

Establishment of a radiation- and estrogen-induced breast cancer model

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It is well accepted that cancer arises in a multistep fashion in which exposure to environmental carcinogens is a major etiological factor. The aim of this work was to establish an experimental breast cancer model in order to understand the mechanism of neoplastic transformation induced by high LET radiation in the presence of 17 β -estradiol (E). Immortalized human breast cells (MCF-10F) were exposed to low doses of high LET α particles (150 keV/ μ m) and subsequently cultured in the presence or absence of E for periods of up to 10 months post-irradiation. MCF-10F cells irradiated with either a single 60 cGy dose or 60/60 cGy doses of α particles showed gradual phenotypic changes including altered morphology, increase in cell proliferation relative to the control, anchorage-independent growth and invasive capability before becoming tumorigenic in nude mice. In α particle-irradiated cells and in those cells subsequently cultured in the presence of E, increased BRCA1, BRCA2 and RAD51 expression were detected by immunofluorescence staining and quantified by confocal microscopy. These studies showed that high LET radiation such as that emitted by radon progeny, in the presence of estrogen, induced a cascade of events indicative of cell transformation and tumorigenicity in human breast epithelial cells.

Introduction

Breast cancer may be induced with relatively high frequency by radiation, for example: the Japanese female survivors of the A-bomb attacks; women from a Nova Scotia sanatorium subjected to multiple fluoroscopies during artificial pneumothorax for pulmonary tuberculosis; women treated for acute post-partum mastitis and benign diseases; young women treated by radiotherapy for Hodgkin's lymphoma; women treated for ankylosing spondylitis, tinea capitis, enlarged thymus glands and skin hemangioma (1–10).

It is well accepted that cancer arises in a multistep fashion and that environmental exposures to physical and chemical agents are major etiological factors. Exposure to carcinogens plays a major and probably an etiological role in the initiation of this human disease. In experimental animal models, ionizing radiation induces mammary carcinomas both *in vivo* and *in vitro*, however, the cellular and molecular mechanisms of

radiation-induced carcinogenesis are not known. To understand the mechanisms, it is necessary to determine the conditions that modulate the susceptibility of this target tissue to ionizing radiation. The assessment of the transforming ability of ionizing radiation on breast epithelium requires a highly proliferating cell population, which in turn has to express normal breast phenotypes. Since ionizing radiation induces features of neoplastic transformation in human breast cells, the identification of malignant phenotypes involved in breast cancer are of critical importance in understanding the pathogenesis of the disease. *In vitro* systems allow us to test the sensitivity of human breast epithelial cells to different carcinogens, including ionizing radiation.

Neoplastic transformation of human diploid cells by ionizing radiation has been previously reported in various virally immortalized human epithelial cells, including human epidermal keratinocytes (11), in thyroid epithelial cells by single and fractionated exposure (12) and in a human papilloma virus immortalized human breast epithelial cell line (13). Hei *et al.* (14) have demonstrated the oncogenic transforming effects of single versus multiple doses of radon-simulated α particles using human papillomavirus immortalized human bronchial epithelial cells and were able to assess the various stages of transformation. They showed that immortalized human cells could be malignantly transformed by a single 30 cGy dose of α particles, which produced progressively growing tumors when inoculated into nude mice. However, there is little information on how radiation induces neoplastic changes in human breast epithelial cells.

Transformed cells can usually be identified by changes in their morphology. Treatment of cells with virus, chemicals or radiation can dramatically change their growth properties in culture. Such changes and the subsequent development of tumor-forming capability are referred to as malignant transformation, which is not only related to morphology but also to other aspects of cell growth control such as cell–cell interaction, invasiveness, gene expression, etc. The human breast epithelial cell line MCF-10F, spontaneously immortalized and derived from the breast tissues of a 36-year-old female, is unique in the sense that it has the morphological characteristics of normal breast epithelial cells. These characteristics include dome formation in confluent cultures, three-dimensional growth in a collagen gel, dependence upon hormones and growth factors for growth *in vitro* (15), lack of anchorage independence or invasive capability and tumorigenicity in nude mice (16). In addition, these cells present all the characteristics of breast epithelium by their ultrastructural features and by their immunocytochemical reactivities towards keratin and sialomucin (15).

