Adipocyte culture medium stimulates invasiveness of MDA-MB-231 cell via CCL20 production

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Abstract. We explored whether adipocyte culture medium affects the secreted chemokine profile of tumor cells, because adipocytes stimulate progression or metastasis of breast cancer cells, and chemokines secreted from tumor cells are involved in these processes. CCL20 expression was dramatically increased, and an NF-κB blocker completely inhibited adipocyte culture medium-induced CCL20 expression in MDA-MB-231 cells. We showed that adipocyte culture medium increased the production of TNF-α in MDA-MB-231 cells, which stimulated CCL20 expression in an autocrine fashion. Our data also showed that CCL20 increased the migration and invasiveness of MDA-MB-231 cells, but did not affect the proliferation of these cells.

Introduction

Adipose tissue produces a variety of adipokines, including adiponectin and leptin, as well as chemokines (1). These adipokines are normally involved in the regulation of glucose and lipid metabolism and inflammation. However, expression levels of adipokines change markedly during the progression of various diseases including insulin resistance, cardiovascular disease, and cancers (2-8). For example, leptin levels are increased in obese patients, whereas adiponectin levels are decreased, compared to normal subjects. In breast cancer patients, leptin enhances tumor cell proliferation, but adiponectin has a protective function (5,9). Thus, obesity is a risk factor for lifestyle diseases, and a growing body of evidence suggests that obesity is an important risk factor for cancer progression and metastasis (10-12). Although adipocytes are the most abundant stromal cell types, especially in breast tissue, little is known about the impact of adipokines on the interaction between adipocytes and breast cancer cells.

Chemokines, a family of small (8-14 kDa) cytokines, are classified into four categories based on the location of the principal cysteine residues (13-15). The subclasses are the CXC, CC, C, and CX3C chemokines (16). The chemokine receptors are G-protein-coupled receptors with seven transmembrane domains, and chemokines exert their various functions by interacting with specific receptors (17). Cancer cell-derived chemokines are responsible for the tumor infiltration of various types of leukocytes, including macrophages (16). The chemokine-directed accumulation of leukocytes within cancer tissue plays a crucial role in progression and metastasis of various cancers, including sarcomas, gliomas, melanomas, and breast adenocarcinoma (18). Chemokines and their receptors are key mediators of communication between tumor cells and the tumor microenvironment, including stromal and immune cells. Some chemokines may assist in tumor development and progression whereas others may enhance anti-tumor immunity (19). The dual effects of chemokines on the development or progression of tumor cells should be interpreted in the context of the tumor environment. In the present study, we examined chemokine production and explored chemokine profile changes in adipocyte culture medium-treated breast cancer cells, to understand signaling from adipocytes to such cancer cells.

Materials and methods

Cells and reagents. The MDA-MB-231 human breast cancer cell line and the OP9 mouse preadipocyte cell line were purchased from the American Type Culture Collection (ATCC). MDA-MB-231 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% (v/v) FBS, and OP9 cells were maintained in αMEM supplemented with 20% (v/v) FBS, at 37˚C under 5% (v/v) CO2. CCL20, IL-1β, TNF-α, and anti-TNF-α neutralizing antibody were purchased from R&D Systems, Inc. (Minneapolis, MN). The following sequence was used for the construction of siRNAs: human TNF-α receptor (TNFRSF1A) siRNA;
5'-GCUGCUCUAAUGCCGAAA-3'; Mouse TNF-α siRNA: GUCCAACUCUGUGCUCAUA were synthesized (Bioneer Corporation, Daejon, Korea).

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

Chemokine antibody array. MDA-MB-231 cells were treated with preadipocyte-CM or adipocyte-CM for 16 h, and the supernatants were centrifuged to remove cells. Chemokines in supernatants were analyzed using a Human Chemokine Antibody Kit (Ray Biotechnology Inc., Norcross, GA), according to the manufacturer's instructions.

