ACCELERATED PAPER

DNA damage in breast epithelial cells: detection by the single-cell gel (comet) assay and induction by human mammary lipid extracts

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The presence of DNA damage in primary cultures of human mammary epithelial cells (HMECs), and the ability of extracts of human mammary lipid to cause such damage, has been investigated. Lipid extracts, prepared by a solid-phase procedure, and HMECs were obtained from breast tissue removed from healthy women (ages 18–50 years) who were resident in the UK and undergoing elective reduction mammoplasties. DNA single strand breaks (SSBs) were detected using the single-cell gel assay (comet assay) with alkaline electrophoresis (pH 12.3) and quantified by measuring comet tail length (CTL) (µm). Untreated HMECs and HMECs incubated (30 min, 37°C) with a mammary lipid extract, with or without DNA-repair inhibitors hydroxyurea (HU) and cytosine arabinoside (ara-C), were examined. Ionizing radiation was used as a positive control. An active lipid extract gave a linear dose–response over the range 2.0–12.2 g equivalents. When MCL-5 cells, a line of metabolically-competent human lymphoblastoid cells, were used to compare the DNA-damaging properties of lipid extracts from six different donors, significant inter-individual variations (median CTLs were 15.0, 53.5, 32.5, <4.0, <4.0 and 77.5 ±µm respectively) were observed. In eight subjects, the donors’ HMECs were examined both before and after treatment with extracts of that donor’s own lipid. Pre-existing DNA damage was detected in untreated HMECs from some donors (median CTLs 22.0–37.5 µm) that was not present in others (median CTLs 4.0–11.5 µm), and increases in CTL could be induced by incubation with the matching lipid extract (8 g equivalent) in more than half (five out of eight) the subjects examined (median CTL up to 111.0 µm). There was a tendency for the most active lipid extracts to be those obtained from donors whose HMECs also contained the most pre-existing DNA SSBs. The results of this pilot study may prove to be significant in relation to the initiation of breast cancer.

Introduction

Breast cancer incidence varies widely across the globe, with a much higher incidence in Western countries than in, for example, the Far East. Migration studies show that breast cancer risk in Japanese-American women increases to match the resident incidence by the second or third generation (1). This suggests that diet or environment plays a significant part in breast cancer initiation. High-penetration breast cancer susceptibility genes are likely to account for only 5% of cases, while the aetiology of the majority of cases remains obscure. The only environmental exposure proven to induce breast cancer is ionizing radiation (2).

With some 600 000 new cases each year, accounting for nearly 20% of all cancers diagnosed, breast cancer remains the most frequently-occurring malignancy amongst the female population (3). Total cumulative exposure to oestrogens has been linked with increased breast cancer risk, but oestrogens probably act as promoters rather than as complete carcinogens (3). Human exposures to carcinogens via the diet and the environment include exposure to polycyclic aromatic hydrocarbons (PAHs*), nitro-PAHs and heterocyclic aromatic amines (‘food mutagens’), many of which are fat soluble and capable of inducing mammary tumours in rodents (4). Recent studies have shown that when the ‘food mutagens’, 2-amino-3-methylimidazo[4,5-b]quinoline (IQ) or 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP), are given orally to lactating rats they, or their metabolites, can be detected in the milk (5).

It has proved difficult to detect the early genetic alterations that occur in the mammary epithelial cells from which most breast cancers develop. However, the mutational spectrum of the p53 gene in human mammary tumours differs from that attributable to endogenous/background mutagenic processes, suggesting a significant role for xenobiotic mutagens (6). We have recently shown that extracts of human mammary lipid are mutagenic in the Ames test and can induce micronuclei in MCL-5 cells (7).

The single-cell gel electrophoresis assay (comet assay) is a sensitive, simple and rapid method for visualizing and measuring DNA strand breaks in single cell preparations (8). In the assay a small number of treated cells suspended in a thin agarose sandwich are lysed, electrophoresed at high pH, and stained with a fluorescent DNA binding dye (9). Relaxed and broken DNA fragments migrate at different, size-related rates according to the extent of damage and form a comet tail, whose length can be measured.

