Biochemical Pharmacology, Vol. 60, pp. 937–946, 2000.

ISSN 0006-2952/00/$–see front matter
PII S0006-2952(00)00426-3

Preferential Inhibition by (−)-Epigallocatechin-3-Gallate of the Cell Surface NADH Oxidase and Growth of Transformed Cells in Culture

D. James Morré,† Andrew Bridge,* Lian-Ying Wu‡ and Dorothy M. Morré‡*

Departments of *Medicinal Chemistry and Molecular Pharmacology and ‡Foods and Nutrition, Purdue University, West Lafayette, IN 47907, U.S.A.

ABSTRACT. A drug-responsive and cancer-specific NADH oxidase of the mammalian plasma membrane, constitutively activated in transformed cells, was inhibited preferentially in HeLa and human mammary adenocarcinoma by the naturally-occurring catechin of green tea, (−)-epigallocatechin-3-gallate (EGCg). With cells in culture, EGCg preferentially inhibited growth of HeLa and mammary adenocarcinoma cells compared with growth of mammary epithelial cells. Inhibited cells became smaller, and cell death was accompanied by a condensed and fragmented appearance of the nuclear DNA as revealed by fluorescence microscopy with 4′,6-diamidino-2-phenylindole, suggestive of apoptosis. Mammary epithelial cells recovered from EGCg treatment even at 50 μM, whereas growth of HeLa and mammary adenocarcinoma cells was inhibited by EGCg at concentrations as low as 1 μM with repeated twice-daily additions and did not recover from treatment with 50 μM EGCg. The findings correlate inhibition of cell surface NADH oxidase activity and inhibition of growth with EGCg-induced apoptosis. BIOCHEM PHARMACOL **60**;7:937–946, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. plasma membrane; NADH oxidase; (−)-epigallocatechin-3-gallate; tea catechins; apoptosis

A growth factor- and hormone-stimulated NADH oxidase activity of rat liver plasma membranes [1, 2] has been described that is potentially involved in the control of cell proliferation [3]. The cell surface NADH oxidase activity in transformed cells and tissues is different in that the growth factor- and hormone-responsiveness is lost [4, 5] and the activity is responsive to inhibitors and activators other than growth factors and hormones [3, 6–8] including the quinone analog capsaicin (8-methyl-*N*-vanillyl-6-noneamide) [9] and an antitumor sulfonylurea, *N*-(4-methylphenylsulfonyl)-*N′*-(4-chlorophenyl)urea (LY181984) [10]. The results have suggested a fundamental difference in response to drugs between the NADH oxidase activities of normal and transformed cells and tissues that correlated with inhibition of growth and induction of apoptosis in the transformed cells. In the present report, we extended these observations to EGCg,§ the putative active anticancer (chemopreventative) catechin polyphenol of green tea.

Focused research on the health benefits of tea is relatively recent [11]. The major interest stems from high levels of antioxidant tea phenols, including the predominant catechin polyphenol EGCg [12]. Epidemiological and human studies have been carried out. The majority have been of case-control design and have been reviewed [13–18]. Cancer onset of patients in Japan who had consumed 10 cups of green tea per day is 8.7 years later among females and 3 years later among males, compared with patients who had consumed under 3 cups per day [19]. As such, a possible relationship between high consumption of green tea and the low incidence of prostate and breast cancer in Asian countries where green tea consumption is high has been postulated [20, 21]. Also reported has been preferential inhibition of growth and induction of apoptosis by EGCg in cancer cell lines compared with non-cancer cell lines [12, 22].

MATERIALS AND METHODS

Materials

All chemicals were purchased from the Sigma Chemical Co. unless otherwise specified. EGF was from mouse, culture grade, obtained from Upstate Biotechnology, Inc. Tea infusions were prepared by sequential steeping of ca. 2-g bags of tea in 10 mL of water for 10 min each. At the end of the infusion, bags were pressed to remove liquid.

