Antisense Inhibition of Amphiregulin Expression Reduces EGFR Phosphorylation in Transformed Human Breast Epithelial Cells

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Abstract. *The activation of epidermal growth factor receptor (EGFR) by its ligands constitutes an important step in the metastatic process but the clinical response to its inhibition in breast cancer patients has so far been very low. In this work, we investigated the role of the EGFR ligand amphiregulin (AR) in modulating EGFR activation. For this, transformed epithelial mammary tumor cells NS2T2A1 were used in which AR or EGFR expression was down-regulated by antisense cDNA technique. This down-regulation was associated with a significant inhibition of matrix metalloproteinase-9 production as well as cell proliferation, but this inhibition was only minimally reversed by exogenously added AR or EGF. EGFR protein levels were not affected but EGFR-tyrosine phosphorylation in response to EGF was markedly reduced. Thus, the inhibition of AR expression, which impairs EGFR response to its exogenously available ligands, may represent an alternative anti-EGFR therapeutic strategy in breast cancer.*

Growth factors exert their effect by interacting with particular cell surface receptors. They can also control migration and invasion of malignant cells. Some breast carcinomas when compared with normal breast epithelium have shown an overexpression of the epidermal growth factor receptor (EGFR), and its levels correlate with aggressive behaviour and increased metastasis in human breast cancer (1, 2). Surprisingly however, overall clinical response rates to EGFR tyrosine kinase inhibitors (EGFR TKIs) in breast cancer have so far been extremely low (3). The therapeutic resistance to EGFR TKI, such as gefitinib in non-small cell lung cancer has been attributed to specific mutations in the EGFR TK domain (4), but such EGFR mutations have not been reported in breast carcinomas (5). A role for EGFR ligands in modulating EGFR TKI efficacy in breast cancer has been also put forward as an alternative explanation (5). A large number of ligands for EGFR have been characterized, including transforming growth factor α (TGFα) and amphiregulin (AR). They are synthesised in a proform as type I transmembrane proteins and are released in a soluble form by extracellular proteolytic cleavage and become available to activate EGFR (6). However, membrane-bound AR precursor is also able to activate EGFR *via* juxtacrine interaction (7, 8), implying that AR–EGFR membrane interaction may be important in controlling EGFR signaling. AR activation of EGFR appears to play an important role in the developing breast (9) and is associated with breast cancer progression. It can act as an autocrine growth factor for normal and oncogene transformed human mammary epithelial cells (10, 11). It was also found in infiltrating breast carcinoma and adjacent non-involved breast tissues (10). AR has been shown, by functional gene screening, to mediate EGF-independent growth in a breast cancer cell line overexpressing EGFR and to confer EGF independence on normal human mammary epithelial cells (12). A highly tumourigenic cell line, NS2T2A1, was previously obtained, after immortalisation of normal human mammary epithelial cells with SV40 T antigen and successive passages in nude mice (13). It was shown that the inhibition of amphiregulin expression by stable transfection of this malignant cell line with antisense cDNA to AR reversed the malignant phenotype of this cell line when injected in nude mice (13). This reversal in tumour formation was associated with a significant decrease in tumour vascular density implying a role for AR in angiogenesis. However, there is little mechanistic information to elucidate how AR may actually contribute to

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the molecular pathogenesis of breast cancer. Proteolytic degradation of extracellular components by matrix metalloproteinases (MMPs) has been accepted as a major event in tumour invasion and angiogenesis (14, 15) and MMP-2 and MMP-9 are the members of the MMP family believed to be particularly important in these processes. It has been shown that MMP-9 is up-regulated by AR and other EGF-like ligands in breast cancer cells, and head and neck squamous carcinoma cells (16, 17).

The present study was aimed at defining the role of AR in the regulation of MMP-9 by using the transformed human breast epithelial cells NS2T2A1 in which AR expression was inhibited by stable transfection with AR antisense (AS) plasmid.