In the present study we have used the comet assay to examine fresh preparations of human mammary epithelial cells (HMECs) for the presence of DNA damage and to determine if treatment of such cells with extracts of human mammary lipid, often obtained from the donor of those cells, can induce DNA strand breaks. The biological activities of mammary lipid extracts prepared from different donors have also been compared in MCL-5 cells, where treatment and comet assays were carried out in the presence of the DNA repair inhibitors hydroxyurea (HU) and cytosine arabinoside (ara-C) (10). These permit the recognition and incision stages of nucleotide excision repair, but inhibit DNA resynthesis, giving rise to an

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Abbreviations: HMECs, human mammary epithelial cells; CTL, comet tail length; HU, hydroxyurea; ara-C, cytosine arabinoside; SSB, single strand break; PAHs, polycyclic aromatic hydrocarbons; IQ, 2-amino-3-methylimida-zo-[4,5-b]quinoline; PhIP, 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine.
accumulation of single strand breaks (SSBs) (10). This study shows for the first time the use of the comet assay in conjunction with primary cultures of HMECs, from which the majority of breast cancers arise.

Materials and methods

Mammary tissue

Mammary tissue was obtained from healthy women resident in the UK and undergoing elective reduction mammoplasty. All tissues were examined for the presence of macroscopic pathological abnormalities, and were found to be normal. Tissues were digested enzymically in order to separate the mammary lipid from epithelial cell aggregates (11).

Mammary lipid

Mammary lipid, obtained by digestion, was subjected to a solid-phase tandem extraction procedure, originally developed for the extraction of heterocyclic aromatic amines (12). Mammary lipid (4 g) was homogenized in NaOH (1 M, 10 ml) at high speed for 1–2 min with an Ultra Turrax homogenizer and then extracted exactly as described (12). Column eluates in methanolic ammonia (9:1, v/v, 3 ml) were evaporated to dryness in a Savant Speed Vac concentrator and the residues resuspended in DMSO (10 µl).

Mammary epithelial cells (HMECs)

Cells were prepared as organoids following collagenase digestion of breast tissues (11). The organoids were then seeded into 175-cm² flasks containing 1 ml essential medium with 5% foetal calf serum.

Results

Ionizing irradiation was used as a positive control. For these experiments, cells were irradiated at room temperature using a 333Bq 32P-Co source at a dose rate of 1–2 Gy/min over an acute dose range of 0–12 Gy, then lysed and subjected to electrophoresis.

Statistical analysis

CTLs were compared using a non-parametric (Mann–Whitney) test. All P-values given are two-tailed.

Examples of the fluorescence microscopy images obtained with HMECs are shown in Figure 1. Compared at low magnification are a population of untreated cells, which had a low level of pre-existing comets (Figure 1a), with cells that had been treated with an active extract of mammary lipid (Figure 1b). At higher magnification (Figure 1c and d) the comparative lengths of the comets are more readily apparent. When the comet assay is carried out under alkaline conditions, the extent of DNA SSB formation is quantified by measuring the extent of DNA migration (CTL); the greater the CTL, the more numerous the strand breaks.

The typical distribution of CTLs for a preparation of HMECs showing very low levels of pre-existing DNA damage is shown in Figure 2a. This preparation (from donor 1) had a median CTL of 12.0 µm, when the analysis was carried out in the presence of DNA repair inhibitors HU and ara-C. When an aliquot of these cells was treated with an active extract of mammary lipid (Figure 1b), a significant amount of DNA damage was induced (median CTL 96.5 µm) (Figure 2b). However, inclusion of an inhibitor of cytochrome P450 activity, SKF 525A (13), was found to inhibit
most of the comet-forming activity of this extract (median CTL 29.0 µm) although a significant amount of DNA damage still occurred (Figure 2c). This result implies that the majority of the genotoxic components present in this mammary lipid extract require metabolic activation before they can exert their DNA-damaging effects and are not directly acting.

Treatment of another preparation of HMECs (from donor 2) with ionizing radiation, which is known to cause DNA strand breaks and breast cancer (2,9), increased CTLs (Figure 2d and e). At a dose of 8 Gy, the median CTL increased from 11.0 µm in untreated cells to 117.0 µm; in other experiments increases in CTL were found to be dose-dependent (data not shown).