Growth of Cells

HeLa (ATCC CCL2) cells were grown in 175-cm^2 flasks in Minimal Essential Medium (Gibco), pH 7.4, at 37° with

† Corresponding author: D. James Morré, Ph.D., Department of Medicinal Chemistry and Molecular Pharmacology, Purdue University, West Lafayette, IN 47907. Tel. (765) 494-1388; FAX (765) 494-4007; E-mail: Morre@pharmacy.purdue.edu

§ *Abbreviations*: DAPI, 4′,6-diamidino-2-phenylindole; EGF, epidermal growth factor; EGCg, (−)-epigallocatechin-3-gallate; NOX, plasma membrane-associated NADH oxidase; tNOX, cancer-associated forms of NOX; CNOX, constitutive, normal forms of NOX.

Received 11 January 2000; accepted 24 March 2000.

10% fetal bovine serum (heat-inactivated), plus 50 mg/L gentamycin sulfate (Sigma). Cells were harvested by scraping and taken up in 140 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, and 25 mM Tris, pH 7.4, to a final cell concentration of 0.1 g wet weight (gww) per mL.

MCF-10A human mammary epithelial cells were cultured in a 1:1 mixture of Ham's F12 medium and Dulbecco's Modified Eagle's medium containing glutamine (292 mg/L), gentamycin sulfate (50 mg/L), insulin (10 μg/mL), hydrocortisone (0.5 μg/mL), EGF (20 ng/mL), and 5% horse serum. Medium was renewed every 2–3 days.

BT-20 human breast adenocarcinoma cells were cultured in Eagle's minimal essential medium containing 0.1 mM nonessential amino acids with 10% fetal bovine serum and gentamycin sulfate (50 mg/L). Medium was renewed as for MCF-10A cells.

Growth was determined microscopically by counting the number of cells over defined areas consisting of a grid of 1-mm squares. Cell lines were obtained from the American Type Culture Collection.

Purification of Plasma Membranes from Cultured Cells

Cultured cells were collected by centrifugation for 6 min at 1000 *g*. The cell pellets were resuspended in 0.2 mM EDTA in 1 mM $NaHCO_3$ in an approximate ratio of 1 mL per 10^8 cells and incubated on ice for 10–30 min to swell the cells. Homogenization was achieved in 7- to 8-mL aliquots with a Polytron homogenizer (Brinkmann) for 30–40 sec at 10,500 rpm, using a PT-PA 3012/23 or ST-10 probe. To estimate breakage, the cells were monitored by light microscopy before and after homogenization. At least 90% cell breakage without breakage of nuclei was achieved routinely.

The homogenates were centrifuged for 10 min at 175 *g* to remove unbroken cells and nuclei, and the supernatant was centrifuged a second time at 1.4×10^6 *g* · min (e.g., 1 hr at 23,500 *g*) to prepare a plasma membrane-enriched microsome fraction. The supernatant was discarded, and the pellets were resuspended in 0.2 M potassium phosphate buffer in a ratio of ≈1 mL per pellet from 5×10^8 cells. The resuspended membranes then were loaded onto the two-phase system constituted on a weight basis, consisting of 6.6% (w/w) Dextran T-500 (Pharmacia) and 6.6% (w/w) Polyethylene Glycol 3350 (Fisher) in a 0.2 mM potassium phosphate buffer (pH 7.2) for aqueous two-phase separation as previously described [23, 24]. The upper phase, enriched in plasma membranes, was diluted 5-fold with 1 mM sodium bicarbonate, and the membranes were collected by centrifugation. The purity of the plasma membrane was determined to be >90% by electron microscope morphometry. The yield was 20 mg of plasma membrane protein from 10^{10} cells.

Preparation of HeLa Cells and Cell-Free Extracts

HeLa S cells were collected by centrifugation and shipped frozen in 0.1 M sodium acetate, pH 5, in a ratio of 1 mL of packed cell volume to 1 mL of acetate (Cellex Biosciences). The cells were thawed at room temperature, resuspended, and incubated at 37° for 1 hr to release the protein [25]. The cells were removed by centrifugation at 37,000 *g* for 60 min, and the cell-free supernatants were refrozen and stored in 1-mL aliquots at −70°.

