Effect of Silencing Neutrophil Gelatinase-Associated Lipocalin in Ovarian Cancer Cells on Epithelio-Mesenchymal Transition

Ratana Lim1,2*, Martha Lappas1,3, Nuzhat Ahmed1,4, Niels Borregaard5, Michael A. Quinn4 and Gregory E. Rice1,2,3,6

1Department of Obstetrics and Gynecology, University of Melbourne, Victoria, Australia
2Translational Proteomics, Baker IDI, Melbourne, Victoria, Australia
3Mercy Perinatal Research Centre, Mercy Hospital for Women, Victoria, Australia
4Women’s Cancer Research Centre, Royal Women’s Hospital, Parkville, Victoria, Australia
5Department of Hematology, University of Copenhagen, Denmark
6University of Queensland Centre for Clinical Research, Brisbane, Queensland, Australia

Abstract

Objectives: Previously, we reported that neutrophil gelatinase-associated lipocalin (NGAL) is present in epithelial ovarian cancer cells, but not mesenchymal cancer cells. Furthermore, we reported that epithelial growth factor (EGF) induced epithelio-mesenchymal transition (EMT) in epithelial ovarian cancer cells and concomitantly suppressed NGAL expression. We hypothesised that inhibition of NGAL expression induces EMT in these cells.

Methods: NGAL knockdown was achieved using siRNA in ovarian cancer cell line OVCA 429. NGAL siRNA-treated cells [n=3] were assayed for EMT markers such as migration and invasion assays, and E-cadherin, N-cadherin, Snail, β-catenin and vimentin expression by Western blot.

Results: There were no significant differences between NGAL siRNA-treated cells and controls for the protein markers. There was an increase in cell migration for NGAL siRNA-treated cells compared to control (96 h post transfection), however there was no change in cell invasiveness between treatments.

Conclusions: We conclude that direct NGAL inhibition by siRNA does not induce a mesenchymal transition of ovarian cancer cells.

Keywords: Ovarian cancer; Epithelio-mesenchymal transition; Neutrophil gelatinase-associated lipocalin

Introduction

Epithelial ovarian cancer is the most lethal gynaecological cancer and the fifth most common cause of cancer death in women in the Western world [1]. It has an overall five-year survival of 42%, due to most cases being diagnosed at an advanced stage of disease [2]. There is a paucity of information concerning the pathophysiology of ovarian cancer. It is by understanding the pathways that are intrinsic to disease progression and metastasis that new insights are realised and improvements in patient management and treatment affected.

Epithelial-mesenchymal transition (EMT) has recently been recognised as a mechanism for cancer progression and malignant transformation. EMT by cancer cells does not follow an orderly program and is different from physiological and developmental EMT [3]. EMT induced by growth factors and cytokines requires reprogramming of epithelial cells in order to reshape for locomotion and degradation of the extracellular matrix (ECM). Epithelial growth factor (EGF) has been described as a powerful mitogen for human ovarian surface epithelium (OSE) [4], where 90% of all ovarian carcinomas originate. The over-expression of EGF receptors (EGFRs) is common in human ovarian carcinoma-derived cell lines and tumours, thereby suggesting that the growth factor family plays a critical role in ovarian tumour aetiology and progression [5]. EGF has been shown to induce EMT in epithelial cells [6] and epithelial breast cancer cells [7].

Loss of the epithelial phenotype is characteristic of EMT; the shift from E-cadherin to N-cadherin is a central event mediating EMT [8]. Crucial to E-cadherin activity is its interaction with β-catenin, which links E-cadherin to the actin cytoskeleton. Two members of the Snail family of transcription factors, Snail and Slug, have been shown to down-regulate E-cadherin by binding the E-boxes present in its promoter. During EMT, Snail up-regulates mesenchymal molecules such as fibronectin and vimentin. The mesenchymal N-cadherin interacts with members of the fibroblast growth factor receptor (FGFR) family, thereby inducing pro-migratory and invasive signalling cascades [9]. This promotion of cell motility and migration is the opposite effect of E-cadherin. Thus, hallmarks of EMT include the loss of E-cadherin and β-catenin, and the gain of N-cadherin, Snail and vimentin.

