Involvement of adiponectin and leptin in breast cancer: clinical and *in vitro* studies

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Abstract

Obesity is a risk factor for breast cancer development. A recent hypothesis suggests that the adipokines, adiponectin and leptin, are involved in breast cancer development. This prompted us to investigate the role of adiponectin and leptin in mammary carcinogenesis. Adiponectin receptors (AdipoR1 and AdipoR2) and leptin receptor (Ob-Rt, representing all the isoforms of Ob-R) proteins were detected by immunohistochemistry in *in situ* ductal carcinoma, invasive ductal malignancy, and healthy adjacent tissue. In addition, mRNA expression of adiponectin, AdipoR1, AdipoR2, leptin, Ob-Rt, and Ob-RI (the long isoform of Ob-R) was observed in MCF-7 breast cancer cells. Interestingly, leptin mRNA expression was 34.7-fold higher than adiponectin mRNA expression in the MCF-7 cell line. Moreover, adiponectin (10 µg/ml) tended to decrease the mRNA expression of leptin (–36%) and Ob-RI (–28%) and significantly decreased Ob-Rt mRNA level (–26%). In contrast, leptin treatment (1 µg/ml) significantly decreased AdipoR1 mRNA (–23%). Adiponectin treatment (10 µg/ml) inhibited the proliferation of MCF-7 cells, whereas leptin (1 µg/ml) stimulated the growth of cancer cells. In addition, adiponectin inhibited leptin-induced cell proliferation (both 1 µg/ml). Using microarray analysis, we found that adiponectin reduced the mRNA levels of genes involved in cell cycle regulation (mitogen-activated protein kinase 3 and ATM), apoptosis (BAG1, BAG3, and TP53), and potential diagnosis/prognosis markers (ACADS, CYP19A1, DEGS1, and EVL), whereas leptin induced progesterone receptor mRNA expression. In conclusion, the current study indicates an interaction of leptin- and adiponectin-signaling pathways in MCF-7 cancer cells whose proliferation is stimulated by leptin and suppressed by adiponectin.

Endocrine-Related Cancer (2009) 16 1197–1210

Introduction

Obesity is related to many metabolic disorders like type 2 diabetes mellitus, coronary heart disease, and hypertension, and is notably associated with an increased risk for breast cancer in postmenopausal women (Klein *et al.* 2002). Numerous factors (obesity-associated hyperinsulinemia and high adipose tissue-induced estrogen levels) have been suggested to explain the relationship between obesity and breast cancer, but none have been totally conclusive. Adipose cells were initially considered as a fat-storing tissue but are now known to have much more complex and

dynamic functions, notably acting as an endocrine organ secreting a range of adipokines, including adiponectin and leptin.

Adiponectin is a 224 amino acid-long polypeptide with several metabolic activities, including anti-diabetic, anti-inflammatory, and anti-atherogenic properties (Maeda *et al.* 1996, Kershaw & Flier 2004). This cytokine is the most abundant protein synthesized by adipose cells, with plasma concentrations ranging from 2 to 30 µg/ml (Maeda *et al.* 1996). Paradoxically, obese subjects present strongly decreased levels of circulating adiponectin

(Arita *et al.* 1999, Matsubara *et al.* 2002). Leptin is a 16 kDa polypeptide hormone encoded by the *obese* gene (Zhang *et al.* 1994) that is involved in the regulation of energy balance (De Vos *et al.* 1995), reproduction, and immunity (Mounzih *et al.* 1997, Caldefie-Chezet *et al.* 2003), and acts as a pro-inflammatory factor (Lago *et al.* 2007). Despite the numerous nonadipose sources described (Mix *et al.* 2000, Solberg *et al.* 2005), the main source of leptin in the body remains adipose tissue. In stark contrast with adiponectin, plasma leptin concentration increases with body mass index (Ruhl & Everhart 2001).

Current hypotheses suggest that adiponectin and leptin could play a role in breast cancer development. Several studies have demonstrated that low serum adiponectin levels and high serum leptin levels are associated with increased risk for breast cancer (Miyoshi *et al.* 2003, Mantzoros *et al.* 2004, Han *et al.* 2005, Chen *et al.* 2006b). Furthermore, Chen *et al.* (2006b) reported that the ratio between serum leptin and serum adiponectin correlated positively with tumor size.

To date, only two studies have examined the expression of adiponectin and its receptors (AdipoR1 and AdipoR2) in breast cancer biopsies. Takahata *et al.* (2007) observed that adiponectin mRNA was not expressed in breast tumor tissue or in breast cancer cell lines, whereas AdipoR1 and AdipoR2 mRNAs were both expressed. Korner *et al.* (2007) reported that AdipoR1 and AdipoR2 proteins were detected in 30.4 and 26.3% of breast cancer biopsies respectively. In previous studies, we demonstrated that leptin and leptin receptor (Ob-Rt) were involved in breast cancer, since both markers were expressed in mammary tumor (Caldefie-Chezet *et al.* 2005, Jarde *et al.* 2008a).

