Growth factor biomarkers associated with estrogen- and radiation-induced breast cancer progression

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Abstract. Breast cancer is the most common cancer in women worldwide. Transformation of a normal cell to a malignant one results from mutations in genes that encode key regulatory proteins. Growth factors are proteins secreted by a variety of transformed cells and tumors and function as autocrine regulators of growth. Biomarkers associated with cancer were examined in human breast epithelial cells transformed by high-LET radiation in the presence of 17ß-estradiol. An established cancer model was used in these studies. The MCF-10F cells that were irradiated with double doses of α-particles in the presence of estrogen (60 cGy + E/60 cGy + E, named Alpha 5) showed gradual phenotypic changes relative to control, including tumorigenicity in heterologous animals. Protein expression was determined by quantification of immunofluorescence staining coupled with confocal microscopy. The transforming growth factor α, epidermal growth factor, ERK1 and fibroblast growth factor-1 (Int2) protein expression was analyzed. Increased protein expression was observed in non-tumorigenic and tumorigenic α-irradiated and estrogen-treated cells. However, Stat-1α and pS2 protein expression was only increased in the tumorigenic Alpha 5 and Tumor 2 cell lines. It can be concluded that high-LET radiation in the presence of estrogen-induced changes in the proteins associated with growth factors and their over-expression may be a critical step in the cascade of events that characterize progression in breast cancer.

Introduction

Any component of a pathway that links a growth factor to a critical biochemical step and regulates cell division has oncogenic potential. Even cell division alone, therefore, increases the risk of genetic error (1). Since prolonged stimulation by steroid hormones may increase cell division, it may also increase the risk of breast cancer (2,4). Estrogens have been suggested to play a role in regulating the proliferation of breast cancer cells (3,5).

Normal cells proliferate in response to an array of external, mostly locally produced, growth factors in a paracrine fashion. These factors include transforming growth factor α (TGFα), epidermal growth factor (EGF), ERK1, fibroblast growth factor-1 (Int2), signal transducer and activator transcription (STAT), Stat-1α and pS2. Activation and/or enhanced expression of growth factor regulators and various cellular oncogenes have been implicated in the progression of breast cancer (2,5). These factors are supposed to be involved in the evolution of breast cancer by exerting their proliferative action after binding to appropriate cell surface receptors and inducing a cascade of responses, most of which involve phosphorylation. Cancer cells are independent of these external growth factor signals (5).

The proliferation of many squamous and mammary carcinoma cell lines *in vitro* is in part regulated by the synthesis and autocrine action of TGFα that binds to and mediates tyrosine phosphorylation of the EGF receptor (5,6). It also promotes anchorage-independent growth of cells in soft agar in the presence of transforming growth factor ß (6-8). TGFα is secreted by a variety of transformed cells and tumors (9). It functions as an autocrine regulator of growth in various tissues, including breast, and exhibits biphasic growth kinetics (10-12).

Another important growth factor, EGF, is an acid- and heat-stable 53-amino-acid protein of 6.0 kDa that shares 33% homology with TGFα (11). EGF has been shown to be a potent mitogen for a variety of cell types, both *in vivo* and *in vitro* (13-15). It binds to the 170,000 Da EGF receptor on the cell surface and mediates phosphorylation of the receptor. The EGF-like growth factor also binds to the EGF receptor (15).

The activation of signal transduction pathways by growth factors and hormones is mediated through two closely related mitogen-activated protein (MAP) kinases; p44 and p42, designated extracellular signal related kinase 1 (ERK1), and extracellular signal related kinase 2 (ERK2), respectively (16-19). ERK1 and ERK2 are proline-directed kinases that are activated through concomitant phosphorylation of tyrosine

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and threonine residues. Their activation by mitogens leads to the induction of cyclin D1 and the initiation of cell-cycle progression during breast cancer progression (19). Furthermore, activation of the Raf/MEK/ERK pathway was also found to be essential for cell survival and proliferation (19).

Fibroblast growth factors (FGFs) share 30-55% of their sequence identity and a similar gene structure and are capable of inducing transformation via an autocrine mechanism when introduced into cells expressing the appropriate FGF receptor (20). Acidic FGF (also designated as FGF or FGF-1) and basic FGF (FGF or FGF-2) are prototypes of this expanding family of regulatory molecules of epithelial origin (21), also well known for their ability to stimulate diverse processes like angiogenesis, control of cell growth and differentiation, and others (21). Fibroblast growth factor-1 (Int2) is amplified in up to 22% of human breast carcinoma (20). Members of the FGF family are capable of transforming cultured cell lines when overexpressed in transfected cell lines (22).