RT-PCR analysis. MDA-MB-231 cells were treated with preadipocyte-CM or adipocyte-CM for the indicated time periods or treated with 50 nM TNF-α receptor siRNA or 50 nM GFP siRNA for 24 h and then preadipocyte-CM or adipocyte-CM was treated for 6 h. Total RNAs were prepared using RNAzolB (Tel Test, Inc., Friendswood, TX). Five microgram amounts of total RNA were reverse-transcribed using M-MuLV reverse transcriptase (Promega, Madison, WI) at 37°C for 1 h. PCR for the amplification of mRNAs encoding CCL20, CCR6, TNF-α, and IL-1α was then performed using appropriate primer pairs. These were CCL20 forward: 5'-CAGAAGCAGCAAGCACT-3', reverse: 5'-AGTGAGTGAGGCAAAA-3'; CCR6 forward: 5'-GAGCGTCAGGAGTCCACG-3', reverse: 5'-GCTGCCTGGTTGTGTATT-3'; TNF-α receptor 1 forward: 5'-GGGTATTGTGAGGTCTCC-3', reverse: 5'-CTGCAATTTGAAGCAGTGGAA-3'; TNF-α receptor 2 forward: 5'-GGTGACAGCCCTGTAGGCCCA-3', reverse: 5'-AAGGATGAGCCTGCCCAGAC-3'; and IL-1β forward: 5'-GGGCTCTCAAGGAAAGAATC-3', and reverse: 5'-TCTGCTGTGAGGCTGCTGTA-3'. PCR was performed with cycles of 30 sec of denaturation at 94°C, 30 sec of annealing at 55°C, and 30 sec of extension at 72°C. β-actin mRNA was amplified as a control. The intensity of bands was measured using a DNR Bio-Imaging system (Kiryat Anavim, Jerusalem, Israel).

Western blot analysis. MDA-MB-231 cells were treated with 50 nM TNF-α receptor siRNA or 50 nM GFP siRNA for 24 h and then treated with preadipocyte-CM and adipocyte-CM for 30 min and total cell lysates were separated on a 12% SDS-polyacrylamide gel, and then transferred to a PVDF membrane (Millipore, Marlborough, MA). 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-IκBα and phosphor-IkBε (Cell Signaling Technology Inc., MA) overnight. After washing three times with TBST (0.05% Tween-20, Tris-HCl, pH 7.5, 137 mM NaCl), the membrane was incubated with the anti-rabbit/mouse IgG secondary antibody conjugated with horseradish peroxidase. The signal was detected using the Amersham ECL system (Amersham Pharmacia Biotech, Arlington Heights, IL).

Migration assays. For migration assays, MDA-MB-231 cells were grown to confluence in a 6-well plate and cells were then removed using a scraper. The medium was changed to DMEM with 1% (v/v) FBS and cells were incubated in the presence of 0, 25, or 100 ng/ml of recombinant CCL20, for 24 h.

Proliferation assays. MDA-MB-231 cells were seeded onto 96-well plates at a concentration of 4×10^3 cells/well and incubated overnight to permit attachment. Next, the medium was changed to DMEM with 10% (v/v) FBS, and cells were cultured in the absence or presence of 25 or 100 ng/ml recombinant CCL20 for 24, 48, or 72 h. The cell proliferation rate was measured using the EZ-Cytos-enhanced cytotoxicity assay kit (Daeil Laboratory Service, Seoul, Korea), according to the manufacturer's instructions.

Invasion assay. The ability of cells to migrate through matrigel-coated filters was determined using a Matrigel coated transwell (8-μm pore size; Corning Costar, Cambridge, MA), as described previously (20). The lower compartment of transwell was filled with DMEM containing 10% (v/v) FBS, and cells were cultured in the absence or presence of 25 or 100 ng/ml recombinant CCL20 for 24, 48, or 72 h. The cell proliferation rate was measured using the EZ-Cytos-enhanced cytotoxicity assay kit (Daeil Laboratory Service, Seoul, Korea), according to the manufacturer's instructions.

Results

Adipocyte-CM increases CCL20 production by MDA-MB-231 cells. Adipocytes are the most abundant stromal cell type in breast tissues and the interaction between stromal cells and tumor cells is known to play a major role in progression and metastasis of tumors. However, little is known about the effect of adipocyte-CM on chemokine gene induction in tumor cells. To examine changes in the chemokine expression profile of adipocyte-CM-treated MDA-MB-231
cells, MDA-MB-231 cells were treated with preadipocyte-CM or adipocyte-CM for 16 h, and changes in chemokine production profile were analyzed by chemokine blot. Notably, CCL20 and CCL13 production increased in adipocyte-CM-treated MDA-MB-231 cells, compared to cells treated with preadipocyte-CM (Fig. 1A). Although CXCL12 production was significantly decreased in adipocyte-CM cultured with MDA-MB-231 cells, similar decrease of CXCL12 level was observed between preadipocyte-CM and adipocyte-CM incubated without MDA-MB-231 cells, indicating that decrease of CXCL12 production is due to adipogenesis. Firstly, we focused on CCL20 in this study. To determine
if the increase in CCL20 production was related to increased transcription of the CCL20 gene, MDA-MB-231 cells were treated with preadipocyte-CM, adipocyte-CM, or proteinase K-treated adipocyte CM, for 6 h. Total RNA was isolated and subjected to RT-PCR analysis. The expression of CCL20 mRNA was markedly increased in adipocyte-CM-treated cells compared with cells exposed to preadipocyte-CM (Fig. 1B). Next, the kinetics of CCL20 mRNA induction by adipocyte-CM were examined. MDA-MB-231 cells were treated with adipocyte-CM and RT-PCR analysis was performed on total RNA isolated after incubation at the indicated times. The expression of CCL20 mRNA dramatically increased as early as 3 h after exposure to conditioned medium, and started to decrease slightly at 18 h (Fig. 1C).

