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Total and High-Molecular-Weight Adiponectin in Breast Cancer: *In Vitro* and *in Vivo* Studies

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Background: Obesity is a major risk factor for breast cancer. We hypothesized that obesity-induced decreases in total and/or high-molecular-weight (HMW) adiponectin levels may underlie this association.

Methods: We measured serum total and HMW adiponectin in a hospital-based case-control study of 74 female breast cancer patients and 76 controls. In parallel, expression of adiponectin and its receptors AdipoR1/R2 were measured in tissue samples using RT-PCR, and protein expression of AdipoR1/R2 was localized and quantified using immunohistochemistry. Finally, we documented AdipoR1/R2 expression in several breast cancer cell lines and studied adiponectin signaling and the effect of adiponectin on proliferation in the T47D breast cancer cell line *in vitro*.

Results: Women with the highest adiponectin levels had a 65% reduced risk of breast cancer ($P = 0.04$). This association became stronger after adjustment for age, body mass index, and hormonal and reproductive factors ($P = 0.02$). Modeling HMW instead of total adiponectin produced similar results and did not offer any additional predictive value. Breast cancer cells expressed AdipoR1/R2 but not adiponectin. Expression of AdipoR1, but not AdipoR2, was higher in tumor tissue than both adjacent and control tissues. Exposure of T47D cells to adiponectin significantly inhibited the percentage of viable cells to 86% and proliferation to 66% but had no effect on apoptosis. These effects were associated with activation of ERK1/2 but not AMP-activated protein kinase or p38MAPK.

Conclusion: These studies suggest that adiponectin may act as a biomarker of carcinogenesis and may constitute a molecular link between obesity and breast cancer. (***J Clin Endocrinol Metab* 92: 1041–1048, 2007**)

OBESITY HAS BEEN associated with the development of several malignancies, particularly hormone-dependent gynecological cancers such as endometrial, ovarian, and breast cancer (1, 2). It has been proposed that hormonal changes due to obesity-associated hyperinsulinemia and/or increased estrogen production in adipose tissue may underlie the observed relationship between obesity and cancer (1, 2). These factors do not fully explain the observed biological variability, however, and the precise underlying mechanisms are not completely understood.

Adipose tissue is now widely considered to be an active endocrine organ, secreting several bioactive adipokines, including adiponectin, that exert distinct metabolic functions (3, 4). Adiponectin has profound antiatherogenic, antidiabetogenic, and antiinflammatory actions, thereby protecting against the development of obesity-related disorders such as cardiovascular disease and diabetes (5). Beyond these metabolic effects, adiponectin has also been shown to suppress proliferation of macrophages (6) and endothelial cells and has recently been shown to be associated with impaired growth of osteosarcomas due to decreased neovascularization (7). Because mammary epithelial cells are embedded in adipose tissue, the immediate contact between epithelial cells and adjacent adipocytes allows for direct functional interactions between adipose tissue and mammary cells in a paracrine manner in addition to its exposure to circulating hormones (8, 9). Thus, we recently proposed that decreased adiponectin levels may partially explain the increased risk of breast cancer in obesity (10, 11). In support of this hypothesis, several case-control studies reported decreased adiponectin levels in patients with breast cancer (10–13). It remains unknown, however, whether high-molecular-weight (HMW) adiponectin has a better predictive value than total adiponectin, adiponectin receptors are differentially expressed in breast cancer cells, and adiponectin may have potential direct actions on cancer pathophysiology at the cellular level.

Thus, the objectives of the present study were to investigate associations of total and HMW adiponectin concentrations with breast cancer and controlling for established risk factors, identify adiponectin receptors (AdipoR1/R2) in breast cancer cell lines and tissue samples studied *ex vivo*, and evaluate direct effects of adiponectin on breast cancer cells *in vitro*.

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Abbreviations: AdipoR, Adiponectin receptor; AMPK, AMP-activated protein kinase; BMI, body mass index; BrdU, bromodeoxyuridine; ER, estrogen receptor; FBS, fetal bovine serum; HMW, high molecular weight; OR, odds ratio; T-TBS, Tris-buffered saline with Tween 20; WST1, water-soluble tetrazolium-1.

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Subjects and Methods

Clinical studies

Study subjects. We recruited 74 patients with newly diagnosed breast cancer before systemic treatment at the Theaghenio Cancer Hospital (Thessaloniki, Greece). Diagnosis was based on pathohistological confirmation of breast cancer in all patients. Patients with previous or present neoplastic disease at any other site, previous breast cancer, other major chronic disease, or use of antidiabetic drugs were excluded from the study. Histological classification revealed 58 ductal carcinomas, 13 lobular carcinomas, and three otherwise differentiated carcinomas (medullary, mucinous, papillary, or tubular). The 76 control subjects were concurrently recruited among women undergoing routine preventive breast cancer screening who displayed no evidence of breast cancer using identical diagnostic criteria on the basis of mammogram and/or biopsy tests. Follow-up information was available from hospital charts of all participants. The study protocol was approved by the Theagenion Cancer Hospital Scientific Committee and subsequently by Beth Israel Deaconess Medical Center. All participants provided written informed consent.