Among various transformed markers of human breast cells, *BRCA1* is a tumor suppressor gene that has been implicated in hereditary forms of breast cancer (17–22). It is transcriptionally regulated in a proliferation-dependent manner (23). Mutations in these breast cancer susceptibility genes are believed to

Abbreviations: BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle's medium; E, 17 β -estradiol; LET, linear energy transfer; PBS, phosphate-buffered saline.

account for ~45% of families with high incidence of breast cancer and at least 80% of families with increased incidence of early onset of breast cancer (18–20). Likewise, carriers of mutations in the breast cancer susceptibility gene *BRCA2* have a highly increased risk of developing breast cancer (24,25). However, it is unclear how mutated genotypes predispose to cancer. A role of *BRCA2* protein in repair of double-strand breaks through its regulation of the recombination pathway has been suggested, including the direct association of *BRCA1* protein with *RAD51* protein (26–29). In addition, deregulation of mutated *BRCA2* protein may lead to deficient DNA repair and genomic instability (27). Association of *BRCA1* protein with the DNA repair protein *RAD51* in relation to changes in the cellular localization of the protein after exposure to DNA-damaging agents has been reported (27). There is evidence that mouse embryonic stem cells deficient in *BRCA1* are defective in the ability to carry out transcription-coupled repair of oxidative damage (29). These cells are hypersensitive to ionizing radiation and hydrogen peroxide, suggesting that *BRCA1* participates, directly or indirectly, in transcription-coupled repair of oxidative damage (27).

The objective of this work was to determine whether high linear energy transfer (LET) radiation, such as α particles, transform human breast epithelial cells in a step-wise fashion and to determine whether estrogen is a confounding factor in breast cancer development. Thus, immortalized MCF-10F and irradiated cells were analyzed for different parameters indicative of neoplastic transformation, including cell growth, anchorage-independent growth, invasiveness, tumorigenicity and changes in different protein expression.

Materials and methods

Cell culture

MCF-10F (15) cells have been in culture for >200 population doublings. The present study was conducted using cells in their 190th passage and they retained all the characteristics of the normal epithelium *in vitro*, including anchorage dependence, non-invasiveness and non-tumorigenicity in nude mice. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM)/F-12 (1:1) medium supplemented with 100 U/ml penicillin, 100 μ g/ml streptomycin, 2.5 μ g/ml amphotericin B, 10 μ g/ml insulin (all from Life Technologies, Grand Island, NY), 5% equine serum (Biofluids Inc., Rockville, MD), 0.5 μ g/ml hydrocortisone (Sigma, St Louis, MO) and 0.02 μ g/ml epidermal growth factor (Collaborative Research, Bedford, MA). The plating efficiency of the cells was ~55–60%, with an average population doubling time of 13 h. In cultures where the effects of estrogen were to be assessed, 17β -estradiol (E) was added to the culture medium at 10^{-8} M, a dose which gave maximal growth stimulatory effect.

Irradiation

Exponentially growing MCF-10F cells were plated 3 days before irradiation at a density of 3×10^5 cells in 60 mm diameter stainless steel rings with a 6 μ m mylar bottom. Cells were irradiated with graded doses of 150 keV/ μ m 4 He ions accelerated with the 4 MeV van de Graaff accelerator at the Columbia University Radiological Research Facilities as described previously (14). These high energy particles have a LET value comparable to the α particles emitted by radon progeny. MCF-10F cells irradiated with either a single or double dose of 30, 60 or 100 cGy of 4 He ions were prepared by subculturing for 10–15 passages and 12–14 weeks between doses. Irradiated cultures were subcultured immediately to determine growth kinetics and expanded in culture to assay for transformed phenotypes and, at the same time, samples were frozen as future stock. Remaining cells were then sampled for various transformed phenotypes and further passaged for additional radiation treatment (Figure 1). Cells were subsequently cultured in the presence or absence of E. Irradiated cultures were assayed for cell growth kinetics, anchorage-independent growth, invasiveness, tumorigenicity and determination of *BRCA1*, *BRCA2* and *RAD51* protein expression.

Growth kinetics assay

To determine the growth rate and saturation density of MCF-10F cells and their putatively transformed variants, 2×10^5 cells from the various

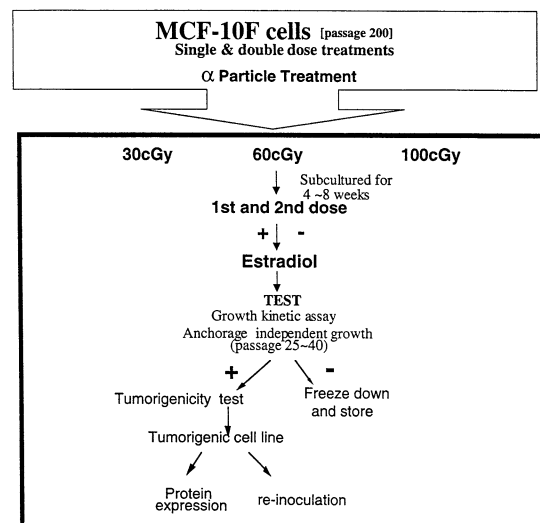


Fig. 1. Schematic diagram showing experimental protocol for assessing the neoplastic transforming effect of α particles in the MCF-10F cell line at passage 200.

exponentially growing cultures were replated in 60 mm diameter dishes in either the presence or absence of E, with lack of serum in certain experimental groups. At each time point studied, triplicate dishes from each group were trypsinized and the total number of cells per dish were determined.