Previously, we have reported that normal ovaries lack neutrophil gelatinase-associated lipocalin (NGAL) expression and that NGAL expression occurs in benign tumours and increases in early grade ovarian tumours [10]. Furthermore, it was also demonstrated that compared to normal healthy women and women with advanced-stage disease, NGAL concentration is higher in the serum of patients with benign and grade 1 tumours. The mechanisms and biology of NGAL in ovarian cancer, however, are not known. Our previous study established that NGAL is only expressed in epithelial ovarian cancer cells and the expression of NGAL is suppressed in ovarian cancer cells induced with EGF to undergo EMT [10]. The aims of this current study were to assess the effect of NGAL knockdown by siRNA transfection

*Corresponding author: Dr. Ratana Lim, Department of Obstetrics and Gynaecology, University of Melbourne, Level 4/163 Studley Road, Heidelberg, Victoria, 3084, Australia, Tel: 61-3-8458 4363; Fax: 61-3-8458 4380; Email: ratana@unimelb.edu.au

Received July 22, 2011; Accepted August 22, 2011; Published August 26, 2011

Citation: Lim R, Lappas M, Ahmed N, Borregaard N, Quinn MA, et al. (2011) Effect of Silencing Neutrophil Gelatinase-Associated Lipocalin in Ovarian Cancer Cells on Epithelio-Mesenchymal Transition. J Mol Biomark Diagn 2:114. doi:10.4172/2155-9929.1000114

Copyright: © 2011 Lim R, et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited
on the expression of EMT markers in ovarian cancer cell line OVCA 429. As EGF is an inducer of EMT in ovarian cancer cells and NGAL expression was decreased in EGF-treated cells, NGAL may play a role in the EMT of ovarian cancer cells. Thus, the hypothesis to be tested in this study was that inhibition of NGAL expression in OVCA 429 cells induces EMT.

Materials and Methods

Cell culture experiments

Cell lines: The ovarian cancer cell line OVCA 429 has been described previously [11]. Cell culture media consisted of a 1:1 ratio of medium 199 and MCDB 105 (Sigma Aldrich, MO, USA), with 10% FBS and supplemented with 2.4 mM L-glutamine and 60 U/ml penicillin/streptomycin solution. All cells were maintained at 37°C in the presence of 5% CO₂. Viability of the cells was checked routinely by the trypan blue exclusion method using a haemocytometer.

siRNA Transfection of ovarian cancer cells

Gene knockdown of NGAL in OVCA 429 ovarian cancer cells (n=3) was achieved by using siRNA transfections. siRNA against NGAL was used, along with a Negative control siRNA and β-actin siRNA (Qiagen, MD, USA). Mock transfected cells consisted of cells treated with Lipofectamine 2000 (Invitrogen, CA, USA) but not siRNA. Ovarian cancer cells were grown in media free of antibiotics in 6 well plates until 30% confluent. For a total transfection complex of 200 μl per well, 2 μl Lipofectamine 2000 was added dropwise to pre-warmed OPTI-MEM medium, vortexed and incubated at room temperature for 10 min. siRNA was added to diluted Lipofectamine 2000 transfection reagent for a final concentration of 150 nM and incubated at room temperature for 15 min. The volume of normal growth medium in each well was adjusted to 800 μl and the siRNA complex was then added. Cells were incubated for 24 h then washed in sterile PBS. Fresh complete medium was added and cells were incubated for up to 72 h. Cells were collected and lysates prepared and frozen until analysis by Western blot or subjected to wounding or invasion assays, whereby data was collected 72 or 96 h post transfection.

Wounding assay

siRNA transfected cells were serum-starved for 24 h before a wound was created with the tip of a sterile 1000 μl pipette. The wound was marked and measurements were taken of distances between wound edges using an ocular micrometer. Six representative fields were marked and measured. Wounds were measured after 24 and 48 h (72 and 96 h post transfection). Three independent experiments were performed.