Serum measurements. Fasting blood samples were obtained in the morning and immediately centrifuged and stored at −70 C until assaying. Commercially available RIAs were used to determine serum levels of total adiponectin, insulin, and leptin (Linco Research, St. Charles, MO), as well as total and HMW adiponectin (ALPCO Diagnostics, Salem, NH). All samples were measured together in one assay. Medical and technical assistants were blinded with respect to case or control status.

Relative expression of adiponectin and AdipoR1/R2 by RT-PCR. Among study participants who provided serum samples, 44 of the 74 cases and 26 of the 76 controls also consented to provide tissue samples. Tissue samples were obtained by needle biopsy of tumor tissue itself (referred to as tumor) and from adjacent macroscopically normal tissue (adjacent) in breast cancer patients, and nontumor tissue samples were obtained from the control group (controls) as well. The excised specimens were immediately dissected before freezing and stored in liquid nitrogen and RNAlater (Ambion, Inc., Austin, TX) medium to prevent degradation of RNA. Clinical and histopathological characteristics were recorded at the time of primary surgery according to standard diagnostic classification (14) with ductal carcinoma in 32, lobular carcinoma in six, and otherwise differentiated carcinomas in four patients. Of nontumor tissue samples from control subjects, 10 were fibroadenoma, three papillomatosis, and 13 fibrocystic.

Adiponectin and AdipoR1/R2 expression of these samples were measured using real-time quantitative PCR (RT-PCR) with human-specific gene expression assays (Applied Biosystems Inc., La Jolla, CA). RT-PCRs were performed, in triplicate, in an automated Stratagene Mx3000 QPCR system (Stratagene, La Jolla, CA) using Taqman Universal PCR master mix (Applied Biosystems).

AdipoR1/R2 expression assessment by immunohistochemistry. In an independent experiment, a separate sample of 96 tumor and 25 nontumor breast tissues in the form of archival paraffin-embedded tissue array slides were available for immunohistochemical analysis. Immunohistochemical staining for AdipoR1/R2 was performed on 30 specimens (15 benign lesions and 15 adenocarcinomas) in sections of two representative 4-μm paraffin blocks as described previously (15). An additional 81 breast cancer and 10 healthy 5-μm, formalin-fixed tissue samples mounted to standard silanized slides (Imgenex, San Diego, CA) were also available and analyzed according to prior documentation (16). Expert pathologists evaluated the intensity and distribution of positive staining for all samples on a scale from 0 to +++.

Experimental studies

Chemicals and reagents. Cell culture media, supplements, antibiotics, and fetal bovine serum (FBS) were obtained from Life Technologies Inc. (Karlsruhe, Germany). Other chemicals and reagents were purchased from Sigma (Munich, Germany). Human adiponectin (produced in HEK293 cells) was obtained from Biovendor (Heidelberg, Germany). Antibodies against phosphorylated and nonphosphorylated protein kinases were obtained from Cell Signaling Technologies (Beverly, MA).

Cell culture. Cell culture experiments were performed in T47D cells (American Type Culture Collection, Manassas, VA) that were grown in RPMI 1640 medium supplemented with glucose to total concentration of 4.5 g/liter, 10 mM HEPES, 1 mM sodium pyruvate, 0.01 mg/ml bovine insulin, FBS 10%, penicillin 100 U/liter, streptomycin 0.1 mg/liter, and L-glutamine 2 mM.

RT-PCR and quantitative RT-PCR from cell lines. The procedures used for conventional (Roche, Mannheim, Germany) and quantitative (TaqMan) RT-PCR have been reported in detail previously (15). For absolute quantification of adiponectin receptor cDNA, full-length AdipoR1/R2 cDNA was amplified by PCR from MCF-7 cDNA using the following primer pairs containing adapter sequences for restriction sites: AdipoR1 forward, gaatgcggccgcctcttcccacaaaggatctgtg, reverse, ttggtacctcagagaagggtgtcatcag; AdipoR2 forward, gaatgcggccgccaacgagccaacagaaaaccg, reverse, ttggtacctcacagtgcatcctcttcactgc. The PCR product was digested with *Not*I and *Kpn*I (New England Biolabs, Ipswich, MA) and ligated into the eukaryotic expression vector p3xFLAG-myc-CMV 26 (Sigma Aldrich) using the rapid DNA ligation kit (MBI Fermentas, Burlington, Canada). The plasmids were applied as template for standard curve in a serial dilution of 10^1 to 10^7 copies confirming a sensitivity of TaqMan PCR of 100 copies and linearity of the standard curve between 10^2 and 10^7 copies ($R^2 > 0.99$). All samples were run in triplicate. Amplification of 18s rRNA (Applied Biosystems) was used as an internal control and the amounts of target gene were normalized to the amount of 18s.

