Apoptotic ratios and mitotic abnormalities in 17-β-estradiol-transformed human breast epithelial MCF-10F cells

*Cruz, LMS., Ferreira, JCV. and Mello, MLS.**

Departamento de Anatomia, Biologia Celular e Fisiologia e Biofísica, Instituto de Biologia,
Universidade Estadual de Campinas – Unicamp,
Rua Charles Darwin, s/n, Cidade Universitária Zeferino Vaz, CEP 13083-863, Campinas, SP, Brazil

*e-mail: mlsmello@unicamp.br

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(With 1 figure)

Abstract

Treatment of human breast epithelial cells MCF-10F with 17-β-estradiol has been reported to result in E2-transformed cells which have given rise to highly invasive C5 cells that in turn generate tumors in SCID mice. From these tumors, various cell lines, among which C5-A6-T6 and C5-A8-T8, were obtained. Although different phases of the tumorigenesis process in this model have been studied in molecular biology and image analysis assays, no cytological data on apoptotic ratios and mitotic abnormalities have been established to accompany the various steps leading to 17-β-estradiol–treated MCF-10F cells to tumorigenesis. Here we detected that the apoptotic ratio decreases with the transformation and tumorigenesis progress, except for the tumor cell line C5-A8-T8, probably on account of its more intense proliferation rate and a more rapid culture medium consumption. Increased frequency of mitotic abnormalities contributed by triple- and tetrapolar metaphases, and by lagging chromosomes and chromosome bridges observed at the anaphase found by transformation and tumorigenesis progress. However, no difference was found under these terms when the C5-A6-T6 and C5-A8-T8 tumor cell lines were compared to each other. Present findings are in agreement with the nuclear instability and enrichment of dysregulated genes in the apoptotic process promoted by transformation and tumorigenesis in 17-β-estradiol-treated MCF-10F cells.

Keywords: MCF-10F cells, estradiol, transformation, apoptotic ratios, mitotic abnormalities.

Índices apoptóticos e anormalidades mitóticas em células epiteliais mamárias humanas MCF-10F transformadas pelo 17-β-estradiol

Resumo

O tratamento das células epiteliais mamárias humanas MCF-10F com 17-β-estradiol tem sido relatado como resultando nas células transformadas E2, que deram origem às células C5, altamente invasivas, e que geraram tumores em camundongos SCID. A partir desses tumores foram originadas em cultura células tumorais, dentre as quais C5-A6-T6 e C5-A8-T8. Embora diversas fases do processo tumorigênico neste modelo tenham sido estudadas por ensaios de biologia molecular e análise de imagem, não foram ainda estimados dados citológicos referentes a índices apoptóticos e anomalias mitóticas que acompanhassem os vários passos que levam as células CF-10F tratadas com 17-β-estradiol à tumorigênese. Neste trabalho detectamos que o índice apoptótico decresce com a transformação e o avanço da tumorigênese, exceto na linhagem celular tumoral C5-A8-T8, provavelmente por causa de sua velocidade de proliferação mais intensa, que poderia levá-la a um consumo mais rápido do meio de cultura presente e à morte celular. Um aumento na frequência de anomalias mitóticas contribuídas por metáfases tripolares e tetrapolares e por pontes cromossômicas e cromossomos desgarrados, identificáveis na anáfase, foi observado com a transformação e o progresso da tumorigênese. Contudo não foram detectadas diferenças nesses parâmetros quando se compararam as linhagens tumorais C5-A6-T6 e C5-A8-T8 entre si. Os presentes achados estão de acordo com a instabilidade nuclear e o enriquecimento em desregulação de genes que atuam no processo apoptótico, promovidos pela transformação e tumorigênese nas células MCF-10F tratadas com 17-β-estradiol.

Palavras-chave: células MCF-10F, estradiol, transformação, índices apoptóticos, anomalias mitóticas.

