Genotoxicity of human breast milk from different countries

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Dietary and/or environmental factors appear to play a key role in the international variations that exist in breast cancer incidence. The genotoxicity of breast milk extracts is being examined as a possible indicator of in vivo exposure of mammary epithelial cells to DNA-damaging agents. Breast milk samples were obtained from the UK (n = 32), a high risk country, and from Hong Kong (n = 10), India (n = 20) and Singapore (n = 20), countries of lower breast cancer incidence. The abilities of breast milk extracts to induce DNA damage detected as single-strand breaks (SSBs) in the alkaline Comet assay and to induce micronuclei in MCL-5 cells and mutations in Salmonella typhimurium YG1019 were investigated. In the Comet assay 18 of 32 (56%) UK samples induced significant increases in DNA SSBs compared with 2 of 10 (20%), 5 of 20 (25%) and 8 of 20 (40%) of the samples from Hong Kong, India and Singapore, respectively. The proportion of positive samples was significantly higher in the UK group than in the combined low breast cancer incidence group and significantly higher than in the Indian group (P < 0.05, Fisher’s exact test). In the micronucleus assay 9 of 32 (28%) UK samples showed significant activity compared with 0 of 10 (0%), 2 of 20 (10%) and 3 of 20 (15%) of the samples from Hong Kong, India and Singapore, respectively. Extracts of all the aforementioned milk samples were also tested for bacterial mutagenicity. Nine of 32 (28%) UK samples induced significant activity with a dose–response effect. Although activity was detected in samples from the other countries, comparable dose–response data could not be obtained because of a lack of material. This pilot study suggests that genotoxic components occur more frequently in UK breast milk than in milk from some other countries with a lower incidence of cancer. More work is required to confirm these initial findings and to examine their relevance to variations in breast cancer incidence.

Introduction

Breast cancer is the most commonly occurring malignancy in women (Higginson et al., 1992) but its incidence varies considerably from country to country. Recent estimates from the IARC are that, in 1990, there was a 3-fold higher age-standardized rate (ASR) in women in developed countries than in developing countries (56.4 versus 20.33 per 100 000) (Ferlay et al., 1998; Parkin et al., 1999).

Although the factors responsible for such differences have not been identified, epidemiological studies on cancer incidence rates in migrants appear to implicate dietary and/or environmental changes. Data from 1962–1971 show that mortality from breast cancer amongst recent Italian migrants to Australia was low but that among immigrants in residence for more than 17 years it matched that of the Australian born population (McMichael and Armstrong, 1988). Similarly, studies on Chinese, Japanese and Filipino females who migrated to the USA showed that breast cancer risk in immigrants who had lived in the USA for more than a decade was 80% higher than in recent immigrants (Ziegler et al., 1993).

Whilst a proportion (~5%) of breast cancer cases are attributable to the inheritance of high penetrance breast cancer susceptibility genes, the majority of cases are described as sporadic (Higginson et al., 1992; Lancaster et al., 1996). Oestrogen most probably acts as a tumour- and growth-promoting agent rather than as a complete carcinogen. Total oestrogen exposure does influence breast cancer risk and early menarche and/or late menopause are known, but weak, risk factors. However, such risk factors do not appear to account for the total of breast cancer incidence (Kelsey and Berkowitz, 1988).

The human breast consists of 70–90% adipose tissue and it has been suggested that fat-soluble carcinogens could accumulate therein (Beer and Billingham, 1978), thus exposing the adjacent epithelial cells to DNA damage. The mutational spectrum found in the p53 gene of human breast cancers differs from that expected to result purely from mutations characteristic of endogenous processes (Biggs et al., 1993).

An examination of mammary lipid extracts obtained from UK resident women undergoing elective reduction mammoplasty for the presence of genotoxins, using a range of short-term tests, showed that some 40% were positive (Martin et al., 1996, 1997). These studies also showed that when freshly isolated human mammary epithelial cells (HMECs) were examined for the presence of DNA damage, detected as single-strand breaks (SSBs) using the single cell gel electrophoresis (Comet) assay, the cells with the most SSBs tended to come from donors whose lipid extracts contained the highest levels of genotoxic activity.