Anchorage-independent assay

To test for cell growth in semi-solid medium, cells in passages 8–10 after radiation treatment were trypsinized and replated in DMEM/F-12 medium containing 10% bovine serum plus 1.2% methocel. Control and irradiated MCF-10F cells were plated in 24 multiwell plates in which the polystyrene flat bottom had been precoated with an agar layer prepared by adding 5% agar to DMEM/F-12 (2 \times) medium, without supplements, to give a final agar concentration of 0.9%. Cells were seeded in 0.8% methylcellulose at a concentration of 1×10^4 cells/well. The plates were incubated at 37°C with weekly feedings. After 2 weeks of incubation, colonies with >50 cells were counted under a dissecting microscope and the cloning efficiency was determined.

Cell invasion assay

Invasiveness was carried out as described previously (16,30) using modified Boyden's chambers (Transwell; Costar, Cambridge, MA) constructed with multiwell cell culture plates and cell culture inserts. Cell culture inserts were converted into invasion chambers by applying a layer of basement membrane onto the surface of microporous filters in each unit. Briefly, 8 mm diameter filters (8 μ m pore) of cell culture inserts were coated with 60 μ g/filter basement membrane matrigel (Collaborative Research) reconstituted with 100 μ l of MEM with 0.1% bovine serum albumin (BSA) (Collaborative Research) and separated by a porous polycarbonate filter (8 mm pore size, Nucleopore, Pleasanton, CA). The filters were coated and dried overnight. Exponentially growing cells were trypsinized, harvested, resuspended in DMEM plus 5% horse serum and passed repeatedly through a 25 gauge needle to produce a single cell suspension. After determination of the cell count and viability in a hemocytometer by the trypan blue exclusion test, the cells were added to the upper compartment of the chamber (3×10^5 cells/chamber). Fibronectin (Collaborative Research) was used as chemoattractant at a concentration of 1 μ g/ml/chamber in 0.5 ml of MEM with 0.1% BSA and placed in the lower chamber. After incubation for either 12 or 20 h at 37°C in a 5% CO₂ incubator, the cells on the upper surface of the filter were completely removed by wiping with a cotton swab and the cells that had traversed the matrigel and attached to the lower surface of the filter were studied. The filters were fixed, stained with Diff Quick (Sigma Chemical Co.), cut out and mounted on glass slides. The total number of cells that crossed the membrane were counted under a light microscope. Experiments were performed three times with 4 chambers/cell line. Clone BP1Tras (16) and breast epithelial cells derived from reduction mammoplasties were used as controls.

Tumorigenic assay

Female CB17 SCID mice (Taconic, Germantown, NY) and nude mice (Harlan Sprague Dawley, Indianapolis, IN) were used in these studies. Each animal was injected s.c. at two different sites with 8×10^6 cells in 0.2 ml saline in

the fat pad of the right and left side of the abdominal mammary gland. Animals were maintained under sterile conditions for 7–8 months and palpated for tumor appearance once a week. Control animals were inoculated with either MCF-10F cells or with a tumorigenic cell line, clone BP1Tras (16). Animals were killed as soon as the tumor nodules attained 0.5–0.9 cm in size.

Establishment of tumor cell lines

To establish cell lines, tumors were resected under aseptic conditions and washed three or four times with phosphate-buffered saline (PBS) to remove blood cells and tissue debris. Each tumor was then finely minced with a sterile scalpel blade into small fragments no larger than 1–2 mm in size. The pieces were then digested with 1% collagenase (Type IV; Sigma) in buffered solution containing Ca^{2+} and Mg^{2+} for 6 h at 37°C. Following centrifugation at 200 g for 10 min, the resultant pellets were washed once with medium and trypsinized for 5 min with 0.05% trypsin–EDTA solution (Life Technologies). The cell suspension was centrifuged again before being suspended in medium and plated out in culture dishes.

