Zinc-α2-glycoprotein: A proliferative factor for breast cancer? *In vitro* study and molecular mechanisms

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Abstract. Zinc-α2-glycoprotein (ZAG) is a new adipokine whose gene expression is downregulated in obese patients. We recently reported ZAG expression in breast tumor or healthy breast tissue and detected this expression at high levels in ductal carcinoma and in normal epithelial adjacent tissue but not in normal tissue of healthy women. In the present study, we used two human breast tumor cell lines (MCF-7 and MDA-MB-231) and one fibrocystic breast cell line (MCF-10a) to examine whether recombinant ZAG has an effect on proliferative/apoptotic response in breast cancer cell lines. ZAG seemed to exert a proliferative effect on breast cancer cell proliferation [+11 to 27% in MCF-7 with (ZAG) = 5-20 μg/ml; +13% in MDA-MB-231 with (ZAG) = 5 μg/ml] and, on the contrary, an anti-proliferative effect in the fibrocystic breast cell line [-5 to -8% in MCF-10a with (ZAG) = 5-10 μg/ml]. ZAG was able to modulate gene and protein expression involved in the apoptotic response. However, further studies are required to fully elucidate the effects of ZAG on the proliferation of mammary cells.

Introduction

Obesity is related to several metabolic disorders such as type 2 diabetes mellitus, coronary heart disease and hypertension, and is associated with cancer development in different tissues including colon, prostate and breast (1). It has been clearly demonstrated that obesity is a risk factor for breast cancer development in postmenopausal women (2,3). Moreover, an excess of adipose tissue favors breast cancer recurrence and is associated with higher mortality (4). Thus, overweight or obese women with breast carcinoma have a 2.5-fold increased risk of mortality within five years of diagnosis compared with lean women (5). Numerous factors have been proposed to explain the relationship between obesity and breast cancer (6-8), however, none has been completely conclusive.

Emerging data suggest that adipose tissue, which is an endocrine organ producing a large range of factors, may interfere with cancer development. These factors, mainly secreted by the adipose tissue, known as adipokines, include angiogenic factors, paracrine mitogens and anti-mitogens, growth factors and pro-inflammatory cytokines (IL-1, TNF-α and IL-6) involved in the mediation or the coordination of inflammatory diseases and obesity (9,10). Adipokines are produced by different fat depots, including subcutaneous, visceral and mammary adipose tissue. Of note, adipokines may act on breast tissue in an endocrine manner (via external adipose sources), in a paracrine pathway (via mammary adipose tissue secretion and non adipose sources including stromal cells and inflammatory cells) and in an autocrine manner (via the mammary tumor by itself). The structure of the mammary gland favors a close interaction between mammary adipose tissue and breast tissue, and suggests that adipokines produced by mammary adipose tissue and the tumor microenvironment may be the major link between obesity and disease progression and metastasis (11-14). We previously investigated simultaneously the *in vitro* and *in vivo* molecular mechanisms by which leptin induces and, conversely, adiponectin suppresses, tumor proliferation in breast cancer cells (15,16). We suggested that these two adipokines have antagonistic properties in breast cancer development by modulating differentially both proliferative and apoptotic signaling pathways (17).

Zinc-α2-glycoprotein (ZAG) is a new adipokine whose gene expression is reduced in subcutaneous fat of obese patients (18,19). This expression is positively correlated with adiponectin expression and negatively correlated with leptin expression, suggesting a protective role for ZAG in breast cancer (20). ZAG is also a sound immunohistochemical marker of breast cell differentiation since ZAG tissue levels are associated with histological grades of tumors and vary from 4.6 μg/mg in well-differentiated tumors to 0.9 μg/mg in poorly differentiated tumors (21,22). However, other studies