For heat treatment, 1-mL aliquots of the supernatant material described above were thawed at room temperature and heated to 50° for 10 min. The denatured proteins were removed by centrifugation (1500 *g*, 5 min). Full activity was retained from this step [25].

For protease treatment, the pH of the heat-stable supernatant was adjusted to 7.8 by addition of 0.1 M sodium hydroxide. *Tritirachium album* proteinase K (Calbiochem) was added (4 ng/mL) and incubated at 37° for 1 hr with full retention of enzymatic activity and drug response [25]. The reaction was stopped either by freezing for determination of enzymatic activity or by addition of 0.1 M phenylmethylsulfonyl fluoride in ethanol to yield a final concentration of 10 mM phenylmethylsulfonyl fluoride.

Spectrophotometric Assay of NADH Oxidase

NADH oxidase activity was determined as the disappearance of NADH measured at 340 nm in a reaction mixture containing 25 mM Tris–methylethanesulfonate buffer (pH 7.2), 1 mM KCN to inhibit low levels of mitochondrial oxidase activity, and 150 μM NADH at 37°, with stirring. Activity was measured using a Hitachi U3210 or SLM Aminco DW2000 spectrophotometer with continuous recording over two intervals of 5 min each. A millimolar extinction coefficient of 6.22 was used to determine specific activity. EGCg was added at the final concentrations indicated at the beginning of the assay and was present during the assay period.

Proteins were estimated by the bicinchoninic acid method [26] with BSA as standard.

Fluorescence Microscopy

Cells were grown for 72 hr on glass coverslips placed in small culture dishes with medium containing 100 μM EGCg in ethanol or an equivalent amount of ethanol alone. The coverslips were rinsed, and the cells were fixed in methanol followed by the addition of the fluorescent dye DAPI as described [27]. Cells were observed and photographed at a primary magnification of 400X.

Determination of EGCg

EGCg was determined in the hot water extracts using the standardized chromatographic procedure described by Kati-

