Increased Oxidative DNA Damage in Mammary Tumor Cells by Continuous Epidermal Growth Factor Stimulation

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Background: Growth factors can enhance the malignant potential of tumor cells. To examine the relationship between growth factors and tumor progression, we previously established a weakly malignant cell line, ER-1. We found that a 24-hour exposure of ER-1 cells to epidermal growth factor (EGF) induced malignant properties (tumor progression) that were reversible but that, after a 1-month exposure, these changes were irreversible. In this study, we investigated the irreversible changes induced in ER-1 cells by a 1-month exposure to EGF and the possible involvement of oxidative stress.

Methods: ER-1 cells were treated with EGF (100 ng/mL) for 1 month in the presence or absence of an antioxidant, N-acetylcysteine or selenium, and compared with untreated control ER-1 cells. We assessed tumor progression by measuring intracellular peroxide levels, 8-hydroxydeoxyguanosine (a marker for oxidative DNA damage) levels, in vitro invasiveness, and in vivo tumorigenicity and metastatic ability. All statistical tests are two-sided.

Results: After ER-1 cells were treated for 1 month with EGF, levels of intracellular peroxide and 8-hydroxyguanosine in the DNA of treated cells were higher than those in the DNA of control cells, and treated ER-1 cells were more tumorigenic and metastatic in vivo and more invasive in vitro than untreated control cells (all P<.001). Levels of 8-hydroxyguanosine in DNA increased as the length of the EGF treatment increased (P<.001). However, when N-acetylcysteine or selenium was added with EGF for 1 month, levels of intracellular peroxide and 8-hydroxyguanosine in DNA were comparable to those in control cells (r = .795). Both tumorigenicity (P = .008) and metastatic ability (P<.001) decreased after addition of N-acetylcysteine or selenium. Conclusion: The irreversible changes caused by continuous EGF stimulation of ER-1 cells result from increased oxidative damage in the DNA, which generates tumor cells with more malignant characteristics. [J Natl Cancer Inst 2001;93: 214–9]

Tumor progression is the process by which tumor cells acquire malignant properties, such as aggressive growth and the capability for invasion and metastasis (1). The microenvironment of the tumor is believed to affect tumor progression. Indeed, microenvironmental factors, including growth factors, cytokines, hormones, oxygen, nitrogen, and extracellular matrix components, induce genetic or epigenetic changes in tumor cells (2,3). Various growth factors transiently enhance malignant properties of tumor cells when cells are exposed to them for a short period in vitro. Although tumor cells are continually exposed to growth factors in vivo, little is known about the effect of persistent stimulation by growth factors on tumor cells.

We isolated (4) a weakly malignant cell line, ER-1, from the rat mammary carcinoma cell line c-SST-2 to identify factors involved in tumor progression and found (5) that epidermal growth factor (EGF) or transforming growth factor-β enhanced the tumorigenic, metastatic, and in vitro invasive capacities of these cells. We observed (6) that a 1-month exposure to EGF induced irreversible changes (tumor progression) in ER-1 cells but that a 24-hour exposure did not. We also hypothesized (6) that the increased intracellular oxidative stress induced by EGF might play an important role in both irreversible and reversible tumor progression. Thus, the ER-1 cell line appears to be a good model for investigations of tumor progression induced by continuous growth factor stimulation.

In this report, we investigate the mechanisms of irreversible tumor progression by using ER-1 cells treated with EGF in vitro for 1 month.

Materials and Methods

Animals

SHR rats were purchased from Charles River Japan (Yokohama, Japan). Female SHR rats aged 7–10 weeks were used for all experiments.

