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Oncoprotein expression in human breast epithelial cells transformed by high-LET radiation

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Abstract.
Purpose: The aim of the present work was to analyze the expression of oncoproteins that are frequently altered in breast cancer with specific phenotypic stages in the neoplastic process.
Materials and methods: Expression of c-myc, c-jun, c-Ha-ras and the tumor suppressor gene p53 oncoproteins were examined by immunohistochemical staining coupled with confocal microscopy in transformed and tumorigenic human breast epithelial cells induced by high-LET α-particles (150 keV/μm).
Results: MCF-l0F cells, irradiated with single and double doses of 60 cGy α-particles and subsequently treated with estrogen, showed gradual phenotypic changes including altered morphology, increased cell proliferation relative to control, anchorage-independent growth, invasive capabilities and tumorigenicity in nude mice. MCF-10F cells irradiated with a second dose of 60 cGy α-particles after estrogen treatment (60 cGy + E/60 cGy + E) showed tumorigenicity both in SCID and nude mice. Alterations in the protein expression of several oncogenes including c-*myc*, c-*jun*, c-Ha-*ras* and the tumor suppressor gene *p53* were detected in α-particle-irradiated cells and in those cells subsequently cultured in the presence of estrogen. The expression level of these oncoproteins correlated with the progressive nature of the neoplastic process.
Conclusion: These studies suggest that overexpression of several oncoproteins is important in the neoplastic transformation of human breast epithelial cells induced by high-LET radiation. In addition, use of endocrine factors such as estrogen allows the examination of various aspects of protein expression providing the basis for understanding the complex interactions of hormones and genes.

1. Introduction

Breast cancer is a complex disease in which numerous genetic alterations occur. It is unclear which of these abnormalities are causative for breast tumorigenesis. Specific sequences of genetic change that are relevant to tumor progression often involve the accumulation of multiple abnormalities in individual cells (Field and Spandidos 1990, Janocko *et al.* 1995). On the basis of the currently accepted view of breast cancer as a multistep process, it is possible that specific abnormalities may be required to illustrate the progression from normal to invasive tumor cells (Sato *et al.* 1991). The knowledge of specific genetic changes in tumorigenesis is critical to an understanding of the natural history of breast tumors. These changes may involve specific genetic loci that directly contribute to one or more attributes of transformation, i.e. deregulated proliferation and invasion. Other changes may confer genetic instability, which increases the possibility of acquiring subsequent specific lesions relevant to tumorigenesis. Much still remains to be learned to understand the key factors behind the evolution of breast cancer. It is accepted that the transformation of a normal cell to one that is malignant can result from mutations in genes that encode key growth regulatory proteins. Among them, *c-myc*, *c-jun* and *c-fos*, *c-Ha-ras*, and *p53*, along with many others, can lead to aberrant cell growth, hyperproliferation and eventually cancer. Alternatively, there is substantial experimental, epidemiological, and clinical evidence that breast cancer risk is influenced by endogenous hormones (Pike *et al.* 1993). Breast cancer may have both its genesis and cell growth influenced by hormonal factors, since about one-third of breast cancers are responsive to endocrine therapies (Dickson and Lippman 1988, 1998). It has been shown that estrogens modulate cell proliferation and differentiation of human breast epithelium (Dickson and Lippman 1998); however, the exact pathway has not been elucidated.

Expression of c-myc is generally associated with cellular proliferation (Shiu 1988). Overexpression of this early signaling oncogene is thought to contribute to the formation and progression of some human malignancies, including breast cancer, where it is commonly amplified and overexpressed at frequencies ranging from 4% to 52% (Field and Spandidos 1990, Watson *et al.* 1993). Expression of other nuclear protooncogenes such as c-*jun* and c-*fos* are also indicative of an early response event during cell proliferation, but timing of their appearance is uncertain (Sherman *et al.* 1990, Prasad *et al.* 1995). Prasad *et al.* (1995) reported that low doses of ionizing radiation (0.25–2.0 Gy of γ-radiation) can modulate the expression of these early genes in a variety of mammalian cells. The expression of both c-myc

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and c-jun proteins are critical for the growth of both hormone-dependent and hormone-independent breast cancer cells (Shiu 1988, Field and Spandidos 1990, Web *et al.* 1990).