In order to make direct comparisons between lipid extracts obtained from different donors, the comet-forming activities of six extracts were determined in MCL-5 cells, a line of human lymphoblastoid cells genetically engineered to express enzymes that metabolize a wide range of xenobiotic compounds (14). Preliminary experiments had shown that mammary lipid extracts only caused increases in CTL in these cells when incubated in the presence of HU/ara-C (data not shown). Untreated MCL-5 cells in the presence of HU/ara-C exhibit a very low level of DNA migration (Figure 3b) that was not significantly different from that observed in the absence of these inhibitors (Figure 3a). A large inter-individual variation in comet-forming activity was observed following incubation of MCL-5 cells, in the presence of HU and ara-C, with the six extracts (from donors 3–8) (Figure 3c–h). Extracts from donors 3, 4, 5 and 8 (Figure 3c–h) induced highly significant increases in median CTLs (15.0, 53.5, 32.5 and 77.5 µm respectively). The remaining two extracts (donors 6 and 7, Figure 3f and g) did not induce comet formation (median CTL <4.0 µm).

An extension of these experiments was the incubation of an extract of human mammary lipid with the donor’s own mammary epithelial cells. As an example, an untreated epithelial cell population (from donor 9) exhibited a low level of pre-existing DNA damage that was slightly, but not statistically significantly, increased in the presence of HU/ara-C (Figure 4a and b). The addition of increasing concentrations of extract (2.0, 6.1 and 12.2 g equivalents) into the assay clearly increased the CTL (Figure 4c–e). As shown in Figure 4f, this genotoxic extract of human mammary lipid induced a concentration-dependent linear increase in DNA migration in the HMECs prepared from the same individual.

In all, the treatment of HMECs with mammary lipid extract from the same donor has been carried out on preparations from a total of eight donors. In addition to the example shown in Figure 4, the results of six more such analyses are shown in more detail in Figure 5. In each case, CTLs were measured in treated and untreated cells, with and without the addition of HU and ara-C to the cell medium.

In three of these cases, HMECs showed evidence of pre-existing DNA damage (donors 8, 10 and 11). Median CTLs...
were 22.0, 37.5 and 26.0 µm, respectively in the absence of repair inhibitors and 43.0, 42.0 and 19.5 µm, respectively, in their presence. Although the formation of comets in MCL-5 cells required the presence of repair inhibitors, mammary lipid extracts induced comets in HMECs both in the presence and in the absence of HU and ara-C. For cells plus extracts from donors 8 and 11, the increase in median CTLs was significant both with and without repair inhibitors (donor 8, 111.0 and 96.5 µm respectively; donor 11, 38.0 and 39.0 µm). In cells from donor 10, the median CTL was increased in the presence of repair inhibitors (57.5 µm) but not in their absence (35.0 µm).

The cells from three other donors did not show evidence of significant pre-existing DNA damage, either in the presence or absence of repair inhibitors (donors 12, 13 and 14, Figure 5). The median CTLs were, respectively, 11.5, 4.0 and 6.5 µm in the absence of HU and ara-C, and 15.5, 4.5 and 7.0 µm in their presence. When treated with their lipid extracts, only cells from donor 14 showed any increase in DNA damage, observed when the repair inhibitors were included (median CTL 11.0 versus 7.0 µm). This increase was not statistically significant, and no increases in median CTLs in treated HMECs from donors 12 and 13 was observed when treated with their own extracts.

The results of experiments with an additional preparation of HMECs and lipid (from donor 15, 27 years) gave results similar to those obtained with donors 8, 10 and 11; that is, pre-existing damage was present in the untreated cells (median CTL 40.5 µm in the presence of HU and ara-C), and the lipid extract caused a significant increase in CTL (median 59.0 µm) (data not shown).

Thus, there are inter-individual variations in levels of pre-existing DNA damage in HMEC populations from different individuals and differing abilities of extracts of human mammary lipid to induce DNA damage in the donor’s own cells. Five out of eight lipid samples caused a significant increase in CTL in that donor’s own cells. In addition, active lipid extracts were most often obtained from those donors whose epithelial cells show evidence of pre-existing DNA strand breaks.

As part of a series of control experiments conducted in our previous study of extracts of mammary lipid (7), in which mutagenicity in S. typhimurium and micronucleus formation in MCL-5 cells were determined, the possibility of artifactual generation, through autoxidation, of genotoxic components during the processing of lipid was ruled out. We also determined that extracts of beef fat and corn oil were not genotoxic in these assays. In the present study, we have also found that extracts of beef fat and corn oil do not induce comet formation in MCL-5 cells (data not shown).