Proliferation experiments. T47D breast cancer cells were seeded into 96-well plates at a density of 7500 cells/well in 100 μl of culture medium in 6-well plates and were allowed to adhere overnight. Optimal cell number and exposure time for proliferation experiments was determined before actual stimulation tests with adiponectin. Cells were treated with recombinant human eukaryotically expressed adiponectin at a concentration of 20 μg/ml for 48 h in medium with (10% FBS) or without serum (0.1% BSA replacement). Subsequently cells were washed with PBS and cell number/viability was assessed using water-soluble tetrazolium-1 (WST1) test (Roche), and cell number/proliferation was assessed using bromodeoxyuridine (BrdU) colorimetric ELISA (Roche). In the initial experiments, standard dilutions of cell number were measured in parallel to control for discrimination and sensitivity of the assays. Both WST1 and BrdU proliferation tests were confirmed to show a linear correlation with cell number in prior experiments using this cell line. For each stimulation, five wells were measured. After correction of the OD to basal medium without cells, results are given as relative OD of stimulated cells to cell number in respective basal medium. For investigation of apoptosis, 200,000 cells were seeded in 24-well plates and allowed to adhere overnight. After stimulation with adiponectin 15 μg/ml and tamoxifen 1 mM as positive control, cells were stained with Annexin V and propidium iodide (Invitrogen, Karlsruhe, Germany). The percentage of stained cells was quantified by fluorescence-activated cell sorter (EPICS; Beckman/Coulter, Krefeld, Germany).

Assessment of signal transduction by Western blotting. To determine the effect of adiponectin on signaling cascades, 10^6 cells were allowed to adhere in normal culture medium and were then starved in serum-free medium overnight. Cells were washed with PBS and exposed to adiponectin at a concentration of 15 μg/ml for 15 min. For protein preparation, cells were rinsed with ice-cold PBS, and lysis was performed in buffer containing 50 mM HEPES, 100 mM NaCl, 10 mM EDTA, 1% Triton X-100, 1 μg/ml leupeptin, 1 mM NaF, 1 mM NaO vanadate, and protease inhibitors (Complete; Roche). Lysates were centrifuged at 20,000 rpm at 4 C, and protein content was determined using the Bradford method (Bio-Rad, Munich, Germany).

For Western blotting, 20 μg of total protein were incubated in sodium dodecyl sulfate loading buffer containing β-mercaptoethanol, boiled for 5 min, and immediately subjected to 10–12% SDS-PAGE. Fractionated proteins were transferred to a nitrocellulose membrane (Bio-Rad) and transfer was controlled by Ponceau staining. To block unspecific antibody binding, the membrane was incubated in 5% dry milk or 5% BSA in Tris-buffered saline containing 0.1% Tween 20 (T-TBS) for 60 min. Specific antiserum against phosphorylated isoforms of signaling kinases were diluted 1:1000 in T-TBS with 5% dry milk or BSA. The membrane was exposed to this primary antiserum [phosphorylated (p)-AMP-activated protein kinase (AMPK), p-ERK1/2, p-p38MAPK, p-Akt] over-

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night at room temperature under continuous shaking. After washing in T-TBS, the membrane was incubated with goat antirabbit immunoglobulin labeled with horseradish peroxidase (Pierce, Bonn, Germany) at a dilution of 1:5000 in T-TBS with 5% dry milk. After washing, the immune complex was detected using a commercial ECL detection system (Bio-Rad). After stripping of membranes in 2% sodium dodecyl sulfate in 60 mM Tris with 100 mM mercaptoethanol, immunoblotting for the nonphosphorylated isoform was performed following the same protocol. Quantification was achieved by determining the OD of at least two exposure times of a minimum of three independent blots from independent cell culture experiments using the Multianalyst imager and software (Fluor-S multiimager; Bio-Rad).

Statistical analyses

Descriptive characteristics of the group variables are expressed as mean ± SD in the tables and mean ± SE in the figures. Differences among continuous measures were determined using unpaired *t* tests with logarithmic transformation of hormonal variables. Comparisons of categorical variables were conducted using χ^2 tests. For serum analysis, data were modeled using multiple logistic regression with case or control status as the outcome variable. Adiponectin levels were analyzed in quartile groups based on distributions within the control population. We tested for interaction of body mass index (BMI) and menopausal status on adiponectin levels by testing for significance of the multiplicative interaction terms in the models. Statistical analyses were performed using SPSS 11.5 (SPSS Inc., Chicago, IL). Statistical significance was determined using a two-sided level of $\alpha = 0.05$.