Activation or amplification of c-Ha-*ras* has also been implicated, to varying degrees, in the development and progression of human breast cancer (Field and Spandidos 1990). There is evidence that genetic changes may occur in a preferred sequence in the development of solid tumors including c-Ha-ras and c-myc overexpression (Field and Spandidos 1990, Sherman *et al.* 1990, Janoko *et al.* 1995, Prasad et *al.* 1995). These two oncogenes seem to play a role in the progression of mammary cancer in which an overexpression of a mutated c-Ha-*ras* oncogene has been previously reported in 10% of breast cancer patients (Field and Spandidos 1990).

p53 has been identified as an important tumor suppressor gene in normal cells (Nigro *et al.* 1989, Levine *et al.* 1991, Callahan 1992). It has been suggested that early loss of cell-cycle control in the presence of a mitogenic stimulus allows a cell to continue dividing (Levine 1991). Wild-type *p53* plays a crucial role in maintaining genomic stability by either allowing the repair of damaged DNA through the induction of a transient G1 arrest or eliminating the damaged cells by triggering apoptosis (Nigro *et al.* 1989). Mutant *p53* fails to mediate any of these effects. Uncontrolled proliferation in the absence of wild-type *p53* would expectedly yield a high level of genomic instability, which is a common abnormality detected in primary breast cancer (Nigro *et al.* 1989, Callahan 1992, Band *et al.* 1993). Although the signal transduction pathway(s) leading to induction of the p53 protein has not been fully established, it has been shown that ultraviolet radiation and double-strand DNA breaks strongly induce its expression (Elledge and Lee 1995).

The identification of genes involved in breast cancer is of critical importance to elucidate the progression of this disease. Identification of the factors involved in cell proliferation and transformation has been facilitated by studies using breast cancer cell lines representative of different tumor phenotypes. *In vitro* model systems have been extensively used in the study of radiation-induced transformation (Thraves *et al.* 1990, Hei *et al.* 1994, Riches *et al.* 1994). There is little or no information available on the mechanisms of radiation-induced breast cancer. Therefore, an *in vitro* breast transformation model, utilizing epithelial cells at different stages of the neoplastic process, would provide a unique opportunity for studying radiation carcinogenesis. The objective of the present work is to determine the oncogenes that are frequently altered in breast cancer. MCF-10F, a spontaneously immortalized human breast epithelial cell line derived from primary human diploid mammary epithelial cells, was used in these studies (Soule *et al.* 1990). These cells have the morphological characteristics of normal breast epithelial cells such as dome formation in confluent cultures, epithelial morphology in culture (Calaf *et al.* 2000) and exhibition of three dimensional growth in collagen gel. They depend upon hormones and growth factors for optimal growth *in vitro*. In addition, they are anchorage-dependent, non-invasive and non-tumorigenic in nude or SCID mice (Calaf and Russo 1993, Calaf *et al.* 1994, Calaf and Hei 2000). Calaf and Hei (2000) have recently developed a model in which MCF-10F cells were transformed by high-LET radiation in a stepwise fashion before becoming tumorigenic in nude mice, which is the system used here.

2. Materials and methods

2.1. *Cell culture*

The spontaneously immortalized human breast epithelial MCF-10F cells were used in the present study (Soule *et al.* 1990, Calaf and Hei 2000). The subcutaneous tissue from which MCF-10F cells were derived was acquired from a mastectomy performed in a 36-year-old premenopausal woman. MCF-10F cells have been in culture for more than 200 population doublings and the present study was performed with cells in their 190th passage. These cells have all the morphological phenotypes of a normal epithelial cell line (Russo *et al.* 1991, Calaf *et al.* 2000). The plating efficiency of the cells was $\sim 75\%$. The media used was DMEM/F-12 (1:1) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 2.5 μg/ml amphotericin B, and 5% equine serum (all from Life Technologies, Bedford, NY), 0.5 μg/ml hydrocortisone (Sigma Chemical Co., St Louis, MO), 10 μg/ml insulin and 0.02 μg/ml epidermal growth factor (Collaborative Research, Bedford, MA).

2.2. *Irradiation conditions and cell lines*

Exponentially growing MCF-10F cells were plated in 60 mm diameter specially constructed stainless steel rings with a 6 μm mylar bottom at a density of 3×10^5 cells 3 days before irradiation. Cells were irradiated at 70% confluency with graded doses of 150 keV/μm ^{4}He ions accelerated with the 4 MeV van de Graaff accelerator at the Columbia University Radiological Research Accelerator. The dose rate of these ^{4}He ions averaged ~ 4.5 Gy/min as described previously (Hei *et al.* 1994). These high-energy par-

ticles were used in the present study because of their high oncogenic transforming and mutagenic potential shown previously (Hall and Hei 1986, Zhu *et al.* 1996) and their relevance to high altitude flying and to the space environment.