Numerous *in vitro* studies have explored the involvement of adipokines in breast cancer. Adiponectin has been shown to mediate an anti-proliferative response, while leptin enhances proliferation in MCF7, MDA-MB-231, SK-BR-3, and T47D breast cancer cells (Dieudonne *et al.* 2002, 2006, Hu *et al.* 2002, Kang *et al.* 2005, Korner *et al.* 2007, Ray *et al.* 2007, Grossmann *et al.* 2008). The activities of both adiponectin and leptin in breast cancer cell growth are in part mediated by cell cycle inhibition or stimulation respectively, (Nakayama *et al.* 2007, Saxena *et al.* 2007) and by the induction of apoptosis or down-regulation of pro-apoptotic p53 mRNA respectively (Chen *et al.* 2006a, Dieudonne *et al.* 2006).

In order to better understand the potential involvement of these two adipokines in breast cancer, we investigated the expression of adiponectin and leptin receptors in biopsies of different grades of epithelial

ductal breast lesions and adjacent normal tissue. We also evaluated *in vitro* the effect of adiponectin, leptin, or both on breast cancer cell proliferation, on the adiponectin and leptin pathways, and on the expression of molecular markers used to diagnose and gauge prognosis of breast cancer.

Materials and methods

Clinical study on breast cancer biopsies

Breast cancer biopsies

A total of 45 breast cancer tissue samples from women not treated by radiotherapy or chemotherapy were obtained from the tumor bank at the Jean-Perrin Anti-Cancer Center (Clermont-Ferrand, France). Primary breast cancer tissue samples were fixed in alcohol–formalin–acetic acid and embedded in paraffin.

Tissue classification

Diagnoses were made on paraffin-embedded 4 µm tissue sections after hematoxylin–eosin–safran staining. Tissues were classified according to histological subtypes as malignant lesions corresponding to *in situ* ductal carcinoma ($n=14$) or invasive ductal carcinoma of different grades ($n=45$). Invasive tumors were evaluated according to the SBR grade classification modified by Elston & Ellis (1991). Normal tissue adjacent to breast cancer was also analyzed ($n=40$).

Immunohistochemistry

AdipoR1, AdipoR2, and Ob-Rt protein expression was investigated by immunohistochemical staining using affinity-purified polyclonal biotinylated antibodies raised against AdipoR1 (Phoenix Pharmaceuticals, Burlingame, CA, USA), AdipoR2 (Phoenix Pharmaceuticals), and Ob-Rt (recognizing all six isoforms of Ob-R; Roche).

Sections were deparaffinated in xylene and rehydrated in graded alcohols. Antigen retrieval was performed for AdipoR1 and AdipoR2 localization. The immunohistochemical procedure was performed as previously described (Jarde *et al.* 2008a).

Microscopic examination

The immunostaining was assessed by a pathologist blinded to the clinical data. AdipoR1, AdipoR2, and Ob-Rt expression in tumor and normal adjacent tissues was classified as either negative (<5% labeled cells) or positive ($\geq 5\%$ labeled cells). Expression intensity was graded as 0 (none), 1+ (mild), 2+ (moderate), or 3+ (intense).

In vitro studies

Cell culture

The human breast cancer cell line MCF-7 was obtained from the American Type Culture Collection (ATCC). These cells were routinely cultured in a phenol-red-free RPMI 1640 medium supplemented with 10% heat-inactivated FCS, L-glutamine (2 mM), and gentamicin (50 µg/ml) at 37 °C under 5% CO₂ atmosphere.

Immunohistochemistry

MCF-7 cells were grown on plastic slides for 48 h in a 37 °C humidified atmosphere under 5% CO₂ before fixing with acetone for 10 min. Immunohistochemical detection of AdipoR1, AdipoR2, and Ob-Rt proteins was performed as described above.

Proliferation assay

MCF-7 cells were plated at a density of 5×10^3 cells in 96-well plates in a complete medium, and were allowed to adhere in an incubator. After 48 h, cells were washed with PBS and exposed to fresh medium containing 5% heat-inactivated FCS with full-length human trimeric adiponectin at 0.1, 1 (obese concentration), and 10 µg/ml (physiological concentration; Neumeier *et al.* 2006), with human recombinant leptin (Roche) at 0.01 (physiological concentration), 0.1 (obese concentration), and 1 µg/ml, or both, adiponectin and leptin (1 µg/ml each). The percentage of FCS in the medium was reduced to minimize the effect of other growth factors on breast cancer cells. After 24, 48, 72, and 96 h, cells were washed with PBS and 200 µl of a 25 µg/ml solution of resazurin in RPMI 1640 medium were added to each well. The plates were incubated for 2 h at 37 °C in a humidified atmosphere containing 5% CO₂. Fluorescence was then measured on an automated 96-well plate reader (Fluoroskan Ascent FL, Thermo Fisher Scientific, Wilmington, DE, USA) using an excitation wavelength of 530 nm and an emission wavelength of 590 nm. Under these conditions, fluorescence was proportional to the number of living cells in the well (Debiton *et al.* 2003). The cell proliferation assay was performed five times in replicates of six wells for each concentration tested.

