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Cancer-Associated Adipocytes Exhibit an Activated Phenotype and Contribute to Breast Cancer Invasion

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Abstract

Early local tumor invasion in breast cancer results in a likely encounter between cancer cells and mature adipocytes, but the role of these fat cells in tumor progression remains unclear. We show that murine and human tumor cells cocultivated with mature adipocytes exhibit increased invasive capacities in vitro and in vivo, using an original two-dimensional coculture system. Likewise, adipocytes cocultivated with cancer cells also exhibit an altered phenotype in terms of delipidation and decreased adipocyte markers associated with the occurrence of an activated state characterized by overexpression of proteases, including matrix metalloproteinase-11, and proinflammatory cytokines [interleukin (IL)-6, IL-1β]. In the case of IL-6, we show that it plays a key role in the acquired proinvasive effect by tumor cells. Equally important, we confirm the presence of these modified adipocytes in human breast tumors by immunohistochemistry and quantitative PCR. Interestingly, the tumors of larger size and/or with lymph nodes involvement exhibit the higher levels of IL-6 in tumor surrounding adipocytes. Collectively, all our data provide in vitro and in vivo evidence that (i) invasive cancer cells dramatically impact surrounding adipocytes; (ii) peritumoral adipocytes exhibit a modified phenotype and specific biological features sufficient to be named cancer-associated adipocytes (CAAs); and (iii) CAAs modify the cancer cell characteristics/phenotype leading to a more aggressive behavior. Our results strongly support the innovative concept that adipocytes participate in a highly complex vicious cycle orchestrated by cancer cells to promote tumor progression that might be amplified in obese patients. Cancer Res; 71(7); 2455-65. ©2011 AACR.
Obesity, wherein the normal balance of adipose tissue secretory proteins is disturbed, has been recently identified as a negative prognosis factor for breast cancer (9, 10). This poor prognosis seems to be independent of menopausal status, tumor stage, and tumor hormone-binding characteristics (for review, see refs. 11–13). Several studies pointed out that obese women exhibit at diagnosis an increase in lymph nodes involvement and a higher propensity to distant metastasis (10, 11, 14–17). Furthermore, obesity is also associated with the occurrence of tumors of high grade with increased local and distant invasion in prostate cancer (18). Taken together, these compelling observations suggest that excess adiposity may favor tumor invasiveness by yet uncharacterized mechanism(s). We show here that tumor-surrounding adipocytes exhibit profound phenotypic changes in vitro and in human tumors, and these modified adipocytes promote tumor cell invasion and metastasis.

Materials and Methods

Cell culture

The murine 3T3-F442A preadipocyte cell line was differentiated as described previously (19). To estimate the adipocyte phenotype, triglyceride (TG) content was quantified using a colorimetric kit (Wako Chemicals), and red oil staining was carried out as previously described (20). The human breast cancer cell line ZR 75.1 (provided by Prof. K. Bystricky, Laboratoire de Biologie Moléculaire Eucaryote, Toulouse, France) and the murine 67NR and 4T1 breast cancer cell lines (ref. 21; provided by Dr. S. Vagner, INSERM U563, Toulouse, France) were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% (ZR 75.1 and 67NR) and 5% (4T1) fetal calf serum (FCS). The human breast cancer cell line SUM159PT (provided by Dr. J. Pietet, IGMM, Montpellier, France) was grown in Ham F12 (50:50) medium supplemented with 10% (SUM159PT, 4T1), or 3% (ZR 75.1) fetal calf serum (FCS). The human breast cancer cell line SUM159PT was cultured in Ham F12 (50:50) medium supplemented with 5% FCS, 1 μg/mL hydrocortisone (Sigma), and 0.2 UI/mL insulin. All the cell lines were used within 2 months after resuscitation of frozen aliquots. Cell lines were authenticated on the basis of viability, recovery, growth, and morphology. For ZR 75.1 and SUM159PT cell lines, the expression status of estrogen receptor (ER) α, E-cadherin, N-cadherin, and vimentin was confirmed by immunoblotting before they were used in the experiments (22). For all cell lines, their invasive behavior was confirmed using Boyden chamber assays before they were used in the experiments. Cell lines 67NR and 4T1 were also tested for their ability to form (4T1) or not (67NR) metastatic tumors when injected in syngenic BALB/c mice (21).