Results

Serum adiponectin levels and breast cancer

Ages ranged from 39 to 82 yr for breast cancer cases and 30 to 80 yr for controls. Cases had significantly higher mean age and tended to report a later age of menopause and age at first birth, compared with controls (Table 1). There were no significant differences between groups on BMI or other risk factors. Cases had 21.6% lower total and 12.3% lower HMW adiponectin than control subjects but had similar insulin and leptin levels. Women who consented to tissue biopsy tended to be younger than those who provided only serum samples but had similar measures of other risk factors.

In age-adjusted models, women in the second highest and highest quartiles of adiponectin had 54 and 65% reduced risk of breast, respectively, compared with women in the bottom two quartiles (Table 2). Adjusting further for classical breast cancer risk factors and insulin (to control for differences in insulin resistance) and leptin (to account for variation in fat mass) strengthened the association, with risk reductions of 62% for quartile 3 and 77% for quartile 4. A similarly reduced risk of breast cancer was observed when HMW adiponectin was modeled in place of total adiponectin.

To determine whether the effect of adiponectin on breast cancer risk is consistent with respect to BMI and menopausal status, we tested for interaction by these factors on risk of breast cancer. Interaction terms of adiponectin with BMI and menopausal status did not achieve statistical significance ($P = 0.08$ for BMI and $P = 0.10$ for menopausal status), but because these results are suggestive of a potential effect, exploratory stratified analyses were performed. These results showed a stronger beneficial effect of adiponectin in premenopausal [odds ratio (OR) = 0.42, $P = 0.03$] and obese (OR=0.47, $P = 0.006$) women, compared with postmenopausal (OR = 0.88, $P = 0.47$) and nonobese women (OR = 0.85, $P = 0.98$). Excluding metastatic cases and/or restricting the sample to ductal carcinomas did not appreciably alter

TABLE 1. Demographic, anthropometric, and clinical data of breast cancer patients and controls, expressed as mean ± SD or n (%)

Variable	Serum samples			Tissue biopsies		
	Cases (n = 74)	Controls (n = 76)	*P* value	Cases (n = 42)	Controls (n = 26)	*P* value
Case characteristics						
Metastasis	9 (12.2)			5 (11.9)		
Stage						
I	19 (25.6)			9 (16.6)		
II	32 (43.2)			19 (42.8)		
III	14 (18.9)			9 (21.4)		
IV	9 (12.2)			5 (11.9)		
ER+/PR+	40 (54.9)			27 (64.2)		
ER+/PR−	11 (14.9)			7 (16.6)		
ER−/PR+	3 (4.0)			1 (2.3)		
ER−/PR−	17 (23.0)			7 (16.6)		
Descriptive characteristics						
Age (yr)	62.5 ± 11.6	55.6 ± 11.6	0.004	59.4 ± 12.8	49.2 ± 14.1	0.003
BMI (kg/m^2)	29.1 ± 4.6	29.8 ± 6.1	0.40	29.5 ± 5.3	28.4 ± 6.2	0.43
Risk factors						
Family history of breast cancer	5 (6.8)	4 (5.3)	0.70	5 (7.3)	1 (3.8)	0.38
Family history of any cancer	20 (27)	21 (27.6)	0.93	8 (11.8)	4 (15.4)	0.42
Age at menarche (yr)	12.9 ± 1.3	12.9 ± 1.5	0.50	12.9 ± 1.4	12.8 ± 1.4	0.68
Age at first birth (yr)	24.6 ± 4.0	23.4 ± 4.4	0.09	24.6 ± 4.1	24.9 ± 4.5	0.81
Age at menopause (yr)	49.3 ± 4.0	47.6 ± 5.1	0.04	48.8 ± 3.7	47.7 ± 3.1	0.35
Premenopausal	13 (17.6)	20 (26.3)	0.24			
Obese (BMI ≥ 30 kg/m^2)	35 (47.3)	34 (44.7)	0.87			
Hormones						
Adiponectin (μg/ml)	9.1 ± 4.0	11.3 ± 4.7	0.007			
HMW adiponectin (μg/ml)	3.8 ± 2.3	4.3 ± 2.5	0.09			
Leptin (ng/ml)	11.0 ± 4.9	11.3 ± 5.2	0.81			
Insulin (μUI/ml)	13.0 ± 6.9	14.4 ± 8.3	0.30			

PR, Progesterone receptor.

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TABLE 2. Odds ratio for risk of breast cancer by quartile of adiponectin concentration for all subjects (n = 150)

	Q1 and Q2	Q3	Q4	*P* value
Total adiponectin, range (μg/ml)	0.13–9.95	10.13–15.37	15.46–20.04	
Model 1	1.0	0.46 (0.19–1.13)	0.35 (0.14–0.88)	0.04
Model 2	1.0	0.46 (0.19–1.13)	0.35 (0.14–0.87)	0.04
Model 3	1.0	0.40 (0.16–1.03)	0.30 (0.11–0.80)	0.03
Model 4	1.0	0.37 (0.14–1.00)	0.28 (0.10–0.75)	0.02
Model 5	1.0	0.38 (0.14–1.04)	0.23 (0.08–0.66)	0.02
HMW adiponectin, range (μg/ml)	0.60–3.74	3.76–5.66	5.80–15.24	
Model 1	1.0	1.02 (0.46–2.27)	0.30 (0.11–0.82)	0.05
Model 2	1.0	1.02 (0.46–2.29)	0.31 (0.11–0.84)	0.05
Model 3	1.0	0.95 (0.42–2.15)	0.29 (0.10–0.83)	0.06
Model 4	1.0	0.77 (0.33–1.83)	0.25 (0.09–0.75)	0.04
Model 5	1.0	0.78 (0.32–1.88)	0.27 (0.09–0.83)	0.07