Microarray analysis

MCF-7 cells were exposed to adiponectin (10 µg/ml) or leptin (1 µg/ml) for 96 h as described above. Total RNA was extracted using TRIzol according to the manufacturer's guidelines (Invitrogen). The quality and quantity of total RNA were evaluated by measuring the A260/A280 ratio (at least 1.9) using a NanoDrop 8000 spectrophotometer (Thermo Fisher

Scientific) coupled with gel electrophoresis. Using the TrueLabeling-AMP 2.0 kit (Superarray, Frederick, MD, USA), the mRNAs were reverse transcribed into cDNA and converted by transcription to obtain biotin-labeled cRNA using biotin-16-UTP (Roche). The biotinylated cRNA probes were purified with the ArrayGrade cRNA cleanup kit (Superarray) and then hybridized overnight to the pretreated Oligo GEArray Human Breast Cancer Biomarker Microarray (Superarray), which profiles the expression of 264 genes that are considered useful molecular markers in the diagnosis and prognosis of breast cancer. Following several washing steps, biotinylated cRNA probes hybridized to the array were detected by a Chemiluminescent Detection kit (Superarray), using alkaline phosphatase-conjugated streptavidin and CDP-Star as chemiluminescent substrate. Chemiluminescence was detected on a CCD camera Versadoc Imaging System (Bio-Rad Laboratories). Data acquisition and quantification of spot intensities were performed using GEArray Expression Analysis Suite 2.0 software (Superarray). Data evaluation included background correction (subtraction of minimum value) and house-keeping gene normalization. Each determination corresponds to the mean value $\pm$ s.d. of four independent assays to ensure reproducibility of the results. Only genes whose expression changed similarly in all four comparative analyses were considered adipokine-responsive genes ($P < 0.1$, statistical trend; $P < 0.05$, significant difference, *t*-test), as previously described (Le Corre *et al.* 2006).

Quantitative RT-PCR

The GEArray results were validated by confirming the adipokine-induced modification of gene expression using quantitative reverse transcription-PCR (qRT-PCR). We also investigated the effect of adiponectin and leptin on selected genes (adiponectin, leptin, AdipoR1, AdipoR2, Ob-Rt, and Ob-R1), which were not spotted on microarrays.

RT was conducted from 1 µg total RNA (extracted for GEArray analysis), yielding 20 µl cDNA as previously described (Goncalves-Mendes *et al.* 2004). The cDNAs were amplified using the primers summarized in Table 1. The qPCR was performed using Light Cycler Fast Start DNA Master SYBR Green I following the manufacturer's instructions, on a LightCycler Instrument (Roche Diagnostics). Sample mRNA copy numbers were extrapolated from standard curves obtained with serially diluted purified PCR products. The real-time qPCR program was 50 °C for 2 min, 95 °C for 10 min, and 50 cycles of 95 °C for 15 s

Table 1 Primers used in quantitative reverse transcription-PCR assays

Gene	Sequence	
	Forward	Reverse
Adiponectin	5'-ATGGTCCTGTGATGCTTTGA-3'	5'-GAGAGTAAATGCACCAATAAG-3'
Leptin	5'-TGAGCACCTGCTTCATGCTC-3'	5'-TGAGTGCGGTTTGACCACTG-3'
AdipoR1	5'-AATTCCTGAGCGCTTCTTTCCT-3'	5'-CATAGAAGTGGACAAAGGCTGC-3'
AdipoR2	5'-TGCAGCCATTATAGTCTCCAG-3'	5'-GAATGATTCCACTCAGGCCTAG-3'
Ob-Rt	5'-CATTTTATCCCCATTGAGAAGTA-3'	5'-CTGAAAATTAAGTCCTTGTGCCAG-3'
Ob-Rl	5'-GATAGAGGCCAGGCATTTTTTA-3'	5'-ACACCACTCTCTCTCTTTTGATTGA-3'
ACADS	5'-GCTGCCATGCTGAAGGATAA-3'	5'-GGCATCTCTGTACAGTAGCC-3'
ATM	5'-TGGATCCAGCTATTTGGTTTGA-3'	5'-CCAAGTATGTAACCAACAATAGAAGAAGTAG-3'
BAG1	5'-CACAGCAATGAGAAGCACG-3'	5'-GTGTTTCCATTTCCTTCAGAG-3'
BAG3	5'-ATGACCCATCGAGAACTGC-3'	5'-AATTGGGATGTGTCCAGGAG-3'
CIRBP	5'-GAGGGCTGAGTTTTGACACC-3'	5'-TGGGTCTCCCTGTCTTTTAC-3'
CYP19A1	5'-CAAGGTTATTTTGATGCATGG-3'	5'-AATCCTTGACAGACTTCTCAT-3'
DEGS1	5'-TTGAGGGCTGGTCTTCTGT-3'	5'-AATTGGTTTGGGGTTGATGA-3'
ESR1	5'-GTGTACAACACCCGAGGGC-3'	5'-AAACCCCCCAGCCGTTGGAG-3'
EVL	5'-TTCCGTGATGGTCTACGATG-3'	5'-GCTGGCAGTGTTGTGGTAGA-3'
HMGB3	5'-TCAGCAGCCTTCCAAGGTAT-3'	5'-CATTTGGCCCCACAAAATAG-3'
KIAA1324	5'-AACACCCACTGCTGGAATC-3'	5'-GTTTGAGGCAAGCAGAAAGG-3'
KRT18	5'-TGAGACGTACAGTCCAGTCCTT-3'	5'-GCTCCATCTGTAGGGCGTAG-3'
MAPK3	5'-TGCTGAACCTCAAGGGCTAT-3'	5'-TAGTGCTTGCCAGGGAAGAT-3'
MUC1	5'-GTGCCCCCTAGCAGTACCG-3'	5'-GACGTGCCCCTACAAGTTGG-3'
MX1	5'-AGCTCGGCAACAGACTCTTC-3'	5'-GGATGATCAAAGGGATGTGG-3'
PGR	5'-CGCGCTCTACCCTGCACTC-3'	5'-TGAATCCGGCCTCAGGTAGTT-3'
PSMD7	5'-GAATGACATTGCCATCAACG-3'	5'-GTAGGCAGCCCTAGTCTCT-3'
TP53	5'-GCGCACAGAGGAAGAGAATC-3'	5'-AGAGGAGCTGGTGTGTTGG-3'
18S	5'-GTCTGTGATGCCCTTAGATG-3'	5'-AGCTTATGACCCGCACTTAC-3'