Stat-1α is a membrane receptor that binds various ligands, including growth hormones such as EGF. Stat-1α also induces activation of Jak kinases, which leads to tyrosine phosphorylation of the various STAT transcription factors (23,24). The STAT mediates some of the effects on gene expression initiated by many cytokines and growth factors (24). Growth factors can also activate STAT-signaling pathways. Stat-1α is part of a transcription factor complex (24,25). The involvement of Stat-1α in EGF-stimulated tumorigenesis in various cell types also suggests they play a specific role in mammary gland functioning but genetic deletion of Stat-1α does not alter mammary function (26-28).

The pS2 is a putative growth factor, first isolated from MCF7, the estrogen-dependent breast cancer cell line that acts as an estrogen-induced protease inhibitor in carcinoma cells (29-31). Absent in normal breast tissue, pS2 is highly expressed in a number of carcinomas, including cancers of the breast, pancreas and stomach (29,30). As pS2 expression is induced by estrogen, it may act as an estrogen-regulated autocrine mitogen in breast tumor cells (31). This raises the possibility that the beneficial effects of anti-estrogen therapy may be due in part to a reduction in the expression of pS2 (30).

At present, little information is available on growth factor proteins associated with cell transformation and the progression of breast cancer. The radiation-induced breast cancer model, an *in vitro* breast cell transformation system that utilizes epithelial cells at different stages of the neoplastic process, provides a unique opportunity for studying growth factor markers related to radiation carcinogenesis.

In the previous work of this lab, the spontaneously immortalized MCF-10F breast epithelial cell line (32) was malignantly transformed by double doses of α-particles in the presence of estrogen (33,34). Several studies reported a variety of genetic alterations in the course of mammary tumor progression and have shown that such changes result in the accumulation of multiple abnormalities in individual cells (35-38).

An established cancer model was used in these studies. The immortalized human breast MCF-10F cell line was irradiated with a 60 cGy or 60 cGy/60 cGy dose of α-particles either in the presence or absence of estrogen. Such transformed cells showed gradual phenotypic changes relative to control, including altered morphology, increased cell proliferation, anchorage-independent growth and invasive capabilities. These transformed cell lines were non-tumorigenic after injection into a heterologous animal, whereas MCF-10F cells irradiated with double doses of α-particles in the presence of estrogens (60 cGy + E/60 cGy + E) showed tumorigenicity in both SCID and nude mice.

The identification of growth factor markers associated with breast cancer is of critical importance in understanding the progression of this disease. The aim of this work, therefore, was to determine growth factor markers associated with the radiation-induced transformation of human breast epithelial cells.

Materials and methods

Cell lines. The spontaneously immortalized human breast epithelial cell line, MCF-10F, was used in this study (32). The MCF-10F cell line has a near diploid karyotype and has a luminal epithelial origin. This cell line retains all of the characteristics of normal epithelium *in vitro*, including anchorage dependence, non-invasiveness and non-tumorigenicity in SCID mice (33). The model of the MCF-10F cell line recently established to study breast carcinogenesis was used in this study (33). The MCF-10F cell line was irradiated with graded doses of 150 keV/μm α-particle, accelerated with a 4 MeV van de Graaf accelerator at the Columbia University Radiological Research Facilities as described previously (33). In this study, the MCF-10F cell line and the MCF-10F cell line treated with 17ß-estradiol (E) (Sigma Chemical Co., St. Louis, MO), named MCF-10F + E, were used as controls. The MCF-10F cell line was irradiated with either a single 60 cGy dose of α-particles (60 cGy), named Alpha 1, or a double dose of radiation (60 cGy/60 cGy), named Alpha 3, and then treated with E (60 cGy + E), named Alpha 2 or (60 cGy/60 cGy + E) Alpha 4 respectively. Other cultures were treated with a double dose of 60 cGy α particles with estrogen after each dose (60 cGy + E/60 cGy + E), named Alpha 5. This cell line gave rise to tumors after injection into the nude mice, named Tumor 2. Control and treated MCF-10F cells were tested for cloning efficiency, as well as for invasiveness by using Transwell chambers, as described previously (33). Cells irradiated with single or double doses of α-particles, in the presence of E, formed agar-positive clones after 25 passages. However, invasiveness assays showed that, after such passages, the number of cells that crossed the chamber membrane was significantly greater in the experimental cell lines than in the control cells (33). The tumorigenic cell lines, Alpha 5 and Tumor 2, were positive for anchorage independency, invasiveness and tumorigenicity in nude mice (33).