MCF-10F cells were irradiated with either a single or double dose of 60 cGy of ^{4}He ions. Cells were prepared by subculturing for 10–15 passages and for 12–14 weeks between doses. Cell lines were named 60 cGy and 60 cGy/60 cGy, respectively. The MCF-10F cells irradiated with either a single or double dose of 60 cGy of ^{4}He ions with continuous treatment of 10^{-8} M estradiol 17β (E) (Sigma) were named 60 cGy + E and 60/60 cGy + E, respectively (Calaf and Hei 2000). Control and irradiated cells were subcultured immediately to determine both plating efficiency and survival levels. Cultures were maintained for periods of up to 6–8 months during which they were assayed for the expression of transformed phenotypes, while representative samples were frozen-down periodically as future stock. The clone BP1Tras cell line originated after treatment of MCF-10F cells with benzo(a)pyrene and transfection with the c-Ha-*ras* oncogene as previously described (Calaf *et al.* 1994). These cells were used as a positive control because they were tumorigenic in the SCID mice. The carcinoma-derived MCF-7 cell line (Soule *et al.* 1973) was also used as a positive control because of its breast origin and its tumorigenic nature in the SCID mice.

2.3. *Anchorage-independent assay*

To test for cell growth in semi-solid medium, cells were trypsinized and replated in DMEM/F-12 medium containing 10% horse serum plus 1.2% methocel. Control and treated MCF-10F cells were plated in 24 multi-well plates in which the bottom was precoated with an agar layer prepared by adding 5% agar to DMEM/F-12 (2×) medium 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. After 2 weeks of incubation, colonies were counted to determine cloning efficiency.

2.4. *Cell invasion assay*

Tests for invasiveness were carried out using the modified Boyden's chambers (Transwell, Costar Cambridge, MA) as described previously (Bonfil *et al.* 1989, Calaf and Russo 1993). Briefly, cell culture inserts were converted into invasion chambers by applying a layer of basement membrane onto the surface of microporous filters in each unit. Filters of 8 mm in diameter (8 μm pore) of cell culture inserts were coated with 60 μg/filter basement membrane matrigel (Collaborative Research, Bedford, MA) reconstituted with 100 μl of MEM with 0.1% bovine serum albumin (Collaborative Research) and separated by a porous polycarbonate filter (8 mm pore size) (Nucleopore, Pleasanton, CA). Cells were added to the upper compartment of the chamber (3×10^5 cells/chamber) and fibronectin (Collaborative Research) was used as chemoattractant (1 μg/ml per chamber in 0.5 ml of MEM with 0.1% BSA) in the lower chamber. After 20 h of incubation, cells on the upper surface of the filter were removed with a cotton swab. Such filters were fixed, stained with Diff Quick (Sigma), cut out and mounted onto glass slides. The number of cells that traversed the matrigel membrane and attached to the lower surface of the filter were counted. This is the number of cells that cross the membrane.

2.5. *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 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.

2.6. *Determination of c-myc, c-jun, c-Ha-ras, and mutant p53 protein expression by confocal microscopy*

Exponentially growing control and irradiated cells, at similar passage numbers, 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% confluence. The cells were washed twice with buffer solution and fixed with 1% buffered paraformaldehyde (pH 7.4) at room temperature for 10 min, washed twice with buffer solution, incubated with 1% H_2O_2 in methanol for 30 min to block endogenous peroxidase, and washed twice with buffer solution. Slides were then covered with 3% bovine serum for 30 min at room temperature and then incubated with the corresponding primary antibodies at 1:500 dilution overnight at 4°C. Antibodies included c-*myc*, mutant *p53* (Pab 240, mouse monoclonal antibody: Oncogene Res. Products, Cambridge, MA; and mouse monoclonal antibody: Biotechnology Inc., Santa Cruz, CA), Ha-*ras* (mouse monoclonal antibody: Biotechnology Inc., Santa Cruz, CA) and c-*jun*/AP1 (goat polyclonal antibodies: Biotechnology Inc.). After washing in buffer solution, cultures were incubated for 60 min at room temperature with either antimouse (for c-*myc*, mutant *p53* and Ha-*ras*) or antigoat

