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Abstract. Obesity is a growing worldwide medical problem, as it pre-disposes the affected hosts to a number of severe diseases, including postmenopausal breast cancer. Obesity development is characterised, amongst others, by aberrant secretion of adipokines. White fat tissue infiltrating macrophages secrete tumour necrosis factor-α (TNF-α) so that its circulating levels correlate positively with body mass index (BMI). In the study presented here, the effect of TNF-α on cell proliferation, cell signalling pathway activation and cell cycle in two breast cancer cell lines and one breast epithelial cell lines was assessed to determine the contribution of TNF-α on breast cancer progression and aetiology, respectively. TNF-α acted differently on all three cell lines. In MDA-MB-231 breast cancer cells, cell proliferation and PI3-kinase activation were not affected, while MAP-kinase activation and cell cycle progression were decreased, with indications of increased apoptosis. This suggests a growth inhibitory function of TNF-α in these cells. In SK-BR-3 breast cancer cells, cell proliferation and cell signalling pathway activation increased, while cell cycle progression decreased, which contradictorily suggests both growth promoting and growth inhibiting properties of TNF-α on these cells. This makes TNF-α an unlikely candidate for a general contribution to the link between obesity and breast cancer progression, however, individual tumours may be responsive to a proliferative signal of TNF-α. In MCF-10A breast epithelial cells, cell proliferation and MAP-kinase activation increased, while cell cycle progression was unaffected. This suggests a strong proliferative response in these cells, suggesting the possibility that TNF-α may contribute to breast cancer aetiology as a novel link between obesity and increased risk of breast cancer development.

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Introduction

Obesity is a risk factor for the development of post-menopausal breast cancer (1-3). Despite numerous studies, the molecular mechanism by which obesity may contribute to breast cancer aetiology and progression is still poorly understood, suggesting a complex multi-mechanism of interaction. Obesity is characterised, amongst others, by a change in adipocyte secreted adipokines (4-6). Plasma concentrations of several well-known adipokines are positively correlated with BMI, such as leptin (7), interleukin-6 (IL-6) (8) and tumour necrosis factor-α (TNF-α) (9). In the study presented here, the contribution of TNF-α in the link between obesity and progression or aetiology of breast cancer is assessed.

TNF-α is a cytokine involved in the induction of inflammation and initiation of an acute phase immune response. When cells are stimulated to secrete TNF-α, membrane-bound precursor molecules on the surface of secreting cells are cleaved to form 51 kDa circulating TNF-α, consisting of three 17 kDa TNF-α molecules (10). Its name derived from the initial observation that this cytokine was able to induce cell death in the murine fibrosarcoma L-929 cell line (11). Endocrine signalling is accomplished by circulating TNF-α binding to and activating its specific receptor TNF-receptor 1 (TNF-R1) on target cells. The receptor is expressed ubiquitously in all human tissues, including breast epithelial cells (12). Thus TNF-α signalling in breast epithelial cells and breast cancer cells could be affected by obesity associated increases in TNF-α circulatory concentration.

Previous *in vitro* studies observed conflicting results as to the effect of TNF-α on growth promotion in breast cancer cells. It was found that 20 ng/ml TNF-α increased cell proliferation in T47D human breast cancer cells and induced significant activation of PI3-kinase pathway, MAP-kinase pathway and JAK/STAT pathway (13), suggesting a growth promoting effect of TNF-α on these cells. Conversely, treatment with 10 ng/ml TNF-α for 24 h increased G1-phase population, while reducing the cell population in S-phase and G2-phase (14), suggesting a cell cycle inhibiting effect on these cells. Thus TNF-α had different, indeed opposing effects on T47D breast cancer cells. In MCF-7 breast cancer cells 10 ng/ml TNF-α decreased cell proliferation (15) and an increase in G1-phase cell population was observed after treatment with 10 ng/ml TNF-α for 36 h in MCF-7 cells (16),

suggesting a growth inhibiting effect in this cell line. In different MCF-7 cell subtypes, however, opposing effects of TNF-α on cell proliferation were observed (17). Thus, the effect of TNF-α on breast cancer cell growth is not elucidated satisfactorily, while the effect of TNF-α on human breast epithelial cells has not been investigated at all.