and 60 °C for 1 min. Relative levels of gene expression were calculated by target gene levels normalized to the endogenously expressed housekeeping gene (18S rRNA). The second derivative maximum method was used to determine the crossing point (Cp) for each sample (realized in triplicate). The mean value was used to calculate the ΔC_p (Cp of the target gene minus Cp of the 18S gene). Gene expression levels were obtained according to the transformation ($2^{-\Delta C_p}$). Results were expressed as the mean $\pm$ S.E.M. of five independent experiments.

To confirm the identity of adiponectin, leptin, AdipoR1, AdipoR2, Ob-Rt, and Ob-Rl PCR products, the PCR products were sequenced in both directions using the same primers as described above for amplification and a DNA dye terminator cycle sequencing kit (Applied Biosystems, Courtaboeuf, France). Sequence analysis was performed on an Applied Biosystems model 377 DNA Sequencer.

Statistical analysis

Statistical analysis was performed using the χ^2 test for the immunohistochemical procedure and paired Student's *t*-test for *in vitro* experiments. A *P* value

of <0.05 (flagged as *) was considered as statistically significant, whereas $P < 0.1$ (flagged as #) was considered as a trend.

Results

Expression of AdipoR1, AdipoR2, and Ob-Rt in breast tissue

AdipoR1, AdipoR2, and Ob-Rt were detected by immunohistochemical analysis in the cytoplasm of invasive ductal cancer cells and adjacent normal cells (Table 2A). AdipoR1 expression was positive in 18% (8 out of 45) of breast cancer tissues and 8% (3 out of 40) of normal adjacent tissues (Fig. 1A and D). AdipoR1 appeared to be induced in breast cancer tissue versus normal tissue, but the difference was not statistically significant (χ^2 test, $P = 0.16$). AdipoR2 expression was more pronounced in malignant cells than normal tissue, since AdipoR2 was detected in 82% (37 out of 45) of tumours but in only 53% (21 out of 40) of healthy tissue (χ^2 test, $P < 0.01$; Fig. 1B and E). Ob-Rt protein was detected in 38 out of the 45 (84%) invasive ductal tissues and in 29 out of the 40 (73%) normal adjacent tissues studied, without significant difference between the two tissues (χ^2 test, $P = 0.18$; Fig. 1C and F).

Table 2 The expression of AdipoR1, AdipoR2 and Ob-Rt (A), the percentage of labelled cells, and staining intensity (B) in breast ductal cancer and in adjacent normal tissue

Total number of cases studied									
Tissue	n	AdipoR1 expression		AdipoR2 expression		Ob-Rt expression			
		n ¹	%	n ²	%	n ³	%		
(A)									
Normal tissue adjacent to breast cancer	40	3	8	21	53	29	73		
<i>In situ</i> ductal carcinoma	14	2	14	11	79	11	79		
Invasive ductal carcinoma	45	8	18	37	82	38	84		
Grade 1	13	2	15	11	85	12	92		
Grade 2	17	4	24	15	88	12	71		
Grade 3	15	2	13	11	73	14	93		
(B)									
Tissue	AdipoR1			AdipoR2			ObRt		
	Positive AdipoR1 expression n ¹	Labeled cells %	Staining intensity	Positive AdipoR2 expression n ²	Labeled cells %	Staining intensity	Positive ObRt expression n ³	Labeled cells %	Staining intensity
			1+ 2+ 3+			1+ 2+ 3+			1+ 2+ 3+
Normal tissue adjacent to breast cancer	3	55±15	3 0 0	21	80±5	16 5 0	29	85±5	18 10 1
<i>In situ</i> ductal carcinoma	2	100±0	2 0 0	11	70±10	9 2 0	11	85±10	5 6 0
Invasive ductal carcinoma	8	55±15	8 1 0	37	75±5	31 6 0	38	85±5	25 12 1
Grade 1	2	40±30	1 1 0	11	80±10	9 2 0	12	90±5	6 5 1
Grade 2	4	65±20	4 0 0	15	80±5	13 2 0	12	90±5	9 3 0
Grade 3	2	40±30	2 0 0	11	60±15	9 2 0	14	80±10	10 4 0