(for c-*jun*/AP1 polyclonal antibody)-Rhodamine-conjugated secondary antibodies (Jackson ImmunoResearch Lab., West Grove, PA) at a 1:1000 dilution. Following several washes of 5 min each with buffer solution, slides were mounted with Vectashield mounting media (Vector Laboratories, Burlingame, CA). Controls included cultures stained with either the primary or secondary antibodies alone to monitor the background staining levels. Cells were viewed on a Ziess Axiovert 100 TV microscope (Carl Zeiss, Thornwood, NY) using a 40 × 11.3 NA objective lens equipped with a laser scanning confocal attachment (LSM 410; Carl Zeiss, Thornwood, NY). Fluorescent images were collected by using an argon/krypton mixed gas laser (488 nm) to excite the Rhodamine. Composite images were generated using the Adobe Photoshop program, and printed on a Kodak DS 8650 printer (Rochester, NY). A semi-quantitative estimation based on the relative staining intensity of the various oncoproteins expressed by the control and transformed cells was determined by the computer program, which gives the area and the intensity of the staining. The data presented in the figures was normalized to cell area by considering the amount of protein per area. The experiments were repeated three times with cells of similar passages. The number of immunoreactive cells (30 cells/field) were counted in several randomly selected microscope fields (× 400) per sample. Statistical analysis was performed using the *F*-test for multiple comparisons and Bonferroni (Dunn) *t*-test with significance at the 0.05 level between groups.

3. Results

MCF-10F cells and those cultured in the presence of E (MCF-10F + E) grew as contact-inhibited monolayers with a population doubling time of ~13 h. After exposure to radiation, cells showed no differences in morphology relative to controls for the first 20 passages. The cells grew closely juxtaposed, forming flat monolayers without overlapping. After the twentieth passage, all irradiated cells exhibited focal morphological alterations that were extended to the remaining population. Irradiated MCF-10F cells exhibited a pleomorphic appearance, many of them acquired a spherical shape, and others became elongated. Irradiated cells with one or two doses of α-particles, either in the presence of E, before or after irradiation, formed agar-positive clones after 25 passages in culture with a colony-forming efficiency in agar of 1–3%.

The invasiveness assays showed that the number of cells that crossed the membrane in control MCF-10F and MCF-10F + E cell lines were significantly lower than the irradiated and transformed cell lines ($p < 0.05$). The tumorigenic 60 cGy + E/60 cGy + E cell line as well as the clone BP1Tras demonstrated the highest invasive characteristics, which was consistent with their malignant phenotypes in comparison to the other groups ($p < 0.05$). Among the irradiated cell lines, only cells irradiated with double doses of 60 cGy α-particles in the presence of E (60 cGy + E/60 cGy + E) induced tumors in both the SCID and nude mice. Table 1 shows the list of cells used in these studies and their phenotypic alterations, illustrating the gradual, stepwise transformation in the α-irradiated and E-treated cells before they formed tumors in the SCID (1/3) and nude mice (4/3). No tumors were observed in any of the 12 nude mice injected with control MCF-10F and MCF-10F + E cells. Hematoxylin and eosin staining of the tumor tissues indicated that they were adenocarcinomas.

Using exponentially growing cultures, an increase in c-myc protein expression was detected afterwards in all the irradiated populations compared with control MCF-10F cells after 20 passages. Such increases were irrespective of E treatment, as seen in figure 1a. There was no significant difference in c-myc expression between cells irradiated with either a single 60 cGy dose or with a double dose of α-particles. The tumorigenic breast cell line MCF-7 showed an expression level that was significantly higher than the control MCF-10F and MCF-10F + E cells ($p < 0.05$). In contrast, the c-myc expression level in the positive control BP1Tras cells was similar to the rest of the irradiated population. Similarly, expression of the c-myc protein among the tumorigenic cell lines MCF-7, clone BP1Tras, and the 60 cGy + E/60 cGy + E cell line was not significantly different from the non-tumorigenic 60 cGy/60 cGy + E cells, as seen in figure 1a.

Figure 1b represents the quantification of c-Ha-ras protein expression in α-irradiated MCF-10F cells with or without pre-and post-treatment with E. The tumorigenic breast carcinoma MCF-7 cells and the positive control clone BP1Tras showed a similar expression level of c-Ha-ras oncoproteins, at a level significantly higher than the control MCF-10F cells ($p < 0.05$). In contrast, most irradiated cells without E treatment showed low expression levels of c-Ha-ras, and there was no significant difference in expression level between those receiving single versus double doses of α-particles. The tumorigenic 60 cGy + E/60 cGy + E cell line had higher c-Ha-ras protein expression than the non-irradiated cells with or without E treatment ($p < 0.05$).