Materials and Methods

Cell culture. A transformed NS2T2A1 cell line was derived from normal human breast epithelial cells following immortalisation by SV40 T Ag and nude mouse tumour selection (18, 19). These cells which can form colonies in soft agar, were shown to be derived from a unique clone and demonstrated high expression of EGFR and AR both *in vitro* and *in vivo* (13, 19). Transfection of the NS2T2A1 cells with the 1.1 kb *AR* cDNA fragment and the 1.8 kb *EGFR* cDNA resulting in amphiregulin antisense RNA expressing AR-AS cell clone and EGFR-antisense expressing EGFR-AS cell clone respectively as described previously (13, 19). AR was reduced by approximately 60% and 95% in the AR-AS cells and EGFR was inhibited by 80% in the EGFR-AS cells. Control cell line NS2T2A1-V was obtained by transfection of the parental NS2T2A1 cells with vector alone. *AR* RNA and *EGFR* RNA antisense expressing cell lines and control cell line NS2T2A1 V were maintained in DMEM/F12 (1:1) medium without calcium (Biological Industries, Kibbutz Beit Haemek, Israel) supplemented with 2 mM glutamine, 10 μg/ml insulin, 5 μg/ml hydrocortisone, 2 ng/ml EGF (Upstate Biotechnology, Millipore, Guyancourt, France), 30 μg/ml hygromycin B (Roche Diagnostics, Meylan, France) and 5% horse serum Ca^{2+}-chelated by Chelex 100. When approximately 50-60% confluent, the cells were transferred to growth factor-free DMEM/F12 containing 2% of carbon-dextran depleted fetal bovine serum, 2 mM glutamine and 30 μg/ml hygromycin B (M2) for further 24 h. The effect of EGF and AR (R&D Systems Europe, Lille, France) was examined in M2 in all studies except for zymography analysis, where cells were first incubated in M2 for 24 h and then transferred to serum free medium (M0) prior to treatment. In some experiments, control cells were treated with 10 μM of the specific tyrosine kinase inhibitor of EGFR gefitinib (Iressa, kind gift from AstraZeneca France, Rueil-Malmaison, France) for 2 h before the addition of growth factors.

Cell proliferation. Proliferation was assayed using a colorimetric 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenol)-2-(4-sulfophenyl)-2H-tetrazolium assay (MTS) (CellTiter 96 AQ; Promega, Charbonnières-les-Bains, France). Cells were plated at a density of 2×10^3/ well in a 96-well flat-bottomed plate. After 48 h in culture, MTS solution was added to each well, plates re-incubated at 37˚C for further 4 h and the absorbance was determined at 490 nm using a plate reader.

Quantitative RT-PCR. Total RNA (1 μg) extracted from the cells using Trizol reagent (Invitrogen, Cergy Pontoise, France) was transcribed with random hexamer and M-MLV reverse transcriptase (Invitrogen). MMP-9 mRNA expression levels was quantified using Perfect MasterMix Probe (AnyGenes, Créteil, France) on LightCycler 2.0 (Roche Diagnostics) according to the manufacturer protocol. Primers and probes (Eurogentec, Angers, France) are detailed in Table I. Gene expression levels were determined using standard calibration curves prepared from gene-specific PCR products cloned using the TOPO II TA cloning kit (Invitrogen). The expression levels of interest transcripts were normalised to the housekeeping β2 microglobulin (B2M) gene transcripts. All experiments were performed in duplicate.

Samples preparation and Western blot analysis. When approximately 50-60% confluent, cells were transferred to M2 media for 24 h and then treated with 10 nM EGF for different time periods. Cells were then lysed in Laemmli sample buffer containing 50 mM dithiothreitol, centrifuged and 25 μg of the supernatants were loaded on a 7% SDS-PAGE gel and electrophoretically separated and immunoblotted with EGFR and tyrosin-1045 phospho-EGFR rabbit polyclonal antibodies (Cell Signaling Technology, Ozyme, Saint-Quentin-en-Yvelines, France). The proteins were visualised with Lumi-Light Western blotting substrate (Roche Diagnostics) and the protein amount was controled by β-actin visualisation.

Gelatin zymography. The presence of MMP-9 in the serum-free conditioned media was analysed by zymography in 10% polyacrylamide gels containing 1 mg/ml gelatin (Sigma Aldrich, Saint-Quentin-Fallavier, France) as previously described (20). Briefly, samples were mixed with Laemmli sample buffer without reducing agents or heating and were subjected to SDS-PAGE. The gels were incubated for 30 minutes at 22˚C in renaturating buffer (2.5% Triton X-100), rinsed in distilled H_2O, and then incubated in developing buffer (50 mM Tris buffer pH 7.5, 200 mM NaCl, 5 mM $CaCl_2$) for 48 h at 37˚C. The gels were stained with 0.2% Coomassie Blue R250 in a solution of 20% isopropanol and 10% acetic acid and then destained in 20% isopropanol, 10% acetic acid.