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found that circulating levels of ZAG are significantly higher in cancer patients (51 mg/l) as compared with levels in healthy women (44 mg/l), particularly in patients with advanced and node-positive breast cancer (23). Moreover, Bing *et al* (18) showed that ZAG expression is upregulated in mice with cancer cachexia. Overexpression in white adipose tissue of tumor-bearing mice suggests that ZAG plays a role in the substantial reduction of adiposity in cancer cachexia. ZAG is also considered a prognostic marker in breast cancer (24). We recently reported ZAG expression in breast tumor or healthy breast tissue and detected this expression at high levels in ductal carcinoma and in normal epithelial adjacent tissue, but not in normal tissue of healthy women (25). We also found ZAG expression was positively correlated to leptin receptor and negatively correlated to adiponectin receptor in cancer tissues. Our previous data suggested both a potential prognostic role for ZAG in breast cancer and a close interaction between ZAG and other major adipokine pathways.

The aim of the present study was to characterize the involvement of ZAG in breast cancer proliferation. Thus, we explored the *in vitro* potential effect of human recombinant ZAG on i) proliferative/apoptotic response, and ii) the modifications of gene expression in different breast epithelial cell lines.

Materials and methods

Cell culture. The human breast cancer cell lines MCF-7 and MDA-MB-231, and the human fibrocystic breast cell line MCF-10a were obtained from the American Type Culture Collection (ATCC). MCF-7 cells were routinely cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS), L-glutamine (2 mM) and gentamicin (50 μg/ml) at 37˚C under a 5% CO_2 atmosphere. MDA-MB-231 cells were cultured in Leibovitz's L-15 medium with 15% heat-inactivated FCS, L-glutamine (2 mM) and gentamicin (50 μg/ml) at 37˚C in humidified conditions. MCF-10a cells were cultured in DMEM HAM's F12 medium supplemented with 10% heat-inactivated horse serum (HS), EGF (0.02 μg/ml), cholera toxin (0.1 μg/ml), hydrocortisone (0.5 μg/ml), insulin (0.25 UI/ml) and L-glutamine (2 mM) at 37˚C under a 5% CO_2 atmosphere (16).

Recombinant human ZAG (rh-ZAG). Recombinant human (rh)-ZAG was kindly provided by Mracek *et al* (20) (Obesity Biology Research Unit, School of Clinical Sciences, University of Liverpool) and its production was recently described.

Proliferation assay. The human mammary cell lines (MCF-7, MDA-MB-231 and MCF-10a) were seeded at the density of $5x10^3$ cells in 96-well plates in a complete medium. After 24 h of incubation, cells were washed with PBS and exposed to fresh medium (control) or to fresh medium containing different concentrations of rh-ZAG: 1.2, 2.5, 5, 10 and 20 μg/ml. After 96 h, cells were washed with PBS and incubated with 200 μl of a 25 μg/ml solution of resazurin in RPMI-1640 medium for 2 h at 37˚C. 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 (16). The cell proliferation assay was performed three times in triplicates for each concentration tested.

Table I. Primers used in qRT-PCR assays.

BAG1	F: 5'-CACAGCAATGAGAAGCACG-3'
BAG1	R: 5'-GTGTTTCCATTTCCTTCAGAG-3
BAG3	F: 5'-ATGACCCATCGAGAAACTGC-3'
BAG3	R: 5'-AATTGGGATGTGTCCAGGAG-3'
MX1	F: 5'-AGCTCGGCAACAGACTCTTC-3'
MX1	R: 5'-GGATGATCAAAGGGATGTGG-3'
TP53	F:5'-GCGCACAGAGGAAGAGAATC-3'
TP53	R: 5'-AGAGGAGCTGGTGTTGTTGG-3'
18S	F: 5'-GTCTGTGATGCCCTTAGATG-3'
18S	R: 5'-AGCTTATGACCCGCACTTAC-3'

F, forward; R, reverse.

Analysis of gene expression. Total RNA was extracted with Trizol according to the manufacturer's recommendations (Invitrogen). The quantity and quality of RNA was assessed by 260/280 ratio using a NanoDrop 8000 Spectrophotometer (Thermo Fisher Scientific). cDNAs were obtained with HighCap cDNA RT kit RNAse inhibitor (Applied Biosystems).