**Discussion**

Although circumstantial evidence suggests the involvement of environmental factors in the aetiology of breast cancer, no

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**Fig. 3.** Inter-individual variation in comet-forming activity of human mammary lipid extracts measured in MCL-5 cells. MCL-5 cells were treated with vehicle control (DMSO): (a) in the absence of HU/ara-C; (b) in the presence of HU/ara-C. Cells were also treated in the presence of HU/ara-C with mammary lipid extract (8 g equivalent) added as solutions in DMSO as follows: (c) donor 3 (age 41 years); (d) donor 4 (24 years); (e) donor 5 (26 years); (f) donor 6 (23 years); (g) donor 7 (18 years); (h) donor 8 (24 years). Incubations and the comet assay were carried out as described in Materials and methods.
We have previously demonstrated the genotoxicity of extracts of human mammary lipid in strains of *S. typhimurium* (mutagenic activity) and in MCL-5 cells, a genetically engineered mammalian cell line (micronucleus formation) (7). We found that 16 out of 40 lipid samples were mutagenic and there was a close correlation with micronucleus-forming activity. We have now extended these studies to the application of the comet assay as a measure of genotoxic activity. This assay offers some distinct advantages for the study of DNA damage. As discussed by Tice (9) and by Fairbairn et al. (8) it enables damage to be detected at the level of the single cell, allowing heterogeneity in cell populations to be observed. It is rapid, relatively simple and can be carried out on small numbers of cells. It is also sensitive and quantitative and, if carried out under alkaline conditions, detects overt strand breaks, alkali-labile lesions such as apurinic sites and also excision repair-induced strand breaks. The assay is applicable to almost any type of mammalian cell and does not require cell division to occur. To our knowledge, this is its first application to primary cultures of HMECs. Also, our results demonstrate that the DNA repair inhibitors, HU and ara-C, which were previously shown to enhance the frequency of mutagen-induced micronucleus formation in human lymphocytes (20), can increase the sensitivity of the comet assay.

Ionizing radiation, known to cause comet formation in many cell types (9), was used as a positive control. Thus the assay is positive for HMECs treated with a known human mammary carcinogen (2), which lends credence to the belief that the genotoxic activity observed with the mammary lipid extracts may also be of potential relevance to the aetiology of breast cancer.

Our study has demonstrated, firstly, that some samples of HMECs contain measurable levels of pre-existing damage. This implies that they have been subjected to some source of genotoxic insult *in vivo*. A similar phenomenon has been observed with gastric mucosa and nasal mucosa cells (21). Secondly, extracts of mammary lipid show differing amounts of activity in increasing the extent of comet formation in these HMECs (Figure 5).

Since the inter-individual variations in comet-forming ability of lipid extracts in HMECs could have been due either to variations in activity of the extracts or to differences in metabolic activation or DNA repair activity of the cells, a series of extracts were tested in MCL-5 cells. The inter-individual variation found in these experiments (Figure 3) demonstrates that the lipid extracts from different individuals do indeed vary in their genotoxic potential. Moreover, HMECs from individuals not showing pre-existing damage do form comets when exposed to active lipid extracts (Figure 2), so their lack of initial damage is not due to an inability to metabolically activate lipid components. Where there is pre-existing damage, this is more frequently seen with an individual whose lipid extract is active in the assay (Figure 5); conversely, undamaged cell DNA is more frequently associated with inactive extracts. This suggests that, although in our experiments DNA damage could be induced by lipid extract *in vitro*, there is a possibility that DNA damage had already occurred, by a similar mechanism, in the HMECs *in vivo* as a result of their proximity to the same adipose tissue from which the lipid extracts were prepared.

With MCL-5 cells, comets were observed only in the presence of the DNA repair inhibitors, HU and ara-C, indicating that these cells are very repair-proficient. With HMECs, comets were observed in the absence of the repair inhibitors, but CTLs

![Fig. 4](image_url) Dose-related increases in comet-forming activity induced by an extract of human mammary lipid in the donor’s own HMECs (donor 9, 23 years). (a) Untreated HMECs (no HU or ara-C); (b) untreated HMECs in the presence of HU and ara-C; (c–e) HMECs treated with lipid extract in the presence of HU and ara-C; (f) dose–response curve of mean CTL versus g equivalent of lipid extract added.

