Effects of aluminium chloride and aluminium chlorohydrate on DNA repair in MCF10A immortalised non-transformed human breast epithelial cells

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Use of underarm aluminium (Al)-based antiperspirant salts may be a contributory factor in breast cancer development. At the 10th Keele meeting, Al was reported to cause anchorage-independent growth and double strand DNA breaks in MCF10A immortalised non-transformed human breast epithelial cells. We now report that exposure of MCF10A cells to Al chloride or Al chlorohydrate also compromised DNA repair systems. Long-term (19–21 weeks) exposure to Al chloride or Al chlorohydrate at a $10^{-4}$ M concentration resulted in reduced levels of BRCA1 mRNA as determined by real-time RT-PCR and BRCA1 protein as determined by Western immunoblotting. Reduced levels of mRNA for other DNA repair genes (BRCA2, CHK1, CHK2, Rad51, ATR) were also observed using real-time RT-PCR. Loss of BRCA1 or BRCA2 gene function has long been associated with inherited susceptibility to breast cancer but these results suggest that exposure to aluminium-based antiperspirant salts may also reduce levels of these key components of DNA repair in breast epithelial cells. If Al can not only damage DNA but also compromise DNA repair systems, then there is the potential for Al to impact on breast carcinogenesis.

1. Introduction

Application of aluminium (Al)-based antiperspirant salts to the underarm region has been implicated in the rising incidence of breast cancer [1–4]. Breast cancers originate with a disproportionately high frequency (>50%) in the upper outer quadrant of the breast [5,6] for which an explanation remains lacking other than that there is more target epithelial tissue in that region [7,8]. However, coincidentally, this is also the region of the breast to which antiperspirant products are applied. Al can be absorbed from application of antiperspirant to the skin of the underarm [9], and procedures equivalent to shaving have been demonstrated to increase dermal absorption [10]. Al has been measured in several compartments of the human breast including breast tissue [11–16], breast cyst fluid [17] and nipple aspirate fluid [18].

Genomic instability has been reported in outer regions of human breast tissue compared with inner regions [19,20] which is interesting in the light of the report of Al also being measured at greater levels in human breast tissue (but not the fat) in outer than inner regions of the breast [13]. The cancer field theory, proposed many years ago, suggests that cancer may arise stochastically in regions of genomic instability [21] and the hypothesis that antiperspirant use may contribute to generating a region of genomic instability in the human breast [1–4] is worthy of testing.

DNA damage and loss of DNA repair capability are fundamental components of the mechanisms of carcinogenesis [22] leading to genomic instability which is a hallmark of cancer cells [23]. Al is known to possess DNA damaging capability [3,24], and at the 10th Keele meeting, Al was reported specifically to induce double strand breaks in the DNA of MCF10A immortalised, non-transformed human breast epithelial cells [25] at concentrations measured in nipple aspirate fluids [18] and where control experiments showed no mutagenic activity in bacteria or similar DNA damage in keratinocytes or fibroblasts [25]. Loss of function of genes involved in repair of DNA double strand breaks, most notably BRCA1 and BRCA2, is known to be linked to inherited susceptibility to breast cancer [26,27] but the increasing penetrance of these genes [28] indicates another environmental component as well. In this context, it is of interest to investigate whether Al might influence expression of these or any other components of the DNA damage sensing or repair protein complexes. In this paper we describe how long-term exposure of MCF10A human breast epithelial cells to the Al-based antiperspirant salts Al chloride and Al chlorohydrate can result in downregulation of BRCA1 mRNA/protein. We also report downregulation of mRNAs for BRCA2, CHK1, CHK2, Rad51 and ATR which are other components of the DNA double strand break repair processes associated with increased risk of breast cancer when lost [27,29].
2. Methods

2.1. Stock culture of MCF10A human breast epithelial cells

MCF10A human breast epithelial cells were purchased from the American Tissue Culture Collection at passage number 98. The cells were maintained as monolayer cultures in a medium containing 1:1 (v/v) Dulbecco’s modified Eagle’s medium and Ham’s F12 (Invitrogen) supplemented with 5% (v/v) horse serum (Invitrogen), 500 ng/ml hydrocortisone (Sigma), 10 μg/ml insulin (Sigma) and 20 ng/ml epidermal growth factor (Sigma) in a humidified atmosphere of 10% carbon dioxide in air at 37 °C.