Invasion assay

The invasive potential of siRNA treated OVCA 429 cells was determined by the ability of the cells to invade a synthetic basement membrane [12]. Briefly, polycarbonate filters (8 μm pore size) pre-coated with Matrigel were placed in a modified Boyden chamber. OVCA 429 cells transfected with siRNA for 48 h were trypsinized and resuspended in SFM. Culture medium containing 5% FBS as a chemoattractant was added to each well of a 24 well plate. Additional wells contained SFM as a negative control. Inserts were placed in the wells and resuspended cells were added. After 24 h, non-invading cells were removed from each membrane by running a sterile cotton tip over the membrane on the inside of the chamber; invading cells appeared on the outer side of the membrane and were fixed in 100% methanol, stained with Diff-Quik and mounted on slides. Total numbers of invading cells were counted using a light microscope. Each experiment was performed in triplicate.

Western blotting

Cell lysates were prepared by adding 150 μl RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Igepal, 0.1% SDS, 0.25% Na deoxycholate, 1 mM EDTA, pH7.4, 1mM PMSF and 10 μl/ml aprotinin) to cell pellets, vortexed, incubated at 4°C for 1 h, then centrifuged at 10,000 X g for 20 min. Supernatants were collected as cell lysates. Equal amounts of protein were electrophoresed on 7.5% and 12% SDS-PAGE gels. Western blotting was performed as described previously [10]. The following antibodies and concentrations were used: NGAL (Clone 211-1 [13], at 0.2 μg/ml), E-cadherin (BD Biosciences, at 50 ng/ml), Snail (Abcam, at 0.5 μg/ml), β-catenin (BD Biosciences, at 50 ng/ml), vimentin (Dako, at 2 μg/ml) and β-actin (Sigma Aldrich, at 0.1 μg/ml). Western blots were the product of at least two independent experiments.

Statistical analyses

Statistical analysis of the data was performed using commercially available statistical software (Statgraphic Plus version 3.1, statistical graphics corporation, Rockville, Maryland, USA). The data are presented as the mean ± standard error of the mean (SEM). Statistical significance was assigned to P<0.05, unless otherwise stated. The data were initially analysed for homogeneity of variance using Bartlett’s test and when significant (P<0.05) the data were logarithmically transformed and homogeneity of variance confirmed before further analysis. Where logarithmic transformation of the data was necessary, the mean values before transformation have been presented. In cases where homogeneity was established, two-sample comparisons were performed using Student’s t-tests, and three or more sample analysis was performed using one-way analysis of variance (ANOVA).

Results

Effect of NGAL knockdown by siRNA on EMT markers

siRNA Transfection of OVCA 429 cells: OVCA 429 cells were transfected with NGAL siRNA, Negative control siRNA or no siRNA (mock cells). Western blot showed that NGAL protein expression was unaffected by β-actin and negative control siRNA-treated cells (Figure 1). NGAL siRNA successfully decreased NGAL protein expression in OVCA 429 cells compared to mock transfected cells by ~70% (P<0.05). Likewise, a decrease in β-actin protein expression was found in cells treated with β-actin siRNA; however, there was no effect on β-actin protein expression on the NGAL siRNA-treated cells.

Protein expression of EMT markers

The effect of NGAL knockdown in OVCA 429 cells on markers of EMT was assessed by Western blot. The EMT markers investigated were E-cadherin, β-catenin and Snail (Figure 2). Blots were also probed for β-actin. A431 lysate and HEY day 4 spheroid cell lysates were included as positive controls [14]. Human recombinant Snail protein provided a positive control for the Snail antibody. The NGAL siRNA-treated cells were compared to negative and mock siRNA treated cells and there was no effect for E-cadherin, β-catenin or Snail proteins. There was also no change for N-cadherin and vimentin proteins [data not shown].

Cell motility

The invasive potential of siRNA-treated OVCA 429 cells was explored using a wounding assay (Figure 3). The average percentage
wound closure after 72 h [24 h post wound] for NGAL siRNA-treated cells was 26 ± 6; after 96 h (48 h post wound) it was 55 ± 3. For mock transfected cells, the wound closure after 72 and 96 h was 21 ± 1 and 42 ± 3 respectively. There was a statistical significance between 72 and 96 h for each treatment (P < 0.05), but there was no difference between treatments at 72 h. However, at 96 h, NGAL siRNA-treated cells had a greater percentage wound closure compared to mock transfected cells (P < 0.05).