The study presented here aimed to assess the effect of TNF-α on cell proliferation, PI3-kinase and MAP-kinase cell signalling pathway activation and on changes in the distribution of the cell population across cell cycle stages in an *in vitro* cell culture model. It was of particular interest to determine the possible different impact of TNF-α on breast cancer cells compared to normal breast epithelial cells as a novel way of assessing the molecular mechanisms involved in the link between obesity and breast cancer progression and aetiology. In order to assess the contribution of TNF-α on breast cancer progression, two breast cancer cell lines were selected, MDA-MB-231 and SK-BR-3 breast cancer cells. The contribution of TNF-α on breast cancer aetiology was assessed using normal MCF-10A breast epithelial cells. Previous experiments were performed in oestrogen receptor (ER) positive cells. In the study presented here, ER negative cell lines were used.

Materials and methods

Materials. Human Caucasian breast adenocarcinoma cells MDA-MB-231 (Cat No. 92020424, passage No. 36) were purchased from the European Collection of Cell Cultures (ECACC, Salisbury, UK). Human Caucasian breast adenocarcinoma cells SK-BR-3 (ATCC No. HTB-30, passage No. 28) were purchased from the American Type Culture Collection (ATCC, Manassas, USA). MDA-MB 231 cells and SK-BR-3 cells were routinely cultured in RPMI-1640 Medium (including 25 mM HEPES, 1x Glutamax) [Gibco (Invitrogen), Paisley, UK, Cat No. 72400] supplemented with 10% FCS (Pierce Biosciences, Cramlington, UK, Cat No. CHD0413) and 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco, Cat No. 15140). MDA-MB-231 breast cancer cells have mutations in genes coding for RAS and RAF, increasing their kinase activity, while SK-BR-3 breast cancer cells possess wild-type genes coding for RAS and RAF (18). RAS and RAF are kinase members of the MAP-kinase cell signalling pathway. Human Caucasian breast epithelial cells MCF-10A (ATCC No. CRL-10317, passage No. 102) were purchased from ATCC and were cultured in DMEM/F-12 Medium (BioWhittaker, UK [Lonza Biologics], Slough, UK, Cat No. BE12-7199) supplemented with 5% Horse Serum (Sigma-Aldrich, Gillingham, UK, Cat No. H1138), 10 μg/ml human insulin (Sigma, Cat No. I9278), 0.5 μg/ml hydrocortisone (Sigma, Cat No. H0888), 20 μg/ml human epidermal growth factor (Invitrogen, Cat No. 13247-051), 100 ng/ml cholera toxin (Sigma, Cat No. 8052), 50 U/ml penicillin and 50 μg/ml streptomycin (Gibco). Starvation medium was RPMI-1640 (25 mM HEPES, 1x Glutamax) (Gibco) supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco) for MDA-MB 231 cells and SK-BR-3 cells and DMEM/F12 (BioWhittaker) supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin for MCF-10A cells. Human recombinant TNF-α was purchased from Sigma (Cat No. T0157). Cell culture conditions were 37˚C in humidified air supplemented to contain 5% CO_2.

BrdU-incorporation assay. Cell proliferation was detected using a colorimetric Cell Proliferation ELISA Kit (Roche Diagnostics, Penzberg, Germany, Cat No. 11 647 229 001), which assesses DNA replication by measuring bromodeoxyuridine (BrdU) incorporation. Cells ($5x10^3$ cells/well) from each cell line were plated in 96-well plates (Fisher Scientific, Cat No. 167008) with 100 μl/well growth medium and incubated for 24 h at 37˚C. Cells were washed once in 100 μl/well sterile PBS and incubated in starvation medium for 24 h. After starvation, cells were washed as before and treated for 24 or 48 h with 10 ng/ml TNF-α in 100 μl/well starvation medium. During treatment, medium was supplemented with 10 μM BrdU. Incorporated BrdU was detected according to the manufacturer's instructions. In brief, cells were fixed and incubated with supplied peroxidase conjugated anti-BrdU antibody. Bound antibody was detected by colorimetric turn-over. Colour development was quantified on μQuant microplate spectrophotometer (BioTek, UK) by measuring absorption at 450 nm with a reference wavelength of 690 nm. Three experiments were performed for each cell line and each time point. Each experiment consisted of six replicates for each treatment, i.e. six wells for control and six wells for treatment.