2.2. Long-term growth with Al

Cells were maintained in stock culture medium as above with no further addition (control), 10−4 M Al chloride (AlCl3) (Sigma, UK) or 10−4 M Al chlorohydrate (AlChloro) (Alembic Ltd., UK). Al salts were made as stock solutions in distilled water and diluted 1 in 1000 (v/v) into culture medium. Controls contained the same volume of distilled water.

2.3. Real-time RT-PCR

Following long-term (18–20 weeks) growth without or with Al (see above), cells were plated at a density of 12.8 × 105 cells/9 cm culture dish (16 mL), and grown for a further 7 days in stock medium without or with the respective concentration of Al salt. This enabled cells to be harvested (using a rubber policeman) at similar densities after a total exposure time of 19–21 weeks, and whole cell RNA was prepared using the RNeasy mini kit with a Qiashredder column according to manufacturer instructions (Qiagen). First strand cDNA synthesis was performed using the QuantiTect reverse transcription kit (Qiagen) and polymerase chain reactions were carried out using the QuantiTect SYBER Green PCR kit (Qiagen) together with BRCA1 (QT00039305), BRCA2 (QT00008449), CHK1 (QT00006734), CHK2 (QT01016155), Rad51 (QT00072688), ATR (QT00030779) or β-actin (QT01680476) QuantiTect primers (Qiagen) according to manufacturer protocols. Quantitative values were obtained from the threshold cycle (Ct) number at which the exponential increase in fluorescent signal from the PCR product was at the midpoint (50%). All reactions were performed in triplicate. The Ct number for each gene target (BRCA1, BRCA2, CHK1, CHK2, Rad51, ATR) was normalised to the Ct number for the corresponding β-actin reaction and values from cells grown with Al normalised to control values of cells grown without Al using the \(2^{-\Delta\Delta C_{t}}\) method of relative gene expression analysis [30]. Average values for the triplicate reactions were then calculated. This was then repeated for each of the three biological replicates generated from independent cell cultures after 19, 20 or 21 weeks of culture, and results presented show the average ± SE (n = 3) of the three biological replicates. According to the \(2^{-\Delta\Delta C_{t}}\) method, results are presented relative to the control value of 1.0 for cells grown in the absence of Al. Statistically significant differences were determined using ANOVA Dunnett in Minitab17.

2.4. Western immunoblotting

Cells were grown for RT-PCR and lysates prepared as described previously [31] but using lysis buffer [50 mM Tris–HCl pH 7.4, 250 mM NaCl, 5 mM EDTA, 0.3% (v/v) Triton-X-100, 4-(2-aminophenyl)benzenesulfonyl fluoride (AEBSF 0.3 mM), leupeptin (10 μg/ml) and aprotonin (2 μg/ml)] (1 μl buffer/107 cells). Lysates were incubated on ice 30 min, passed through needles 19G–25G, run on 10% SDS-PAGE BioRad stain-free gels (25 μg protein per track) and transferred onto BioRad PVDF membranes using the BioRad Trans-Blot-Turbo semi-dry transfer system according to manufacturer’s instructions. Membranes were blocked and immunoblotted as published [31] but using Tris-buffered saline (TBS) throughout. Primary antibody to BRCA1 or β-actin (Cell Signaling) was diluted 1/1000 and HRP-linked secondary antibody (rabbit (Cell Signaling)) was diluted 1/2000. Bands were detected using enhanced chemiluminescence (ECL) (GE Healthcare) according to manufacturer’s instructions and quantitation was performed digitally using the GE ImageQuant LAS4000mini luminescent image analyser. Band signals were normalised relative to digitally quantified total protein using the BioRad stain-free system according to manufacturer instructions. All results show the average ± SE (n = 3) of biological replicates generated from three independent cell cultures and were analysed for statistical significance using ANOVA Dunnett in Minitab17.