Determination of BRCA1, BRCA2 and RAD51 protein expression

Exponentially growing cells were plated on a glass chamber slide (Nunc Inc., Naperville, IL) at a density of 1×10^4 cells in 1 ml of medium and allowed to grow for 2–3 days until they reached 70% confluency. Cells were washed twice with PBS and fixed with 3.7% paraformaldehyde in PBS (pH 7.4) at room temperature. Cells were incubated with the corresponding primary antibodies: anti-BRCA1 (mouse monoclonal antibody; Santa Cruz Biotechnology Inc., Santa Cruz, CA), anti-BRCA2 (rabbit polyclonal antibody; Oncogene Research Products, Cambridge, MA) and anti-RAD51 (Santa Cruz Biotechnology Inc.) used at a dilution of 1:500. Incubations with the primary antibodies were performed overnight at 4°C. After washing in PBS, dishes were incubated for 60 min at room temperature with FITC-conjugated secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) at a 1:1000 dilution. Following several washes of 5 min each with PBS, slides were mounted with Vectashield mounting medium (Vector Laboratories, Burlingame, CA) and cells were viewed on a Zeiss Axiovert 100 TV microscope (Carl Zeiss, Thornwood, NY), using a 40×1.3 NA objective lens equipped with a laser scanning confocal attachment (LSM 410; Carl Zeiss). Fluorescent images were collected using an argon/krypton mixed gas laser. Composite images were generated using Adobe Photoshop and printed on Kodak DS 8650 (Rochester, NY). A semi-quantitative estimation based on the relative amount of BRCA1, BRCA2 and RAD51 protein expressed by cells was determined by the use of Adobe Photoshop, which gives the area and the intensity of staining. The MCF7 (31) and BP1Tras (16) cell lines were used as controls. The number of immunoreactive cells (30 cells/field) were counted in 15 randomly selected microscope fields ($400 \times$) per sample. Statistical analysis was done with the *F*-test and comparisons between groups with the Bonferroni (Dunn) *t*-test with significance at $P < 0.05$.

Results

The immortalized MCF-10F cells grew as a contact-inhibited monolayer with a population doubling time of ~ 13 h. At confluency, these cells had a saturation density of $\sim 8 \times 10^6$ cells/60 mm dish, as shown in Figure 2. Addition of a 10^{-8} M dose of E, which modulated the neoplastic transforming response of MCF-10F cells to ionizing radiation, did not appear to affect the normal growth of the cells (Figure 2). In contrast, cell kinetics assays performed on MCF-10F cells irradiated with either a single dose or two equal fractions of 60 cGy α particles, either in the presence or absence of E, showed altered morphology and higher saturation density of growth. Irradiated cells became elongated, overlapped each other and demonstrated a 2- to 3-fold increase in cell density after 20 passages in comparison with control cultures. The population doubling time of the α particle-irradiated cells was not much different from the control. Furthermore, MCF-10F cells grown in serum-free medium had a much slower growth rate, thus the growth dependence of MCF-10F cells on serum was clearly illustrated (Figure 2).

Table I lists the anchorage-independent and tumorigenic characteristics of various MCF-10F cells irradiated with either a single or double doses of radon-simulated α particles. Only cells irradiated with one or double doses of 60 cGy α particles

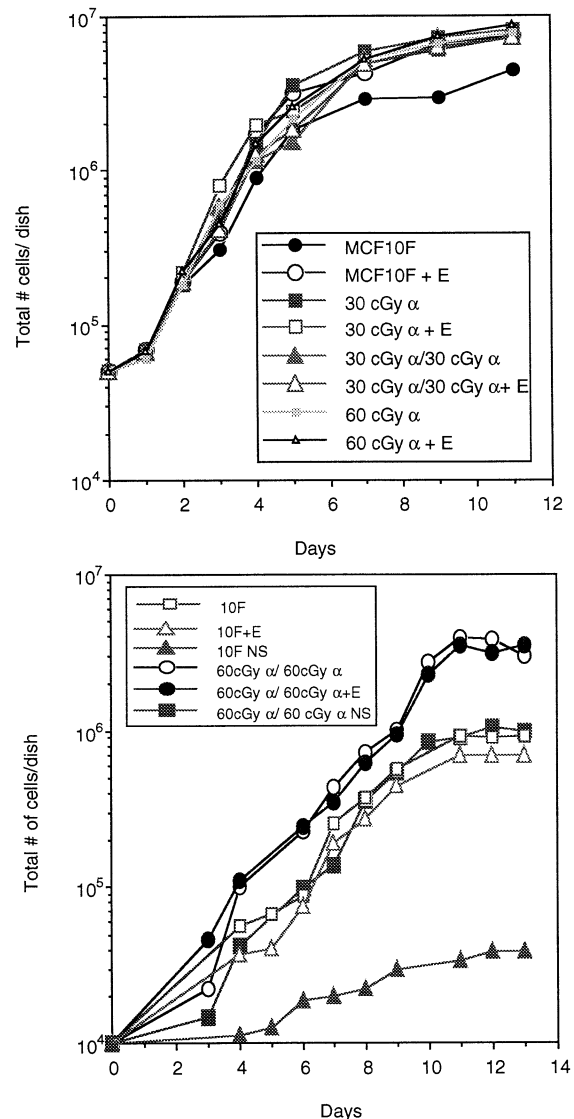


Fig. 2. Growth kinetics of control MCF-10F cells and their variants treated with single and double doses of 30, 60 and 100 cGy doses of α particles in either the presence or absence of E or lack of serum in the medium. Data shown are from one experiment and are representative of a series of growth studies. The population doubling time of the non-tumorigenic 60/60NS cGy α particle-treated cells was not much different from that of MCF-10F cells. See text for details.