Figure 1 Immunohistochemical detection of AdipoR1, AdipoR2 and Ob-Rt in breast tissue. Invasive ductal cancer cells express AdipoR1 (A), AdipoR2 (B) and Ob-Rt (C). Healthy adjacent tissue presents immunopositivity for AdipoR1 (D), AdipoR2 (E) and Ob-Rt (F).

The *in situ* ductal carcinoma showed low expression of AdipoR1 (14% of cases studied) and high expression of AdipoR2 and Ob-Rt (79% of cases studied; Table 2A).

Finally, in cases expressing AdipoR1, AdipoR2, or Ob-Rt, the percentage of labeled cells and staining intensity were similar between invasive ductal carcinoma and adjacent normal tissue (Table 2B).

Expression of adiponectin, leptin, and their receptors in MCF-7 breast cancer cells

The mRNA of adiponectin, leptin, and their receptors was detected in MCF-7 cancer cells (Fig. 2A). Interestingly, leptin expression was radically higher (35-fold) than adiponectin expression. In addition, AdipoR1 expression was 15-fold higher than AdipoR2 expression. Ob-Rt expression was sevenfold higher than the expression of the full-length isoform Ob-Rl. Finally, AdipoR1 and AdipoR2 were highly significantly more expressed than Ob-Rt (120- and 8-fold higher expression respectively) and Ob-Rl (849- and 58-fold higher expression respectively; *t*-test, $P < 0.01$).

Immunohistochemical staining detected adiponectin, leptin, and their receptors in the cytoplasm of MCF-7 breast cancer cells (Fig. 2B).

Effect of adiponectin, leptin, or both adipokines on breast cancer cell proliferation

Adiponectin (10 $\mu\text{g/ml}$) significantly decreased the proliferation of MCF-7 cells by 11.1, 11.2, 14.4, and 15.6% at 24, 48, 72, and 96 h respectively versus controls (*t*-test, $P < 0.05$; Fig. 3A). At 1 $\mu\text{g/ml}$, adiponectin significantly inhibited cellular growth by

7.8 and 7.6% at 72 and 96 h respectively versus controls (*t*-test, $P < 0.05$). Moreover, the effect of adiponectin at 10 $\mu\text{g/ml}$ was significantly more pronounced at all time points analysed compared with the effects of 1 $\mu\text{g/ml}$ (*t*-test, $P < 0.05$), whereas 0.1 $\mu\text{g/ml}$ had no effect.

Leptin at 1 $\mu\text{g/ml}$ enhanced the growth of MCF-7 breast cancer cells by 10.5, 13.4, and 16.3% at 48, 72, and 96 h respectively versus controls (*t*-test, $P < 0.05$; Fig. 3B). The lower leptin concentrations (0.01 and 0.1 $\mu\text{g/ml}$) were inefficient in reducing cell proliferation.

Interestingly, adiponectin at 1 $\mu\text{g/ml}$ significantly inhibited leptin-stimulated breast cancer cell proliferation at 48, 72, and 96 h versus controls (*t*-test, $P < 0.05$; Fig. 3C).

Interaction of adiponectin and leptin pathways

These studies were performed using the most efficient concentrations of adiponectin (10 $\mu\text{g/ml}$) and leptin (1 $\mu\text{g/ml}$) on MCF-7 cancer cells that were incubated for 96 h (Fig. 4). Adiponectin significantly upregulated (88% induction) adiponectin mRNA and downregulated (−26%) Ob-Rt mRNA expression. In addition, adiponectin tended to downregulate leptin (−36%) and Ob-Rl (−28%) expression. Adiponectin treatment had no effect on AdipoR1 and AdipoR2 mRNA abundance.

Leptin significantly upregulated (+30%) Ob-Rl mRNA and downregulated (−23%) AdipoR1 mRNA expression. Leptin treatment also tended to induce leptin mRNA expression (+31%). Leptin had no effect on the expression of adiponectin, AdipoR2, or Ob-Rt mRNA.

Figure 2 Expression of adiponectin, leptin and their specific receptors in MCF-7 breast cancer cells. The expression of adiponectin, leptin and their receptors was analysed by quantitative RT-PCR as described in Materials and Methods (A). Bars are the means $\pm$ S.E.M. obtained from five independent experiments. The protein expression of adiponectin, leptin and their receptors was investigated by immunohistochemistry (B).

Effect of adipokines on gene expression

We used microarray analysis to explore the effects of adiponectin and leptin on numerous pathways involved in breast cancer development. Table 3 summarizes the genes showing significant differences ($P < 0.05$) or statistical trends ($P < 0.1$) versus controls. We identified 15 genes that were altered by adiponectin treatment and 3 genes that were modified by leptin incubation. These results were confirmed by qRT-PCR (Fig. 5).