1. Introduction

MCF-10 F, estrogen receptor α-negative immortalized human breast epithelial cells acquire cell transformation properties similarly to those elicited by in vitro treatment with the carcinogen benzo[a]pyrene, when subject to treatment using17-β-estradiol (E2) (Russo et al., 2002a,b; Mello et al., 2007a). This estrogen-elicited event is not abrogated by the estrogen antagonist, ICI 182,780, confirming its independence from the presence of estrogen receptor α (Lareef et al., 2005; Mello et al., 2007a).

E2-transformed MCF-10F cells (E-2), selected for high invasive potential in Matrigel chambers, have given rise to C5 cells. These cells, if containing a 4p15.3-16 deletion, are tumorigenic in SCID mice (Russo et al., 2006). Adenocarcinomas generated in SCID mice by injecting C5 cells have made various cell lines, among which C5-A6-T6 and C5-A8-T8 cells, which differ in terms of proliferation aggressiveness (Russo et al., 2006).

The neoplastic progression in E-2-transformed MCF-10F cells is characterized by anchorage-independent growth, colony formation in agar-methocel, an increase in cell proliferation and in invasive capability, loss of heterozygosity in chromosomes 13 and 17, loss of a putative tumor-suppressor gene-containing 9p11-13 locus, and of chromosome 4, deletions in chromosomes 3, 8, 9 (including p15 and p16 genes), and 18 (including one or more tumor-suppressor genes), and gains in chromosomes 1 and 5 (Russo et al., 2002a,b, 2006; Huang et al., 2007). These events are accompanied by changes in DNA content and nuclear sizes and by supraorganization chromatin remodeling in interphase cells (Mello et al., 2007b).

Although several geometric, densitometric and textural characteristics of the interphase cell chromatin image have been evaluated in MCF-10F cells with the E2-induced neoplastic progression (Mello et al., 2007a,b, 2009), no cytological data on apoptotic ratios and mitotic abnormalities were reported for these cells under sequential steps of the transformation/tumorigenesis process. Apoptosis rates are expected to diminish while mitosis abnormalities contributing to nuclear disturbances are expected to become more representative with the E2-induced transformation/tumorigenesis progress (Huang et al., 2007). Thus, in the present study, apoptotic ratios, mitotic indices, and the frequency of abnormal mitoses were cytologically compared in 17-β-estradiol–transformed E-2, C5, C5-A6-T6, and C5-A8-T8 cells, and non-transformed MCF-10F control cells.

2. Material and Methods

2.1. Cells

Spontaneously immortalized human breast epithelial MCF-10F cells, E2-treated MCF-10F cells (E2), the tumorigenic C5 cell line selected from the E2-treated MCF-10F cells, and C5-A6-T6 and C5-A8-T8 tumor-derived cells were kindly supplied by Dr. Jose Russo (FCCC, Philadelphia, USA).

MCF-10F cells were cultivated in DMEM:F-12 medium containing 1.05 mM calcium, antibiotics, antimycotics, hormones, growth factors and equine serum as described elsewhere (Calaf and Russo, 1993). E2 cells consisted of MCF-10F cells (123rd passage) treated with 70 nM 17-β-estradiol as reported previously (Russo et al., 2006); E2 cells at passage 10 were used. C5 were cells expanded from the 10th passage of E2; these cells, when injected into SCID mice, induced the development of poorly differentiated adenocarcinomas (Russo et al., 2006) from which C5-A6-T6 and C5-A8-T8 tumoral cell lines derived. The tumor-derived cell lines were used in passage three.

2.2. Cell preparation and staining

The cells were cultivated for 96 hours (MCF-10F cells) and 48 hours (the other cell types) on Permanox® plastic chamber slides of 4.2 cm^2 and 1.2-2.0 mL working volume (bi-chamber) (Mello et al., 2005) and then fixed in absolute ethanol-acetic acid mixture (3:1, v/v) for 1 minute, rinsed in 70% ethanol for 3-5 minutes, and air dried at room temperature. The preparations were subjected to the Feulgen reaction (4 M HCl at 24 °C, 75 minutes) and counterstained with acid fast green (Mello et al., 2004).