Figure 1c represents the quantification of the immunofluorescent imaging of c-jun protein expres-

Table 1. Biological properties of α-particle-irradiated and 17β estradiol (E)-transformed human breast epithelial cells.

Cell line[a]	Passage[b]	Anchorage independency[c]	Invasion assay[d]	Tumorigenicity[e]
MCF-10F	+ 46	−	−	0/6
MCF-10F + E	+ 14	−	−	0/6
60 cGy	+ 25	−	−	0/6
60 cGy	+ 43	−	+	0/6
60 cGy + E	+ 24	−	−	0/6
60 cGy + E	+ 40	+	+	0/6
60 cGy + /60 cGy	+ 25	−	−	0/6
60 cGy + /60 cGy	+ 45	+	+	0/6
60 cGy/60 cGy + E	+ 25	−	−	0/6
60 cGy/60 cGy + E	+ 45	+	+	0/6
60 cGy + E/60 cGy	+ 46	+ +	+ +	0/6
60 cGy + E/60 cGy + E	+ 27	+ +	+ + +	0/6
60 cGy + E/60 cGy + E	+ 45	+ + + +	+ + +	5/6

[a] Dose in cGy. Number of exposures. E: 17β estradiol treatment (10^{-8} M).
[b] Number of passages in culture after radiation treatment when tests were performed.
[c] Colony-forming efficiency in agar fluctuated from 1% to 3%.
[d] Invasive characteristics of control and MCF-10F cells after the various treatments were scored 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. The experiments were repeated with three very similar passages. Positive signs represent the results in relation to the number of cells that crossed the filters.
[e] An average of six animals were used per group. See text for details.

sion in α-irradiated MCF-10F cells with or without pre- and post-treatment with E. It was evident that cells receiving a double dose of α-particles had significantly higher expression levels of c-jun than cells irradiated only once. The c-jun expression level among MCF-10F cells exposed to α-particles once was similar to those of control cells. The E treatment after one dose of 60 cGy of α-particles did not enhance c-jun expression among irradiated cells. However, c-jun expression among MCF-10F cells irradiated twice with α-particles was significantly higher than controls ($p < 0.05$). Among the tumorigenic control cell lines, the MCF-7 cells showed an expression level that was significantly greater than that of the control MCF-10F cells ($p < 0.05$).

Quantification of the immunofluorescent imaging showed an increase in mutant p53 oncoproteins in MCF-10F cells irradiated with a double dose of α-particles either in the presence or absence of E (in comparison with control MCF-10F, as seen in figure 1d). There was no significant difference among those irradiated with single or double doses of α-particles. The tumorigenic 60 cGy + E/60 cGy + E cell line showed an expression level that was significantly greater than the non-tumorigenic cell lines, 60 cGy, 60 cGy + E, 60 cGy/60 cGy and 60 cGy/60 cGy + E ($p < 0.05$), and at a level 10-fold higher than the control MCF-10F cells. Among the tumorigenic control cell lines, the MCF-7 cells showed an expression level not significantly different from control MCF-10F cells. The clone BP1Tras had a p53 protein expression level similar to the 60 cGy + E/60 cGy + E cells. The cells irradiated with one dose of 60 cGy α-particles, either in the presence or absence of E, or with double doses of 60 cGy α-particles, had p53 protein levels not significantly different from the control MCF-10F cells. Figure 2 shows a representative confocal image of the immunofluorescent intensity and distribution of p53 oncoprotein in the nucleus of control MCF-10F cells (left panel) and the tumorigenic cell line (60 cGy + E/60 cGy + E) (right panel). The granular distribution was homogeneous among the cells; however, the intensity was remarkably greater in the tumorigenic cell line. The staining pattern was uniform among the cells in the population.

4. Discussion

It is well known that carcinogenesis is a multistep process of initiation, promotion, and progression. Identification of genes involved in these stages in radiation-induced carcinogenesis is of critical importance for a better understanding of the mechanisms involved. An experimental breast cancer model has recently been established where normal human breast epithelial cells underwent a stepwise transformation into malignant cells by the effect of low doses of α-particles in the presence of estrogens (Calaf and Hei 2000).