Protein expression determination. 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 $1\text{x}10^4$ cells in 1 ml of medium and allowed to grow for 2-3 days until they reached 70% confluency. The cells were treated as previously reported (33,34). Primary antibodies (1:500 dilution) were used for TGFα (C18; sc1338;

Table I. Phenotypic alterations and biomarkers of progression of α-particle irradiated and 17ß-estradiol (E)-transformed human breast epithelial cell.

Cell lines	MCF-10F	MCF10F + E	Alpha 1	Alpha 2	Alpha 3	Alpha 4	Alpha 5	Tumor 2
AIA	N	N	N	N	P	P	P	P
IA	N	N	N	P	P	P	P	P
TA	N	N	N	N	N	N	P	P
			↑c-myc	↑c-myc	↑c-myc	↑c-myc	↑c-myc	↑c-myc
			↑c-jun	↑c-jun	↑c-jun	↑c-jun	↑c-jun	↑c-jun
			↑c-fos	↑c-fos	↑c-fos	↑c-fos	↑c-fos	↑c-fos
			↑m p53	↑m p53	↑m p53	↑m p53	↑m p53	↑m p53
			↑TGFα	↑TGFα	↑TGFα	↑TGFα	↑TGFα	↑TGFα
			↑EGF	↑EGF	↑EGF	↑EGF	↑EGF	↑EGF
			↑ERK1	↑ERK1	↑ERK1	↑ERK1	↑ERK1	↑ERK1
			↑Int2	↑Int2	↑Int2	↑Int2	↑Int2	↑Int2
							↑BRCA1	↓BRCA1
							↑Rad51	↑Rad51
							↑c-Ha-ras	↑c-Ha-ras
							↑Stat-1α	↑Stat-1α
							↑pS2	↑pS2

AIA: anchorage-independent assay, colony-forming efficiency in agar fluctuated from 1-3%. N, no colony formation in agar-methocel; P, colony formation in agar-methocel. IA: invasion assay, invasive characteristics of control and treated cell lines. TA: tumorigenic assay, tumors formed in the nude and SCID mice. Average of 6 animals/group. Five out of 6 animals presented mammary tumors.

goat polyclonal), EGF (Z12; sc275; rabbit polyclonal), ERK1 (K23; sc94; rabbit polyclonal), Int2 (H125; sc7910; rabbit polyclonal), pS2 (C20; sc7842; goat polyclonal), and Stat-1α (P91/C111; sc417; mouse monoclonal) (all from Santa Cruz Biotechnology Inc., Santa Cruz, CA). After washing in buffer solution, cultures were incubated for 60 min at room temperature with anti-mouse, rabbit or goat Rhodamine-conjugated secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) at a 1:1,000 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. Cells were quantified and viewed on Zeiss Axiovert 100 TV microscope (Carl Zeiss, Thornwood, NY) using a x40 11.3 NA objective lens equipped with a laser scanning confocal attachment (LSM 410 Carl Zeiss, Thornwood, NY) as previously described (33,34). An argon/krypton mixed gas laser, set by exciting the Rhodamine at 488 nm, collected fluorescent images. The number of immunoreactive cells was counted in several randomly selected microscopy fields per sample. A semi-quantitative estimation based on the relative staining intensity of protein expression by the different cell lines was determined. A computer program was used to obtain the area and the intensity of staining of the cells present in the culture dishes. The experiments were repeated with cells from three similar passages. Standard errors of mean are shown in the different figures. Statistical analysis was performed using the F-test (randomized block) and comparisons between groups using the Bonferroni t-test with significance at p-value <0.05.

Results

We have demonstrated that MCF-10F cell lines showed a very complex pattern of protein expression when exposed to double doses of α-particles and then treated with estrogen as compared to a single dose of radiation and control (24). During the transformation process several changes were induced, such as anchorage independency and invasive capabilities by single or double doses of radiation, either in the presence or absence of estrogens. These changes paralleled the appearance of new biomarkers. The cell line was exposed to 60 cGy-α particles, treated with estrogen, subsequently treated with a second dose of radiation and again exposed to estrogen-induced tumors in the nude mice. Table I summarizes the phenotypic characteristics and the biomarkers of progression of the established model with the MCF-10F cell line used in these studies.