2.3. Parameters

Apoptotic ratios and mitotic indices were determined in 2000 cells per preparation of each cell line. Three to four preparations (chambers) of each cell line were examined. Apoptosis was identified based on morphological criteria (Wyllie et al., 1980). As abnormal mitoses, triple-polar and tetra-polar metaphases, metaphases/anaphases showing lagging chromosomes, and chromosomal bridges were considered in total 100 metaphases and 100 anaphases, respectively per cell line. Counts were done in a Zeiss binocular microscope equipped with a 100/1.25 objective.

2.4. Statistics

Non-parametric Mann-Whitney and Kruskall-Wallis tests were used to assess the statistical significance of comparisons. The critical level to reject the null hypothesis was considered to be a P value of 5%. Calculations and statistical analyses were performed using the Minitab 12™ software (State College, PA, USA).

3. Results and Discussion

Cells with an apoptotic morphology (Figure 1a-c) were observed in non-transformed and all transformed MCF-10F cells analyzed here. The apoptotic ratio was found to decrease significantly by the transformation and tumorigenesis progress in the transformed cells, with the exception of the C5-A8-T8 cell line (Table 1). In this case, the apoptotic ratio was 100% higher than that of control MCF-10F cells. On the other hand, C5-A8-T8 cells showed the highest mitotic index values amongst those evaluated here (Table 1). Increase in mitotic index for the case of the C5-A8-T8 cells may have been associated with enhancement in apoptotic ratios, since a higher proliferative rate under in vitro conditions may have lead cells to

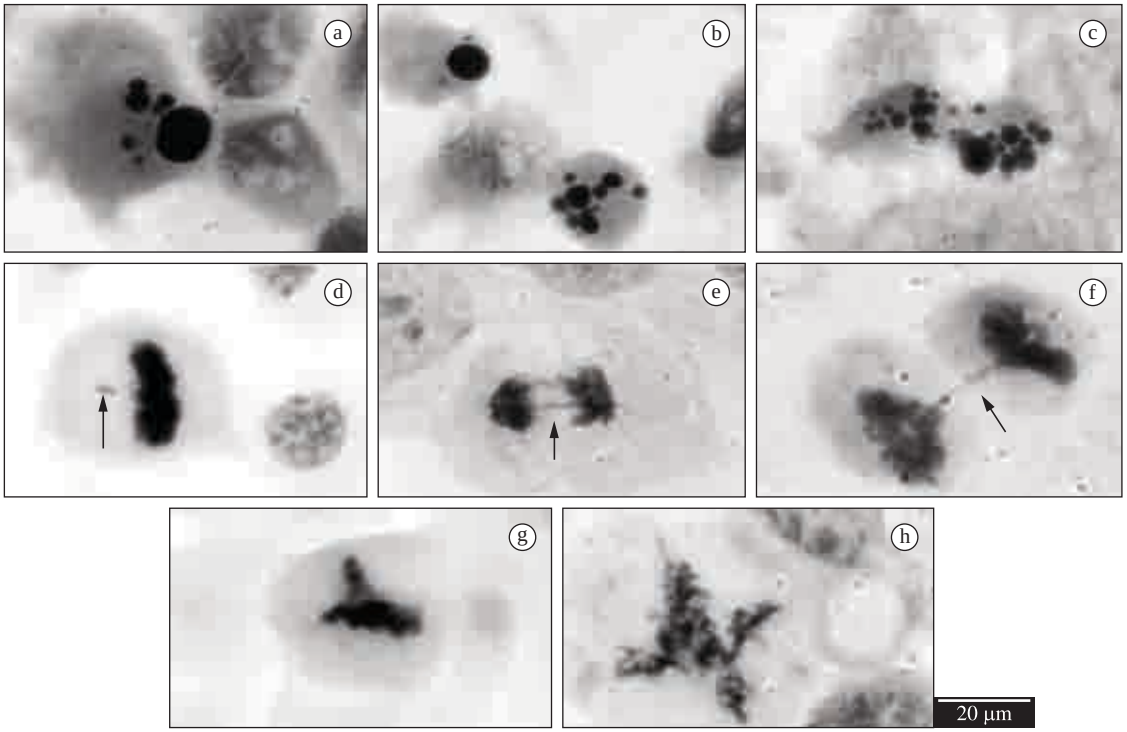

Figure 1. a-c) Examples of apoptotic cells; and d-h) mitotic abnormalities in transformed MCF-10F cells stained with the Feulgen reaction and counterstained with acid fast green. d) The arrow indicates a lagging chromosome; e, f) Chromosome bridges; g) Tripolar metaphase; and h) Tetrapolar metaphase. Bar, 20 µm.