Discussion

Obesity is an established risk factor for breast cancer in postmenopausal women (Chu *et al.* 1991, Lahmann *et al.* 2004). This study provides evidence that adiponectin and leptin, which are both adipocyte-secreted hormones that are deregulated in obesity, may be involved in breast cancer development.

Using an immunohistochemical approach, we detected AdipoR1, AdipoR2, and Ob-Rt in breast cancer tissue and in normal tissue adjacent to malignant lesions. However, AdipoR2 and Ob-Rt were expressed in more than 80% of breast cancer tissues studied, in contrast with AdipoR1 that was found in only 18% of cancer tissues. Korner *et al.* (2007) recently have investigated adiponectin receptor expression in breast cancer biopsies and showed that AdipoR1 and AdipoR2 were expressed in about 30% of cancer tissues studied. Our study suggests that AdipoR2 may be the effective receptor for *in vivo* adiponectin signaling in cancer tissue. However, this notion remains uncertain, since other studies obtained conflicting results. Nakayama *et al.* (2007) noted that siRNA against AdipoR1 completely abrogated the growth inhibition of adiponectin in T47D cells, and limited the anti-proliferative activity of adiponectin in MDA-MB-231 cells to about 50%. In contrast, two studies reported that siRNA against AdipoR1,

Figure 3 Effect of adiponectin, leptin and both adipokines on MCF-7 breast cancer cell proliferation. Breast cancer cells were exposed to adiponectin (A), leptin (B) or both adipokines (C) for the times indicated. Bars are the means $\pm$ S.E.M. obtained from five determinations. *Significant difference compared to control ($P < 0.05$, t -test).

AdipoR2, or both had no effect on adiponectin-mediated inhibition of the proliferation in MDA-MB-231 cells (Wang *et al.* 2006, Dos Santos *et al.* 2008).

We also observed that AdipoR2 expression was higher in breast cancer tissue than in normal adjacent tissue. A recent study has reported higher levels of adiponectin receptors in colorectal carcinomas versus nontumor specimens (Williams *et al.* 2008). In another investigation, AdipoR1 expression tended to be more pronounced in breast malignant cells than normal tissue (Korner *et al.* 2007). The relevance of these observations is still unclear, but may indicate an upregulation of adiponectin receptors in carcinogenesis.

In the current study, adiponectin mRNA and protein were identified in MCF-7 breast cancer cells for the first time, whereas Takahata *et al.* (2007) did not

observe adiponectin mRNA expression in breast cancer cell lines. Interestingly, leptin mRNA expression in MCF-7 cells was dramatically higher (34.7-fold) than adiponectin mRNA expression. This finding is in accordance with our previous immuno-histochemical study, where adiponectin-positive staining was noted in 15% of the 45 breast cancer tissues studied and leptin-positive staining was observed in 80% of the 45 breast cancer tissues (Jarde *et al.* 2008b). These results suggest that leptin is much more abundant in the local breast cancer environment than adiponectin. As previously described (Dieudonne *et al.* 2002, Nakayama *et al.* 2007), adiponectin and leptin receptors were identified in the MCF-7 cells, with the latter being significantly lower. Interestingly, we noted that adiponectin induced adiponectin mRNA ($P < 0.05$) and that leptin increased leptin ($P < 0.1$) and Ob-R1 ($P < 0.05$) mRNA expression in MCF-7 cells. Chen *et al.* (2006a) also reported that leptin treatment induced leptin and Ob-R1 mRNA expression in ZR-75-1 breast cancer cells. Thus, these adipokines may autoregulate their pathways to amplify the signal. Moreover, in the present study, adiponectin down-regulated the leptin pathway by decreasing leptin, Ob-Rt, and Ob-R1 mRNA expression in MCF-7 cancer cells. Similarly, AdipoR1 mRNA expression was reduced by leptin treatment, a finding that is similar to a recent study where leptin treatment decreased AdipoR1 and AdipoR2 mRNA expression in MDA-MB-231 cells (Dos Santos *et al.* 2008).

Therefore, these adipokines appear to exert antagonistic activities by downregulating each other's signaling pathway. These results are particularly important where obese women are concerned, since hyperleptinemia can decrease the sensitivity of cancer cells to adiponectin via downregulation of AdipoR1

Figure 4 Effect of adiponectin and leptin on their own pathways. MCF-7 breast cancer cells were exposed to adiponectin (10 μg/ml) or leptin (1 μg/ml) for 96 h. The expression of adiponectin, leptin and their receptors was analysed by quantitative RT-PCR as described in Materials and methods. Bars are the means $\pm$ S.E.M. obtained from five determinations. *Significant difference compared to control ($P < 0.05$, t -test), #Statistical trend compared to control ($P < 0.1$, t -test).