Model 1: adjusted for age; model 2: adjusted additionally for BMI; model 3: adjusted additionally for age at menarche, nulliparous status, menopausal status, and family history of breast cancer; model 4: adjusted additionally for insulin; model 5: adjusted additionally for leptin. Q, Quartile.

reported associations. Stage of disease was not associated with adiponectin concentration ($P = 0.17$).

Tissue expression of adiponectin and adiponectin receptor mRNA

Adiponectin expression in breast cancer patients was substantially higher in the adjacent adipose tissue, compared with tumor tissue, in which expression was only marginal (Fig. 1). Adiponectin expression was 3.3 times higher in adjacent case tissue, compared with control breast tissue. AdipoR1 expression was also markedly increased in the adjacent tissue of patients, compared with control tissues, and was even more increased in tumor tissues, whereas AdipoR2 was not significantly increased in adjacent or tumor tissue, compared with control tissue.

Immunohistochemistry analysis for localized adiponectin protein expression in 96 tumor and 25 healthy mammary tissue samples revealed a strong positive signal in epithelial and ductal cells for AdipoR1 (Fig. 2). AdipoR1 expression was marginally more pronounced in malignant cells than normal tissue, with 30.4% of cancerous tissues *vs.* 13.0% of healthy tissues showing strong positive signals ($P = 0.09$). Staining against AdipoR2 revealed no significant difference in the signal between malignant and normal tissue (strong expression in 26.3% of cancerous and 29.2% of healthy tissue, $P = 0.78$).

Adiponectin receptor expression in breast cancer cell lines

We identified the classical AdipoR1/R2 receptors and the putative T-cadherin receptor (17) in several breast cancer cell (MCF-7, MDA MB 231, MDA MB 435, MDA MB 436). None of the cell lines showed intrinsic expression of adiponectin itself. We quantified the ratio of AdipoR1/R2 receptor mRNA expression. In accordance with the tissue sample data, AdipoR1 expression was higher in all cell lines and exceeded that of AdipoR2 expression by 2.7- to 4.2-fold.

Effect of adiponectin on proliferation on breast cancer cell lines

To evaluate direct biological actions of adiponectin on breast cancer cells, we exposed cells of the estrogen receptor (ER)-positive T47D cell line derived from a ductal carcinoma to adiponectin for 48 h and determined cell viability and proliferation. Adiponectin significantly inhibited proliferation to 86%, compared with basal medium under serum-free conditions as determined by WST1 test (Fig. 3A) and even more so in the BrdU test (Fig. 3B). This effect was also seen in normal culture conditions containing 10% FBS, although it was not significant in the BrdU test, which may be attributed to masking the effect by optimal conditions for proliferation through FBS. To assess the extent by which adiponectin reduces cell number is attributable to apoptosis, we

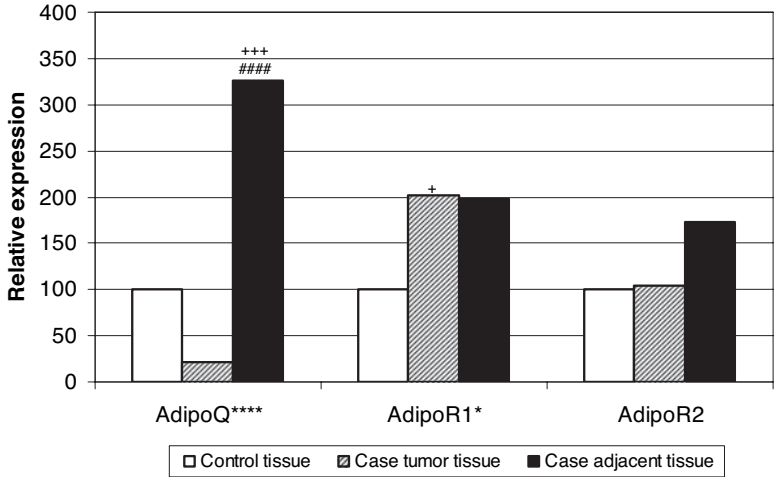

FIG. 1. Expression of adiponectin and adiponectin receptors in breast cancer patient and control tissues. Mean tissue expression for adiponectin (AdipoQ), AdipoR1, and AdipoR2 in 42 women with breast cancer and 26 controls quantified by TaqMan PCR. Data are corrected for 18s. ****, $P < 0.0001$, *, $P < 0.05$ from one-way ANOVA; +++, $P < 0.0001$, +, $P < 0.05$ for Bonferroni corrected *post hoc* comparison with control tissue; ####, $P < 0.0001$ for Bonferroni corrected *post hoc* comparison with case tumor tissue.