Our attention turned recently to breast milk since the possible presence of genotoxins in the breast can be investigated non-invasively in larger cohorts using this material. The initial studies showed that extracts of UK breast milk had levels of comet-forming activity similar to those of UK mammary lipid extracts, although they were less mutagenic (Martin et al., 1999a, 2000). Exfoliated cells recovered from some milk samples contained DNA damage and these breast milk samples tended to yield extracts that were more genotoxic.

A pilot study has now been carried out comparing the
genotoxic activities of breast milk samples obtained from women resident in Hong Kong, India and Singapore, countries where breast cancer incidence rates are lower than in the UK, with samples from UK resident women. The results of Comet and micronucleus assays carried out with MCL-5 cells and of some bacterial mutagenicity tests are reported here.

Materials and methods

Human milk

Samples of human milk were obtained from healthy nursing mothers in different countries. The UK samples (n = 32) were from mothers who donated milk to a milk bank at a London maternity hospital. Nineteen of the mothers were Caucasian, three were Asian, two were Afro-Caribbean, two were from other ethnic groups and six were of unstated ethnicity. The Hong Kong samples (n = 10) were all donated by ethnic Chinese and selected randomly from surplus milk samples expressed by mothers for their hospitalized babies. Six samples were from mothers who had pre-term babies ranging from 28 to 34 weeks gestation; the other four were from mothers of term babies. The age of the mothers ranged from 29 to 44 years (mean 35.5). The Indian samples (n = 20) were collected from mothers at a general maternity hospital in Kerala in Southern India. All donors were recruited at random from among ethnic Indians from the district. The samples from Singapore (n = 20) were also donated by mothers recruited at hospitals and represent an ethnically mixed group of donors. All samples were frozen following expression, and extracted using a solid phase tandem extraction (Martin et al., 1999a). Data from analysis of UK milk samples 1–20 have appeared in a previous publication (Martin et al., 1999a).

Extraction of milk

Milk was saponified and extracted using a solid phase tandem extraction procedure originally described by Gross (1990) and used in previous studies on human mammary lipid (Martin et al., 1996, 1997, 1998) and breast milk (Martin et al., 1999a, 2000). Final eluates in methanolic ammonia (9:1 v/v, 3 ml) were evaporated to dryness and the residues were resuspended in DMSO for incorporation into assays for biological activity. Samples of cow’s milk and corn oil were extracted under the same conditions and served as controls. The heterocyclic aromatic amine 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) was used as a positive control in all assays.

Single cell gel electrophoresis (Comet) assay

Comet formation by milk extracts was investigated in MCL-5 cells, a line of human lymphoblastoid cells genetically engineered to express enzymes that metabolize a wide variety of xenobiotic compounds (Crespi et al., 1991). MCL-5 cells were treated with extracts (8 g equivalent) of each human milk sample in a total volume of 110 µl for 30 min at 37°C as described previously (Martin et al., 1999a). Treated cells were then examined in the Comet assay (Tice, 1995; Martin et al., 1999b), carried out under alkaline conditions and in the presence of 10 mM hydroxyurea (HU) and 1.8 mM cytosine arabinoside (ara-C) (Martin et al., 1997, 1999b). PhIP (2.03 mM) and extracts of corn oil (8 g equivalent) and cow’s milk (8 g equivalent) were used as controls. Images were visualized by epifluorescence using a Leitz Laborlux S microscope. Images were digitized and DNA damage expressed as comet tail length (CTL) (µm) using a comet analysis system (Komet 3.1; Kinetic Imaging, Liverpool, UK). Tail length was measured from the edge of each individual nucleus head. A total of 50 comets/data point, 25 from each of two duplicate slides, was measured.

Trypan blue exclusion/cell counts

Individual cell suspensions were gently mixed 50:50 with trypan blue (0.4% solution in 0.85% saline; Flow Laboratories, Irvine, UK), allowed to stand for 5–10 min and applied to a haemocytometer with a coverslip. The percentage of cells that excluded trypan blue was used as an indicator of cell viability and was estimated both before and after treatment of cells.