Human samples

Breast adipose tissue samples were collected from reduction mammoplasty (RM) or tumorectomy (TU), according to the guidelines of the Ethical Committee of Toulouse-Rangueil. All subjects gave their informed consent to participate in the study, and investigations were conducted in accordance with the Declaration of Helsinki as revised in 2000. In cases of TU, the fat tissue samples obtained were immediately close to the tumor mass and were devoid of fibrosis, as assessed macroscopically (data not shown). Adipose tissue pieces were immediately used for collagenase digestion as previously described (23) and centrifuged (20 minutes, room temperature, 1,000 rpm) to separate adipocytes from the stroma vascular fraction (pellet, SVF).

Coculture and invasion assays

Tumor cells and adipocytes were cocultured using a Transwell culture system (0.4-μm pore size; Millipore). A total of 5 × 10^5 (67NR), 1.5 × 10^5 (SUM159PT, 4T1), or 3 × 10^5 (ZR 75.1) cells were seeded in the top chamber of the Transwell system in the culture medium of adipocytes and cocultivated with or without mature adipocytes in the bottom chamber for the indicated times. Adipocytes or tumor cells cultivated alone in similar conditions served as controls. To evaluate the effect of adipocyte-conditioned medium (Ad-CM) on tumor cell invasion, mature adipocytes were cultivated overnight with serum-free medium containing 1% of delipidated bovine serum albumin (Sigma) and medium was collected. CM was also collected from adipocytes previously cocultivated during 3 days with tumor cells [cancer-associated adipocytes conditioned medium (CAA-CM)]. After 3 days of coculture in the presence of mature adipocytes, Ad-CM, or CAA-CM (complemented with 10% FCS), tumor cells were used to conduct Matrigel invasion assays as previously described (24). A total of 35 to 40 fields per filter were counted using an optical microscope. Invasion assays were also conducted with tumor cells previously cultivated for 3 days with recombinant human IL-6 (PeproTech).

Western blot analysis

Whole cell extracts and immunobots were prepared as previously described (25). All the antibodies used in our study are presented in Supplementary Information.

Tail vein metastasis assays

A total of 1 × 10^6 4T1 cells, previously cocultivated either with or without mature adipocytes for 4 days, were injected intravenously in BALB/c mice (6 animals per condition). Mice were sacrificed after 14 days, and lungs were taken for analyses. Nodules were scored from 1 to 5 according to their likelihood to represent metastasis to lung (0, normal lung tissue; 1, tissue with 1–5 nodules; 2, 6–10 nodules; 3, 11–20 nodules; 4, 21–30 nodules; and 5, >31 nodules). Lungs were then fixed in 4% paraformaldehyde and embedded in paraffin. Sections were stained with hematoxylin and eosin (H&E) and nodules were observed. The mean number of nodules per lung area and the mean nodule area as a function of lung area (that defined the nodule area index) were measured by ImageJ software program. The local Institutional Animal Care and Use Committee approved the experimental protocols described in the study.

Immunofluorescence, immunohistochemistry, and determination of adipokines secretion

Immunofluorescence and immunohistochemistry were carried out as previously described (8, 24). Concentration of
Key Role of Mature Adipocytes in Breast Cancer Progression

secreted factors in supernatants was determined using the xMAP Technology (Luminex), with a Milliplex kit (Millipore).

RNA extraction and quantitative PCR
Gene expression was analyzed using real-time PCR as described previously (23). The sequences of the primers are presented in Supplementary Information.

Statistical methods
Quantitative expression of biological markers measured by quantitative PCR (qPCR) in isolated human adipocytes was assessed using estimations of means and SDs. Hence, a normal distribution of these measures was assumed. However, the size of subpopulations was limited in most cases. Thus, we tested differences about the expression of biological markers between subpopulations of interest, using nonparametric tests (i.e., Wilcoxon’s nonparametric rank test). Quartiles were also computed; box plots permitted to appreciate the presence of outliers and to illustrate differences in the expression of biological markers. The threshold of statistical significance was fixed at alpha risk at 5%. SPlus statistical software was used for the data analysis (26). For the in vitro experiments, the statistical significance of differences between the means (at least 3 independent experiments) was evaluated using Student’s t test, done using Prism (GraphPad Inc.). The values of *P < 0.05, **P < 0.01, and ***P < 0.001 were deemed as significant whereas ”NS” stands for not significant.