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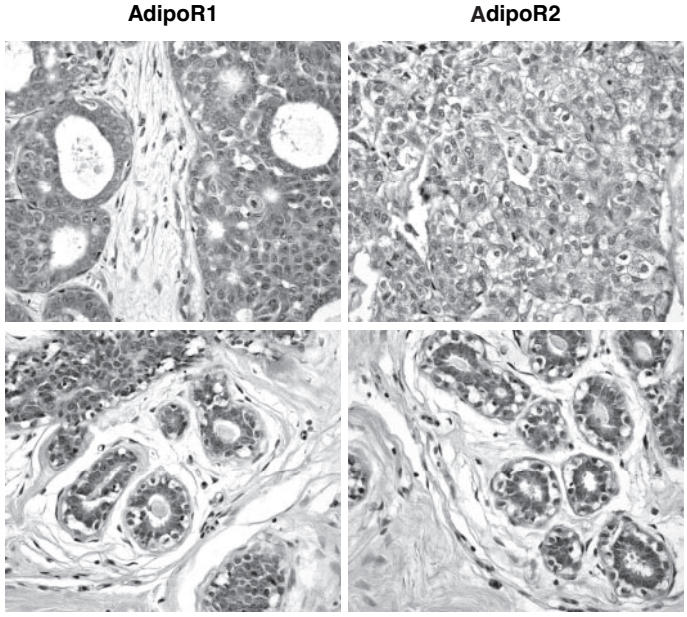

FIG. 2. Localization of adiponectin receptor expression by immunohistochemistry. Immunohistochemical staining for AdipoR1/R2 in tumor and healthy breast tissues (original magnification, ×400).

determined annexin V-positive cells in fluorescence-activated cell sorter analysis. Adiponectin did not significantly induce apoptosis in T47D.

Adiponectin signaling in breast cancer cell lines

We detected a subtle but significant activation of ERK1/2 but not AMPK or p38MAPK (Fig. 4). To exclude potential effects of insulin, a component also present in the serum free medium, we repeated Western blots under withdrawal of insulin during the serum starving before adiponectin stimulation. These experiments produced identical results.

Discussion

Obesity is an established risk factor for breast cancer, with an estimated 50% increased risk for obese compared with normal-weight women (18), but the mechanisms underlying this association have yet to be fully elucidated. In this study, we provide clinical and experimental evidence that the adipocyte-secreted hormone adiponectin might be directly involved in this process. Consistent with previous case-control studies (11, 13), we report decreased adiponectin levels in breast cancer patients and extend prior data by showing that this association is independent of age, BMI, and hormonal and reproductive factors.

Adiponectin occurs in several isoforms. The homotrimeric isoform that aggregates to hexameric and multimeric complexes of higher molecular weight is assumed to be essential for its bioactivity in the liver, whereas bacterially expressed globular or full-length adiponectin has a more limited bioactive availability (5). We evaluated for the first time the predictive value of HMW adiponectin using a recently published ELISA (19) and found that HMW is significantly and inversely associated with breast cancer risk but does not provide any additional information over measures of total adiponectin. Hence, both low serum total and HMW adiponectin concentrations predict breast cancer independent of other classical epidemiological risk factors in this primarily postmenopausal sample.

Besides previously suggested endocrine action via altering circulating insulin and IGF-I levels (18), it is possible that circulating adiponectin may act directly on breast cancer cells to influence risk of carcinoma. Adiponectin may also have paracrine and/or autocrine effects, potentially influencing breast cancer development locally through direct interactions between adipocytes and/or adjacent mammary epithelial cells. In our study, adiponectin was not expressed in breast cancer cells, excluding an autocrine mechanism, but adiponectin expression was elevated in adjacent nontumor adipose tissue from cases, compared with control tissue. Whether this is due to hyperplasia of adipose tissue due to increased differentiation rates with concomitant increase in adiponectin expression (15) remains to be studied.