3. Results

3.1. The experimental model

MCF10A human breast epithelial cells are an immortalised but non-transformed cell line [32] and therefore offer a model system for investigating mechanisms of carcinogenesis [33]. Published research has demonstrated that exposure to Al enables anchorage-independent growth of these cells [25], which is a property of epithelial cells in vitro acknowledged to be closely correlated with transformation in vivo [34]. In the research reported here, Al chloride and Al chlorohydrate were chosen as relevant antiperspirant salts for study [35]. Since Al has been measured in many samples of human breast tissue [11–18], Al is likely to be present long-term in the human breast and so effects were studied over the long-term on expression of DNA repair genes. A time period of 19–21 weeks was chosen in line with previously published gene expression studies on Al in human breast cells [36] and mRNA/protein samples were harvested from independent cell cultures after 19, 20 and 21 weeks to ensure biological replicates. The concentration of 10−4 M Al was chosen for study as the highest concentration of Al which had previously been shown to have no detrimental effect on proliferation of human breast cells in the long term [3].

3.2. Effect of Al on levels of BRCA1 mRNA and protein in MCF10A cells

Effects of long-term exposure to Al were investigated on levels of BRCA1 mRNA and BRCA1 protein. Biological replicate cell lysates were prepared after 19, 20 and 21 weeks of exposure to 10−4 M Al chloride or 10−4 M Al chlorohydrate. Using real-time RT-PCR, reduced levels of BRCA1 mRNA were observed after the long-term exposure to 10−4 M Al chloride (P < 0.001) or 10−3 M Al chlorohydrate (P < 0.001) (Fig. 1). Western immunoblotting showed that levels of BRCA1 protein were also reduced after long-term exposure to aluminium and an example of a Western immunoblot after 20 weeks is shown in Fig. 2A. Calculated averages of biological replicates (19, 20, 21 weeks) normalised to total protein showed that levels of BRCA1 protein were reduced following long-term exposure to 10−4 M Al chloride (P = 0.043) or 10−3 M Al chlorohydrate (P < 0.008) (Fig. 2B).

3.3. Effect of Al on expression of mRNAs for other DNA repair proteins in MCF10A cells

Effects of long-term exposure to 10−4 M Al chloride or 10−4 M Al chlorohydrate were then investigated on levels of mRNA coding for other DNA repair proteins. Using real-time RT-PCR and the same collected samples as above, levels of BRCA2 mRNA, CHK1 mRNA, CHK2 mRNA, Rad51 mRNA and ATR mRNA were also all reduced following long-term exposure to the Al salts (Fig. 3).
These results demonstrate that long-term exposure to Al causes a reduction in cellular levels of BRCA1 mRNA and BRCA1 protein in MCF10A immortalised non-transformed human breast epithelial cells. Inheritance of loss of function of the BRCA1 gene, which encodes a protein involved in homology directed repair of DNA, is well established as a risk factor for susceptibility to breast cancer development [26,27,29] but this is the first report that BRCA1 gene expression can also be reduced by long-term exposure to Al in cultured breast epithelial cells. The finding also of downregulated mRNAs for BRCA2, CHK1, CHK2, Rad51 and ATR indicates a more generalised reduction in DNA repair processes is essential to the maintenance of genomic integrity, and impaired processes for sensing or repair of damaged DNA are associated with development of genomic instability which is an enabling characteristic of cancer cells [23].

Although the Al concentration of $10^{-4}$ M used in these experiments might be considered as high, it is not out of line with measurements of Al in human breast tissues which are also high. Levels of Al in human breast tissue have been reported as ranging from 4–437 nmol/g dry weight [13]: assuming tissue is about 80% water and 1 g of tissue has a volume of 1 ml, these concentrations equate to $0.80 \times 10^{-6}$ M–$0.87 \times 10^{-4}$ M. Levels of Al have been reported in type I breast cyst fluids as ranging from 150–520 µg/l (mean $268.4 \pm 28.1$ µg/l) [18] which equate to concentrations of $0.56–1.93 \times 10^{-3}$ M. Levels of Al have been reported in nipple aspirate fluids as ranging from 80–330 µg/l (median 150 µg/l) [17] which equate to concentrations of $0.30–1.22 \times 10^{-5}$ M. This shows that the concentrations of Al used in this study could be reached in some human breast tissues and therefore that this study has biological relevance. However, there is clearly a need to now repeat these time courses using lower concentrations of Al in order to complete a fuller understanding of effects across the whole range of concentrations measured in human breast structures.