^{125}I-Labelled EGF binding studies. Plasma membranes were prepared from proliferating cells maintained for 24 h in serum-free, EGF-free media. Cells were disrupted with a polytron homogenszer (Kinematica, Lucerne, Switzerland), centrifuged at 1000 ×g for 10 min and the membrane fraction was then obtained by centrifugation at 25000 ×g for 30 min. Binding experiments were performed with 100 μg/ml plasma membrane protein in 0.2 ml binding assay medium containing 50 mM Tris HCl (pH 7.4), 5 mM $MgCl_2$, 0.1% bacitracin, 1% BSA and 0.2 nM ^{125}I-labelled EGF (Amersham Life Science, GE Healthcare Europe, Orsay, France). Incubations were performed at room temperature for 1 h (^{125}I-EGF binding was shown to reach equilibrium at 1 h) and stopped by the addition of 1 ml ice-cold 50 mM Tris HCl (pH 7.4) and 5 mM $MgCl_2$. Membranes were then washed twice in the same buffer and the radioactivity bound to membranes was determined in a gamma counter (LKB, Wallach, Bromma, Sweden). Specific binding was determined as the difference between the total radioactivity of ^{125}I-labelled EGF bound in the absence (total binding) or presence (non-specific binding) of 100 nM unlabelled EGF. The specific binding data were Scatchard plotted.

Table I. *Oligonucleotide primer and probe sequences used.*

Gene and oligonucleotide	Location	Sequence	PCR product size (bp)
MMP9			
Upper primer	610U	5'-CATTCAGGGAGACGCCCA-3'	
Lower primer	673L	5'-AACCACGACGCCCTTGC-3'	64
Probe	629U	5'-TTCGACGATGACGAGTTGTGGTCCCT-3'	
β2 Microglobulin			
Upper primer	20U	5'-CGCTCCGTGGCCTTAGC-3'	
Lower primer	86L	5'-GAGTACGCTGGATAGCCTCCA-3'	67
Probe	39U	5'-TGCTCGCGCTACTCTCTCTTTCTG-3'	

Results

AR-antisense transfected breast tumour cells NS2T2A1 have a reduced MMP-9 expression which is not restored by exogenous AR or EGF. To investigate the effects of AR on invasiveness of breast cancer cells, we examined the expression level of MMP-9 in the AR-AS expressing cells and their response to the addition of exogenous AR or EGF. Since AR expression can be induced by EGF (data not shown), cells were cultured in EGF-free medium containing 2% of carbon-dextran depleted fetal bovine serum for 24 h prior to AR addition.

As shown in Figure 1, the empty vector-transfected control NS2T2A1 V cells responded to exogenously added AR and EGF by stimulating MMP-9 production, shown by both zymography and real-time PCR analysis. However, the AR-AS transfected cells, as well as the EGFR-AS-expressing cells, demonstrated a greatly reduced constitutive expression of MMP-9. The inhibition of MMP-9 observed in the AS cells was comparable to that obtained in the presence of the specific inhibitor of the EGFR TK, gefitinib (Figure 1B). However, the addition of exogenous 10 nM AR or EGF to the AR-AS clone did not restore normal expression of MMP-9 as it did in control cells. The PCR plots in Figure 1B represent values obtained at 6 h after addition of the growth factor where maximal stimulation was obtained in the NS2T2A1V control cells. Similar results were obtained at different times of incubation, ranging from 1 to 24 h, and when using different concentrations of AR and EGF, ranging from 1 to 200 nM (data not shown).

Exogenous AR only partially restored the proliferation rate in the AR-AS cells. To examine whether other biological effects of AR are also refractive to exogenous AR effects in the AR-AS cells, the proliferation rate of these cells in response to AR was also measured. The results in Figure 2 show that although AR did stimulate growth in the AR-AS cells, its effects were much reduced in comparison to the vector-transfected control cells.