Real-time PCR assays of *BAG1*, *BAG3*, *MX1*, *TP53* and *18S* mRNA expression levels were performed using the StepOne instrument (Applied Biosystems) with Power SYBR-Green (Applied Biosystems) following the manufacturer's instructions. The cDNAs were amplified using the primers summarized in Table I. The PCRs were set up in 48-well plates in a total volume of 20 μl and 20 ng of cDNA. The PCR conditions were as follows: 2 min at 50˚C, 10 min at 95˚C, 40 cycles of 15 sec at 95˚C and 30 sec at 62˚C.

The comparative cycle threshold (CT) method ($2^{-\Delta\Delta CT}$) was used to calculate the relative gene expression of ZAG-treated cells normalized within the sample to an endogenous reference gene (18S), and relative to the expression of the same gene in untreated cells: $2^{-\Delta\Delta CT}$ method with $\Delta\Delta CT$ = [ΔCT (ZAG-treated cells) - ΔCT (untreated cells)] and ΔCT = [CT (target gene) - CT (reference gene)].

Statistical analysis was performed using the paired Student's t-test. A P-value <0.05 was considered to indicate a statistically significant difference.

Western blotting. Cells lysates (containing 10 μg of total proteins) were separated on 4-12% SDS-PAGE gels (Invitrogen), transferred to nitrocellulose and blotted with various antibodies. We used primary antibodies (Santa Cruz Biotechnology, Inc.,) against the following proteins: p53 (DO-1), p21, Bax, c-Myc and cyclin D1. Secondary horseradish peroxidase (HRP)-conjugated antibodies were obtained from Santa Cruz Biotechnology, Inc., (anti-rabbit) or Dako (anti-mouse). Immunoreactive bands were visualized by incubation with DURA Western blotting detection system from Thermo Fisher Scientific. Glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) monoclonal antibody was used as a loading control. Developed films were scanned

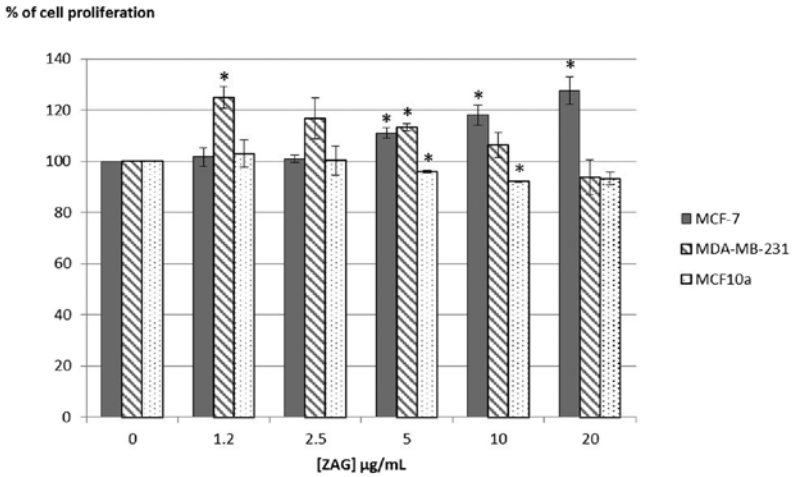

Figure 1. Effect of rh-ZAG on mammary cell proliferation. Cells were seeded in 96 well-plates with complete medium for 24 h and were then exposed to rh-ZAG (1.2, 2.5, 5, 10 and 20 μg/ml) for 96 h. Bars are means ± SEM obtained from three experiments. Significant difference compared to control ($^*P<0.05$, t-test).