Phospho-kinase ELISA. Cell-based ELISA phospho-AKT (S473) immunoassay (Cat No. KCB887) and phospho-ERK1/ERK2 (T202/Y204) immunoassay (Cat No. KCB1018) were purchased from R&D Systems (Abingdon, UK). Cells from all three cell lines were plated into a supplied clear bottom black 96-well plate at a density of $5x10^3$ cells/well with 100 μl/well growth medium and incubated for 24 h at 37˚C. Cells were washed once in 100 μl/well sterile PBS and incubated in starvation medium for 24 h. After starvation, cells were washed as before and treated with 10 ng/ml TNF-α in 100 μl/well starvation medium for times indicated in Fig. 2. Phosphorylation of AKT and ERK1/2 was then assessed following the manufacturer's instructions. In brief, after fixing, the cells were incubated with anti-phospho-AKT or anti-phospho-ERK1/2 specific antibody in conjunction with anti-total AKT or anti-total ERK1/2 specific antibody, respectively. Phospho-specific antibodies were of mouse origin, while antibodies for the total protein were produced in rabbits. Each antibody was identified by mouse or rabbit specific secondary antibodies, which were tagged with HRP or AP, respectively, allowing for simultaneous quantification of phosphorylated and total AKT or ERK1/2 protein. After antibody incubation, excess secondary antibody was removed by several washes in supplied wash buffer and PBS, before two supplied fluorescent substrates were added. Fluorescence was measured on Fluoroskan Ascent microplate reader (Labsystem, UK) with excitation at 544 nm and emission at 590 nm (phosphorylated protein), followed by a second read with excitation at 355 nm and emission at 460 nm (total protein). For each cell line two experiments were performed to assess AKT-phosphorylation and two to assess ERK1/2-phosphorylation after TNF-α treatment. Each experiment included two replicates for each treatment time and the control.

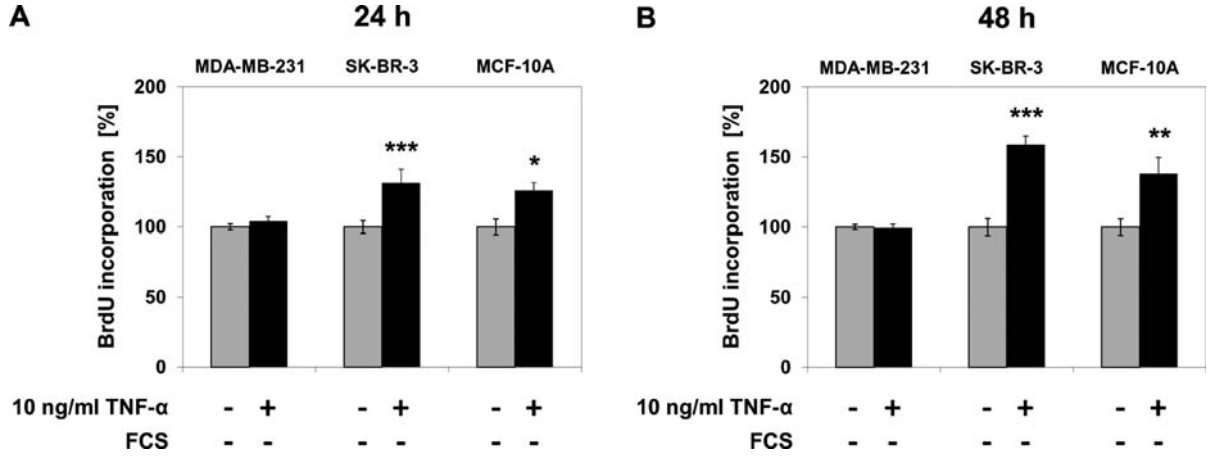

Figure 1. Changes in cell proliferation after treatment with 10 ng/ml TNF-α (A) 24 h or (B) 48 h in MDA-MB-231 breast cancer cells, SK-BR-3 breast cancer cells and MCF-10 breast epithelial cells. Bars represent BrdU-incorporation in relation to the respective control and are expressed as a percentage thereof. Error bars represent ± SEM of three experiments, each consisting of six replicates, i.e. 18 data points for each bar. *Significance value compared to control, obtained using one-way ANOVA analysis. (*0.05>p>0.01; **0.01>p>0.001; ***p<0.001).