Since c-*myc*, *jun* and *fos* oncogenes are often found to be involved in breast cancer, their expression

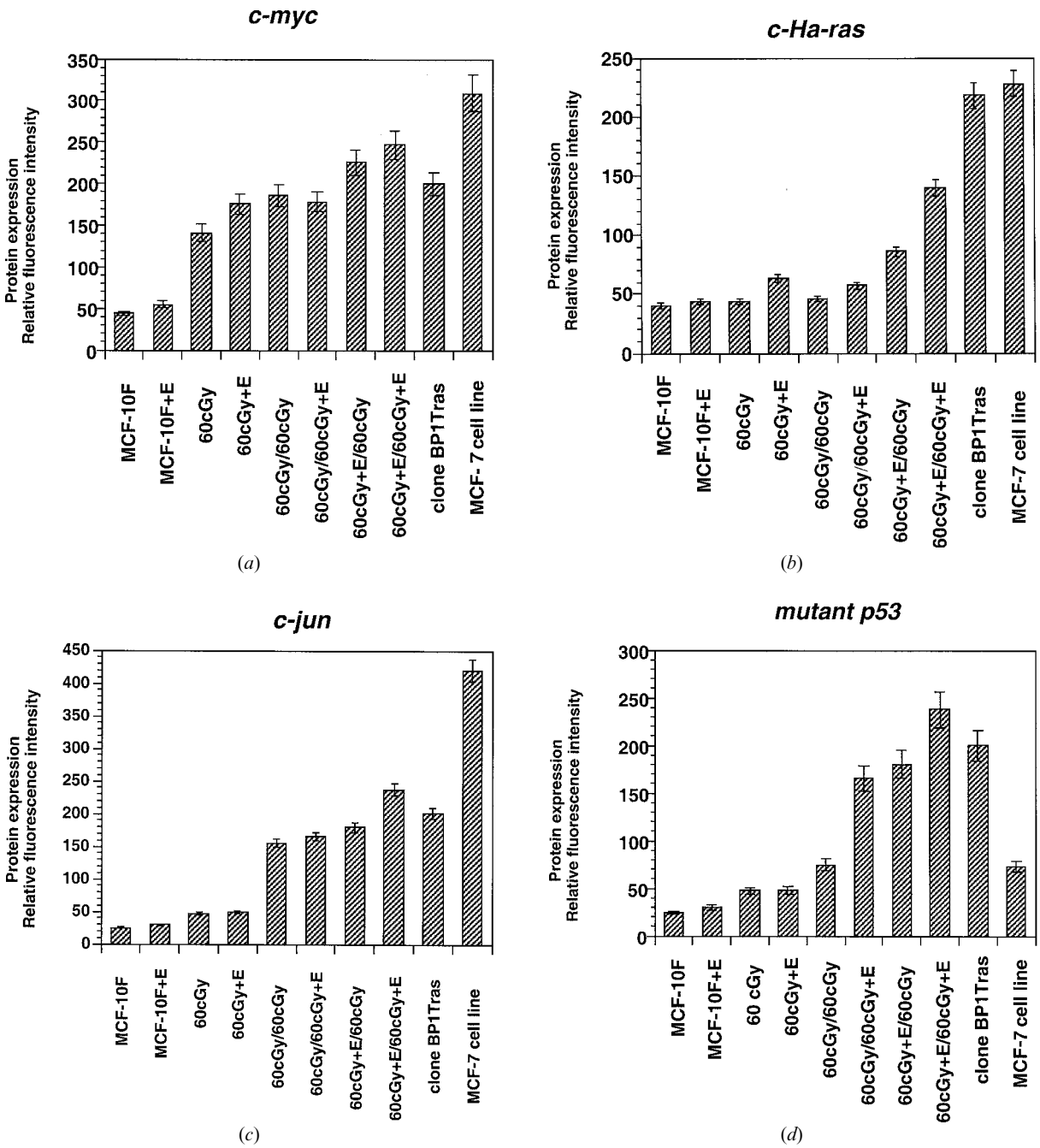

Figure 1. Average relative amounts of immunofluorescent staining of proteins expressed by cells and determined by fluorescence microscopy for c-myc (*a*), c-Ha-ras (*b*), c-jun (*c*) and mutated p53 (*d*). Bars in the figure indicate the standard error of the mean.

levels in transformed MCF-10F cells were examined at different stages of the neoplastic process in order to identify stage-specific alterations. Current results indicate that the irradiated cell lines had a higher level of c-myc expression than the control MCF-10F cells. These results corroborate other previous findings that c-myc expression was induced by ionizing radiation during stimulation of cell proliferation or cell transformation (Mothersill *et al.* 1994). The c-myc has been shown to be overexpressed in many clinical breast tumor samples (Shiu *et al.* 1988, Watson *et al.* 1993) and its expression is an early event during activation of cellular proliferation, although timing of its appearance is uncertain. The finding that c-myc is overexpressed in irradiated cells exhibiting none of the transformed phenotypes, apart from morphological changes, is consistent with the notion that c-*myc* participates in the stimulation of cell