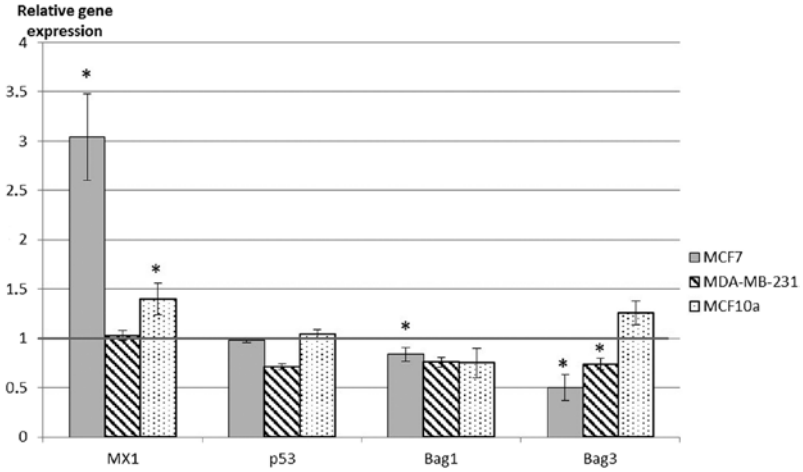

Figure 2. Effect of ZAG on mRNA expression in breast cell lines after 72 h of treatment. The gene expression of two apoptotic genes (*Mx1* and *p53*) and two anti-apoptotic genes (*Bag1* and *Bag3*) was evaluated in the three breast cell lines (MCF-7, MDA-MB-231 and MCF-10a) after treatment of rh-ZAG for 72 h. The concentration of rh-ZAG used corresponded at the respective concentration points leading to an optimal effect on cell growth: 20 μg/ml in MCF-7 cells, 1 μg/ml in MDA-MB-231 cells and 10 μg/ml in MCF-10 cells. Significant difference compared to control ($^*P<0.05$, t-test).

as JPEG images, and the pixel intensities within a band were measured with ImageJ software. The intensity of each gene was then reported to GAPDH intensity.

Results

Mammary cell proliferation. The proliferation of estrogen receptor positive (ER+) mammary cancer cells MCF-7 was increased at the highest concentrations tested [11 to 27% with (ZAG) = 5 to 20 μg/ml] (Fig. 1). In ER- mammary cancer cells MDA-MB-231, rh-ZAG had a proliferative effect at the lowest concentrations tested [+24% with (ZAG) = 1.2 μg/ml; +13% with (ZAG) = 5 μg/ml]. By contrast, rh-ZAG had an anti-proliferative effect on fibrocystic breast cells MCF-10a when used at the concentrations of 5 and 10 μg/ml (-5 and -8% in proliferation decrease).

Gene expression. qRT-PCR was performed to study the effects of a 72-h rh-ZAG treatment on the expression of two pro-apoptotic genes (*p53*, *mx1*) and two anti-apoptotic genes (*Bag1* and *Bag3*) in the three studied breast cell lines. We used rh-ZAG at the respective concentration points leading to an optimal effect on cell growth: 20 μg/ml for MCF-7 cells, 1 μg/ml for MDA-MB-231 cells and 10 μg/ml for MCF-10 cells.

Bag1 and *Bag3* expressions were downregulated by rh-ZAG in MCF-7 and MDA-MB-231 cells, but remained unaltered in MCF-10a cells (Fig. 2). Concomitantly, *Mx1* gene expression was upregulated by rh-ZAG in MCF-7 and MCF-10a and more strongly in MCF-7 cells.

Protein expression. Fig. 3 shows the results obtained in cells treated for 24, 48 or 72 h with rh-ZAG (0, 1, 10 ng/ml). In MCF-7 cells treated with rh-ZAG, an increase in p53 protein expression at 24 h [+100% with (ZAG) = 1 ng/ml; +300% with (ZAG) = 10 ng/ml] but a decrease at 72 h (-32% with (ZAG) = 10 ng/ml) were observed. The expression of c-Myc was increased only at 24 h (+160%) whereas the expression of p21 and Bax slightly increased during the experiments (p21: +21, +39 and 28% at 24, 48 and 72 h, respectively; Bax: +58, +37 and +64% at 24, 48 and 72 h, respectively). In MDA-MB-231