Flow cytometry. Changes in the cell distribution across cell cycle stages were assessed by measurement of DNA-content in cells. The DNA specific dye was propidium iodide (PI) (Sigma, Cat No. P4170). Cells were plated at $5x10^5$ cells/well in 6-well plates with 3 ml growth medium and incubated for 24 h at 37˚C. Cells were starved for 24 h and then treated with 10 ng/ml TNF-α for 24 h. Cells were harvested by trypsin, centrifuged at 500 x g for 5 min and each sample was resuspended in 100 µl PBS and 900 µl 70% (v/v) ice-cold ethanol before being incubated at 4˚C for 30 min. Samples were centrifuged at 10,000 x g for 5 min, resuspended in 1 ml PBS and again centrifuged at 10,000 x g for 5 min. Pellet was resuspended in 500 µl extraction buffer (4 mM citric acid, 0.2 M Na_2HPO_4, pH 7.8) and 500 µl PBS and incubated at room temperature for 5 min. Cells were centrifuged at 10,000 x g for 5 min and the pellet was resuspended in 500 µl DNA staining solution (20 µg/ml propidium iodide and 0.2 mg/ml DNase free RNase). The cells were incubated at room temperature for 30 min. Flow cytometry of PI stained cells was performed using a Coulter Epics XLMCL flow cytometer (Beckman Coulter, UK). Data were captured and analysed using EXPO 32 Software (Applied Cytometry Systems, Sheffield, UK). Cell cycle distribution was assessed from linear FL-2 area vs. width plots. The percentage of cells in the three cell cycle phases (G1, S and G2) was calculated from histograms of linear FL-2 area plots after assessing 10,000 events. Additionally, percentage of cells with a DNA content <2N (sub-G1) was measured as an indication of apoptosis. Three experiments were performed for each cell line, containing two replicates for control and treatment.

Statistical analysis. Cell proliferation results were assessed using one-way ANOVA. Cell signalling pathway activation and cell cycle results were assessed using Dunnett's post-hoc t-test following univariate analysis of variance. Changes were defined as significantly different at p<0.05.

Results

Cell proliferation. Cell proliferation of all cell lines was assessed after 10 ng/ml TNF-α treatment for 24 or 48 h (Fig. 1). Untreated control cells were incubated in starvation medium (Control) at all times. All results are presented as percentage change from this control. In MDA-MB-231 breast cancer cells, cell proliferation did not significantly change after 24- or 48-h treatment with 10 ng/ml TNF-α. In SK-BR-3 breast cancer cells, cell proliferation increased significantly by 31% after 24-h treatment (p=0.016) and by 59% after 48-h treatment (p<0.001) with 10 ng/ml TNF-α. In MCF-10A normal breast epithelial cells, cell proliferation increased significantly by 26% after 24-h treatment (p<0.001) and by 38% after 48-h treatment (p=0.002) with 10 ng/ml TNF-α.

Cell signalling pathway activation. Phosphorylation of AKT or ERK1/2 was measured after treatment with 10 ng/ml TNF-α for between 5-20 min (Fig. 2). Untreated control cells were incubated in starvation medium (Control) at all times. All results are presented as percentage change of this control. In MDA-MB-231 breast cancer cells, AKT-phosphorylation did not change significantly after treatment between 5 and 20 min with 10 ng/ml TNF-α. ERK1/2-phosphorylation was significantly decreased by 22% after 5-min treatment (p=0.044) with 10 ng/ml TNF-α (Fig. 2A). In SK-BR-3 breast cancer cells, AKT-phosphorylation increased significantly by 66, 72 and 91% after 5-min (p=0.04), 10-min (p=0.025) and 20-min treatment (p=0.006) with 10 ng/ml TNF-α, respectively compared to untreated control. ERK1/2-phosphorylation increased significantly by 47% after 5-min treatment (p=0.027) with 10 ng/ml TNF-α (Fig. 2B). In MCF-10A normal breast epithelial cells, AKT-phosphorylation did not change significantly. A non-significant increase of 77% (p=0.169) after 20 min of treatment with 10 ng/ml TNF-α compared to untreated control was, however, observed. ERK1/2-phosphorylation increased