Table 1. Apoptotic ratios (AR) and mitotic indices (MI) in 17-β-estradiol-transformed MCF-10F cells.

Cells	AR (%)			MI (%)		
	X	S	Statistical comparison	X	S	Statistical comparison
MCF-10F (control)	1.31	0.35	**a**	5.23	0.83	**a**
E-2	1.04	0.15	**b**	5.20	1.31	**a**
C5	0.70	0.23	**b**	5.13	0.35	**a**
C5-A6-T6	0.60	0.07	**b, c**	5.20	1.37	**a**
C5-A8-T8	2.61	0.37	**d**	5.98	1.04	**b**

a-c, different letters in the same column indicate differences that were significant at $P_{0.05}$ (Mann-Whitney); S, standard deviation; X, arithmetic means. Number of cells per chamber, 2000. Number of preparations, 4.

more rapid culture medium consumption and facilitated cell death. A similar phenomenon has been reported for MCF-10F cells transformed by benozo[a]pyrene and subsequently transfected with the c-Ha-*ras* oncogene (Barbisan et al., 1999). Although both C5-A6-T6 and C5-A8-T8 cells are tumor-derived cell lines, C5-A8-T8 has been reported as the most rapidly growing tumor amongst various tumors induced in SCID mice by the C5 cells (Russo et al., 2006). In addition, C5-A8-T8 cells show the smallest chromatin entropy probably associated with global DNA methylation in Feulgen-stained interphase nuclei in comparison with the other above-cited cell lines (Mello et al., 2007b, 2009).

As regards mitotic abnormalities (Figure 1d-h), a tendency of increase in their frequencies was demonstrated statistically in MCF-10F cells with advancing transformation and tumorigenesis (Table 2). No difference in frequencies of abnormal metaphases and anaphases was found when compared to C5-A6-T6 and C5-A8-T8 cells (Table 2).

Present findings on mitotic abnormalities are in agreement with increasing nuclear instabilities accompanying transformation and tumorigenesis induced in the MCF-10F cells by 17-β-estradiol (Russo et al., 2006;

Table 2. Metaphase and anaphase abnormalities in 17-β- estradiol-transformed MCF1-F cells.

Cells	Abnormal metaphases (%)			Abnormal anaphases (%)		
	X	S	Statistical comparison	X	S	Statistical comparison
MCF-10F (control)	34.94	9.19	**a**	11.53	3.98	**a**
E-2	61.11	7.83	**b**	28.22	15.35	**b**
C5	49.10	11.03	**a, b**	41.70	25.80	**b, c**
C5-A6-T6	70.38	8.91	**b, c**	69.47	4.30	**c**
C5-A8-T8	69.19	10.44	**b, c**	71.42	18.02	**c**

a-c, different letters in the same column indicate differences that were significant at $P_{0.05}$ (Kruskall-Wallis/Mann-Whitney); S, standard deviation; X, arithmetic means. Total number of metaphases or anaphases per cell line, 100. Number of preparations, 4.

Huang et al., 2007; Mello et al., 2007a). The diminished frequency of apoptotic ratios by cell transformation and tumorigenesis progress, with the exception of the case for C5-A8-T8 cells, is also an expected finding, considering the nature of the cell lines analyzed here and that Gene Ontology analysis has revealed enrichment of dysregulated genes in the apoptotic process in tumorigenic 17-β-estradiol-transformed MCF-10F cells (Huang et al., 2007).