Table 3 Expression of genes altered by adiponectin or leptin treatment. MCF-7 breast cancer cells were exposed to adiponectin (10 μ g/ml) or leptin (1 μ g/ml) for 96 h and gene expression was quantified using microarray ($n=4$)

Protein function and gene name	Gene symbol	Accession number	Adiponectin Fold change	Leptin Fold change
Cell-cycle regulation				
Ataxia telangiectasia mutated	ATM	NM_000051	0.79 [†]	NS
Mitogen-activated protein kinase 3	MAPK3	NM_002746	0.85*	NS
Apoptosis				
BCL2-associated athanogene	BAG1	NM_004323	0.72*	NS
BCL2-associated athanogene 3	BAG3	NM_004281	0.71 [†]	NS
Myxovirus resistance 1, interferon-inducible protein p78	MX1	NM_002462	1.53*	NS
Tumor protein p53	TP53	NM_000546	NS	0.79 [†]
Transcription factors and regulators				
Estrogen receptor 1	ESR1	NM_000125	0.75 [†]	NS
High-mobility group box 3	HMGB3	NM_005342	0.81 [†]	NS
Progesterone receptor	PGR	NM_000926	NS	1.38 [†]
Potential diagnostic markers				
Cytochrome P450, family 19, subfamily A, polypeptide 1	CYP19A1	NM_000103	0.77 [†]	NS
Keratin 18	KRT18	NM_000224	0.85 [†]	NS
Mucin 1, cell surface associated	MUC1	NM_001018016	NS	2.31*
Potential prognostic markers				
Acyl-coenzyme A dehydrogenase, C-2 to C-3 short chain	ACADS	NM_000017	0.64*	NS
Cold-inducible RNA-binding protein	CIRBP	NM_001280	0.78 [†]	NS
Degenerative spermatocyte homolog 1, lipid desaturase	DEGS1	NM_003676	0.55*	NS
Enah/Vasp-like	EVL	NM_016337	0.74 [†]	NS
KIAA1324	KIAA1324	NM_020775	0.62 [†]	NS
Proteasome 26S subunit 7	PSMD7	NM_002811	0.75 [†]	NS

Significant differences from controls are flagged as * ($P < 0.05$, t -test), and statistically trends versus controls are flagged as [†] ($P < 0.1$, t -test). NS, not significant.

Figure 5 qRT-PCR validation of candidate genes. Data obtained from microarray experiments (open bars) and qRT-PCR analysis (solid bars) are shown. *Significant difference compared to control ($P < 0.05$, t -test), #Statistical trend compared to control ($P < 0.1$, t -test).

and upregulation of the leptin pathway. Furthermore, obesity-associated hypoadiponectinemia may limit the negative feedback of adiponectin on leptin signaling in cancer cells.

We also found that adiponectin inhibited the growth of MCF-7 breast cancer cells, whereas leptin induced a proliferative response. Similar results have been observed in previous studies using MCF-7, MDA-MB-231, SK-BR-3, and T47D breast cancer cells (Kang *et al.* 2005, Dieudonne *et al.* 2006, Nakayama *et al.* 2007). We also noted that adiponectin inhibited the proliferative activity of leptin, which underlines the antagonistic activity of these adipokines in breast cancer. Similar results were obtained in preneoplastic colon epithelial cells (Fenton *et al.* 2008).

To determine the potential pathways involved in the regulation of cell proliferation by these adipokines, we evaluated the effect of adiponectin and leptin on numerous molecular markers of breast cancer using microarray and qRT-PCR analysis. Although an induction of CYP19A1 (aromatase), an enzyme-catalyzing estrogen biosynthesis, and the activation of estrogen receptor- α (ESR1) in MCF-7 cancer cells (Catalano *et al.* 2003, 2004) by leptin were recently described, the mRNA expression of this enzyme was not altered by leptin in the current study. However, we observed that adiponectin downregulated ER α mRNA and tended to downregulate aromatase mRNA. Recently, Treeck *et al.* (2008) have showed that adiponectin increased the mRNA expression of ER β 5 in MCF-7 cells, a protein known to negatively interfere with the transcriptional activity of ER α . These results suggest a potential anti-proliferative role of adiponectin in breast cancer, mediated by the decreased *in situ*

estradiol production and decreased sensitivity of cancer cells. We also observed that leptin induced progesterone receptor mRNA expression in MCF-7 cells. The relationship between progesterone and leptin is unclear, but an interaction between these two pathways is possible, since progesterone-induced inhibition of Ob-R1 mRNA has been described in human endometrium (Koshiba *et al.* 2001).

Mitogen-activated protein kinase 3 (MAPK3) belongs to the family of signal transduction intermediates leading to cell proliferation and is suppressed by adiponectin. Overexpression of MAPK has been observed in breast cancer and metastatic cells (Sivaraman *et al.* 1997). Recently, Dieudonne *et al.* (2006) have observed that adiponectin inhibited MAPK phosphorylation in MCF-7 cells. These results suggest that inhibition of MAPK3 expression and/or phosphorylation may be one of the numerous factors explaining the anti-proliferative activity of adiponectin in cancer cells. The physiological role of adiponectin-mediated inhibition of the ATM pathway remains unclear, because this protein induces the activation of cell-cycle checkpoints and the initiation of DNA repair, and may lead to the induction of apoptosis (Prokopcova *et al.* 2007).