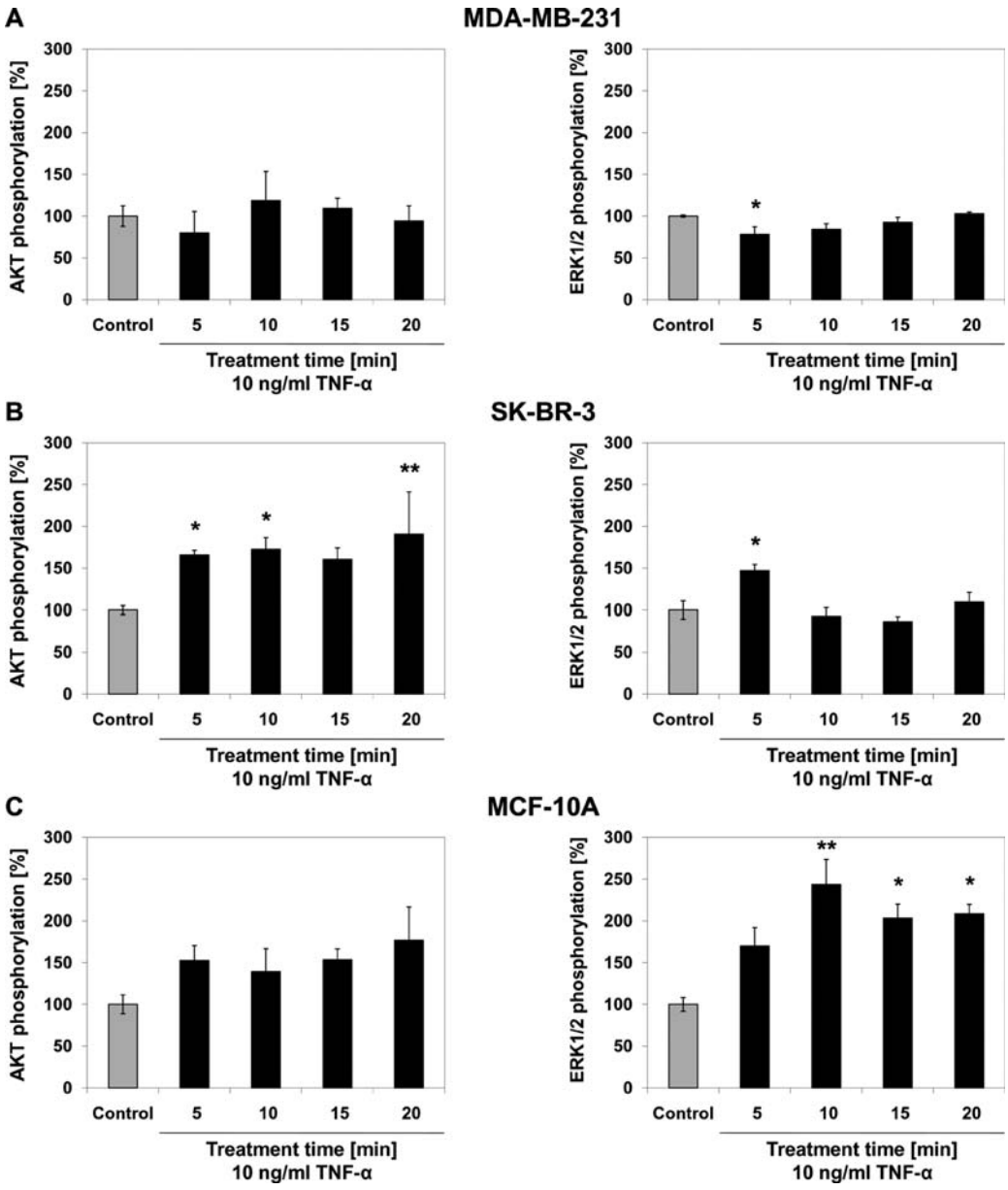

Figure 2. Changes in cell signalling pathway activation [PI3-kinase (left) and MAP-kinase (right))] with 10 ng/ml TNF-α treatment in (A) MDA-MB-231 breast cancer cells (B) SK-BR-3 breast cancer cells and (C) MCF-10A breast epithelial cells. Bars represent AKT-phosphorylation or ERK1/2-phosphorylation in relation to the respective control within each graph and are expressed as a percentage thereof. Error bars represent ± SEM of two experiments, each consisting of two replicates, i.e. four data points for each bar. *Significance value compared to control, obtained using Dunnett's post-hoc t-test following univariate analysis of variance. (*0.05>p>0.01; **0.01>p>0.001).