Fig. 1a and b represents the quantification of the immunofluorescent imaging of TGFα and EGF protein expression in irradiated and estrogen-treated MCF-10F cells. Fig. 1a represents the quantification of TGFα protein expression of cells irradiated with single and double doses of α-particles. The cell lines irradiated with single and double doses of 60 cGy α-particles with or without E treatment exhibited a significantly greater protein expression ($p<0.05$) than the control MCF-10F cell. Furthermore, protein expression in the tumorigenic Alpha 5 and Tumor 2 cell lines was not significantly different from all the other treated cells. Quantification of EGF protein expression in the various MCF-10F cell lines irradiated with single and double doses of α-particles and treated with E is shown in Fig. 1b. The cells irradiated with a single dose of

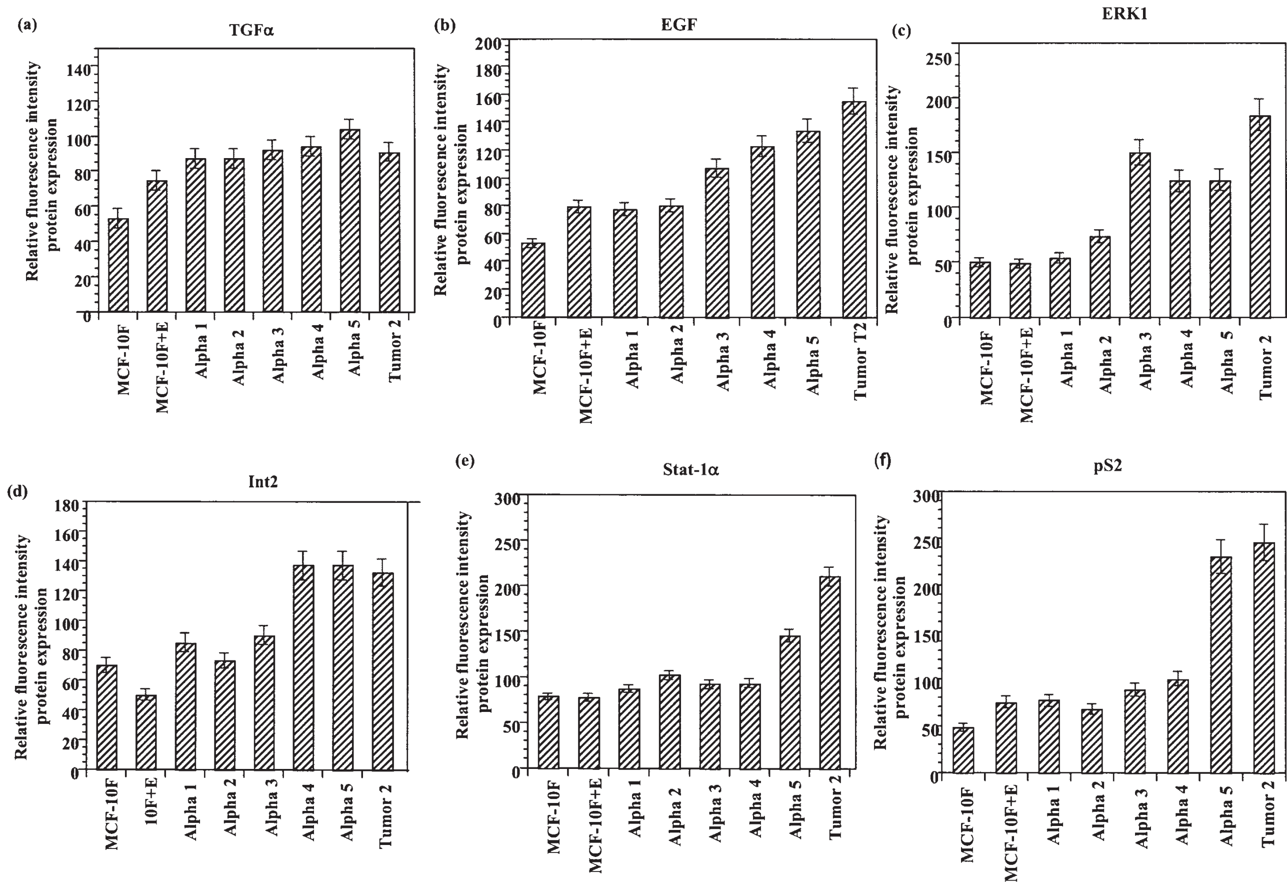

Figure 1. Quantification of immunofluorescent imaging of TGFα (a), EGF (b), ERK1 (c) and Int2 (d) Stat-1α (e) and pS2 (f) protein expression in irradiated MCF-10F cells with or without pre- and post-treatment with E. The bars represent the average relative amounts of protein expression of MCF-10F, MCF-10F + E, Alpha 1, Alpha 2, Alpha 3, Alpha 4 Alpha 5 and Tumor 2. All primary antibodies used were from Santa Cruz Biotechnology Inc., Santa Cruz, CA.

60 cGy α-particles, with or without E treatment, showed a slightly higher EGF protein expression than the control. The non-tumorigenic Alpha 3 and Alpha 4 cell lines showed an expression level greater ($p<0.05$) than the control MCF-10F cell lines and the Alpha 1 cell line. However, the protein expression in Alpha 5 and Tumor 2 cell lines was significantly ($p<0.05$) greater than in the MCF-10F cell line and the non-tumorigenic Alpha 1 cell line.

Cells irradiated with a single dose of 60 cGy α-particles, with or without E treatment, did not exhibit ERK1 protein expression significantly different from the control (Fig. 1c). The non-tumorigenic Alpha 3 and Alpha 4 cell lines and the tumorigenic Alpha 5 and Tumor 2 cell lines all showed an ERK1 expression level 3-3.5 times greater ($p<0.05$) than the control MCF-10F