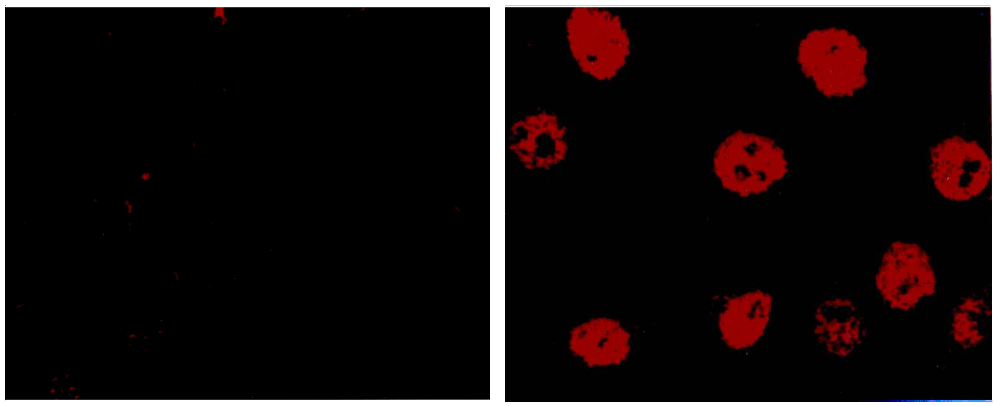

Figure 2. Representative immunofluorescent staining of mutant p53 protein expression in control MCF-10F cells (left) and tumorigenic MCF-10F cells irradiated with a 2 × 60 cGy dose of α-particles followed by treatment with E, 60 cGy + E/60 cGy + E (right). Protein expression was determined by immunofluorescent staining and quantified using confocal microscopy (400 ×). See text for details.

proliferation and the first stages of cell transformation. Overexpression of the c-*myc* gene as a result of gene amplification is probably critical to the genesis of breast cancer.

It would seem that genetic evolutionary changes may occur in a preferred sequence and one of the steps in this sequence includes c-Ha-*ras* and c-*myc* overexpression in breast cancer (Field and Spandidos 1990, Janocko *et al.* 1995). Studies on the evolution of human solid tumors, for example ductal breast carcinomas, have shown that this process includes ras and myc oncogene overexpression (Field and Spandidos 1990). An early manifestation of genomic instability is gene amplification as observed with c-*myc*. This alteration seems to occur throughout all stages of the disease from ductal carcinoma *in situ* onwards.

The involvement of these oncogenes in the multistep process of neoplastic transformation was first demonstrated by several authors when cotransfection with c-*myc* and c-Ha-*ras* oncogenes induced transformation of normal diploid rat embryo primary cells and induced the formation of tumors in nude mice (Land *et al.* 1983, Chen *et al.* 1993). Previous studies (Calaf *et al.* 1994, Zhang *et al.* 1994) have reported that chemically induced neoplastic transformations correlated with alterations in the c-Ha-*ras* oncogene. The c-Ha-*ras* transfected into MCF-l0F cells was able to induce neoplastic phenotypes in 1/14 nude mice tested (Calaf *et al.* 1994) in a manner similar to the role of v-Ha-*ras* in human bronchial epithelial cells (Yoakum *et al.* 1995). There is some indication that c-Ha-*ras* and c-*myc* are found in some radiation-induced tumors via either point mutations or gene amplification, or both. Although the causal roles have not yet been defined, they seem to play an important role in the progression of epithelial tumors (Chen *et al.* 1993). Among them, amplification of the c-*myc* oncogene proved to be an early initiating event resulting from the direct action of radiation on target tissue. It has been reported that ionizing radiation induced the amplification of c-K-*ras* and increased expression of c-myc in the rat skin during the process of transformation (Ruggeri *et al.* 1991).