formed agar-positive clones after 25 passages with a colony-forming efficiency in agar of 1%. When MCF-10F cells were reconstituted from these agar clones and injected into nude mice, only those cells previously irradiated with 60 cGy α particles and cultured for three consecutive months in the presence of E prior to the second 60 cGy dose (60E/60E) produced tumors in one out of the three mice tested. This single tumor developed in the fat pad of the mammary gland with a latency period of 3 months. Routine hematoxylin and eosin staining of paraffin-embedded sections indicated that the tumor was a mammary carcinoma in nature (Figure 3) and metastatic to the liver. Cells established from this tumor were further reinoculated into nude mice and tumor formation was observed in 2/3 animals, producing a total of four tumors with a much shorter latency period of only 4 weeks.

Figure 4 shows the invasive characteristics of control and MCF-10F cells after the various treatments scored at either 12

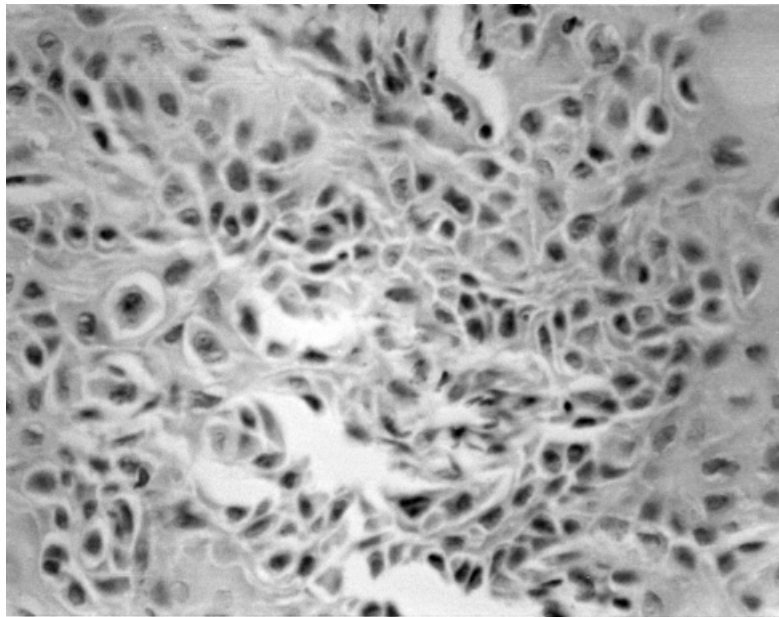


Fig. 3. Hematoxylin and eosin staining of paraffin-embedded tumor tissue showing the carcinoma induced by exposure to 60 cGy α particle- and E-treated cells in SCID mice ($\times 400$). A progressively growing tumor was evident 3 months post-injection of 8×10^6 cells in the fat pad of the mammary gland.

Table I. Characteristics of radon-irradiated human breast epithelial cells

Cell line	Dose (cGy \times exposure)	Passage	Anchorage-independent growth	Tumorigenicity
Non-immortalized normal breast cells	0	+10	-	-
MCF10F	0	246	-	-
MCF10F + E	0	+14	-	-
MCF10F without serum	0	+13	-	-
MCF10F	30 \times 1	+30	-	-
MCF10F + E	30 \times 1	+30	-	-
MCF10F	30 \times 2	+30	-	-
MCF10F + E	30 \times 2	+27	-	-
MCF10F	60 \times 1	+23	-	-
MCF10F	60 \times 1	+25	+	-
MCF10F + E	60 \times 1	+30	+	-
MCF10F without serum	60 \times 1	+20	-	-
MCF10F	60 \times 2	+19	-	-
MCF10F	60 \times 2	+25	+	-
MCF10F + E	60 \times 2	+22	-	-
MCF10F + E	60 \times 2	+25	+	-
MCF10F without serum	60 \times 2	+11	-	-
MCF10F	60E/60	+25	+	-
MCF10F	60E/60E	+25	+	+
MCF10F	100 \times 1	+16	-	ND
MCF10F + E	100 \times 1	+20	-	ND
MCF10F without serum	100 \times 1	+13	-	ND

ND, not determined.

or 20 h after plating onto the matrigel basement membrane. The number of cells which migrated through the membrane was clearly a function of time in addition to E treatment. Neither normal breast epithelial cells nor the immortalized MCF-10F cells showed any significant invasive capability. Addition of E to the growth medium significantly enhanced the invasive phenotype of MCF-10F cells in every treatment group examined. It should be noted that the BP1Tras clone demonstrated the highest invasive behavior and was consistent with its tumorigenic phenotype.