Results and Discussion

Tumor cells cocultivated with mature adipocytes exhibit an enhanced invasive phenotype
Human cancer cells with low (ZR 75.1) or high (SUM159PT) invasive capacities (27) were grown for 3 days on Transwells, allowing the diffusion of soluble factors, in the presence or absence of murine mature adipocytes (28). After 3 days, tumor cells were trypsinized and Matrigel invasion assays were conducted in a medium containing either 0% or 10% FCS. As shown in Figure 1A, the invasive capacity of ZR 75.1 tumor cells in 10% FCS containing medium was increased by 3-fold in the absence of murine mature adipocytes (28). After 3 days, tumor cells were trypsinized and Matrigel invasion assays were conducted in a medium containing either 0% or 10% FCS. As shown in Figure 1A, the invasive capacity of ZR 75.1 tumor cells in 10% FCS containing medium was increased by 3-fold in the absence of murine mature adipocytes (28).

Cocultivated adipocytes exhibit extensive phenotypic changes
Adipocytes have been shown to promote tumor survival and growth in vitro and in vivo (6, 29), but their contribution to tumor cell invasion is an emerging concept. Because our results showed that a cross-talk between the 2 cell populations is necessary to observe the proinvasive effect (Fig. 1C), we next examined the phenotype of cocultivated adipocytes. As shown in Figure 4A (left), mature adipocytes cocultivated with ZR 75.1 tumor cells exhibited a dramatic reduction in
the number and size of lipid droplets that correlated with a significant decrease in lipid accumulation (Fig. 4A, right, *P* < 0.05). These adipocytes lose several terminal differentiation markers exemplified by the strong decrease in HSL (hormone-sensitive lipase), APN, resistin, and aP2 mRNA (ref. 5; Fig. 4B, *P* < 0.05 for all the markers), this loss was also confirmed at protein levels for APN and resistin (Fig. 4C, *P* < 0.05). In cocultivated adipocytes, the level of C/EBPα was dramatically reduced (*P* < 0.001) whereas that of PPARγ was not significantly decreased (Fig. 4B). As previously stated,
MMP-11/stromelysin-3 is induced in vivo in adipose tissue by cancer cells as they invade their surrounding environment (8). Similarly, an upregulation for this protease was noted in the cocultivated adipocytes, highlighting the in vivo relevance of this coculture model (Fig. 4B, \( P < 0.001 \)). In contrast, the levels of MMP-9 (Fig. 4B) and MMP-2 (not shown) remain unchanged whereas a significant increase (10-fold) of plasminogen activator inhibitor-1 (PAI-1) expression was found (Fig. 4B, \( P < 0.01 \)). In cocultivated adipocytes, a 5-fold increase in levels of IL-6, IL-1β, and TNF-α was shown at mRNA levels (Fig. 4B, \( P < 0.001 \) for all cytokines). At protein levels, however, only IL-6 and IL-1β were found to be significantly higher in the supernatant of cocultivated cells (Fig. 4C, \( P < 0.05 \) for both cytokines) whereas the levels of TNF-α remained undetectable in both conditions (data not shown). Of note, these extensive adipocyte phenotypic changes were obtained with other tumor cells lines (Supplementary Fig. S2). Furthermore, these results were confirmed with mammary adipocytes derived from ex vivo differentiation of progenitors purified from mammary adipose tissue of healthy donors undergoing RM (6 independent donors; Supplementary Fig. S3). Therefore, we extensively described tumor-induced changes in adipocytes that are reproducibly displayed by both human and mouse cocultivated adipocytes. Collectively, our observations clearly show that cocultivated adipocytes generally exhibit a loss of lipid content, a decrease in late adipose markers, and overexpression of inflammatory cytokines and proteases. We named these cells “cancer associated adipocytes” (11).

