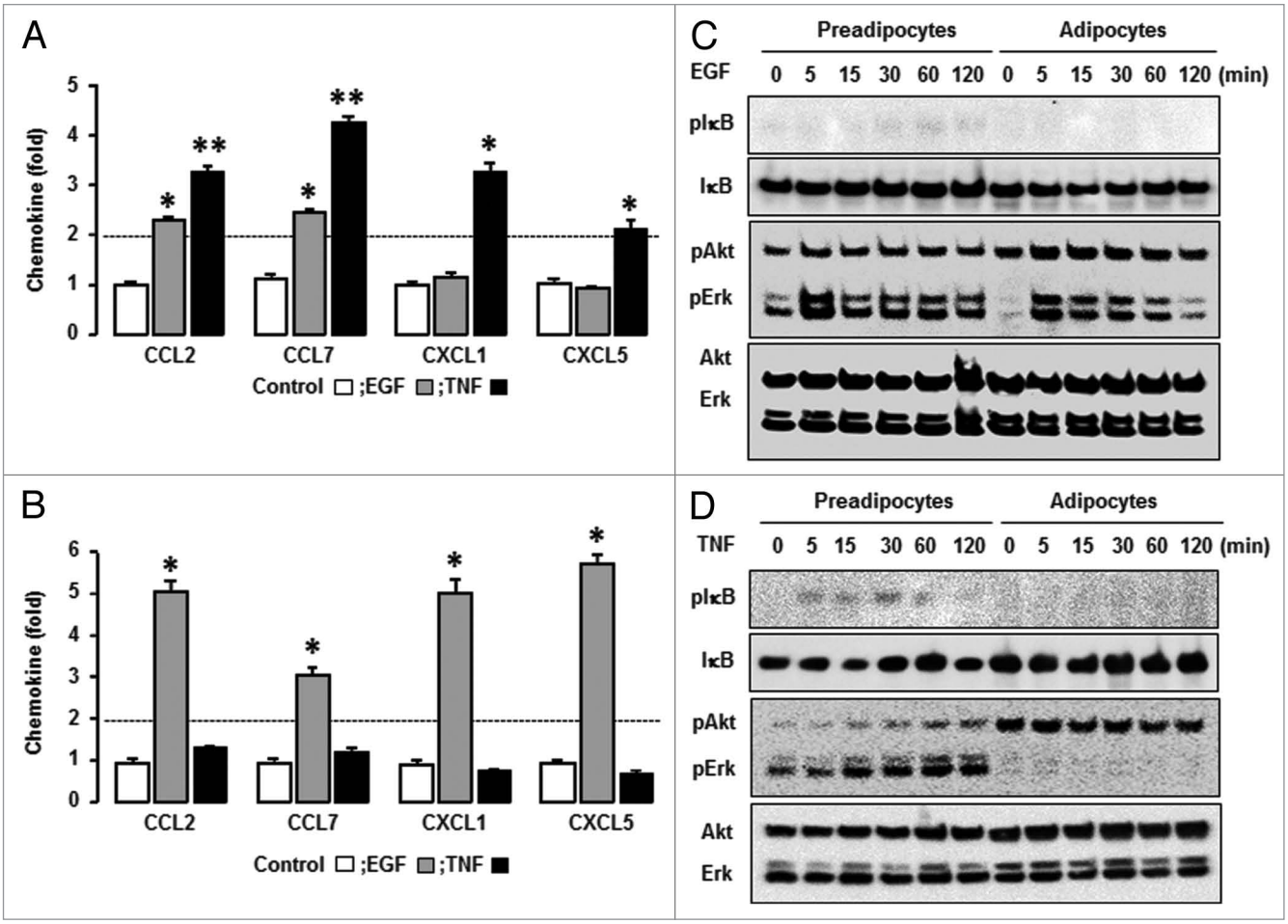

Figure 5. Confirmation of EGF- and TNF-responsive chemokines and comparison of signaling pathways in response to EGF and TNF. (**A**) In preadipocytes and (**B**) adipocytes, confirmation of EGF- and TNF-responsive chemokines. After isolating total RNA, qRT-PCR was performed using primers for CCL2, CCL7, CXCL1, and CXCL5. Fold changes were calculated as a relative value after setting the first vehicle-treated sample of preadipocytes and adipocytes as a control group (1.0), respectively. *, **, and # indicate significant increases and decrease ($P \leq 0.05$), respectively (Student *t* test). Experiments were performed in triplicate and all data are shown as mean ± SEM. (**C**) EGF- and (**D**) TNF-responsive signaling pathways in preadipocytes and adipocytes. Cells were treated with EGF (10 ng/ml) or TNF (10 ng/ml) for 0, 5, 15, 30, 60, and 120 min. The whole cell lysates were prepared and western blots were performed using antibodies specific to IκB, Akt, Erk, and their phosphorylated forms. Experiments were performed in duplicate and a representative result is shown.

Qiagen. Specific PCR primers for chemokines or chemokine receptors were obtained from Eurofins MWG Operon. Chemiluminescent detection kits (sc-2048) were from Santa Cruz Biotechnologies. 3-Isobutyl-1-methylxanthine (IBMX, I-7018), insulin (I-5500), and dexamethasone (D-4902) were purchased from Sigma-Aldrich. All liquid culture media such as FBS (26140) and Dulbecco's modified Eagle's medium (DMEM, 11965) were acquired from Invitrogen.