significantly by 144, 103 and 108% after 10-min (p=0.003), 15-min (p=0.025) and 20-min treatment (p=0.019) with 10 ng/ml TNF-α compared to untreated control (Fig. 2C).

Cell cycle. Cell cycle profiles for all cell lines were established after treatment with 10 ng/ml TNF-α for 24 h (Table I). In MDA-MB-231 breast cancer cells, population of the G1-phase decreased significantly by 3.5 percentage points (a 6% decrease) after 24-h treatment (p=0.006) with 10 ng/ml TNF-α compared to G1-phase population of control cells. S-phase population decreased significantly by 1.8 percentage points (a 15% decrease) after 24-h treatment (p<0.001) with 10 ng/ml TNF-α compared to S-phase population of control cells. No significant difference in G2-phase population after 24-h treatment (p=0.233) with 10 ng/ml TNF-α compared to G2-phase population of control cells was observed. Additionally, the sub-G1 population increased significantly by 4.66 percentage points (a 27% increase) after treatment with 10 ng/ml TNF-α for 24 h (p<0.001). In SK-BR-3 breast cancer cells G1-phase population was not significantly different between 24-h treatment (p=0.811) with 10 ng/ml TNF-α and the control. Population of the S-phase decreased significantly by 1.5 percentage points (a 25% decrease) after 24-h treatment (p=0.011) with 10 ng/ml TNF-α compared to S-phase population of control cells. Population of the G2-phase decreased significantly by 3.8 percentage points (a 24% decrease) after 24-h treatment (p=0.011) with 10 ng/ml TNF-α compared to G2-phase population of control cells. In

Table I. Changes of cell population distribution across cell cycle stages after 24-h treatment with 10 ng/ml TNF-α in MDA-MB-231 cells, SK-BR-3 cells and MCF-10A cells.

	Sub-G1 (%)	G1/G0 (%)	S (%)	G2 (%)
MDA-MB-231				
Control	16.98±1.07	55.12±0.40	7.23±0.65	12.88±0.43
10 ng/ml TNF-α for 24 h	21.64±1.44	51.64±1.14	5.43±0.58	12.38±0.42
UNIANOVA	p<0.001	p=0.006	p<0.001	ns
SK-BR-3				
Control	21.25±1.41	39.90±2.00	6.30±0.45	15.98±0.70
10 ng/ml TNF-α for 24 h	26.62±3.85	37.97±1.14	4.75±0.91	12.19±1.87
UNIANOVA	ns	ns	p=0.011	p=0.011
MCF-10A				
Control	23.17±1.28	65.23±1.50	1.27±0.09	6.60±0.41
10 ng/ml TNF-α for 24 h	27.50±2.33	58.61±2.54	1.54±0.19	7.60 0.76
UNIANOVA	ns	p=0.051	ns	ns

Values represent mean ± standard error of three independent experiments. Each experiment had two replicates, i.e. six data points for control and treatment. ns, not significant.

MCF-10A normal breast epithelial cells no significant difference in any cell cycle phase was observed after 24-h treatment with 10 ng/ml TNF-α compared to control. In the G1-phase population a non-significant decrease of 6.6 percentage points (a 12% decrease) after 24-h treatment (p=0.051) with 10 ng/ml TNF-α compared to G1-phase population of control cells was observed.