**CAAs are found in human tumors**

We next investigate whether these cells are present in primary human tumors. To address this critical issue, we first conducted a histologic analysis of human breast tumors. As exemplified in Figure 5A, we consistently observed at the invasive front of human breast tumors the presence of adipocytes of smaller sizes with a dilated interstitial space, which
proteins were expressed within the rim of cytoplasm of the adipocytes of RM (Fig. 5B, iii and v, respectively), these proteins were expressed within the rim of cytoplasm of the adipocytes located proximally to the invasive cancer cells (Fig. 5B, iv and vi, respectively). To further assess the expression of these markers by a quantitative approach, adipocytes were isolated from fat samples associated with TU or RM (23). A series of 28 samples, with 14 samples in each group (Supplementary Tables S1 and S2 for the clinical characteristics of the patients), was used comparable with respect to age (mean: 51.8 ± 7.3 vs. 60 ± 13) and BMI (26.2 ± 2.8 vs. 25.2 ± 2.3). Adipocytes isolated from tumor samples overexpressed MMP-11 (P = 0.001), PAI-1 (P < 0.01), and IL-6 (P < 0.001), as compared with adipocytes purified from normal mammary gland, whereas the mRNA levels of IL-1β and TNF-α were not statistically different (Fig. 5C). The adipose tissue also contains stromal cells (present in the SVF), such as fibroblasts, endothelial cells, and adipocyte precursors. The levels of expression of MMP-11, PAI-1, IL-6, IL-1β, and TNF-α remained unchanged between normal and tumor-surrounding adipose tissue, highlighting that the observed increase in proinflammatory cytokines and proteases is an adipocyte-dedicated event (Fig. 5C). Furthermore, when the expression levels of IL-6 in adipocytes were analyzed as a function of tumor characteristics, we found that higher levels of this cytokine were associated with the tumors of larger size and with lymph nodes involvement (Fig. 5D). This correlation was not found either for PAI-1 or for MMP-11 (Supplementary Fig. S4). Taken together, these results show that CAAs are found in human tumors.

Our data strongly support the concept that an intimate cross-talk is established between cancer cells and mature adipocytes at the invasive front of the tumor. Invading tumor cells are able to modify adipocytes phenotype, which, in turn, stimulate cancer cells aggressive behavior (30). The mechanism(s) responsible for the transition from mature adipocytes to CAAs remain(s) unclear. Modification resembling to CAAs is also observed in adipose tissue of tumor-bearing mice far from the tumor site. In cachexia, adipocytes also exhibit morphologic modification, decreased in adipogenic transcription factors such as C/EBPα and late differentiation markers such as leptin (31). However, during cancer cachexia, adipocytes exhibit a decrease in resistin expression and upregulation of lipolytic enzymes such as HSL or ATGL (adipose triglyceride lipase) (31), events not found in CAAs (Fig. 4 and Supplementary Fig. S5), alluding to their unique phenotype. Several molecules expressed by tumor cells have been described as strong suppressors of adipogenesis such as IL-1β, TNF-α, or even IL-6 (32), representing one mechanism by which tumor cells might directly influence adipocyte behavior. An alternative mechanism might be adipocytes themselves as the source of autocrine signals that induce the CAAs phenotype. In favor of the latter hypothesis, we showed that tumor CM was not able to reproduce CAAs phenotype, suggesting that either adipocyte factors or inducible factors in tumor cells are necessary to observe this effect (Supplementary Fig. S6).