The expression of several oncoproteins frequently associated with breast cancer was determined among the various immortalized and transformed MCF-10F cells with or without estrogen

treatment as shown in Figure 5. Quantification of the immunofluorescent imaging of stained cells showed a significant increase in BRCA1 and BRCA2 in MCF-10F cells irradiated with α particles and treated with E compared with control cultures (Figure 5A). The staining intensity for BRCA1 among individual cells was fairly uniform (Figure 6) and showed a gradual increase in expression between control MCF-10F cells and their tumorigenic counterparts (60E/60E). The difference in staining intensity for the tumor suppressor proteins between the two groups was highly significant ($P < 0.05$). Irradiated cells that were transformed but non-tumorigenic (e.g. 60 cGy, 60/60) showed an intermediate staining intensity (Figures 5A and 6B). The tumorigenic cell line (60E/60E) showed more

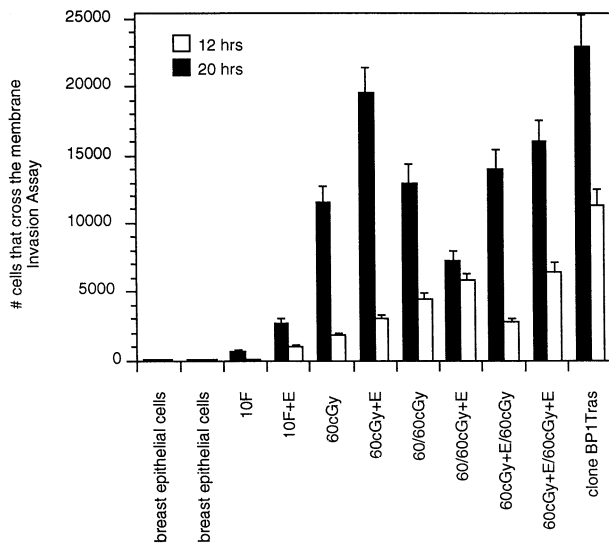


Fig. 4. Invasive characteristics of control and MCF-10F cells after the various treatments were scored either 12 or 20 h after plating onto the matrigel basement membrane. Invasiveness was determined using modified Boyden's chambers constructed with multiwell cell culture plates and cell culture inserts. See text for details.

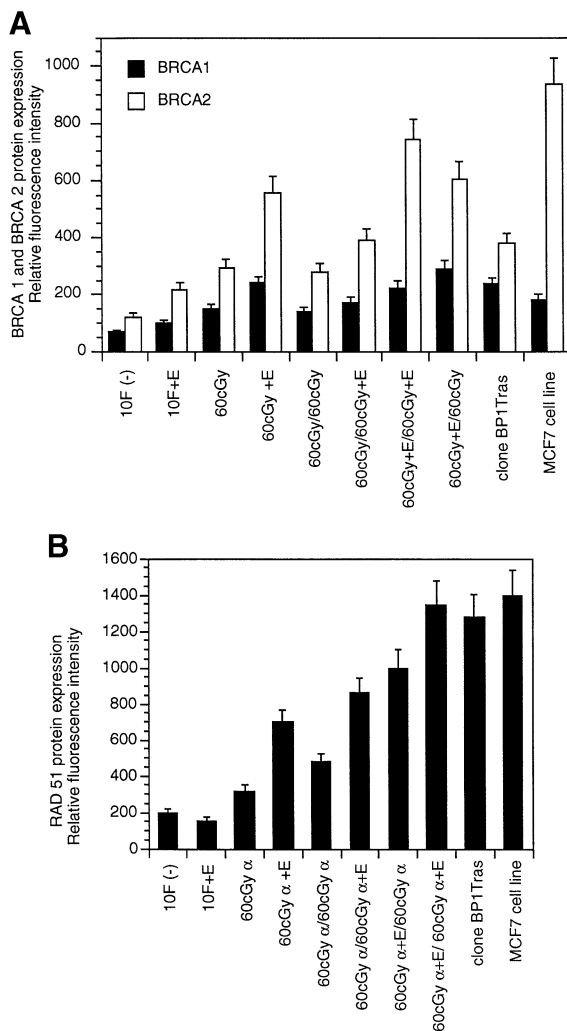


Fig. 5. Relative amounts of BRCA1 and BRCA2 (A) and RAD51 (B) proteins expressed by cells was determined by the use of a computer program which gives the area and intensity of the staining. See text for details.

intense staining (Figure 6C). Furthermore, addition of E enhanced the expression level of all of the oncoproteins examined. It should be noted that BRCA1 expression among MCF-10F cells was lower than the other two tumorigenic cell lines examined (clone BP1Tras and 60E/60E) and might be a reflection of the clonal origin of the MCF-10F cells. In general, expression of BRCA2 paralleled that of BRCA1 except that the expression level was much higher. The noticeable difference was in the expression of BRCA2 in MCF7 cells, which was the highest among the cell lines examined.