cell and Alpha 1. Int2 protein expression of MCF-10F cells irradiated and estrogen-treated is shown in Fig. 1d. The cells irradiated with a single dose of 60 cGy α-particles with or without E treatment showed a slightly higher Int2 protein expression than the control. The non-tumorigenic Alpha 3 and Alpha 4 cell lines showed an expression level greater than ($p<0.05$) the control MCF-10F cell line and Alpha 1 cells. Protein expression of Alpha 5 and Tumor 2 cell lines was significantly ($p<0.05$) greater than the MCF-10F cell line.

The quantification of the tumorigenic cell lines, Alpha 5 and Tumor 2, showed a 5-fold increase in Stat-1α protein expression over the control MCF-10F cell line. However, there was no significant difference in expression level between the cell lines treated with single or double doses of radiation. Fig. 1e represents the quantification of Stat-1α protein expression in the irradiated MCF-10F cell line. Fig. 1f is the quantification of pS2 protein expression of the control MCF-10F cell line and cells irradiated with single or double doses of α-particles. The Alpha 5 and Tumor 2 tumorigenic cell lines showed 5 times higher pS2 protein expression levels than the control MCF-10F cells ($p<0.05$). However, there was no significant difference in protein expression between the cell lines treated with single or double doses of radiation. Fig. 2 represents the immunofluorescence image of TGFα (a), EGF (b), ERK1 (c), Int2 (d), Stat-1α (e) and pS2 (f) protein expression in irradiated MCF-10F, Alpha 3 and Tumor 2 cells.

Discussion

In this study, an established experimental breast cancer model was used in which normal human breast epithelial cells underwent a stepwise transformation into malignant cells by exposure to low doses of high-LET α-particles in the presence of estrogen (33). Progress in molecular biology has revealed that proliferation of breast cancer cells is controlled by various growth factors. The malignant progression of breast cancer involves a transition from normal controlled cell proliferation to highly abnormal deregulation of this process. In this model it has been previously reported that progression