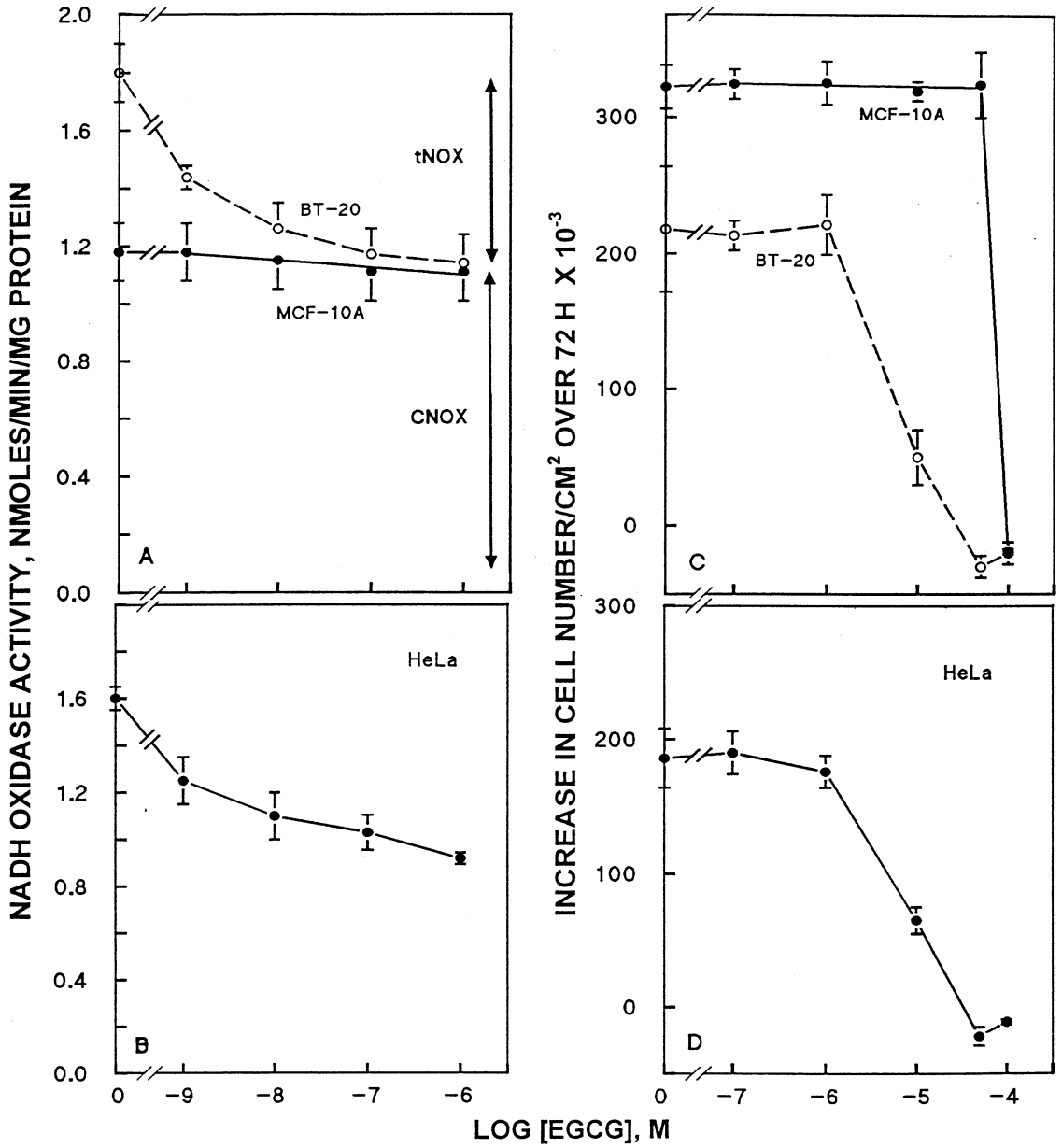

FIG. 1. Concentration–response curves of NADH oxidase of isolated plasma membranes (A, B) and growth of attached cells (C, D) to EGCg. (A, C) MCF-10A human mammary epithelial (non-cancer) cells (●) and BT-20 human mammary adenocarcinoma (cancer) cells (○). (B, D) HeLa (human cervical carcinoma) cells. Values are averages of duplicate determinations in each of three separate experiments (N = 6) ± SD among the experiments (N = 3). NADH oxidase (NOX) activity of non-cancer cells (e.g., MCF-10A) was normally drug-resistant (designated CNOX). Cancer cells (e.g., BT-20 or HeLa) had, in addition, a second NOX component, equivalent to about 40% of the total NOX activity, that was unregulated and anticancer drug-responsive (designated tNOX). Cancer cells contained both the drug-responsive (tNOX) and drug-unresponsive (CNOX) NADH oxidase components. The tNOX was inhibited by EGCg with an EC_{50} of about 1 nM, whereas CNOX was unaffected.

yar *et al.* [28]. Authentic EGCg (Sigma) was used as the standard.

RESULTS

NOX activities of non-cancerous cells and tissues (CNOX) are resistant to the anticancer drugs that affect the drug-responsive NADH oxidase component of cancer cells and tissues. Cancer cells have, in addition to CNOX, a second NADH oxidase activity that is drug-responsive (tNOX). It represents about 40% of the total NOX activity (Fig. 1A). MCF-10A mammary epithelial cells contained only CNOX, whereas BT-20 mammary adenocarcinoma cells contained both CNOX and tNOX (Fig. 1A).

With MCF-10A mammary epithelial cells, EGCg was without effect on the NADH oxidase activity (CNOX) of plasma membrane vesicles (Fig. 1) or NADH oxidase solubilized and partially purified from the cell surface (Fig.