Cell Culture

The ER-1 clonal cell line was derived from a mammary adenocarcinoma that developed spontaneously in a female SHR rat (4). This cell line was grown on tissue culture dishes in Dulbecco’s modified Eagle medium (DMEM) supplemented with 7% fetal bovine serum (FBS). The rat lung endothelial (RLE) cell line was provided by Dr. G. L. Nicolson (The University of Texas M. D. Anderson Cancer Center, Houston) (7). Mesothelial cells were isolated from transparent sheets of mesentery in SHR rats by the method of Akedo et al. (8). RLE and mesothelial cells were grown on gelatin-coated tissue culture dishes in DMEM supplemented with 10% FBS. All cell lines were maintained in a CO2 incubator (5% CO2–95% air).

Treatment of ER-1 Cells With EGF

ER-1 cells were seeded on tissue culture plates or dishes in DMEM containing 3% FBS and human recombinant EGF at 100 ng/mL (Wakunaga Pharmaceutical, Hiroshima, Japan). During the 4-week (1-month) treatment with EGF, culture medium containing EGF was replaced every day. One week before assays for tumorigenicity, metastasis, and in vitro invasion, EGF supplementation was stopped and EGF-treated ER-1 cells were cultured in the absence of EGF.

In some experiments, ER-1 cells were treated with 5 or 10 mM N-acetylcysteine (Wako Pure Chemical, Tokyo, Japan) or with sodium selenite at a dose of 1, 10, or 100 ng/mL (Sigma Chemical Co., St. Louis, MO) in the presence or absence of EGF for 4 weeks (1 month).

Assay for Tumorigenicity and Metastasis

After ER-1 cells were treated with EGF as described above, they were washed in phosphate-buffered saline (PBS) and detached from culture dishes with PBS containing 2 mM EDTA. Recovered cells were washed twice in PBS followed by centrifugation at 600g for 5 minutes at 4 °C. To evaluate the tumorigenicity of the cells, we injected 1 x 106 cells reuspended in PBS intraperitoneally into an SHR rat (five or six rats per treatment group). Four weeks after the injection, the rats were killed under ether anesthesia by cervical dislocation, and
disseminated tumor nodules in the peritoneum were examined. To evaluate the metastatic capacity of the cells, we injected 1 × 10^6 cells resuspended in PBS intravenously into the tail vein of an SHR rat (five rats per treatment group). Five weeks after the injection, the rats were killed under ether anesthesia by cervical dislocation and examined for pulmonary metastases. The experiments were approved by the Animal Care and Use Committee of Hokkaido University School of Medicine.

**Assay for In Vitro Invasion of Mesothelial or Endothelial Cell Monolayer by ER-1 Cells**

The *in vitro* invasion of rat mesenteric mesothelial cell monolayers and RLE cell monolayers by ER-1 cells was assayed as described previously by Akedo et al. (8) and Ohigashi et al. (9). Briefly, when the RLE or mesothelial cells reached confluence in 60-mm tissue culture dishes with grids, 2 × 10^5 ER-1 cells were layered over the RLE or mesothelial cell monolayer. The invasive capacity of ER-1 cells was measured 1 week after the tumor cell seeding by counting the number of colonies per square centimeter formed under the RLE cell monolayer by use of a phase-contrast microscope.

**Assay for In Vitro Invasion of a Matrigel Reconstituted Basement Membrane by ER-1 Cells**

Chemo-invasion of basement membrane-like matrices by ER-1 cells was assayed by use of Matrigel (Collaborative Research, Inc., Bedford, MA) and Transwell chambers (Costar, Cambridge, MA) as described previously (10). Briefly, 2 × 10^5 ER-1 cells suspended in DMEM supplemented with 0.1% bovine serum albumin were placed in the upper compartment of a Transwell chamber that was separated from the lower chamber by a Matrigel-coated filter with 8-μm pores. The lower chamber contained conditioned medium from newborn SHR rat skin fibroblasts as the chemoattractant. After a 3-day incubation, the cells that had invaded the Matrigel and attached to the lower surface of the filter were fixed with 10% formalin, stained with a 5% Giemsa solution, and counted under a microscope. The invasive capacity was calculated from the number of cells that invaded the Matrigel per microscopic field (×200 magnification).