Unknown protein factor from adipocyte-CM increases CCL20 expression in MDA-MB-231 cells via activation of the NF-κB signaling pathway. Adipocytes secrete adipokines including leptin, resistin, and adiponectin, as well as free fatty acids (FFAs). To explore which factors might be responsible for the induction of CCL20 mRNA expression, adipocyte-CM were treated with proteinase K. Adipocyte-CM thus failed to induce CCL20 mRNA expression by adipocyte-CM were examined. MDA-MB-231 cells were treated with adipocyte-CM and RT-PCR analysis was performed on total RNA isolated after incubation at the indicated times. The expression of CCL20 mRNA dramatically increased as early as 3 h after exposure to conditioned medium, and started to decrease slightly at 18 h (Fig. 1C).

MAPK inhibitor, SB203580, or the ERK inhibitor, PD98059, affected CCL20 gene expression induced by adipocyte-CM treatment. However, the NF-κB inhibitor, parthenolide, dramatically inhibited adipocyte-CM-induced CCL20 mRNA expression (Fig. 2B), indicating that a protein activating NF-κB in MDA-MB-231 cells is responsible for the adipocyte-CM-induced rise in CCL20 mRNA.

TNF-α secreted from MDA-MB-231 cells is responsible for CCL20 production. TNF-α and IL-1β are major stimulators of CCL20 production and NF-κB activation (21). To determine whether one or both of these proinflammatory cytokines might be responsible for induction of CCL20 mRNA expression, MDA-MB-231 cells were treated with IL-1β and/or TNF-α and the levels of CCL20 mRNA expression were examined by RT-PCR (Fig. 3A). Both TNF-α and IL-1β were able to induce CCL20 mRNA expression in MDA-MB-231 cells. Next, we examined whether TNF-α and IL-1β expression might be higher in differentiated adipocytes than in undifferentiated OP9 cells. Unexpectedly, no difference in TNF-α and IL-1β levels between differentiated and undifferentiated cells was observed, and IL-1β was not detected in either cell type (Fig. 3B). Thus, we speculate that a specific, unknown, protein factor in adipocyte-CM may induce TNF-α or IL-1β in MDA-MB-231 cells, and the TNF-α or IL-1β thus produced may increase CCL20 expression, in an autocrine fashion, in MDA-MB-231 cells. To test this possibility, MDA-MB-231 cells were treated with preadipocyte-CM, adipocyte-CM, or proteinase K-treated adipocyte-CM for 6 h, and the levels...
of TNF-α and IL-1β mRNAs were analyzed by RT-PCR. TNF-α mRNA was increased by treatment with adipocyte-CM, whereas IL-1β mRNA was not detected (Fig. 4A). The adipocyte-CM-induced TNF-α mRNA expression peaked at 3 h and then started to decrease 6 h after treatment (Fig. 4B).

To determine whether TNF-α secreted from MDA-MB-231 cells indeed stimulated CCL20 production, MDA-MB-231 cells were treated with adipocyte-CM in the presence of TNF-α neutralizing antibody. The antibody dose-dependently inhibited CCL20 mRNA expression (Fig. 4C). We next
obtained adipocyte-CM from TNF-α siRNA-treated adipocytes and MDA-MB-231 cells were treated with TNF-α-depleted adipocytes-CM. CCL20 mRNA expression was induced (Fig. 4D). To further confirm the hypothesis, MDA-MB-231 cells were treated with TNF-α receptor siRNA for 24 h and then adipocyte-CM was treated for 6 h. The treatment of TNF-α receptor siRNA significantly blocked adipocyte-CM-mediated CCL20 mRNA expression, but did not affect TNF-α expression (Fig. 4E) and adipocyte-CM stimulated IKKα/β and IκBα activation in the presence or absence of TNF-α receptor siRNA (Fig. 4F), indicating that a factor activating NF-κB in adipocyte-CM is not TNF-α. Together, these data show that an unknown factor activating NF-κB in adipocyte-CM, which is able to induce NF-κB production in an autocrine fashion, stimulates CCL20 mRNA expression via TNF-α production in an autocrine fashion.