Cell invasiveness

In addition to the wounding assay to measure the invasive potential of siRNA-treated OVCA 429 cells, an invasion chamber assay was performed. This assay measures the number of cells migrating through pores of a polycarbonate filter, simulating a basement membrane. After 24 h (72 h post transfection), the number of cells on the underside of each membrane was counted and the mean calculated. As a negative control, no chemoattractant was added to wells of mock transfected cells; the average number of invading cells (± SEM) was 2 ± 1 (data not shown). With the chemoattractant, the mean number of mock cells that invaded the membrane was 21 ± 7 (Figure 4). The mean number of NGAL siRNA-treated cells that migrated through the membrane was 13 ± 2. No significant difference was found between NGAL siRNA-treated cells and mock cells. NGAL knockdown of these cells were confirmed by Western blot [data not shown].

Discussion

The aim of this study was to test the hypothesis that inhibition of NGAL expression in OVCA 429 cells induces EMT. NGAL expression was effectively suppressed by 70% using siRNA knockdown. Known phenotypic markers of EMT, including E-cadherin, β-catenin, Snail, vimentin, N-cadherin and cell migration metrics were used as experimental endpoints. The data obtained establish that inhibition of NGAL expression in OVCA 429 cells is not obligate for EMT.

Previously, we reported that transformation to a more invasive mesenchymal phenotype was associated with a decrease in NGAL expression in epithelial ovarian cancer cell lines [10]. In this study, however, the effects of independent suppression of NGAL did not induce EMT.

NGAL activity has been studied in colon carcinoma cells [15]. Colon carcinoma cell lines were stably transfected with NGAL (for over-expression) or antisense NGAL (under-expression). Western blot analysis of cell lysates showed no changes in E-cadherin and β-catenin expression, mirroring the results of this study. A change in both proteins, however, was found by immunofluorescence staining; localisation of E-cadherin was found to be critical for maintaining...
NGAL expression has also been identified in breast cancer cells, with a recent study demonstrating that NGAL promotes breast cancer progression by inducing EMT [17]. NGAL over-expressed in human breast cancer cells up-regulated mesenchymal markers vimentin and fibronectin, down-regulated epithelial marker E-cadherin and increased cell motility and invasiveness. NGAL silencing in aggressive breast cancer cells inhibited cell migration and the mesenchymal phenotype. It has been noted that NGAL expression is highest in advanced breast cancer stages [18], contrary to our findings which showed that NGAL expression was highest in well-differentiated ovarian cancers and decreased with tumour grade [10]. Similarly, NGAL has also been characterised in pancreatic cancer (PaCa) cells [19], being highly expressed in early dysplastic lesions in the pancreas. Stable NGAL over-expression in PaCa cells significantly blocked cancer cell adhesion and invasion, with the opposite effects occurring in the NGAL under-expressing PaCa cells. The differences in the results observed in ovarian cancer cells compared to the findings on breast and colon cancer cells may suggest different mechanistic pathways relevant to the physiology of different types of carcinomas.

Although these results are not conclusive in determining the role of NGAL in the metastasis of epithelial ovarian cancer cells, studies in the literature indicate that NGAL does play some part in disease progression. To further our study, it would be beneficial to explore the effect of NGAL inhibition in other ovarian cancer cell lines, and to determine the effects of NGAL over-expression. We demonstrated that EGF was able to induce EMT in ovarian cancer cell lines, and that NGAL expression also decreased in these treated cells, leading to our hypothesis that NGAL was able to induce EMT; however our results suggest that NGAL expression decreased as a result of EMT, i.e. in EGF-treated cells. Our results may best be explained by the recent study by Tong et al. [20], which demonstrated that EGF blocked NGAL expression in pancreatic ductal adenocarcinoma (PDAC). They showed that EGF exposure decreased NGAL expression, an effect that was abolished when cells were further treated with EGF-inhibitor AG1478, the same result as seen in our ovarian cancer cells [10]. E-cadherin expression was also decreased in EGF-treated cells, again in accordance to our previous study [10]. Tong concluded that EGF down-regulated E-cadherin and NGAL by activating EGFR and the mitogen-activated protein kinase (MAPK)-ERK signalling pathway in PDAC cells. Our studies have shown that NGAL is present in epithelial ovarian cancer cell lines, rather than the more aggressive mesenchymal cell lines, and that EMT induced by EGF decreases NGAL expression. While our hypothesis was drawn from these results, that NGAL inhibition is akin to the transition of the mesenchymal phenotype, we found that there were no differences in NGAL siRNA treated cells for typical EMT markers such as vimentin, E-Cadherin, N-cadherin, Snail and β-catenin. There was an increase in cell migration for NGAL siRNA-treated cells and wounded, as described in Materials and Methods. Measurements were taken between wound edges at time of wound (0 h) and 24 h and 48 h after wound (72 and 96 h post transfection). The percentage wound closure was calculated for both time points compared to 0 h. For both NGAL siRNA and mock transfected cells, there was a significant increase in wound closure between 72 and 96 h (*P<0.05 vs. 96 h mock transfected). At 96 h there was a significant increase in wound closure for NGAL siRNA-treated cells (**P<0.05 vs. 96 h NGAL siRNA).