**Measurement of Intracellular Peroxides by Confocal Laser Scanning Microscopy**

Intracellular peroxide levels were measured by use of 2′,7′-dichlorofluorescein diacetate (DiAscan, Rochester, NY) as reported previously by Ohba et al. (11). 2′,7′-Dichlorofluorescein diacetate is a membrane-permeable nonfluorescent compound that is converted into membrane-impermeable nonfluorescent 2′,7′-dichlorofluorescein by alkaline hydrolysis. 2′,7′-Dichlorofluorescein is rapidly oxidized to fluorescent 2′,7′-dichlorofluorescein in the presence of hydrogen peroxide and peroxidases (12). We also used an oxidized probe, 5- (and 6-) carboxy-2′,7′-dichlorofluorescein diacetate (carboxy-DCFDA) (Molecular Probes, Inc., Eugene, OR), to check for differences in esterase activity and efflux of the probe. Briefly, ER-1 cells that had been treated with or without EGF and/or N-acetylcysteine or sodium selenite for 1 month were cultured on an eight-chamber Chamber SlideTM (Nunc, Naperville, IL) in DMEM containing 3% FBS in the presence or absence of N-acetylcysteine (5 or 10 mM) or sodium selenite (1, 10, or 100 ng/mL) for 1–3 days. EGF at a dose of 100 ng/mL was then added to some cultures; 30 minutes later, the medium in all cultures was replaced with DMEM including 5 μM 2′,7′-dichlorofluorescein diacetate or carboxy-DCFDA for 5 minutes, and the cellular fluorescence intensity was measured under a confocal laser scanning microscope. Intracellular peroxide levels were shown by the relative fluorescence intensity, i.e., the fluorescence intensity of a cell treated with 2′,7′-dichlorofluorescein diacetate divided by the fluorescence intensity of a cell treated with carboxy-DCFDA.

**Determination of 8-Hydroxydeoxyguanosine Levels in DNA of ER-1 Cells**

Genomic DNA was extracted from ER-1 cells by use of a SepaGene® kit (Sanko Junyaku, Tokyo, Japan) as described previously (13). In a Eppendorf tube, the extracted DNA was resolved with 250 μL of distilled deionized water, incubated at 95°C for 2 minutes, and immediately placed on ice for 3 minutes. Six microliters of 1 M sodium citrate was then added, and the mixture was vortex mixed. Fifteen microliters of nuclease P1 (EC 3.1.30.1; Yamasa Shoyu, Chohshi, Japan) at 2000 U/mL was added, and the mixture was mixed well and incubated at 60°C for 1 hour. After the incubation, 20 μL of 1 M Tris–HCl (pH 7.5) was added, the mixture was mixed well, 15 μL of alkaline phosphatase at 1000 U/μL (EC 3.1.3.1; Sigma Chemical Co.) was added, and the mixture was mixed again. After a 1-hour incubation at 37°C, enzyme-digested DNA was stored at −20°C until the levels of 8-hydroxydeoxy- guanosine were measured.

8-Hydroxydeoxyguanosine levels in these DNA samples were measured by the use of high-pressure liquid chromatography equipped with an electrochemical detector (HPLC/ECD) (Coulombich II; ESA, Bedford, MA) system, as reported previously by Nakae et al. (13), and standard curves made with authentic 8-hydroxydeoxyguanosine and deoxyguanosine. Data are expressed as the number of 8-hydroxygua nosine nucleosides per 10^6 deoxyguanosine nucleosi des.

**Assay for Glutathione Peroxidase**

Glutathione peroxidase activity was determined by the method of Beutler et al. (14), as modified by Yoshimura et al. (15). The amount of reduced nicotinamide dinucleotide phosphate (NADPH) was followed at 340 nm, with cumene hydroperoxide (0.23 mM) as substrate. One unit of enzyme activity was defined as 1 μmol of NADPH oxidized per minute at 37°C. The enzyme activity was expressed as milliunits per milligram of protein.