Expression of RAD51, which has been shown to be frequently associated with BRCA1 and BRCA2, was determined in control and transformed MCF-10F cells with or without E (Figure 5B). Quantification of the immunofluorescent imaging of stained cells showed a significant increase in RAD51 in MCF-10F cells irradiated with double doses of 60 cGy α particles concurrently treated with E (60E/60E and 60E/60) in comparison with control cultures. Similarly, the tumorigenic cell lines BP1Tras and MCF7 showed high RAD51 expression. Similar to our findings with BRCA1, addition of E significantly enhanced the expression of RAD51 in all irradiated groups.

Discussion

The objective of the present study was to establish a radiation-induced human breast cancer model based on spontaneously immortalized MCF-10F cells. These cells present all the characteristics of normal breast epithelium by their ultrastructural features and by their immunocytochemical reactivities towards keratins and sialomucins (15). Although definitive cell surface markers of human epithelial cell transformation have not been determined yet, there are indicators of cell transformation that have been shown to be common for both human and rodent mammary epithelial cells (33–36).

The progressive and step-wise process of selection of more aggressive phenotypes among the irradiated immortalized MCF-10F cells is consistent with the hypothesis of a multistep process in the progression of carcinogenesis (37). Physical and chemical carcinogens such as radiation and polycyclic aromatic hydrocarbons have been used as both initiators and promoters in rodent cell models and cell lines (14,16,32,36,38). In particular, fractionated exposure to γ -rays has been reported to transform human breast epithelial cells in culture (13). In the present study, we show for the first time that immortalized human breast epithelial cells can be transformed by a single dose of high LET α particles after successive cultivation for 6 months post-irradiation. The first observable sign was morphological changes occurring after the 25th passage post-treatment, suggesting that this phenotype may represent an early manifestation of transformation. The change in the rate of cell proliferation, indicated by a shorter doubling time, which becomes progressively shorter with the number of passages, suggests that in each passage there is selection of highly proliferating cells which boosts the cell growth advantage. Thus each passage resulted in the selection of other subsequent, more aggressive phenotypes, including an increase in saturation density, anchorage-independent growth, *in vitro* invasive capability and tumor formation in nude mice. This gradual increased expression of transformed phenotypes among α particle irradiated MCF-10F cells is consistent with our previous findings with immortalized human bronchial epithelial cells (14,33). A marker for mammary gland epithelial cells, human milk fat globule membrane antigen, and a differentiation

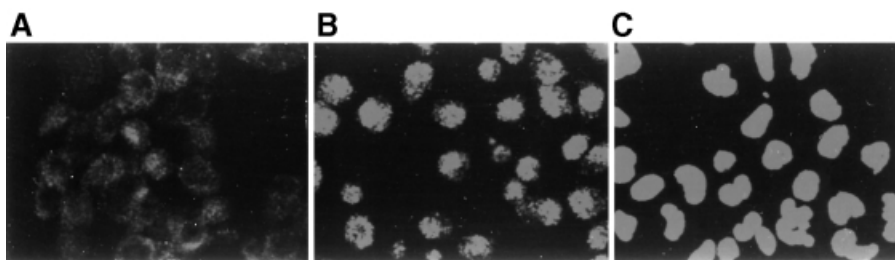


Fig. 6. Representative immunofluorescent staining of BRCA1 in control MCF-10F cells (A), transformed yet non-tumorigenic (B) and tumorigenic MCF-10F cells irradiated with 2×60 cGy doses of α particles followed by treatment with E (C). Protein expression was determined by immunofluorescent staining and quantified using confocal microscopy (400×).

marker for epithelial cells, human keratin 19, continued to be expressed in both the early transformants and in the tumorigenic cell line (not shown).

Since radiation is unlikely to be the only causative agent in the rising incidence of breast cancer, we are concerned about the possible additive effects in those individuals exposed to both radiation and estrogenic hormones. Segaloff and Maxfield (39) demonstrated that continuous administration of diethylstilbestrol produced a high incidence of mammary carcinoma and that those animals irradiated while under the influence of estrogen had many more carcinomas than those given diethylstilbestrol alone. To study the process of transformation we need to determine the conditions that modulate the susceptibility of this target tissue to carcinogenesis. Because of these considerations, we designed a study to determine whether radiation and estrogen are synergistic for the production of breast carcinoma.