Discussion

Adipokines have been hypothesised as molecular explanation of the link between obesity and increased risk of developing postmenopausal breast cancer (19-21). Both leptin (22-26) and adiponectin (22,27-29) have been studied in *in vitro* systems and results have indicated that there is some involvement, even though their effect on breast cancer is diametrically opposed. Their effect on growth promotion in breast cancer cells, however, is small and suggests that additional factors may play a role. The fact that many more adipokines have been discovered recently, the expression of which being affected by obesity, makes the contribution of other adipokines in the molecular link between obesity and postmenopausal breast cancer likely. The effects of TNF-α on a breast cancer cell model were therefore studied to evaluate the contribution of TNF-α in the link between obesity and breast cancer progression. Additionally, a normal breast epithelial cell line was used to examine the potential impact of TNF-α on breast cancer aetiology. The impact of TNF-α on breast epithelial cells has not been assessed previously and represents a novel way of assessing the impact of adipokines on breast cancer aetiology.

In MDA-MB-231 breast cancer cells, cell proliferation did not change after 24- or 48-h treatment with 10 ng/ml TNF-α. Similarly, Mueller and his colleagues observed that 0.25 nM (12.75 ng/ml) TNF-α treatment alone did not change cell proliferation in these cells (30). Thus, there is no indication that TNF-α increases cell proliferation in these cells. In SK-BR-3 breast cancer cells, 10 ng/ml TNF-α treatment increased cell proliferation after 24 and 48 h of treatment. The highest increased was observed after 48-h treatment. Similarly, Rivas and colleagues observed an increase in SK-BR-3 cell proliferation following 20 ng/ml TNF-α treatment for 48 h (31). There is, therefore, good evidence that TNF-α has a proliferative effect on SK-BR-3 breast cancer cells. Hence, cell proliferation of the two breast cancer cell lines reacted differently to treatment with TNF-α. While both cell lines are ER-negative, there is a difference in HER2 expression with SK-BR-3 cells expressing high levels and MDA-MB-231 cell expressing low levels (32). In addition, MDA-MB-231 cells carry mutations in the genes coding for kinases RAS and RAF, that form part of the MAP-kinase cell signalling pathway (18). These differences may contribute to the different effect of TNF-α on these cells, which may also apply to the other differences observed. In MCF-10A breast epithelial cells, treatment with 10 ng/ml TNF-α increased cell proliferation after 24 and 48 h. In rat breast epithelial cells TNF-α treatment similarly increased proliferation (33,34). Our results indicate that TNF-α treatment has a substantial and sustained effect on promoting cell proliferation in MCF-10A cells.

PI3-kinase signalling is involved in breast cancer development, its activation being linked to increased cell growth (35). In MDA-MB-231 breast cancer cells, TNF-α treatment did not change phosphorylation of AKT, while in SK-BR-3 breast cancer cells, 10 ng/ml TNF-α treatment increased phosphorylation of AKT. These are novel findings as no other studies investigated the phosphorylation of AKT after treatment with TNF-α in these cells. In one study, TNF-α mediated activation of AKT has been linked to activation of

proliferation-stimulating transcription factor NF-κB in T47D breast cancer cells (13). Thus, similarly activation of AKT may be a pathway for TNF-α stimulated cell proliferation in SK-BR-3 breast cancer cells. In MCF-10A breast epithelial cells, 10 ng/ml TNF-α treatment did not change AKT-phosphorylation significantly. A non-significant increase of 77% in AKT-phosphorylation after 20-min treatment was, however, observed.

MAP-kinase signalling is involved in promoting cell proliferation and apoptosis evasion in breast cancer cells (36,37). TNF-α treatment significantly decreased phosphorylation of ERK1/2 in MDA-MB-231 breast cancer cells. In the only comparable study, treatment of MDA-MB-231 breast cancer cells with 10 ng/ml TNF-α for 24 h induced expression of MMP-9, which was reduced after inhibition of MEK1/2 by 10 μM U0126, suggesting that TNF-α activates ERK1/2 to exert this effect (38). This study, however, did not examine ERK1/2-phosphorylation directly. Thus, there is no confirmation to this finding. Additionally, ERK1/2-phosphorylation slowly and non-significantly increased between 5 and 20 min of treatment. Extension of treatment time may be needed to determine, if this trend continues and TNF-α treatment has a time-delay in inducing ERK1/2-phosphorylation after increased treatment time. A reduction in ERK1/2-phosphorylation is explained by TNF-α's interference with the, by mutation of RAS and RAF, constitutively activated MAP-kinase pathway. It also indicates, however, a growth inhibitory effect of TNF-α on these cells, which in the previous results had not been observed. In SK-BR-3 breast cancer cells, 10 ng/ml TNF-α treatment significantly increased phosphorylation of ERK1/2. This is a novel finding and further supports the growth promoting effect of TNF-α in this breast cancer cell line. With both PI3-kinase and MAP-kinase pathway activated in response to TNF-α treatment, it can not be ascertained which pathway may promote the observed increase in cell proliferation. In MCF-10A breast epithelial cells, 10 ng/ml TNF-α increased phosphorylation of ERK1/2 significantly after between 10- and 20-min treatment. The highest increase was observed after 10-min treatment. This indicates a sustained activation of at least 10 min. The non-significant increase in AKT-phosphorylation may suggest that the MAP-kinase may be majorly responsible for mediating TNF-α induced cell proliferation in this cell line.