**Statistical Analysis**

Statistical significance was determined by one way analysis of variance followed by Fisher’s prob able least-squares difference analysis as a post hoc test. In all statistical comparisons, P<.01 was used to indicate a statistically significant difference. The incidence of tumorigenicity and of metastasis in Table 1 was analyzed by Fisher’s exact test. Pearson’s correlation analysis was done to determine statistical correlation. All statistical tests are two-sided.

**RESULTS**

Reactive oxygen species (ROS), including peroxides, have at least two targets, intracellular signal transduction pathways and DNA. ROS cause structural alterations in DNA, such as base-pair mutations, rearrangements, deletions, and insertions. In ER-1 cells, ROS are involved in intracellular signal transduction that transiently enhances tumorigenicity, metastasis, *in vitro* invasiveness, and motility (5,10); Hamada J: unpublished data. As shown in Fig. 1, after a 1-month EGF treatment, the intracellular peroxide level in ER-1 cells was approximately 2.5-fold higher than that in untreated ER-1 cells. When ER-1 cells were treated with both EGF and N-acetylcysteine, a chemical antioxidant, the level of intracellular peroxide was equal to that in untreated cells (Fig. 1).

To determine whether the ROS generated in cells stimulated with EGF for 1 month can cause genetic alterations, we measured 8-hydroxydeoxyguanosine levels in their DNA. 8-Hydroxydeoxyguanosine is a useful marker for oxidative DNA damage and is measured by HPLC/ECD. As shown in Fig. 2, after a 1-month EGF treatment of ER-1 cells, the level of 8-hydroxyguanosine in DNA was threefold to fourfold higher than that in untreated cells. The 8-hydroxyguanosine levels in DNA from ER-1 cells were elevated in a time-dependent manner by EGF treatment (Fig. 2, C; r = .927, n = 15, and P<.001 by Pearson’s correlation analysis). However, when cells were treated with both EGF and N-acetylcysteine, no increase in 8-hydroxyguanosine levels was observed (Fig. 2, A). Selenium can enhance the ability of cells to scavenge ROS. Moreover, it is an essential integral component of glutathione peroxidase, which catalyzes the reduction of organic hydroperoxides and hydrogen peroxide (16). Consequently, we used sodium selenite to enhance the ability of ER-1 cells to scavenge ROS. EGF-treated ER-1 cells had decreased glutathione peroxidase activity, whereas ER-1 cells treated with both EGF and sodium selenite (>10 ng/mL) and untreated control ER-1 cells had the same levels of glutathione peroxidase activity (Fig. 3, A). Glutathione peroxidase is consumed as it neutralizes the ROS, which suggests...
ER-1 cells were seeded onto Matrigel-coated filter of a Transwell chamber. Cells that attached to the lower surface of the filter were counted under a microscope 7 days after seeding. Data are the number of colonies under the monolayer (mean ± 95% confidence interval [CI]) per cm² (n/M). 

Table 1. Malignancy of ER-1 cells treated with epidermal growth factor (EGF) in the presence or absence of N-acetylcysteine (NAC) or sodium selenite (Na₂SeO₃)*