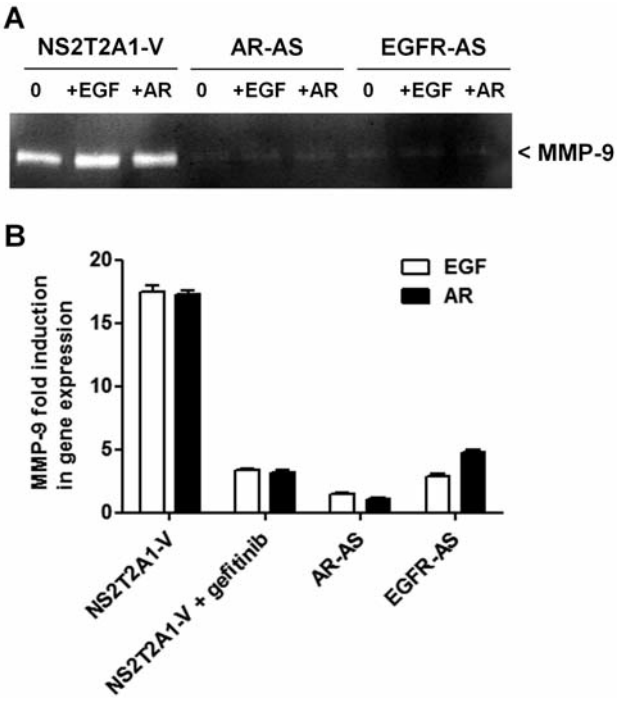

Figure 1. *Expression of MMP-9 is reduced in AR and EGFR-AS transfected NS2T2A1 and is not restored by exogenous AR or EGF. A: Zymography analysis of gelatinolytic activity secreted by the cells incubated 24 h in serum-free medium with or without 10 nM AR or 10 nM EGF. Representative zymogram from at least 3 separate experiments. B: Quantitative real-time PCR measurements of the increase in MMP-9 gene expression following treatment of the cells with either 10 nM AR or 10 nM EGF. Control cells were also pretreated with 10 μM gefitinib for 2 h before ligand addition. In each case, the number of copies of MMP-9 relative to the reference gene β2 microglobin was measured as a function of time from 1-24 h after AR or EGF addition and the ratio of maximum values divided by control values from untreated cells were calculated. Columns represent mean of quantitative change ± standard deviation (n=3).*

EGFR tyrosine phosphorylation induced by EGF was reduced in the AR-AS cells. As the exogenous EGFR ligands were only able to partially reverse the MMP expression or proliferation rate in AR-AS RNA expressing cells, both

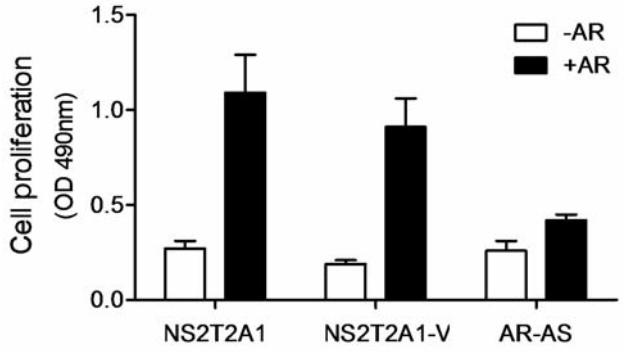

Figure 2. *Effect of AR on the proliferation of AR-AS transfected cells. Cells were incubated in growth factor-free medium for 24 h before the addition of 10 nM AR and proliferation was measured after 48 h incubation. Cell proliferation was determined by the absorbance (OD) at 490 nm of reduced MTS (CellTiter 96 AQ, Promega). Columns represent mean of quantitative change ± standard deviation (n=6).*

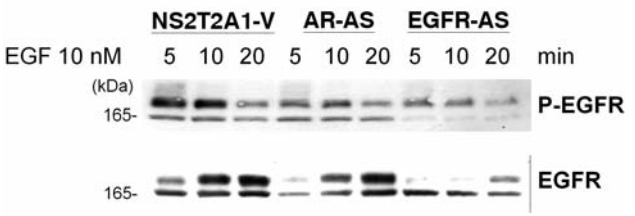

Figure 3. *Reduced EGFR tyrosine phosphorylation in response to EGF in AR-AS cells. EGFR expression and tyrosine phosphorylation in AR-AS- and EGFR-AS-expressing cells at 5, 10 and 20 min after EGF addition. Representative blots from at least three separate experiments.*

Table II. *Membrane EGFR binding.*

Cell line	Low affinity sites		High affinity sites	
	Sites/cell	Kd (M)	Sites/cell	Kd (M)
NS2T2A1 V	33.35	0.50×10^{-9}	4.112	6.4×10^{-12}
AR-AS	28.73	0.33×10^{-9}	4.655	9.5×10^{-12}

EGFR levels and the EGF-induced activation profile of EGFR were analysed by Western blot analysis in the empty vector transfected control cells and the AR-AS cells (Figure 3). In the NS2T2A1V control cells, EGFR tyrosine phosphorylation was induced by 10 nM EGF, reaching a maximum at 5-10 min and diminished at 20 min; EGFR expression was also stimulated by EGF at 10 and 20 min. Compared with these control cells, the AR-AS cells exhibited a weaker phosphorylation response to EGF as judged by the lower intensity of the phosphorylated bands. Total EGFR expression, which was significantly inhibited in the EGFR-AS cells, was however not modified in the AR-AS cells.