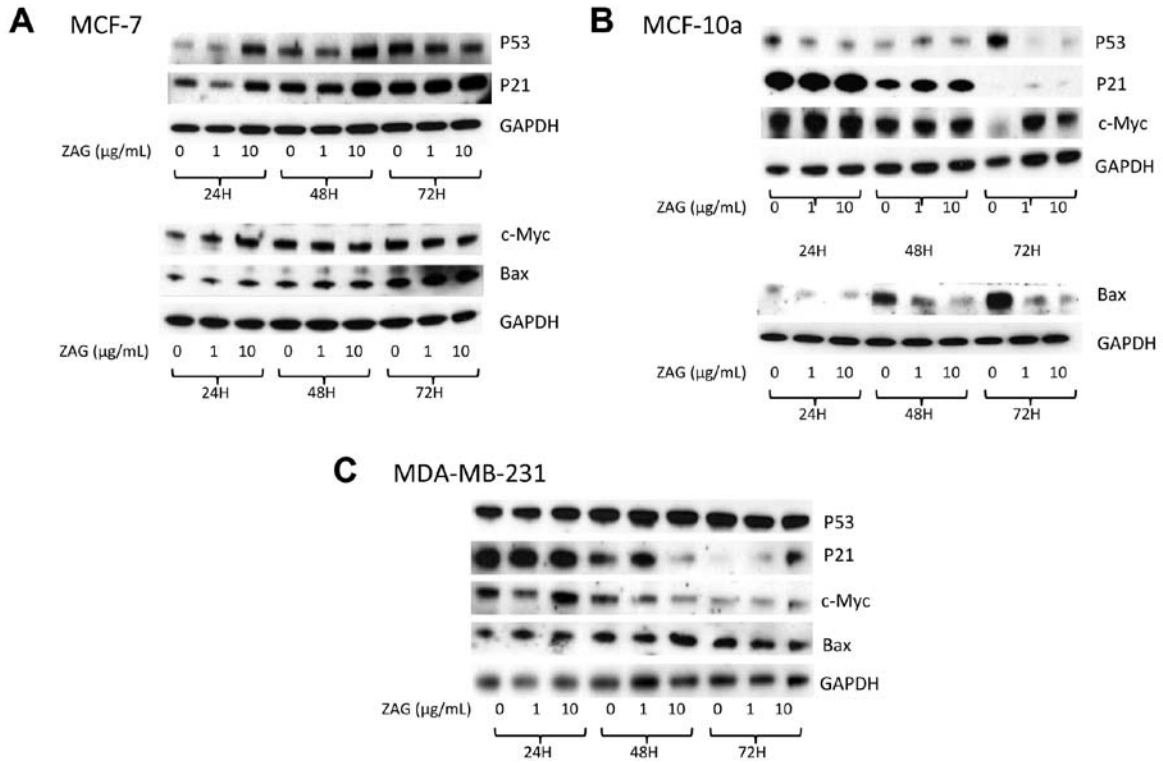

Figure 3. Effect of ZAG on protein expression in breast cell lines. The three mammary cell lines (MCF-7, MDA-MB-231, MCF-10a) were treated for 24, 48 or 72 h with rh-ZAG (0, 1, 10 μg/ml). The expression of P53, P21, C-Myc and Bax was measured.

cells, there was no evidence of ZAG-modulated variations in the expression of the different proteins tested. In MCF-10a cells, rh-ZAG enhanced c-Myc expression at 72 h [+80% with (ZAG) = 10 ng/ml] while downregulating both p53 (-95%) and Bax (-90%) protein expression at the same incubation time.

Discussion

ZAG, a well-known lipid mobilizing factor, which is downregulated in obesity, is now considered as an adipokine. We previously reported *in vivo* ZAG expression in breast malignant cells and in normal epithelial adjacent tissue (25). The present study showed ZAG had a proliferative activity on the two human breast tumor cell lines (MCF-7 and MDA-MB-231), but an anti-proliferative effect on MCF-10a cells derived from a non-cancerous fibrocystic tissue.