In MDA-MB-231 breast cancer cells, 10 ng/ml TNF-α treatment for 24 h significantly decreased the G1- and S-phase population of the cell cycle profile. In addition, cells with a DNA-content below that expected of cells in G1-phase were measured. These cells were indicative of apoptotic cells. In MDA-MB-231 breast cancer cells, this 'subG1' population increased significantly after TNF-α treatment. This suggests that TNF-α may promote apoptosis in these cells. This is a novel finding and in addition to the decrease in ERK1/2 phosphorylation after TNF-α treatment suggests a growth inhibiting and even apoptosis inducing effect of TNF-α on MDA-MB-231 breast cancer cells. In SK-BR-3 breast cancer cells, 10 ng/ml TNF-α treatment significantly decreased S-phase and G2-phase population after 24-h treatment. There was, however, no indication of increased apoptosis. A similar result was observed for T47D breast cancer cells, where S-phase and G2-phase decreased after treatment with 10 ng/ml TNF-α for 24 h (14). These authors concluded that TNF-α induced cell cycle arrest at the G1/S cell cycle checkpoint (14), which may similarly apply to SK-BR-3 breast cancer cells. Cell cycle arrest is typically associated with growth inhibition, thus, the cell cycle results oppose the previous findings, all of which could be interpreted as TNF-α promoting cell growth in SK-BR-3 breast cancer cells. There is no conclusive explanation for this observation. One may be confronted with a similar situation as observed for different subtypes of the MCF-7 breast cancer cell line, which showed opposing effects to TNF-α treatment (17). In MCF-10A breast epithelial cells, 10 ng/ml TNF-α did not change the distribution of the cell population across cell cycle phases. G1-phase population decreased non-significantly after TNF-α treatment ($p=0.051$). The probability value is just above the cut-off point. A decrease in G1-phase population could be interpreted as an increase in cell cycle progression, thus complementing findings from the cell proliferation results and cell signalling pathways. This suggests that all experiments point to an increase in cell growth in MCF-10A cells, following TNF-α treatment.

In conclusion, TNF-α affects the metabolism of breast cancer cells and breast epithelial cells. The effect on breast cancer cells is, however, not as comprehensive as the effect on breast epithelial cells. In MDA-MB-231 breast cancer cells the results generally suggest no effect on cell growth, with some indications of growth inhibition and even increased apoptosis after TNF-α treatment. Conversely, results for SK-BR-3 cells generally indicate a growth promoting effect of TNF-α, while the cell cycle results are contradicting these findings. TNF-α exerts different and even opposing effects between just two breast cancer cell lines, suggesting there is no general mode of action of TNF-α on breast cancer cells, but that different cells, and thus tumours, may react differently to increased TNF-α concentrations. If one assumes cell mosaicism in tumours, one may also speculate that cells of the same tumour could react differently to TNF-α. As the majority of our results indicate growth promotion in SK-BR-3 breast cancer cells, TNF-α can not be excluded as a potential mediator in the link between obesity and breast cancer progression. In MCF-10A breast epithelial cells, TNF-α increases cell proliferation, with no indication of cell cycle arrest or onset of apoptosis. This significant finding introduces a potential and novel way of impact of TNF-α on breast cancer aetiology and may provide a new understanding in the link between obesity and increased risk of breast cancer development.

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