Micronucleus assay

Micronucleus formation in MCL-5 cells was examined exactly as described previously (Martin et al., 1996). Extracts (6 and 10 g equivalents) of each milk sample were tested and PhIP (25 ng/ml cell culture), corn oil extract (6 and 10 g equivalents) and cow’s milk extract (6 and 10 g equivalents) were used as controls in each assay. Following treatment cell cultures (10 ml) were incubated for 24 h at 37°C, after which they were treated with 6 µg cytochalasin B and incubated for a further 24 h. Cells were then fixed to microscope slides and stained (Crofton-Sleigh et al., 1993). Five hundred binucleate cells were scored for micronuclei; the percentage of binucleate cells remaining after each treatment was used as an index of cytotoxicity.

Results

DNA strand-breaking activity of milk extracts

The median CTLs obtained when the comet-forming effects of extracts of milk samples from different individuals (single determinations) were examined are shown in Figure 1. The concentration of 8 g equivalent breast milk extract was chosen from dose–response curves published previously (Martin et al., 1997, 1999a). The four panels in Figure 1 show that the median values for CTLs in untreated MCL-5 cells were stable and low. However, higher median values for CTLs did occur following treatment with breast milk extracts from the four different countries and the proportion of positive samples varied. Eighteen of 32 (56%) UK samples induced significantly higher proportions of positive samples when compared with 2 of 10 (20%) Hong Kong samples, 5 of 20 (25%) Indian samples and 8 of 20 (40%) Singapore samples. A significantly higher proportion of UK samples were found to be positive compared with groups of samples from countries of lower breast cancer incidence combined and India alone (P < 0.05, Fisher’s exact test). No significant activity was observed when extracts of corn oil or cow’s milk were tested (data not shown). PhIP (2.03 mM) gave a median CTL range of 23.0–58.5 µm (12 determinations), while in untreated cells a background CTL range of 5.5–11.0 µm was observed. No discernible differences were observed between UK samples
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Fig. 2. Comet tail length distributions from 50 nuclei obtained when extracts of breast milk were incubated with MCL-5 cells. Cells suspended in PBS (~1 x 10^6 cells/75 µl) were incubated in the presence of the DNA repair inhibitors HU and ara-C (10 and 1.8 mM final concentrations) at 37°C for 30 min and comet tail lengths (µm) were measured. Cells were treated with breast milk extracts (8 g equivalent), added as solutions in DMSO, as follows: (A–E) extracts of breast milk from Hong Kong resident individuals (H.K.6, H.K.7, H.K.8, H.K.9 and H.K.10); (F) an extract of breast milk from a UK resident individual (U.K.26); (G) vehicle control (DMSO only) in the absence of HU/ara-C; (H) vehicle control (DMSO only) in the presence of HU/ara-C. Incubations and the Comet assay were carried out as described in Materials and methods.

Examples of the CTL distributions obtained with extracts of breast milk samples from five Hong Kong resident individuals (H.K.6–H.K.10) and from a UK resident individual (U.K.26) are shown in Figure 2. Two extracts of milk from Hong Kong resident individuals (H.K.7 and H.K.8) can be seen to possess significant comet-forming activity (P < 0.0001), as does the extract of UK breast milk: the other three Hong Kong extracts were inactive.

Micronucleus-inducing activity of milk extracts

The raw data (Figure 3) obtained for micronucleus induction in MCL-5 cells by milk extracts (6 and 10 g equivalents) show that increases in micronucleus formation, over and above the background values obtained for untreated MCL-5 cells, occurred with milk extracts from the four countries in the study. Plotting the mean values (± SD) shows that there is evidence for a dose-related response with the samples obtained from the UK, India and Singapore and that the slopes of the dose–response curves (0.51 ± 0.11, 0.42 ± 0.01 and 0.46 ± 0.01, respectively, linear regression) are similar for these three countries, in contrast to a value of 0.27 ± 0.13 for samples from Hong Kong (Figure 4).