The biological effects of adiponectin are likely mediated through two classical adiponectin receptor subtypes. We identified both classical adiponectin receptors, AdipoR1/R2, in breast cancer tissue samples and localized the expression to epithelial acinary and ductal cells. In both tumor tissue samples and breast cancer cell lines, AdipoR1 expression was 2–4 times that of AdipoR2. Prior studies have reported that AdipoR1 is involved in regulating proliferation and apoptosis of endothelial cells (20) and osteoblasts (21). We subsequently investigated potential direct inhibitory effects of adiponectin on proliferation in the ER-positive breast cancer cell line T47D, an established model for breast cancer research (22). Exposing these cells to human adiponectin resulted in mild inhibition of proliferation without induction of apoptotic pathways. Consistent with our observations, adiponectin limits cellular proliferation in cell lines of the myeolomonocytic lineage (with a similar extent of inhibition) but caused no inhibition of proliferation in lymphoid or erythroid lineages (6). These findings are in agreement with clinical data showing an association of adiponectin with

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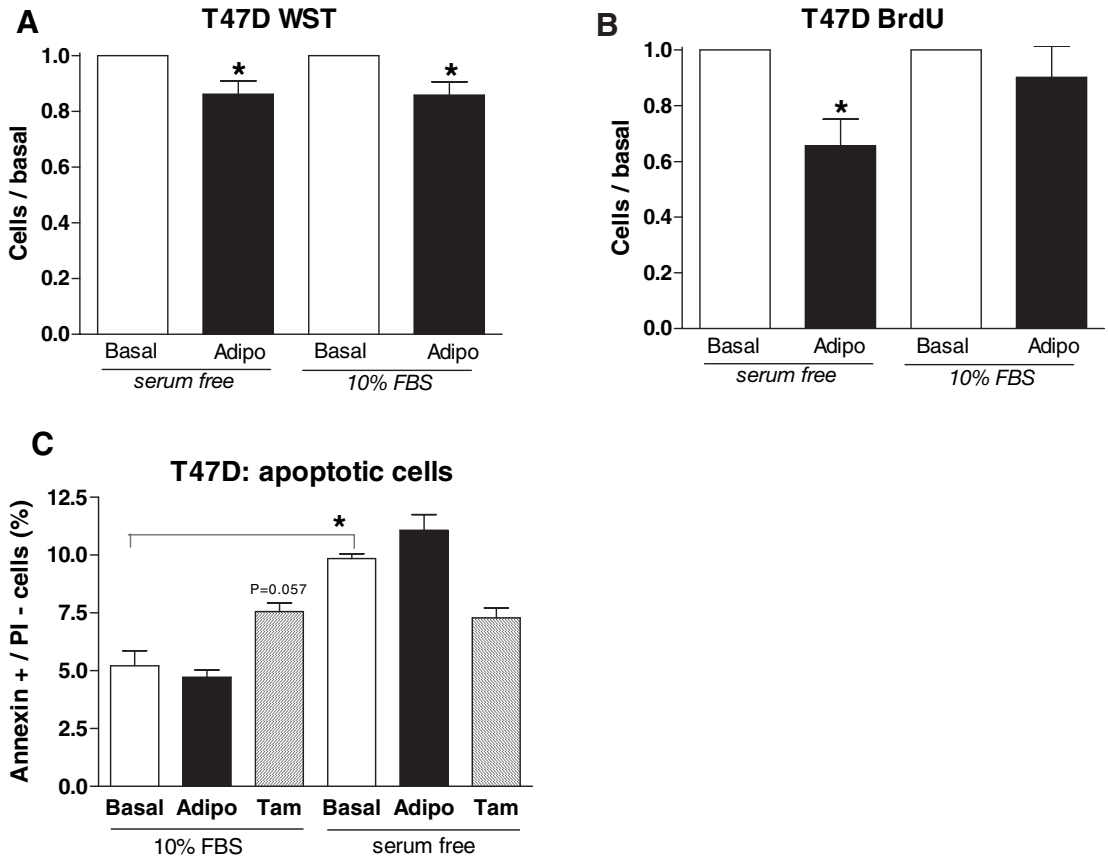

FIG. 3. Effect of adiponectin on proliferation of breast cancer cells. Cells of the T47D cell line were exposed to 20 μg/ml adiponectin (Adipo) for 48 h and viable cell number and proliferation was assessed by WST1 (A) test and BrdU (B) test as indicated. Adiponectin markedly inhibited proliferation in serum-free and serum-containing conditions but did not significantly induce apoptosis. Data are presented as mean ± SE of six independent cell culture experiments with n = 5 wells each. For comparison, data of adiponectin effects (*black bars*) are normalized to respective basal values set 1 (*open bars*). *, $P < 0.05$ from an unpaired *t* test. Tam, tamoxifen; PI, propidium iodide.

acute myelogenous leukemia but not acute lymphocytic leukemia (23).

We employed eukaryotically expressed adiponectin in our *in vitro* studies because this is apparently the most abundant form in human plasma and confers biological activity (5). Among the several signaling molecules that could mediate adiponectin-induced cellular effects, including AMPK, ERK1/2, AKT, and p38MAPK (24), only ERK1/2 phosphorylation was modestly but significantly elevated. Of relevance, recent reports indicate that the degree of ERK1/2 phosphorylation in breast cancer corresponded to smaller tumor size and better survival, hence a less aggressive phenotype (25). The lack of strong activation of these adiponectin inducible molecules raises the possibility that the effect of adiponectin on human cancer cells could also be mediated via alternative molecular/signaling pathways. One such target may be T-cadherin, a proposed additional receptor for adiponectin (17), which has recently been shown to result in activation of ERK1/2 in overexpressing cells (26) and has also been associated with malignant tumor development (27).