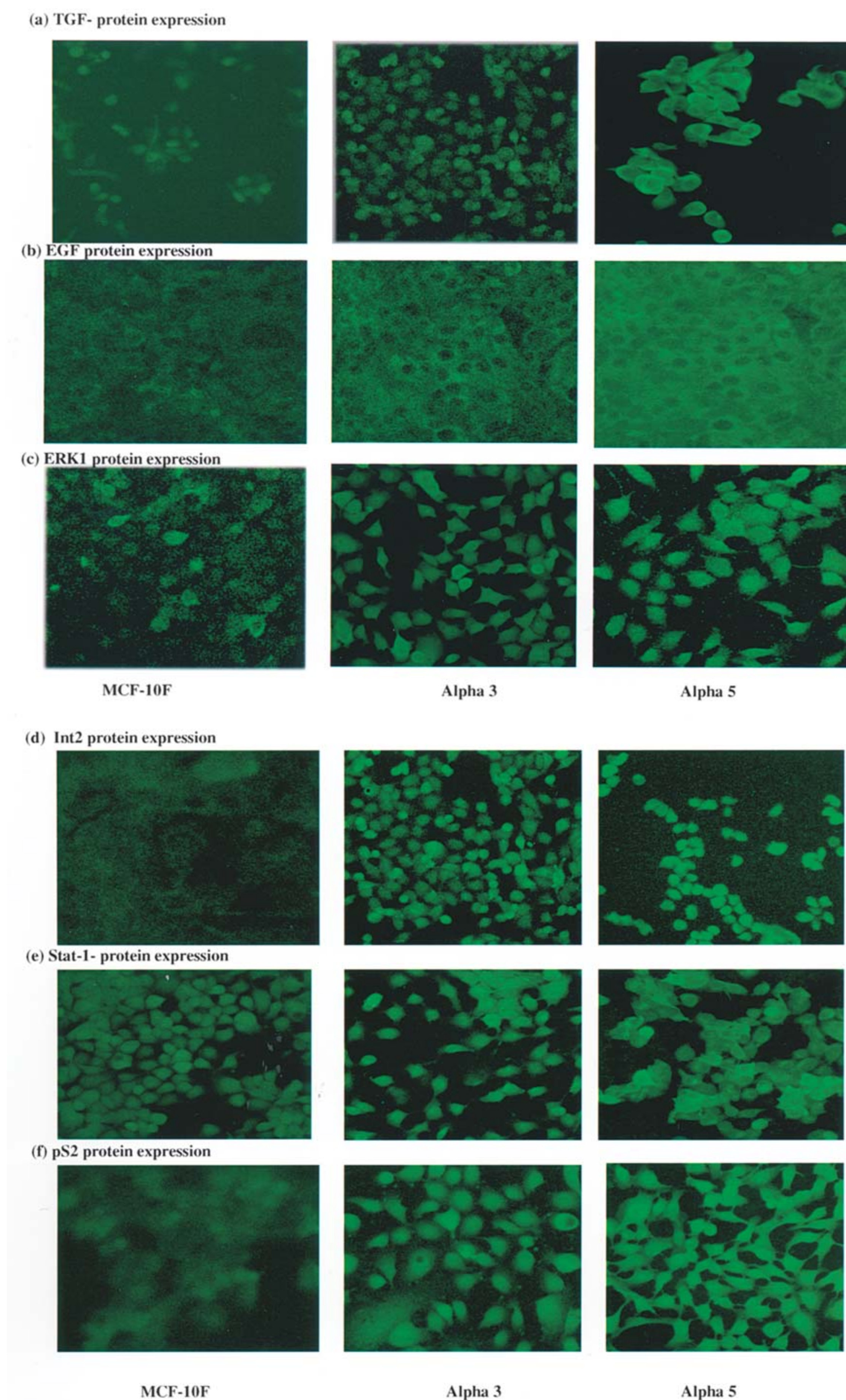

Figure 2. Representative immunofluorescent imaging of TGFα (a), EGF (b), ERK1 (c), Int2 (d), Stat-1α (e) and pS2 (f) protein expression in MCF-10F, Alpha 3 and Tumor 2 cell lines.

correlates with an increase in genetic/genomic instability (35-38). We have previously reported that overexpression of several oncoproteins (33,34) oncogenes and tumor suppressor genes (36-38) are important factors in the transformation of human breast epithelial cells. The present report shows that growth factors that have been linked to human breast cancer were also altered in a progressive manner among transformed human breast epithelial cell lines.

Different growth factors are thought to be involved in the evolution of breast cancer. When the MCF-10F cell lines were exposed to double doses of α-particle radiation and treated with estrogen they had greater protein expression of a variety of growth factor regulators in comparison with cell lines treated with a single dose of radiation without estrogen.