Although extracts of breast milk samples from Hong Kong caused an increase in micronuclei over control values, there was no further increase when the concentration of milk extract in the medium was raised from 6 to 10 g equivalent (Figure 4). No activity was detected with extracts of corn oil or cow’s milk. PhIP (25 ng/ml cell culture) showed a range of 10–22 micronuclei/500 binucleate MCL-5 cells (66–82% binucleate MCL-5 cells, 38 determinations), compared with a spontaneous frequency of 3–11.

Examples of the micronucleus-inducing activities of some individual breast milk extracts and their effects on cell viability, as measured by the percent of binucleate cells remaining after treatment, are shown in Figure 5. Inter-individual variations in activity are present between milk extracts that induce...
**Fig. 5.** Dose–response curves for clastogenicity (micronucleus formation) and cytotoxicity of extracts of breast milk from different individuals. Data obtained with extracts from three breast milk samples from the UK (U.K.1, U.K.2 and U.K.10) are compared with data from extracts of single breast milk samples from Hong Kong (H.K.9), India (Ind.5) and Singapore (Sing.11). (A) Micronucleus formation (micronuclei/500 binucleate cells) and (B) cytotoxicity, expressed as [% binucleate treated cells/% binucleate untreated cells] × 100, were determined in MCL-5 cells following treatment as described in Materials and methods. *Significance in the χ² test for trend, P < 0.05; #significance in the χ² test for trend, P < 0.005.

**Fig. 6.** Bacterial mutagenicity of extracts of breast milk obtained from residents of the UK (n = 32), Hong Kong (n = 10), India (n = 20) and Singapore (n = 20). Bacterial mutagenicity assays were performed in the presence of S9 as described in Materials and methods using *S. typhimurium* YG1019. Where sample size allowed, triplicate measurements (revertants/plate) were made for each concentration of milk extract tested. The data presented include all the data obtained from each individual at each concentration tested. Differences between triplicate measurements obtained with the same extract did not exceed 35%. Revertants occurring in the presence of the vehicle control (DMSO) are referred to as spontaneous.

Most active samples in inducing comets and micronuclei in MCL-5 cells.

With Hong Kong milk samples there was only enough material available to permit four of the 10 samples to be tested at both concentrations. One of these four extracts induced a dose-related increase in revertants, although this was not judged to be significant as a doubling of revertants over background did not occur.

There was only sufficient material available to allow one of the 20 Indian breast milk extracts to be examined at both concentrations and this sample was judged to be mutagenic (i.e. a doubling of revertants over background with evidence of a dose–response effect).

Of the seven Singapore milk extracts examined at both the 6 and 10 g equivalent concentrations two showed significant bacterial mutagenicity. No activity was observed in extracts of corn oil or cow’s milk: PhIP (0.5 µg/plate) gave a range of 824 ± 108–1216 ± 70 (mean revertants ± SD, 11 determinations) compared with a background frequency that ranged from 41 ± 9 to 52 ± 3 revertants/plate.

Examples of representative dose–response curves obtained when six breast milk extracts were tested for bacterial mutagenicity are shown in Figure 7. Inter-individual variations are present between those breast milk extracts that induce significant increases in YG1019 revertants (U.K.1, U.K.10, Ind.18 and Sing.18) and those that are inactive over the concentration range tested (U.K.2 and H.K.9).

**Discussion**

Genotoxic activity has been detected in extracts of mammary lipid and of breast milk obtained from UK resident women (Martin et al., 1996, 1999a; Martin, 2001). The nature of the agents responsible for this activity have not, as yet, been determined. Approximately 40% of UK mammary lipid extracts induced DNA damage (measured as comet-forming activity) in MCL-5 cells and in primary cultures of HMECs (Martin et al., 1997). Pre-existing DNA damage was observed in some...
otherwise untreated primary cultures of HMECs and it was noted that the more active comet-forming lipid extracts tended to be derived from donors whose cells showed evidence of pre-existing DNA damage. Some 40% of UK mammary lipid extracts were also positive in bacterial mutagenicity assays (Ames test) and this activity correlated with activity in a micronucleus assay using human (MCL-5) cells (Martin et al., 1996).