<table>
<thead>
<tr>
<th>Treatment†</th>
<th>Tumorigenicity‡</th>
<th>Metastatic capacity§</th>
<th>In vitro invasiveness to</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of rats with tumor/</td>
<td>Tumor weight, g ± SD</td>
<td>No. of rats with metastasis/No. of rats used</td>
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<tr>
<td>EGF, ng/mL</td>
<td>NAC mM</td>
<td>Na₂SeO₃ ng/mL</td>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>0/6b</td>
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<tr>
<td>100</td>
<td>0</td>
<td>6/6a</td>
<td>5.4 ± 0.7</td>
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<tr>
<td>100</td>
<td>5</td>
<td>1/6d</td>
<td>0.5 ± 1.2</td>
</tr>
<tr>
<td>100</td>
<td>10</td>
<td>1/6d</td>
<td>0.2 ± 0.4</td>
</tr>
<tr>
<td>0</td>
<td>5</td>
<td>0/5d</td>
<td>0.1 (−1.1 to 2.3)†</td>
</tr>
<tr>
<td>0</td>
<td>10</td>
<td>0/5d</td>
<td>0.8 (−1.4 to 3.0)†</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>0/6b</td>
<td>3.2 (0.7 to 5.7)†</td>
</tr>
<tr>
<td>100</td>
<td>0</td>
<td>6/6a</td>
<td>5.5 ± 0.7</td>
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<td>100</td>
<td>1</td>
<td>6/6a</td>
<td>5.0 ± 0.7</td>
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<td>100</td>
<td>10</td>
<td>2/6d</td>
<td>0.9 ± 1.4</td>
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<td>100</td>
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<td>1/6d</td>
<td>0.4 ± 0.9</td>
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<td>0</td>
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<td>0/6b</td>
<td>3.8 (1.4 to 6.2)†</td>
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<td>0</td>
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<td>1/6d</td>
<td>0.2 ± 0.4</td>
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<tr>
<td>0</td>
<td>100</td>
<td>1/6d</td>
<td>0.1 ± 0.3</td>
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</tbody>
</table>

*Statistical significance: †P = .001 compared with untreated ER-1 cells. ‡P = .001, ‡P = .003, or ‡P = .008 compared with EGF-treated ER-1 cells by Fisher’s exact test. ‡P < .001 compared with untreated ER-1 cells; ‡P < .001 compared with EGF-treated ER-1 cells by one-way analysis of variance followed by Fisher’s post hoc analysis. All statistical tests are two-sided.

†ER-1 cells were treated for 1 month with or without EGF in the presence or absence of NAC or Na₂SeO₃, as indicated and then were cultured in medium containing 5% fetal bovine serum without NAC, Na₂SeO₃, and 100 ng/mL EGF for 7 days.

‡ER-1 cells were seeded onto Matrigel-coated filter of a Transwell chamber. Cells that attached to the lower surface of the filter were counted under a phase-contrast microscope 7 days after seeding. Data are the number of colonies under the monolayer (mean ± 95% confidence interval [CI]) per cm² (n = 6).

§ER-1 cells (1 × 10⁶ cells) were inoculated intraperitoneally into an SHR rat. Rats were killed for examination of intraperitoneal tumor growth 28 days after the inoculation. SD = standard deviation.

ER-1 cells (1 × 10⁶ cells) were inoculated intravenously into the tail vein of an SHR rat. Rats were killed for examination of pulmonary metastases 35 days after the inoculation. CI = confidence interval.

ER-1 cells were seeded onto the monolayer of rat mesenteric mesothelial or lung endothelial cells. Colonies formed under the monolayer were counted with a phase-contrast microscope 3 days after seeding. Data are the number of colonies under the monolayer (mean ± 95% confidence interval [CI]) per field (n = 6).

ER-1 cells were treated for 1 month with or without EGF in the presence or absence of NAC or Na₂SeO₃, as indicated and then were cultured in medium containing 5% fetal bovine serum without EGF, NAC, and Na₂SeO₃ for 7 days.

Inhibition of epidermal growth factor (EGF)-induced intracellular peroxides by N-acetylcysteine (NAC), a chemical antioxidant.

Scale bar = 100 μm.

Fluorescence intensity of 20 cells from each of the six treatment groups was measured by confocal laser scanning microscopy. Data are the relative fluorescence intensity per cell (means ± 95% confidence intervals). *, P < .001 compared with untreated ER-1 cells; †, P < .001 compared with EGF-treated ER-1 cells by one-way analysis of variance followed by Fisher’s post hoc analysis. All statistical tests are two-sided.
that addition of selenium to the culture medium provides a sufficient amount of glutathione peroxidase for ER-1 cells to scavenge the ROS. Actually, as shown in Fig. 3, B, the EGF-induced increase in intracellular peroxide levels was statistically significantly reduced when sodium selenite was present at more than 10 ng/mL in the medium (P < .001). In cells treated with both EGF and sodium selenite or with both EGF and N-acetylcysteine, the amount of 8-hydroxyguanosine in DNA correlated well with the levels of intracellular peroxide in ER-1 cells (r = .795, n = 8, and P = .015 by Pearson’s correlation analysis; Fig. 2, B, and Fig. 3, B).  