In addition, adiponectin may be involved in the induction of apoptosis by downregulating BAG1, an anti-apoptotic factor, and upregulating MX1 mRNA, a gene engaged in the induction of apoptosis. However, the relationship between adiponectin and the initiation of apoptosis has not been clearly established, and numerous studies have obtained conflicting results. Three recent studies have failed to detect apoptosis in adiponectin-incubated MCF-7, MDA-MB-231,

and T47D cells (Wang *et al.* 2006, Arditi *et al.* 2007, Nakayama *et al.* 2007), whereas other studies demonstrated a stimulating effect in MCF-7 and MDA-MB-231 cells (Kang *et al.* 2005, Dieudonne *et al.* 2006, Wang *et al.* 2006). Furthermore, Grossmann *et al.* noted that adiponectin activated caspase-8 and PARP in MCF-7 cells (Grossmann *et al.* 2008). Focusing on leptin, we noted that the expression of TP53, an apoptosis-inducible factor, was decreased by leptin treatment. Similar results were obtained in ZR-75-1 breast cancer cells (Chen *et al.* 2006a). This suggests that leptin may stimulate cell proliferation via downregulation of apoptosis in breast cancer cells.

Adipokines may interact with potential diagnosis markers in MCF-7 cells. KRT18 is a gene encoding for keratin 18 components of the intermediate filaments of the cytoskeleton. Overexpression of KRT18 abrogated the ability of MDA-MB-231 to form tumors and metastasize in athymic mice (Buhler & Schaller 2005). In addition, reducing the expression of KRT18 increased the aggressiveness of breast cancer cell lines. Adiponectin downregulated KRT18 mRNA, and this result suggests that adiponectin may not only be considered as an anti-proliferative factor. Moreover, the treatment of MCF-7 cancer cells with leptin induced the expression of another diagnostic marker gene, MUC1. MUC1 expression is related to the aggressive behavior of human neoplasm and to poor patient outcome (Yonezawa *et al.* 2008). MUC1 may function as an anti-adhesion molecule enabling invasion into surrounding tissues (Yonezawa *et al.* 2008).

The present study also provides evidence for an interaction between adiponectin and potential prognostic markers. ACADS proteins are a family of mitochondrial enzymes catalyzing the initial rate-limiting step in the β -oxidation of fatty acyl-CoA. Numerous genes associated with fatty acid metabolism, including ACADS, were upregulated in colorectal cancer versus healthy tissue (Yeh *et al.* 2006), suggesting that ACADS are involved in carcinogenesis. Moreover, the partial loss of the prognostic marker DEGS1, an enzyme that catalyzes the desaturation of dihydroceramide into ceramide, induced an accumulation of dihydroceramide and led to the inhibition of proliferation with cell-cycle arrest in human neuroblastoma cells (Kravaka *et al.* 2007). In our conditions, adiponectin significantly decreased ACADS and DEGS1 mRNA expression in MCF-7 cancer cells. There appears to be a close relationship between adiponectin and fatty acids that may at least

partially explain the anti-proliferative effect of this adipokine in cancer cells.

Finally, we noted that adiponectin significantly decreased the expression of KIAA1324 mRNA and tended to decrease EVL mRNA expression in MCF-7 cells. KIAA1324, an estrogen-induced gene, is overexpressed in MCF-7 cells compared with non-malignant cell lines (Bauer *et al.* 2004). EVL, which belongs to the enabled/vasodilator-stimulated phosphoprotein family, regulates the actin cytoskeleton. Upregulation of EVL correlates positively with the clinical stages of breast cancer (Hu *et al.* 2008). In addition, overexpression of EVL promoted the motility of MCF-7 cells (Hu *et al.* 2008).

In our study, the effects of these adipokines on cell proliferation and gene expression were modest. However, it is important to note that obesity is associated with both hypoadiponectinemia and hyperleptinemia, which suggests a cumulative effect on breast cancer cell growth via proliferative effects of leptin and a decreased anti-proliferative activity of adiponectin. During breast carcinogenesis, the slightly elevated proliferation capacity explained by the dysregulation of adiponectin and leptin in obesity may be highly relevant. These adipokines also alter gene expression of various proteins relevant in breast cancer development.

In conclusion, this study identified new gene targets of adiponectin that may be relevant for the diagnosis and/or therapy of breast cancer.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This work received financial support from the local office of the French Anti-Cancer League (Ligue Contre le Cancer du Puy-de-Dôme). The study was supported by a grant from the Deutsche Forschungsgemeinschaft (BU 1141/3-2). Thierry Jardé was awarded a fellowship from the French Ministry of Research and Technology.

Acknowledgements

The authors thank Prof. C Forestier (Laboratoire de Bactériologie, UFR Pharmacie, Clermont-Ferrand, France) and Prof. G Picard (Laboratoire de Génétique des Eucaryotes et Endocrinologie Moléculaire, Université Blaise Pascal, Clermont-Ferrand, France) for their expert assistance.

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