Mammary lipid extracts were also found to be capable of inducing morphological transformation of C3H/M2 mouse fibroblasts, thus heightening the suspicion that the genotoxic fractions extracted from mammary lipid could be involved in tumour initiation (Martin et al., 1998). We have recently turned our attention to breast milk, a natural lipid-containing secretion of the breast, since it is more readily available than breast lipid. There is evidence that maternal body fat, as opposed to breast lipid (Josephy and Coomber, 1998). Studies on UK breast milk extracts have shown that they do resemble breast milk extracts in this respect (Martin et al., 1999a).

The present small pilot study, in which the genotoxicity of breast milk extracts from four countries has been compared using short-term tests, has been carried out to look into the feasibility of studying the relevance of such genotoxicity to the aetiology of breast cancer. Breast milk samples were obtained from the UK, a high risk country, and from Hong Kong, India and Singapore, countries with lower breast cancer incidence rates. The genotoxicity of milk extracts has been determined using the Comet assay to detect DNA SSBs and micronucleus induction, both carried out in MCL-5 cells, and as mutagenicity in S.typhimurium YG1019. Each point represents mean revertants per plate ± SD from three plates. *Mutagenic breast milk extracts.

![Fig. 7. Dose-response curves for bacterial mutagenicity of extracts of breast milk from different individuals. Data obtained with extracts from three breast milk samples from the UK (U.K.1, U.K.2 and U.K.10) are compared with data from extracts of single breast milk samples from Hong Kong (H.K.5), India (Ind.18) and Singapore (Sing.18). Bacterial mutagenicity assays were performed in the presence of S9 as described in Materials and methods using S.typhimurium YG1019. Each point represents mean revertants per plate ± SD from three plates. *Mutagenic breast milk extracts.](image-url)

clastogenic activity, as measured by micronucleus induction, between the four countries (Figures 3 and 4). Although the data obtained on milk extract mutagenicity were incomplete, Hong Kong samples appeared to be less mutagenic than UK samples (Figure 6). Of the 32 UK milk samples analysed extracts of 21 exhibited activity in at least one genotoxicity assay. Four UK milk extracts were positive in all three assays, two others in the Comet and micronucleus assays, another three were positive in the Comet and bacterial mutagenicity assays and a further two in the micronucleus and bacterial mutagenicity assays. These differences in activity are suggestive of the presence of different genotoxins or mixtures of genotoxins in human breast milk.

Breast cancer incidence rates vary significantly between the four countries from which milk samples were obtained for the present study. Data from the IARC for 1988–1992 give ASRs of 71.74 cases/100 000 population for female breast cancer in the UK (England and Wales) compared with 34.42 for Hong Kong, 26.91 for India and 38.87 for Singapore (31.9–39.5, depending on ethnic origin) (Ferlay et al., 1998).

The breast cancers detected in the period 1988–1992 will have resulted from earlier exposure of mammary epithelial cells to tumour-initiating agents. It is noteworthy that bladder cancer incidence in workers in the UK dyestuffs industry peaked some 10–20 years after occupational exposure to the chemicals responsible (Case and Pearson, 1954; Case et al., 1954) and a latent period (~10 years) pertains for breast cancer after exposure to ionizing radiation (Tokunaga et al., 1987).

Thus, the breast cancer incidence data quoted above are likely to relate to initiating events occurring several years prior to the 1988–1992 recording period. These would, it is supposed, have been influenced by the dietary habits and lifestyle of the earlier period. In contrast, the genotoxic activities detected in breast milk extracts in the present study will be related to the current diet and/or lifestyle of the donors, which may be more ‘westernized’ than was the case in earlier decades.

Although there are no direct links between any of the three genotoxicity assays used here and carcinogenicity, the induction of DNA damage, as measured in the Comet assay, correlated better with the potencies of heterocyclic aromatic amines in a morphological transformation assay than did their activities as mutagens in S.typhimurium or as micronucleus-inducing agents (Pfau et al., 1999). An assessment of the activities of some breast milk extracts in a morphological transformation assay is currently in progress.

The higher activities shown in the Comet assay by UK breast milk extracts, as compared with those from the Far Eastern countries, may perhaps be related to breast cancer initiation, but many more data will be required to establish a firm correlation. In addition, the genotoxins concerned will need to be isolated and characterized and their origins identified.

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References