To assess in vivo malignancy, we cultured ER-1 cells for 1 month in the following media: medium alone; medium with EGF, N-acetylcysteine, or sodium selenite; or medium with the combinations of EGF and N-acetylcysteine or EGF and sodium selenite. Cells were then injected intraperitoneally or intravenously into syngeneic rats (Table 1). EGF-treated ER-1 cells injected intraperitoneally developed into tumors in the peritoneum, and blood-containing ascites fluid was observed in all rats. However, ER-1 cells treated for 1 month with EGF and N-acetylcysteine or with EGF and sodium selenite injected intraperitoneally into rats were as weakly tumorigenic as untreated ER-1 cells. EGF-treated ER-1 cells injected intravenously developed into a statistically significantly greater number of pulmonary metastatic foci than did untreated ER-1 cells or ER-1 cells treated with EGF and N-acetylcysteine or with EGF and sodium selenite (>10 ng/mL) (P < .001). In vitro invasion assays also showed that EGF-treated cells invaded mesothelial cell monolayers, RLE monolayers, or a Matrigel reconstituted basement membranes better than untreated control cells. This enhancement was not observed when cells were treated with EGF and N-acetylcysteine or with EGF and sodium selenite (Table 1).

DISCUSSION

Our data suggest that the irreversible changes induced in ER-1 cells by continuous EGF stimulation for 1 month result from genetic alterations caused by increased oxidative stress, which generates tumor cells with more malignant properties.

Phagocytes, such as macrophages and neutrophils, are known as a source of ROS in tumor microenvironment (17–21). The large amount of ROS produced extracellularly by activated phagocytes can cause cell death or injury, including mutations to target cells that contribute to carcinogenesis and subsequent tumor progression (17–21). The amount of intracellular ROS generated in growth factor-stimulated cells is not high enough to be cytotoxic because it did not reduce the viability and growth rate of the ER-1 cells (data not shown). The 8-hydroxyguanosine levels in DNA from ER-1 cells treated with EGF for 1 week, 2 weeks, and 4 weeks were 1.5-fold, 1.7-fold, 2.2-fold, and 3.6-fold, respectively, greater than those in the DNA from untreated cells. Such a time-dependent increase in the amount of 8-hydroxyguanosine leads us to speculate that the antioxidant system in EGF-treated ER-1 cells is less active. Actually, several reports (21–24) show that some tumor cells have defective antioxidant systems with decreased levels of antioxidant enzymes, such as superoxide dismutase, catalase, and glutathione peroxidases, as well as dysfunctional 8-hydroxyguanosine repair enzymes. Therefore, tumor cells with defective antioxidant systems may be prone to oxidative DNA damage even if a relatively low amount of ROS is generated. 8-Hydroxyguanosine mismatches preferentially with adenosine during replication and thereby causes G:C to T:A transver-
sion mutations (25,26). Therefore, EGF-treated ER-1 cells with the higher levels of 8-hydroxyguanosine in their DNA may be in a mutation-prone state.

The generation of ROS has been detected in various cells stimulated with growth factors and cytokines, including transforming growth factor-β1 (11,27,28), platelet-derived growth factor (29,30), basic fibroblast growth factor (30,31), EGF (5,32), interleukin 1 (33), and tumor necrosis factor-α (31,33). Consequently, other growth factors that are found in the tumor microenvironment may also stimulate the production of ROS, resulting in oxidative DNA damage, genetic alterations, and cellular diversification.

REFERENCES


NOTES

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