Figure 3: Wounding Assay of siRNA-transfected OVCA 429 cells. NGAL siRNA-treated cells and mock transfected cells were grown in a 6-well plate and wounded, as described in Materials and Methods. Measurements were taken between wound edges at time of wound (0 h) and 24 h and 48 h after wound (72 and 96 h post transfection). The percentage wound closure was calculated for both time points compared to 0 h. For both NGAL siRNA and mock transfected cells, there was a significant increase in wound closure between 72 and 96 h (*P<0.05 vs. 96 h mock transfected). At 96 h there was a significant increase in wound closure for NGAL siRNA-treated cells (**P<0.05 vs. 96 h NGAL siRNA).

Figure 4: Invasion chamber assay for siRNA-transfected OVCA 429 cells. NGAL siRNA-treated cells and mock transfected cells were put into modified Boyden chambers to determine the ability of treated cells to invade a synthetic membrane. The total number of invading cells were counted after 24 h (72 h post transfection) and the mean calculated. No significant difference was detected between NGAL siRNA-treated cells and mock transfected cells.

normal epithelial structure. This study explored the effects of NGAL on small Rho GTPases, such as Rac1, as they are implicated in the establishment and maintenance of E-cadherin-mediated cell-cell adhesion as well as in the migration and invasion of epithelial tumour cells [16]. NGAL over-expression in the colon cancer cell line decreased E-cadherin-mediated cell-cell adhesion and increased cell motility and invasion through the alteration of the subcellular localisation of Rac1 [15]. NGAL suppression also decreased colon cancer cell invasion, independent of MMP-9. It was suggested NGAL contributed to colon cancer pathophysiology through the signalling of intracellular small GTPases in cancer migration and invasion. Applying these finding to our own results, the lack of change between NGAL siRNA and control cells for EMT markers may be due to using the total cell lysate and not looking at localisation of proteins. Only further studies will clarify if NGAL has a role in ovarian cancer progression that is different to colon cancer.
Acknowledgements

BOOTS (Breasts, Ovaries, and Other Things Sacred) and All InC provided a PhD scholarship (Dr Ratana Lim) to support this study. Professor Gregory Rice is a National Health and Medical Research Council (NHMRC) Principal Research Fellow. Dr. Martha Lappas is the recipient of a NHMRC RD Wright Fellowship (Grant No. 454777). The authors gratefully acknowledge the assistance of Julene Hallo of the Royal Women’s Hospital for specimen collection and the support of the Fight Cancer Foundation and Jigsaw women’s fashion company.