The role of estrogen in affecting breast cancer risk remains largely unknown. The association between breast cancer development and prolonged exposure to estrogen suggests that this hormone may have an etiologic role in the causation of this disease (2-4). It is likely that the estrogen-induced transformation is based, in part, on the regulation of other genes as well as a variety of growth factors and cell-cycle associated genes. Early defects in proliferation that increase cancer risk, as it occurs in hyperplasia, slightly perturb the pathways usually regulated by systemic hormones, such as estrogen and progesterone, as well as local factors, such as TGFß (3,4).

The TGFα and EGF protein expression was increased in 60 cGy + E/60 cGy + E and Tumor 2 cell lines in comparison to the MCF-10F control cell line. Since TGFα is secreted by a variety of transformed cells as well as tumors (8) it is reasonable to conclude that it may act as an autocrine growth factor for the induction and maintenance of malignancy. The Alpha 5 and Tumor 2 cell lines express *in vitro* growth advantage, anchorage independence and enhanced invasiveness. It has been reported that TGFα also promotes the anchorage-independent growth of cells in soft agar in the presence of transforming growth factor ß (6-9).

Since TGFα binds to EGF receptors and mediates tyrosine phosphorylation of the receptor (6) it is possible that both growth factors regulate signaling pathways leading to tumorigenesis. EGF contains both positive and negative determinants for cell-cycle regulation by interacting with the ErbB2 and ErbB3 heterodimers' (39) growth factor receptors, whose genes are known to be amplified in breast cancer patients.

ERK1 protein expression was higher in Alpha 4, Alpha 5 and Tumor 2 in comparison to the MCF-10F cell line. Activation of mitogen-activated protein kinase (ERK/MAPK) is a critical signal transduction event for estrogen-mediated cell proliferation. Recent studies (40) have shown that persistent activation of ERK plays a major role in cell migration and tumor progression. They showed that estrogen induced activation of MCF-7 breast carcinoma cells, suggesting that estrogen-induced factors play a major role in ERK activation. Our results support these findings and suggest that enhanced MAPK activity could be one important step involved in the transformation and progression of breast cancer.

Results indicated that Int2 protein expression was increased both in the non-tumorigenic and tumorigenic cell lines irradiated with double doses of 60 cGy α-particles in the presence of estrogens but not in any of the singly irradiated cell lines. Int2, located in 11q13, was found more frequently amplified in local recurrences than in primary tumors as well as in tumors of breast cancer patients with lymph node metastases (41,42). Pauley *et al* (41) found that those patients with Int2 and ErbB2 amplification were at a higher risk for tumor recurrence. Further support for these findings is the loss of heterozygosity (LOH) found on the chromosomal arms of 11q13 (42). Furthermore, patients with both LOH at 17q as well as Int2 amplification had a poor prognosis. Around 67% of LOH of chromosome 11q13 in both *in situ* and invasive lesions of the breast have been found in comparison to normal breast epithelium from the same patients (42).

Growth factors can also activate several STAT signaling pathways. Thus, membrane receptor signaling by various ligands, including growth hormones such as EGF, can activate Jak kinases (23). Gao *et al* (43) have shown that BRCA1 interacts with Jak1 and Jak2 signaling cascades and modulates Jak/Jak interaction in prostate cancer cells. The present results also corroborated findings that estrogen regulates pS2 production in breast cancer tumors (30,44) and cell lines (29,31). It has been reported that estradiol increased the pS2 expression in the MCF-10A cell lines, which have the same origin as the MCF-10F cell lines (45). In gastric carcinomas, the strong expression of pS2 is associated with poorly differentiated adenocarcinomas, while well differentiated tumors express it weakly or do not express it at all (30).

In summary, altered oncogenes as well as growth factor protein expression were observed in the radiation-estrogen *in vitro* model used in this study. An increase in c-myc, c-jun, c-fos, mutant p53 (33,34), EGF, TGFα, Int2 and ERK1 were found early in the transformation process. The increased expression levels of these proteins persisted in transformed MCF-10F cells that acquired the tumorigenic phenotype. Others, however, appear to occur late in the neoplastic progression of irradiated MCF-10F cell lines, such as BRCA1, Rad51 and c-Ha-ras (33,34), Stat-1α and pS2. This *in vitro* neoplastic transformation model, developed with human breast epithelial cells treated with α-particles and estrogens, will be helpful to elucidate mechanisms of cancer initiation and progression. This model can be used to correlate the genetic alterations frequently detected in breast cancer with biologically important stages of tumor development.

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