Tumor growth should be viewed as the result of a balance between cell proliferation and cell death. Our aim was to assess whether ZAG can regulate mammary cancer proliferation not only through an inhibitory pathway but also by triggering a programmed cell death. Markedly, ZAG had a proliferative effect in MCF-7 cells while upregulating pro-apoptotic *Mx1* and downregulating the gene expression of anti-apoptotic genes *Bag1* and *Bag3*. Such a modulation on apoptotic markers should result in a slowing process of cell proliferation.

Our data also suggest that ZAG-induced cell-growth inhibition in MCF-10a breast cancer cells could be mediated through the c-Myc pathway. Indeed, we found ZAG upregulates c-Myc protein expression in these cells. Despite its role in promoting tumorigenesis, there is increasing evidence that c-Myc also induces apoptosis in cancer cells (26,27). Epithelial cells have also been shown to be susceptible to apoptosis by c-Myc (28). In addition, it was reported that MCF-7 breast carcinoma cells deprived of glucose exhibit both c-Myc elevation and significant cell death, which can be blocked by the addition of antisense c-Myc oligonucleotides (29).

We previously reported that endogenous ZAG was not associated with Bax and Bcl2 apoptosis biomarkers in breast cancer tissues (25). In the present study, we found exogenous ZAG did not consistently affect Bax expression in the two human breast tumor cell lines (MCF-7 and MDA-MB-231). By contrast, ZAG downregulated the expression of both pro-apoptotic proteins p53 and Bax in MCF-10a cells. We also observed an anti-proliferative effect for ZAG in these cells, therefore, apoptosis may not be the only mechanism to explain the resulting inhibitory effect on cell proliferation. In squamous carcinoma, ZAG inhibits cell proliferation by downregulating cdc2 expression and affecting cell cycle (30). In this study, ZAG did not affect the expression of the genes involved in apoptosis or differentiation (PCNA, p53, c-Myc and Bcl-2). Of note, if adipokines generally modulate the apoptotic response *in vitro*, they may exert their activities through different pathways from one cell type to another since breast cancer cell lines display distinct patterns of apoptosis-regulatory genes (31). For example, MCF-7 cells are caspase-3 negative, ZR-75 cells are Bcl-2 negative and the p53 status is either wild or mutant, depending on the cell line.

Thus, our *in vitro* approach does not seem to reflect the complex regulation that may occur *in vivo*. Metabolic dysregulations associated with obesity (such as hypoadiponectinemia and hyperleptinemia) are likely to promote cancer cell growth via both systemic and local mechanisms. Furthermore, when

mammary cells are engaged in the process of carcinogenesis, they produce adipokines (mainly leptin) able to act on surrounding cancer cells in a paracrine and/or autocrine manner. Adipokines act via their receptors on mammary tumor cells to i) influence tumor cell proliferation, migration and invasion in breast cancer; ii) regulate the production of epithelial-derived proteins, angiogenic proteins and growth factors; iii) stimulate other cells in the tumor microenvironment to invade and proliferate. For example, a study reported a proliferative effect of adipocyte-secreted factors on the MCF-7 breast cancer cell line, through the regulation of genes involved in cell motility, migration, survival, apoptosis and angiogenesis (32). In the same way, Celis *et al* (33) identified in mammary adipose tissue 359 protein components and excreted factors that may provide insight into the close interplay between mammary epithelium, stroma and fat tissue. Among these proteins, they identified several cell cycle regulators, including p53 and p21. Perera *et al* (34) used a proteomic approach and described for the first time the secretion of epithelial-derived proteins in MCF-7 cells in response to leptin. The secretion of such proteins in breast cancer cells in response to ZAG has yet to be reported.

In summary, these preliminary data show that recombinant ZAG has a pro-carcinogenic effect on breast cancer cells and conversely an anti-carcinogenic effect on non malignant breast cells. ZAG clearly modulates signaling pathways involved in proliferation and apoptosis but this modulation cannot fully explain the effects we previously observed with ZAG in breast cancer tissues.

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