The difference in mutant *p53* expression between the control MCF-10F cells and the tumorigenic 60 cGy + E/60 cGy + E cell line (figure 1d) indicates that mutations in the *p53* gene may be closely linked to the development of malignant phenotypes, as shown previously in other human model systems (Mothersill *et al.* 1994) and in carcinogen-treated MCF-10F cells (Calaf *et al.* 1994). Determination of the protein expression depends on the specificity and quality of the antibody used. Although MCF-10F cells have a *p53* mutation in exon 7, which corresponds to codon 254 in one of its alleles (Barnabas *et al.* 1995), immunofluorescent staining using the monoclonal antibody Pab240 showed only marginal staining. In contrast, both the staining intensity as well as the number of positively stained cells increased in the irradiated populations with the highest staining level found in the tumorigenic 60 cGy + E/60 cGy + E cells. This discrepancy is likely due to the fact that Pab240 recognizes conformational changes associated with mutations in codons 212–217 of the *p53* gene, which is not found in MCF-10F cells.

Alterations in the *p53* gene may contribute to the immortalization process of these cells and thus the acquisition of the progressive malignant phenotypes observed among the irradiated cell lines. It is also possible that *p53* mutations may lead to genetic instability, which facilitates the emergence of the other tumor-associated genes leading to malignancy. The progression observed in these studies can be

compared with p53 protein expression reported in breast cancer progress from *in situ* to advanced metastatic lesions. During these events p53 protein expression has been reported to increase from 13% to 50% (Callahan 1992) as well as in mouse skin tumor progression, which increase from 25% to 50% (Ruggeri *et al.* 1991). Alterations in *p53* expression in these irradiated cell lines may complement the high frequency of c-Ha-ras and the c-myc protein overexpression also observed. These results may indicate the cooperation of more than one oncogene in the cancer progression (Land *et al.* 1983).

The authors' studies show for the first time the influence of estrogen in breast carcinogenesis induced by high-LET radiation. With an average nuclear cross-sectional area of $\sim 120\ \mu m^2$ for MCF-10F cells, a 60 cGy dose of 150 keV/μm α-particles corresponds to a mean number of three particle traversals per nucleus. Since none of the cells irradiated with either a single or two doses of α-particles undergoes neoplastic transformation without estrogen co-treatment, the results underline the importance of estrogens in breast cancer. Breast epithelial response to estradiol plays an important role in breast cancer etiology (Dickson and Lippman 1998). Cell division, *per se*, increases the risk of genetic errors and, as a result, prolonged stimulation by steroid hormones may increase the risk of breast cancer. The estradiol and estrone are the major estrogens circulating in the blood, but estradiol is by far the most biologically active in breast tissue (Pasqualini *et al.* 1992). These findings are consistent with earlier reports that demonstrated estrogen's influence on the cell proliferation of epithelial cells derived from benign lesions as well as carcinoma *in situ* obtained from premenopausal women (Calaf *et al.* 1986). It is also known that breast cancer may have both its genesis and cell proliferation influenced by the woman's hormonal and reproductive status at the time of radiation exposure. Higher risks have been reported in nulliparous women and those exposed to radiation before 20 years of age (Kelsey and Bernstein 1996). There is evidence that the hormonal pattern of breast in young premenopausal women seems to render it particularly vulnerable to carcinogenesis (Pike *et al.* 1993). Proliferation in its early stages probably depends upon systemic hormones (estrogens and progesterone) and local growth factors. Critical damage, repair and survival events may ensue in sporadic disease. It is likely that the induction of transformation by estrogens is based, in part, on regulation of other genes such as growth factors and cell-cycle associated genes. Early defects in proliferation that increase cancer risk, as it occurs in hyperplasias, slightly perturbs the pathways normally regulated by systemic hormones, such as estrogens and progesterone, as well as local factors such as TGFα (see Dickson 1999, review). Cancer progression builds up on the accumulation of genetic alterations in transformed cells by amplification of oncogenes. Proteins are involved in DNA replication as components of complex biochemical pathways and amplifications. Mutations of these genes encoding proteins are involved in the cellular processes that could contribute to the process of carcinogenesis.

The present results indicate that transformed MCF-10F cells show aberrant expression of various protooncogenes, oncogenes and tumor supressor genes. An increase in c-myc, c-jun, c-Ha-ras and mutant p53 protein expression in comparison to the control MCF-10F cells was clearly demonstrated in the tumorigenic 60 cGy + E/60 cGy + E cell line. More recent studies have examined interactive effects of co-expression of TGFα/c-myc; TGFα/c-Ha-ras (Dickson 1999). These studies have supported the importance of interactions that promote aberrant cell cycles leading to genomic instability and malignant progression. The p53 protein has been described as a commonly mutated, multifunctional 'guardian of the genome' whose dysfunction may allow accumulation of multiple types of genomic damage.

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