CCL20 increases migration and invasiveness of MDA-MB-231 cells, but does not affect cell proliferation. CCL20 recruits lymphocytes and dendritic cells by binding to the CCR6 receptor expressed on the plasma membranes of such cells. In addition, CCL20 also promotes tumor cell invasion by upregulation of MMP9 in pancreatic cancer cells (22), increases proliferation of colorectal cancer cells (23), and stimulates osteoblast cell growth (24). Thus, we examined the effect of CCL20 on breast cancer cells. Firstly, expression of CCR6 was determined in various breast cancer cell lines including T47D, MCF-7, MDA-MB-435S, and MDA-MB-231 cells. CCR6 expression was observed in all cell lines (Fig. 5A). To determine the effect of CCL20 on cell migration, MDA-MB-231 cells were incubated with recombinant CCL20 for 24 h. This treatment significantly increased migration of MDA-MB-231 cells (Fig. 5B). Next, we sought a possible effect of CCL20 on the invasiveness of breast cancer cells. MDA-MB-231 cells were incubated with 25 or 100 ng/ml of recombinant CCL20 in the upper chambers of transwells, for 24 h. Cells invading the lower chambers were counted after 24 h of incubation with CCL20. Recombinant CCL20 enhanced the invasiveness of MDA-MB-231 cells (Fig. 5C). To examine the effects of CCL20 on proliferation of MDA-MB-231 cells, cells were seeded into 96-well plates and stimulated with 25 or 100 ng/ml of recombinant CCL20 for 72 h. This treatment did not affect proliferation of MDA-MB-231 cells (Fig. 5D).

Discussion

Chemokine receptors are overexpressed in various human cancers including breast cancer, ovarian cancer, and prostate cancer. Chemokine receptor-expressing tumor cells metastasize to chemokine-producing tissues (25,26), but relatively little is known on the roles of chemokines secreted directly by tumor cells. In the present study, we show that a protein secreted from adipocytes stimulates the production of CCL20...
in MDA-MB-231 cells, via autocrine production of TNF-α, and that the CCL20 thus produced was able to increase the migration of MDA-MB-231 cells, without increasing cell proliferation. Co-expression of CCL20 and the CCL20 receptor CCR6 in cancer cells of human pancreatic tumors has been reported, and CCL20 stimulated the growth of one pancreatic cell line and increased the migration of another pancreatic cell line (27). Thus, it is conceivable that CCL20 produced by breast tumor cells acts on the tumor cells, via autocrine and paracrine mechanisms, to contribute to the pathophysiology of human breast cancer. In addition, we showed co-expression of CCL20 and CCR6 in various breast cancer cell lines including MDA-MB-231 cells. This finding indicates that adipocytes, the major stromal cell type, are involved in regulation of chemokine production in breast cancer cells, and that chemokines such as CCL20 are able to contribute to microenvironment preparation for migration and invasiveness of tumor cells.

CCL20-producing breast carcinoma cells recruit immature dendritic cells (DCs) and promote the development of immature DCs into two distinct subpopulations of DCs (28). Importantly, these two subpopulations differ in their capacity to activate T lymphocytes. Thus, infiltration of these stimulated DCs may contribute to an impaired anti-tumor immune response. Moreover, CCL20 expressed by tumors enhances intratumoral lymphocyte infiltration, and facilitates tumor growth. The mechanism involved remains to be further elucidated (29). Collectively, the cited reports, and our data, suggest that enhanced chemokine production in tumor cells stimulated by adipocyte-CM is one mechanism by which adipocytes stimulate tumor progression through immune escape.

Chemokines produced by cancer cells are responsible for the migration and infiltration of tumor-associated macrophages (TAMs). In breast cancer, lower CCL2 expression correlated with longer relapse-free survival time and decreased TAM levels (30), and higher CCL5 expression was associated with an increase in TAMs and lymph node metastasis (31). Matrix metalloproteinases (MMPs) produced by chemokine-recruited TAMs induce invasion of cancer cells, whereas exposure of CD4+, CD8+, T-cells to CXCL16 by chemokine-recruited TAMs induce invasion of cancer cells, whereas exposure of CD4+, CD8+, T-cells to CXCL16 stimulates DCs and promotes the development of other immune cells.

In the present study, we found that adipocyte-CM-treated MDA-MB-231 cells showed increased CCL20 production. Various factors, including proteins and lipids in adipocyte-CM, might be responsible for the increased production of CCL20. Interestingly, an unidentified factor in adipocyte-CM induced TNF-α production in MDA-MB-231 cells, and the induced TNF-α enhanced CCL20 production in an autocrine fashion. As proteinase K-treated adipocyte-CM failed to induce CCL20 production, the unidentified factor is a protein. In a previous study, we showed that FFAs in adipocyte-CM increased MIC-1 production, which stimulated the invasiveness of MDA-MB-231 cells (20). These results imply that both protein factors and lipid components secreted from adipocytes contribute to formation of the tumor microenvironment. As levels of many adipocyte-secreted proteins and lipids are changed in obesity or by diet, detailed study on the roles of each factor, including CCL20, could shed light on the functions of adipose tissue cells serving as stromal cells in breast tumors.

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References