causative agents, with the exception of ionizing radiation (2), have been identified. We are investigating the hypothesis that human mammary lipid acts as a reservoir for genotoxic components to which HMECs may then be exposed, because many rodent mammary carcinogens are lipid-soluble environmental chemicals (4). As these HMECs, in which the majority of human mammary tumours arise, are surrounded by adipose tissue, the breast’s unique structure increases this possibility (15). Previous studies have considered the genotoxicity of a number of related biological fluids, such as nipple aspirates (16–18) and cyst fluid (19). These studies have been confined mainly to measuring the mutagenic activity of such fluids in bacterial cells, with a small percentage of samples (<10%) being found positive. However such studies are limited by the amount of biological material available. Our approach has been to consider the genotoxic properties of mammary lipid for several reasons. Firstly, this is in accord with the hypothesis that this material may accumulate environmental carcinogens. Secondly, the availability of lipid from reduction mammoplasty means that relatively large amounts of material can be obtained for study from healthy women. This, in turn, has enabled us to use sufficient material for analysis in a number of different assays with different genetic end-points.

We have previously demonstrated the genotoxicity of...
Fig. 5. DNA damage in primary cultures of HMECs from six different donors measured before and after treatment with an extract of that donor’s own mammary lipid (a) in the absence of HU and ara-C; (b) in the presence of HU and ara-C. Cells were treated with extract equivalent to 8 g of mammary lipid. The ages of the cell and lipid donors were: donor 8, 24 years; donor 10, 21 years; donor 11, 29 years; donor 12, 23 years; donor 13, 23 years; and donor 14, 25 years.

were greater when the inhibitors were included, indicating that these cells also have a capacity to repair some DNA damage. The presence of pre-existing damage also indicates that the damage that may have occurred in vivo is not very rapidly repaired within the time taken to isolate the epithelial cells. It is of interest to note that deficient DNA repair capacity, manifested as sensitivity of cells to ionizing radiation, has been proposed as a predisposing factor in breast cancer (22,23) and that defective genes that may predispose individuals to breast cancer, including ATM (24) and BRCA2 (25,26), have functions linked to the processing of DNA damage.

The extraction method used on the mammary lipid in this study was originally developed for the extraction of heterocyclic amines from biological matrices (12). However, it is not yet clear whether the activity observed is due to heterocyclic amines or to other genotoxic components that may be extracted by the same procedure. Since a general inhibitor of cytochrome P450, SKF 525A, substantially decreased the comet-forming activity of one extract in HMECs, the predominant components require metabolic activation and direct-acting mutagens do not appear to be as important. Previous studies have demonstrated that HMECs can activate heterocyclic amines and polycyclic aromatic hydrocarbons to DNA binding products (27–29). The need for metabolic activation of the genotoxic components of the mammary lipid extracts was also demonstrated by the requirement for S9 in the Ames assay (7).

The exposure of breast tissue to potentially carcinogenic agents has also been noted in studies that have detected DNA adducts of probable exogenous origin, as well as some arising from apparently endogenous processes. Aromatic and/or hydrophobic adducts have been detected by 32P-postlabelling (30–32). The effect of tobacco smoking was apparent from the detection of an adduct pattern characteristic of smoking-induced DNA damage (31,32). However, retrospective enquiries have indicated that neither the pre-existing DNA damage in HMECs nor the genotoxic activity of lipid extracts detected in the present study is related to tobacco use. In another study (33), differences were found in the levels of adducts induced by malondialdehyde, a product of lipid peroxidation, in normal breast tissue taken from breast cancer patients and in controls.

Newer clues to the aetiology of breast cancer have come from a pilot study of breast cancer risk, tobacco smoking and NAT2 genotype (34). It was found that women who smoked and were designated slow acetylators on the basis of their NAT2 genotype had a four-fold higher risk of breast cancer than non-smokers. Slow acetylators should have a decreased capacity to detoxify carcinogenic aromatic amines in cigarette
smoke, and this is supported by our recent finding that slow acetylators have higher levels of DNA adducts in breast tissue (W. Pfau, E. M. Stone, U. Brockstedt, P. L. Carmichael, H. Marquardt and D. H. Phillips, manuscript in preparation).

In conclusion, the results of this pilot study using the comet assay demonstrate both the presence of pre-existing damage in HMECs from some individuals and the DNA-damaging activity of extracts of mammary lipid. A partial correlation between these two findings suggests that the proximity of HMECs to adipose tissue in vivo may lead to exposure of the HMECs to genotoxic components of the lipid. Since these cells are capable of metabolically activating such components to DNA damaging agents, this could provide a mechanism for the initiation of breast cancer in humans. Additional studies are in progress to test this hypothesis further and to investigate the nature of the genotoxic agents present in human mammary lipid.

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