References

1. Jemal A, Siegel R, Ward E, Murray T, Xu J, et al. (2007) Cancer statistics, CA Cancer J Clin 57: 43-66.
2. Neeham D (2007) Ovarian cancer screening. Aust Fam Physcian 36: 126-128.
3. Vincent-Salomon A, Thiery JP (2003) Host microenvironment in breast cancer development: epithelial-mesenchymal transition in breast cancer development. Breast Cancer Res 5: 101-106.
4. Siemens CH, Auersperg N (1988) Serial propagation of human ovarian surface epithelium in tissue culture. J Cell Physiol 134: 347-356.
5. Mahile NJ, Baron AT, Barrette BA, Boardman CH, Christensen TA, et al. (2002) EGF/ErbB receptor family in ovarian cancer. Cancer Treat Res 107: 247-258.
6. Salamaca CM, Maines-Bandiera SL, Leung PC, Hu YL. Auersperg N (2004) Effects of epidermal growth factor/hydrocortisone on the growth and differentiation of human ovarian surface epithelium. J Soc Gynecol Invest 11: 241-251.
7. Ackland ML, Newsgreen DF, Fridman N, Waltham MC, Arvanitis A, et al. (2003) Epidermal growth factor-induced epithelio-mesenchymal transition in human breast carcinoma cells. Lab Invest 83: 435-448.
8. Thiery JP (2002) Epithelial-mesenchymal transitions in tumour progression. Nat Rev Cancer 2: 442-454.
9. Cavallo R, Christoffi G (2004) Cell adhesion and signalling by cadherins and Ig-CAMs in cancer. Nat Rev Cancer 4: 118-132.
10. Lim R, Ahmed N, Borregaard N, Riley C, Wafai R, et al. (2007) Neutrophil gelatinase-associated lipocalin (NGAL) an early-screening biomarker for ovarian cancer: NGAL is associated with epithelial growth factor-induced epithelio-mesenchymal transition. Int J Cancer 120: 2429-2434.
11. Ahmed N, Pansino F, Clyde R, Murthi P, Quinn MA, et al. (2002) Overexpression of alpha(v)beta6 integrin in serous epithelial ovarian cancer regulates extracellular matrix degradation via the plasminogen activation cascade. Carcinogenesis 23: 237-244.
12. Albini A (1998) Tumor and endothelial cell invasion of basement membranes. The matrigel chemoinvasion assay as a tool for dissecting molecular mechanisms. Pathol Oncol Res 4: 230-241.
13. Kjeldsen K, Koch A, Arriots K, Borregaard N (1996) Characterization of two ELISAs for NGAL, a newly described lipocalin in human neutrophils. J Immunol Methods 198: 155-164.
14. Shield K, Ackland ML, Ahmed N, Rice GE (2009) Multicellular spheroids in ovarian cancer metastases: Biology and pathology. Gynecol Oncol 113: 143-148.
15. Hu L, Hittleman W, Lu T, Ji P, Arfinghaus R, et al. (2009) NGAL decreases E-cadherin-mediated cell-cell adhesion and increases cell motility and invasion through Rac1 in colon carcinoma cells. Lab Invest 89: 531-548.
16. Braga VM, Del Maschio A, Machesky L, Dejana E (1999) Regulation of cadherin function by Rho and Rac: modulation by junction maturation and cellular context. Mol Biol Cell 10: 9-22.
17. Yang J, Bielenberg DR, Rodig SJ, Doiron R, Clifton MC, et al. (2009) Lipocalin 2 promotes breast cancer progression. Proc Natl Acad Sci USA 106: 3913-3918.
18. Bauer M, Eickhoff JC, Gould MN, Mundhenke C, Maass N, et al. (2008) Neutrophil gelatinase-associated lipocalin (NGAL) is a predictor of poor prognosis in human primary breast cancer. Breast Cancer Res Treat 108: 389-397.
19. Furutani M, Ariti S, Mizumoto M, Kato M, Imamura M (1998) Identification of a neutrophil gelatinase-associated lipocalin mRNA in human pancreatic cancers using a modified signal sequence trap method. Cancer Lett 122: 189-214.
20. Tong Z, Chakraborty S, Sung B, Koolwai P, Kaur S, et al. (2010) Epidermal growth factor down-regulates the expression of neutrophil gelatinase-associated lipocalin (NGAL) through E-cadherin in pancreatic cancer cells. Cancer [Epub ahead of print].
21. Berger T, Cheung CC, Elia AJ, Mak TW (2010) Disruption of the Lon2 gene in mice suppresses primary mammary tumor formation but does not decrease lung metastasis. Proc Natl Acad Sci USA 107: 2995-3000.
22. Bolignano D, Donato V, Llacuniqui A, Fazio MR, Bono C, et al. (2010) Neutrophil gelatinase-associated lipocalin (NGAL) in human neoplasias: a new protein enters the scene. Cancer Lett 288: 10-16.