In order to determine whether the reduced phosphorylation of EGFR resulted from a change in affinity to its ligands, the binding of ^{125}I-labelled EGF to plasma membranes prepared from these cells was quantified. No significant difference in binding sites and affinity between AR-AS and NS2T2A1 V cells was observed (Table II). Hence, AR-AS transfection had no effect on EGFR levels or on its ability to bind EGF, but did significantly reduce its activation in response to the added EGF.

Discussion

Accumulated evidence suggests that EGFR and its ligands are involved not only in cell proliferation but also in other aspects of the metastatic phenotype such as cell migration, invasion and angiogenesis although the underlying mechanisms are still unclear. The present data showed that AR contributes to the molecular pathogenesis of breast cancer by increasing cancer cell invasion through upregulation of MMP-9, and that the inhibition of endogenous AR expression realised with antisense strategy not only inhibits this MMP expression but is also accompanied by a reduced EGFR TK activity.

The induction of MMP-9 by the activation of EGFR with its various ligands has already been reported in different cellular models (16, 17, 21). In this study, the reduction obtained in endogenous AR expression by stable antisense transfection of the mammary tumour cells NS2T2A1 was associated with a significant reduction in MMP-9 compared with the cells transfected with the vector alone. The fact that the same down-regulation was also observed when the cells were transfected with the antisense cDNA to EGFR, and when treated with a specific tyrosine kinase inhibitor of EGFR, gefitinib (Iressa), suggests that signaling *via* EGFR is involved. However, when attempting to restore MMP levels in the AR-AS cells to the level found in the mock-transfected cells by exogenously adding AR or EGF, it was surprising to note the minimal response to the added ligands. In another study, it was found that exogenous AR also failed to completely restore expression levels of uPA and PAI-1 and the invasive potential of these cells (22). It is interesting to note that these AR-AS cells were somewhat more responsive to the exogenous AR and EGF in terms of their proliferation rate than their invasive profile, suggesting that AR promotes growth or invasiveness through different mechanisms. Differential response to exogenous EGF in breast cancer cells has already been reported by Kondapaka *et al.* (16). When comparing metastatic SKBR-3 and non metastatic MCF-7 cells, these authors reported that EGF increased proliferation in both cell types but induced MMP-9 only in

the metastatic SKBR-3 cells, suggesting signaling mechanisms mediated by different pathways downstream of EGFR. Evidence for a different pathway for *MMP-9* induction by EGF independently of the mitogenic response has also been shown for head and neck squamous carcinoma cells (17).

EGFR protein levels and ligand affinity were not affected by the AR-AS transfection and cannot account for the diminished MMP activity and proliferation in response to the exogenous ligands. However, the intensity of EGFR tyrosine phosphorylation after stimulation with EGF was markedly reduced in the AR-AS cells, similarly to the EGFR antisense RNA expressing cells in which EGFR level is significantly lower. This suggests that a regulating mechanism exists between endogenous AR and EGFR tyrosine phosphorylation. Spatial regulation of EGFR signaling by AR has already been reported (8). In addition, AR precursor, which is a membrane-anchored protein, was shown to be able to activate EGFR in a juxtacrine fashion and can thus maintain EGFR in an activated state in breast cancer cells without the need for proteolytic shedding (23). It is tempting to speculate that the interaction of the AR precursor with EGFR at the level of the membrane may be necessary for basal EGFR function in a way that cannot be replaced by the extracellular soluble ligand.

The resistance commonly observed in breast cancer patients with the currently used EGFR TKIs inhibitors such as gefitinib (Iressa) was shown to be due to an associated up-regulation of EGFR ligands in the resistant patients (5), pointing to the cellular expression of the ligands as the pitfall of such therapeutic strategy. The present results suggest that targeting endogenous AR may represent a double benefit as it targets both receptor and ligand and thus holds a potential advantage over specific EGFR inhibition. If these results are confirmed in further studies using other cell systems, the inhibition of AR expression by antisense or other techniques could represent an alternative, or combined therapeutic strategy to targeting EGFR directly.

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