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References

BARBISAN, LF., MELLO, MLS., RUSSO, J. and VIDAL, BC., 1999. Apoptosis and catastrophic cell death in benzo[a]pyrene-transformed human breast epithelial cells. *Mutation Research*, vol. 431, p. 133-139. doi:10.1016/S0027-5107(99)00193-1

CALAF, G. and RUSSO, J., 1993. Transformation of human breast epithelial cells by chemical carcinogens. *Carcinogenesis*, vol. 14, p. 483-492. PMid:8453725. doi:10.1093/carcin/14.3.483

HUANG, Y., FERNANDEZ, SV., GOODWIN, S., RUSSO, PA., RUSSO, IH., SUTTER, TR. and RUSSO, J., 2007. Epithelial to mesenchymal transition in human breast epithelial cells transformed by 17-β-estradiol. *Cancer Research*, vol. 67, p. 11147-11157. PMid:18056439. doi:10.1158/0008-5472.CAN-07-1371

LAREEF, MH., GARBER, J., RUSSO, PA., RUSSO, IH., HEULINGS, R. and RUSSO, J., 2005. The estrogen antagonist ICI-182-780 does not inhibit the transformation phenotypes induced by 17-beta-estradiol in human breast epithelial cells. *International Journal of Oncology*, vol. 26, p. 423-429.

MELLO, MLS., BARBISAN, LF., LAREEF, MH., RUSSO, J. and VIDAL, BC., 2004. Cell death evaluation in benzo[a]pyrene-transformed human breast epithelial cells after microcell-mediated transfer of chromosomes 11 and 17. *Mutation Research*, vol. 546, p. 39-43. doi:10.1016/j.mrfmmm.2003.10.005

MELLO, MLS., LAREEF, MH., SANTOS, AB., RUSSO, J. and VIDAL, BC., 2005. Nucleus image properties and cell death in MCF-10F cells grown on slide substrates differing in nature and size. *In Vitro Cellular and Developmental Biology-Animal*, vol. 41, p. 92-96.

MELLO, MLS., VIDAL, BC., RUSSO, IH., LAREEF, MH. and RUSSO, J., 2007a. DNA content and chromatin texture of human breast epithelial cells transformed with 17-β-estradiol and the estrogen antagonist ICI 182,780 as assessed by image analysis. *Mutation Research - Fundamental and Molecular Mechanisms of Mutagenesis*, vol. 617, p. 1-7.

MELLO, MLS., RUSSO, P., RUSSO, J. and VIDAL, BC., 2007b. 17-β-estradiol affects nuclear image properties in MCF-10F human breast epithelial cells with tumorigenesis. *Oncology Reports*, vol. 18, p. 1475-1481.

MELLO, MLS., RUSSO, P., RUSSO, J. and VIDAL, BC., 2009. Entropy of Feulgen-stained 17-β-estradiol-transformed human breast epithelial cells as assessed by restriction enzymes and image analysis. *Oncology Reports*, vol. 21, p. 1483-1487.

RUSSO, J., FERNANDEZ, FS., RUSSO, PA., FERNBAUGH, R., SHERIFF, FS., LAREEF, MH., GARBER, J. and RUSSO, IH., 2006. 17-beta-estradiol induces transformation and tumorigenesis in human breast epithelial cells. *FASEB Journal*, vol. 20, p. 1622-1634.

RUSSO, J., LAREEF, MH., TAHIN, Q., HU, YF., SLATER, C., AO, X. and RUSSO, I., 2002a. 17-beta-estradiol is carcinogenic in human breast epithelial cells. *Journal of Steroid Biochemistry and Molecular Biology*, vol. 80, p. 149-162.

RUSSO, J., TAHIN, Q., LAREEF, MH., HU, YF. and RUSSO, I., 2002b. Neoplastic transformation of human breast epithelial cells by estrogens and chemical carcinogens. *Environmental Molecular Mutagenesis*, vol. 39, p. 254-263. PMid:11921196. doi:10.1002/em.10052

WYLLIE, AH., KERR, JFR. and CARRIE, AR., 1980. Cell death: the significance of apoptosis. *International Review of Cytology*, vol. 68, p. 251-306. doi:10.1016/S0074-7696(08)62312-8