**Adipocyte IL-6 plays a key role in mediating adipocyte-dependent invasive activity of tumor cells**

Our results show that IL-6 is overexpressed in tumor-surrounding adipocytes both in vitro and in vivo. We showed...
that IL-6 (at 10 ng/mL, a dose equivalent to the levels secreted by CAAs, Fig. 4C) significantly increased the invasive capacities in ZR 75.1 and 67NR cells (Fig. 6A, \( P < 0.01 \) for the 2 cell lines). As compared with untreated cells, the epithelial-like ZR 75.1 cells exposed to IL-6 form less compacted colonies and exhibit a more scattered aspect (Fig. 6B). A downregulation of membrane E-cadherin expression resulting into a disorganized state in the cytoplasm, in association with β-catenin redistribution (Fig. 6B), was observed in treated cells in accordance with the literature (33–35). Strikingly, when ZR 75.1 cells were cocultivated with adipocytes in the presence of murine IL-6 blocking antibody, the adipocyte proinvasive effect was significantly decreased (Fig. 6C, \( P < 0.01 \)). mIL-6 blocking antibody specifically...
inhibits the proinvasive effect of CAA-CM in both 67NR and 4T1 models and had no effect on the invasive capacities of cells grown in control medium (Fig. 6D, \( P < 0.01 \) and \( P < 0.05 \), respectively). Altogether, these results indicate that IL-6 plays a key role in the CAAs proinvasive effects.

In breast cancer patients, the extent of the increase in serum IL-6 correlates with poor disease outcome and reduced prognosis (36). Recent evidence shows that fibroblast-derived IL-6 supports breast tumor growth and metastasis (37). Our results show that the stromal IL-6 is also produced by mature adipocytes surrounding the tumors. The absence of IL-6 overexpression in the SVF of human adipose tissue isolated from breast tumor biopsies (Fig. 5C) apparently contradicts a recent study conducted \textit{ex vivo}, and not \textit{in vivo}, using human adipose tissue–derived stromal cells cocultivated with tumor cells (38). Our results obtained in human tumors suggest that within the cellular complexity of the adipose tissue, the crosstalk with tumor cells is rather established with mature, than with precursors, adipose cells (Fig. 5C and D). In addition, because mature adipocytes are the most abundant cells in the breast tumor stroma (3), their cumulative secretion of IL-6 could therefore have profound impact on tumor progression.

Finally, this work paves the way for future clinical and cellular studies aimed at determining the impact of this treatment.
cross-talk in obesity conditions. Indeed, our results suggest that the cross-talk between the CAAs and tumor components might be amplified in obesity in which the normal balance of adipose tissue secretory proteins is disturbed.

Obesity is related to a condition of low chronic inflammation characterized by the abnormal production of inflammatory cytokines involved in insulin resistance (5). Obesity and the now-defined profile of CAAs share inflammation as common

Figure 6. IL-6 is involved in the adipocyte-driven proinvasive effect. A, ZR 75.1 and 67NR cells were cultivated either alone (NT) or in the presence of IL-6, and after 3 days, they were harvested to conduct Matrigel assays in a medium containing either 0 or 10% serum. B, similarly treated ZR 75.1 cells were fixed and stained with the indicated antibodies. Nuclei were labeled with TO-PRO-3; scale bars, 40 μm. C, ZR 75.1 were cocultivated for 3 days in the presence (C) or absence (NC) of murine adipocytes. As indicated, blocking antibody directed against murine IL-6 (aIL-6) or control antibody (IgG) was added in the culture medium. Tumor cells were then harvested to conduct Matrigel invasion assays in a medium containing 10% FCS. D, 67NR and 4T1 cells were cultivated in normal medium (control), normal medium containing control immunoglobulin (control + IgG), or blocking antibody directed against murine IL-6 (control + aIL-6) conditioned medium obtained from adipocytes previously grown in the presence of tumor cells (CAA-CM), CAA-CM containing control (CAA-CM + IgG), or IL-6 blocking antibody (CAA-CM + aIL-6). After 3 days, tumor cells were harvested to conduct Matrigel invasion assay in a medium containing either 0% or 10% FCS.
traits, including overexpression of IL-6 with the noticeable absence of overexpression of TNF-α in CAAs (Fig. 5). Interestingly, the association of high levels of IL-6 in CAAs with high tumor size and enhanced local invasion reflects those traits present in obese patients (10, 16, 17). Therefore, CAAs within a context of obesity should be more prone to amplify the negative cross-talk with tumor cells that we have identified and characterized in this study. This is a hypothesis that remains to be shown in both murine and human models. Such studies will provide unique opportunities to set up specific strategies for the treatment of the subsets of patients exhibiting aggressive diseases.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References


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