Morphologically transformed cells were able to grow in agar-methocel and this latter phenotype was expressed more intensively in later passages of those cells cultured in the presence of E. The transformed cell line, termed the 60 cGy+E/60 cGy+E α particle-treated cell line, exhibited anchorage-independent growth and was tumorigenic in nude mice. Although anchorage-independent growth correlates fairly well with tumorigenicity among rodent cells, such is not the case with human epithelial cells (14). For instance, breast epithelial cells obtained from milk, which were reported to have acquired anchorage-independent growth and immortalization after SV40 infection, did not elicit tumorigenesis in nude mice (40).

It has been shown that chemoinvasion, or the ability of cells to cross basement membranes *in vitro*, is enhanced in transformed cells and correlates with their malignant characteristics *in vivo* (16,30,32). Using a reconstituted basement membrane, we showed that the clones derived from 60 cGy+E/60 cGy+E α particle-treated cells exhibited greater invasive capabilities than the MCF-10F control cells and their invasive character was comparable with the tumorigenic control cells. Tumor formation induced in SCID mice with the 60 cGy+E/60 cGy+E α particle-treated cells suggests to us that tumorigenicity may be related to higher expression of *BRCA1* and *BRCA2* or other altered genes.

Our present results indicate, for the first time, that ionizing radiation induces nuclear localization of BRCA1 and BRCA2 protein in human breast epithelial cells that have previously been treated with estrogen. We observed that the radiation treatment induced a dot-like nuclear staining pattern of BRCA1 in MCF-10F cells (60/60 cGy, Figure 6B) and that estrogen treatment further increased the staining intensity in tumorigenic cell lines as determined by confocal microscopy (60E/60E

cGy, Figure 6C). Studies are now in progress to further characterize these proteins by cell fractionation and immunoblotting in all the cell lines of this experimental model, along with MCF7 cells.

It is not yet clear whether BRCA1 plays a functional role in non-familial breast cancer. No mutations have been detected in sporadic breast cancer suggesting that BRCA1 may play a minor role, if any, in breast cancer. However, a reduction in *BRCA1* mRNA levels in breast cancer relative to normal mammary tissues among non-familial breast cancer has been described (41). Likewise, the expression of BRCA1 protein in breast cancer is still subject to debate. Using an epitope-tagged *BRCA1* cDNA, Chen *et al.* (42) showed previously that the protein was mostly nuclear-associated in normal breast cells (HBL-100), whereas in breast cancer derived cell lines it localized mainly in the cytoplasm. Cytoplasmic localization of BRCA1 protein was demonstrated in 16 out of 17 breast cancer derived cell lines, suggesting that aberrant localization of BRCA1 is an indication of its role in non-familial breast cancer (43). On the other hand, using an immunohistochemical staining assay based on an antiserum raised against the N- and C-termini of human BRCA1, Taylor *et al.* (44) reported that little or no staining for BRCA1 was detected in breast carcinomas, whereas strong staining was observed in non-malignant breast biopsies. While BRCA1 expression was generally reduced in non-familial breast cancer, nevertheless 87 of 142 biopsy cases (61%) showed both nuclear and cytoplasmic BRCA1 staining, with only 20% of the cases studied (28/142) failing to show any staining. These data are consistent with a previous report in which 42/50 breast carcinomas were found to express BRCA1 and none of 17 malignant effusions showed a similar nuclear expression (43). These results seem to indicate a parallel loss of BRCA1 with progression of the disease.

In contrast, Scully *et al.* (26,45) first demonstrated nuclear localization of BRCA1 in breast tumor derived cell lines using BRCA1-specific monoclonal and polyclonal antibodies. Immunostaining of neutral paraformaldehyde-fixed cell lines with anti-BRCA1 antibodies gave rise to a dot-like nuclear staining pattern in all the six breast cancer cell lines tested. They reported that this nuclear dot distribution of BRCA1 was a general cellular characteristic and not the result of a fixation artifact. They also confirmed the presence of the protein in the nuclear fraction by cell fractionation and immunoblotting for BRCA1 with different antibodies in three cancer cell lines, along with MCF7 cells. Our present findings, therefore, are in agreement with these studies since we also fixed the different cell lines with neutral paraformaldehyde, even though we used commercially available monoclonal and polyclonal antibodies for BRCA1 and BRCA2.

We conclude that ionizing radiation and E influence the process of carcinogenesis in human breast epithelial cells. Results generated through these studies could have a great impact on the understanding of the causes and mechanisms involved in the initiation and progression of breast cancer and could provide a basis for future developments to reverse these processes. Understanding the complex interactions of hormones and genes holds great promise for detection and treatment of breast cancer.

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