Since the MCF10 immortalised non-transformed human breast epithelial cell lines have been shown to be a relevant model system for the study of breast carcinogenesis [32,33], validation of the findings in the human breast in vivo is a next challenge. A correlation in vivo is supported from previously published results documenting higher levels of Al in outer than inner regions of the breast [13] where there are reported increases in genomic instability in breast tissue [19,20]. The cancer field theory [21] hypothesises that cancer arises stochastically in an area of genetically unstable cells and it has long been known that breast cancer arises with a disproportionately high incidence in

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**Fig. 1.** Effects of long-term exposure to Al on levels of BRCA1 mRNA in MCF10A human breast epithelial cells as determined using real-time RT-PCR. Cells were assayed after 19–21 weeks with no aluminium, with $10^{-4}$ M Al chloride (AlCl$_3$), or with $10^{-4}$ M Al chlorohydrate (AlChloro). For each RT-PCR assay, BRCA1 values were normalised to $\beta$-actin values, expressed as fold change from control cells grown without Al by the $2^{-\Delta\Delta C_T}$ method and the average of triplicate technical replicates calculated. Results shown are the average ± SE of biological replicate assays ($n = 3$) taken from cells grown for 19, 20 or 21 weeks of culture. Fold change for cells grown with AlCl$_3$ (dark grey bars) or Al Chlor (light grey bars) compared to control cells grown without Al (relative value 1.0). **P < 0.001 from control without Al by ANOVA Dunnett.

**Fig. 2.** Effects of long-term exposure to Al on levels of BRCA1 protein in MCF10A human breast epithelial cells as determined using Western immunoblotting. Cells were assayed after 19–21 weeks with no aluminium, with $10^{-4}$ M Al chloride (AlCl$_3$), or with $10^{-4}$ M Al chlorohydrate (AlChloro). Protein was loaded at 25 µg/track. A. Example of a Western immunoblot for BRCA1 and $\beta$-actin after 20 weeks. B. Quantitated BRCA1 protein was normalised to total protein using BioRad stain-free technology. Results are the average ± SE of the biological triplicate experiments at 19, 20 and 21 weeks. *P < 0.05 from control without Al by ANOVA Dunnett.

**Fig. 3.** Effects of long-term exposure to Al on levels of mRNA for BRCA2, CHK1, CHK2, Rad51 and ATR in MCF10A human breast epithelial cells as determined using real-time RT-PCR. Cells were assayed after 19–21 weeks with no aluminium, with $10^{-4}$ M Al chloride (AlCl$_3$), or with $10^{-4}$ M Al chlorohydrate (AlChloro). For each RT-PCR assay, values for each target gene were normalised to $\beta$-actin values, expressed as fold change from control cells grown without Al by the $2^{-\Delta\Delta C_T}$ method and the average of triplicate technical replicates calculated. Results shown are the average ± SE of biological replicate assays ($n = 3$) taken from cells grown for 19, 20 or 21 weeks of culture. Fold change for cells grown with AlCl$_3$ (dark grey bars) or Al Chlor (light grey bars) compared to control cells grown without Al (relative value 1.0). **P < 0.001, *P < 0.05 from control without Al by ANOVA Dunnett.

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the upper outer quadrant of the breast [5,6]. However, epidemiological studies attempting to correlate usage of Al-based antiperspirant salts with breast cancer development have been few and with mixed results. Two studies reported no association [37,38], whilst one study found within a population of breast cancer patients that those who used more antiperspirant were diagnosed at an earlier age with breast cancer [39]. One major challenge with such studies is the general lack of understanding by consumers of the difference between antiperspirant and deodorant, and therefore the accuracy of recording exposure to Al-based antiperspirant salts specifically.

Al is known to possess genotoxic activity [3,24,25] but specific effects in breast cells are relevant in the context of the widespread use of Al-based antiperspirant salts in the region of the human breast. Recent calculations by regulatory bodies have reported that antiperspirant use, especially in conjunction with dermal damage generated from a prerequisite for development of cancer [23]. However, irrespective then there is the potential for Al to cause genome instability which is externality in instability through simply reduced capacity to repair DNA damaged by any DNA damaging capability of Al, Al could still cause genome instability. Whilst antiperspirant salts are known to enter breast tissue and therefore the accuracy of recording exposure to Al salts from antiperspirant use does add into this complex mixture then reduction in use or reformulation of products could offer a strategy for breast cancer prevention.

Competing financial interests

The authors have no competing financial interests.

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References