Another possibility is that HMW adiponectin specifically binds to growth factors, such as basic fibroblast growth factor and heparin-binding epidermal growth factor, precluding binding to their respective membrane receptors. These interactions resulted in attenuated DNA synthesis and cell proliferation usually induced by these growth factors that was not dependent on adiponectin receptors (28). Nevertheless, the possibility that adiponectin may influence cancer cell proliferation directly should also be considered. The only documented *in vivo* role of adiponectin in tumor pathology so far has been attributed to reduced vascularization subsequent to the inhibiting effects of adiponectin on endothelial cell proliferation, hence restricting the blood supply for the advancing tumor (7).

Our study was not powered to examine differences within various subgroups, and we did not find statistically significant interaction between adiponectin and menopausal status or obesity. However, results were suggestive ($P < 0.10$), meriting exploratory stratified analysis. We found the effect of adiponectin to be stronger in premenopausal and obese women. These potential variations should be further examined in larger studies. Cases were also significantly older than controls in our study. Therefore, we statistically controlled for age in our analyses, which did not alter the reported effect estimates. However, additional case-control studies may consider using an age-matched design. Future studies should also consider central adiposity in examining this relationship because adiponectin has been previously shown to be more closely associated with measures of central obesity (29). Prospective studies are also needed to establish

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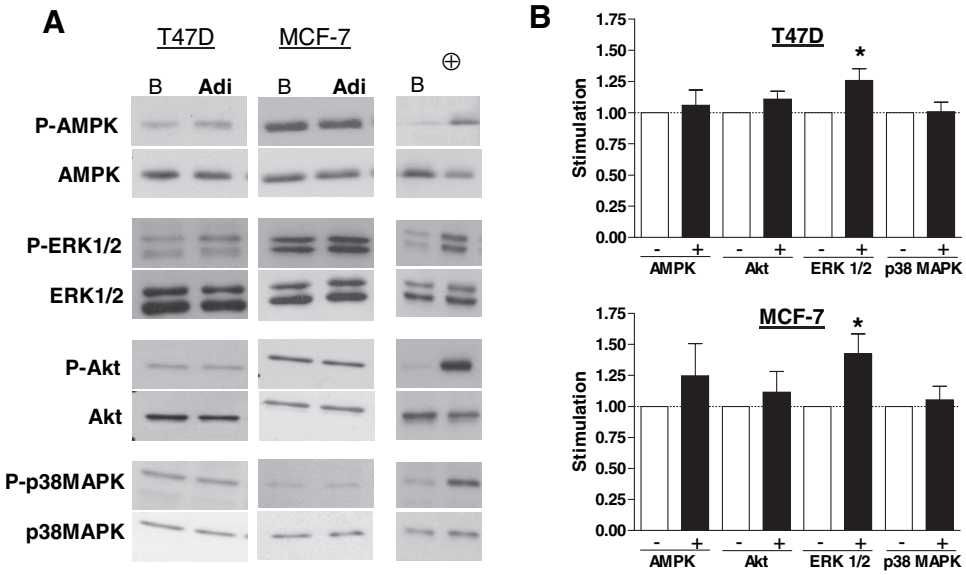

FIG. 4. Investigation of adiponectin signaling in breast cancer cell lines. Western blot was performed after stimulation of the cells with adiponectin 20 μg/ml for 15 min after an overnight serum starvation. Phosphorylated kinases were visualized by Western blot (*upper panel* for each kinase) in both cell lines in younger and older passages as indicated. For each kinase, a positive control was performed (marked ⊕): AICAR for AMPK, IGF-I for ERK1/2 and AKT, 3-isobutyl-1-methylxanthine for p38MAPK. B, Quantification of signal intensity by determination of optical density of at least two exposure times of three independent Western blots from independent cell experiments. *, $P < 0.05$ for t test. B, Basal; Adi, adiponectin stimulated.

a temporal relationship between adiponectin and breast cancer as well as examine whether the association between obesity and breast cancer is modified by adiponectin, indicating that the hormone may in part mediate this association.

In summary, our data indicate that low total or HMW adiponectin associated with risk of breast cancer, independently of classical risk factors, particularly among premenopausal and obese women. Evidence from this study also suggests that adiponectin receptors AdipoR1/R2 are expressed in breast cancer cell lines and tissue samples and that adiponectin may act by not only altering the hormonal milieu but also directly inhibiting the proliferation of breast cancer cells *in vitro*. Hence, adiponectin may not only constitute a biomarker for breast cancer development in obesity but also may act as a molecular mediator linking adipose tissue and carcinogenesis. The mechanisms underlying the actions of adiponectin, as well as its potential diagnostic and/or therapeutic utility, require further investigation.

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