Cell culture and differentiation

The mouse fibroblast cell line 3T3-L1 (CL-173) was purchased from the American Type Culture Collection. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% calf serum (16010159, Invitrogen) at 37 °C in a water-saturated atmosphere of 95% air and 5% CO_2, avoiding situations in which the cells became too confluent (>70%) before the initiation of differentiation. Differentiation was initiated, however, in confluent 3T3-L1 preadipocytes by stimulation with induction media (10% FBS/DMEM with 115 μg/ml IBMX, 1 μg/ml insulin and 1 μmol/l dexamethasone). After 2 d of incubation, cells were maintained in insulin media (10% FBS/DMEM with 1 μg/ml insulin); media was changed every other day thereafter. Differentiated cells (adipocytes) were maintained in 10% FBS/DMEM. Before treatment, the medium was removed and fresh medium without FBS was added to remove the effects of ingredients contained in serum. After at least 4 h of incubation in serum-free media, vehicle (phosphate-buffered saline, PBS), 10 ng/ml EGF or 10 ng/ml TNF was added, and incubations continued for the indicated time periods. Adipogenesis experiments were carried on duplicate or triplicate as appropriate.

PCR array and qRT-PCR

After isolating total RNA and eliminating genomic DNA, reverse transcription reactions were performed at 42 °C for 15 min followed by 94 °C for 5 min. According to the manufacturer's

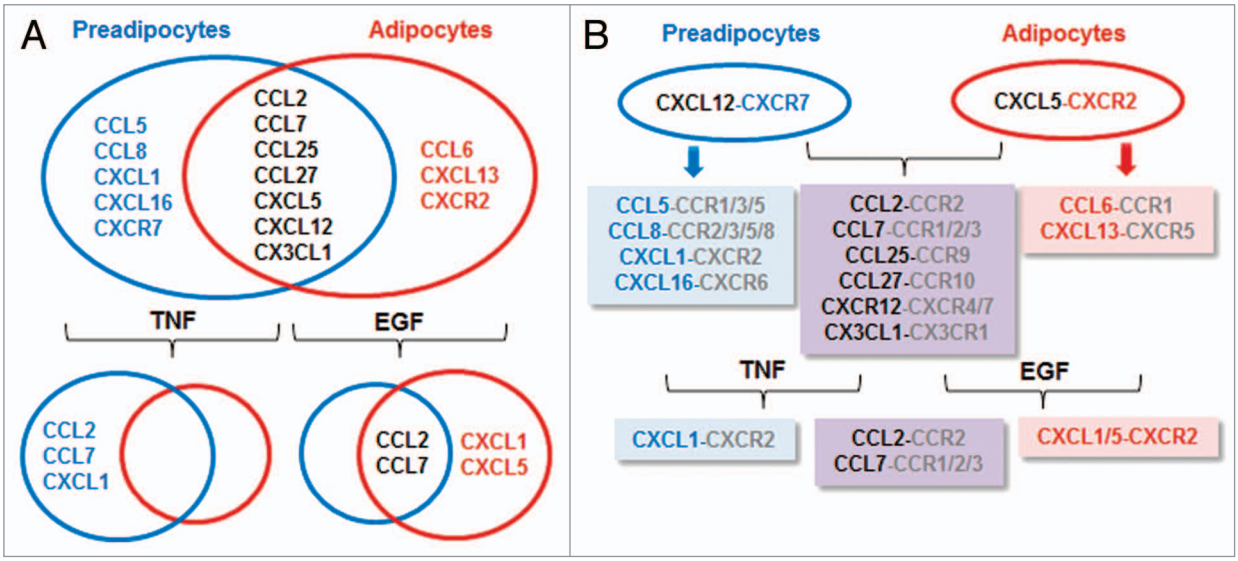

Figure 6. Schematic proposal for chemokine networks between preadipocytes and adipocytes. (**A**) Chemokine networks during 3T3-L1 cell adipogenesis and TNF- and EGF-responsive chemokines between preadipocytes and adipocytes. (**B**) Differential development of chemokine networks between preadipocytes and adipocytes and TNF- and EGF-potentiated chemokine-receptor axes. Black letters, common chemokines for both preadipocytes and adipocytes; blue letters, preadipocyte-derived chemokines; red letters, adipocyte-derived chemokines; gray letters, expected chemokine receptors for chemokines.

instructions, a real-time PCR was performed using a Bio-Rad CFX96 under the following two-step cycling program: 1 cycle at 95 °C for 10 min, 40 cycles at 95 °C for 15 s and at 60 °C for 1 min. Data analysis was performed based on a web-based PCR Array Data Analysis protocol (http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php) provided by SABiosciences/Qiagen. Values for PCR array are the means from duplicate experiments. Primers used in qRT-PCR are described in **Table 1**. Experiments for qRT-PCR were performed at least in triplicate.

Western blot

Cell lysates were prepared, fractionated on SDS-polyacrylamide gels and transferred to nitrocellulose membranes according to established procedures. Blocking of nonspecific proteins was performed by incubation of the membranes with 5% nonfat dry milk in Tris buffered saline Tween-20 (TBST containing 10 mM Tris, 150 mM phosphate buffered saline, 0.05% Tween 20, pH 8.0) for 2 h at room temperature. Blots were incubated with primary antibodies at 1:1,000 dilution in blocking solution overnight at 4 °C. The membranes were washed 3 times with TBST for 10 min and followed by incubation for 1 h with horseradish peroxidase conjugated secondary antibody according to primary antibody, used at 1:2500 in 5% milk/TBST. The membranes were then rinsed 3 times with TBST for 10 min and the bands were visualized by enhanced chemiluminescence.

Statistical analysis

Data were expressed as mean ± SEM. Difference between two groups were analyzed by the paired Student *t* test with statistical significance of $P \leq 0.05$.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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Supplemental Materials

Supplemental materials may be found here:
www.landesbioscience.com/journals/adipocyte/article/28110

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