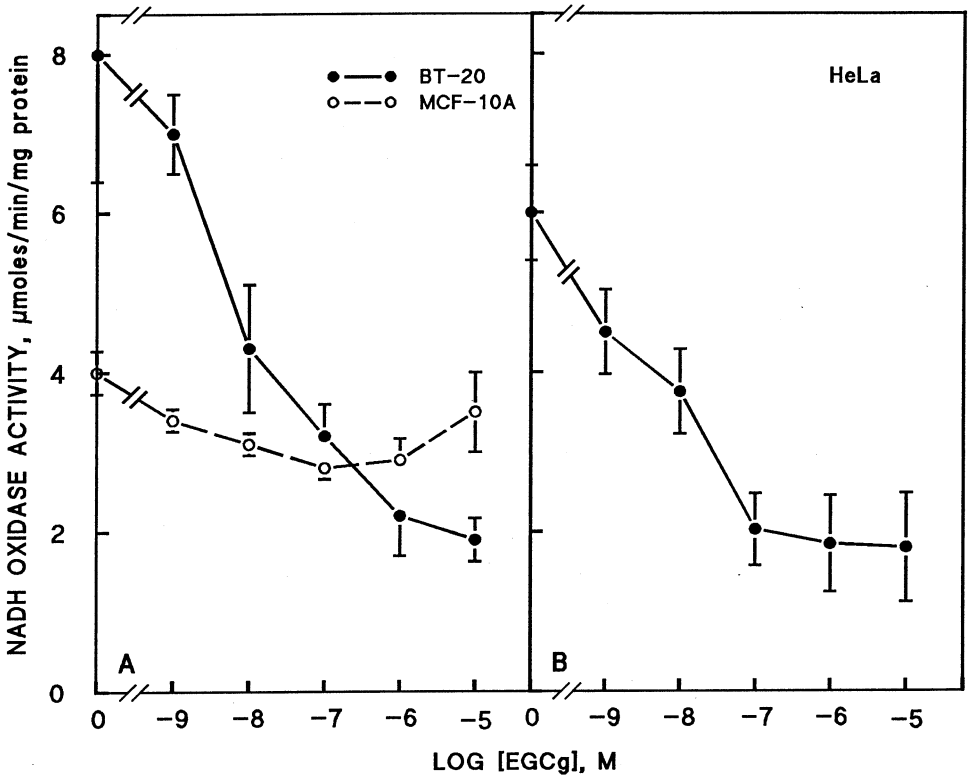

FIG. 2. Concentration–response curves of solubilized and partially purified NADH oxidase to EGCg. (A) NADH oxidase from MCF-10A and BT-20 cells. (B) NADH oxidase from HeLa cells. As with plasma membranes (Fig. 1), the preparations from BT-20 and HeLa cells contained NOX activities both susceptible and resistant to inhibition by EGCg, whereas the preparations from MCF10A cells were resistant to inhibition. Results are averages of duplicate determinations in each of three separate experiments (N = 6) ± SD among experiments (N = 3).

2). However, with plasma membranes from human mammary adenocarcinoma (BT-20) or HeLa (human cervical carcinoma) cells, NADH oxidase activities were inhibited by 30 to 40% with an EC_{50} of about 1 nM (complete inhibition of tNOX but no effect on CNOX) (Fig. 1A). The drug-responsive component of NADH oxidase activity of BT-20 and HeLa cells also was inhibited completely by capsaicin [9] or the antitumor sulfonylurea LY181984 [10]. The responses to EGCg were comparable to those for capsaicin and the sulfonylurea.

With plasma membrane vesicles from the BT-20 mammary adenocarcinoma cell line, the NADH oxidase specific activity was approximately 1.5 times that of the MCF-10A cell line (Fig. 1A). Upon the addition of EGCg, the specific activity of the MCF-10A cells was unchanged, whereas that of the BT-20 was reduced to approximately the same level as that of the MCF-10A cells (i.e., tNOX activity completely inhibited) (Fig. 1A). Also inhibited by EGCg in a similar fashion was the NADH oxidase activity from plasma membranes of HeLa cells (Fig. 1B). Thus, in the plasma membrane vesicles from the BT-20 and HeLa cells, there were both EGCg-resistant (CNOX) and EGCg-susceptible (tNOX) components, whereas in the plasma membrane vesicles from the MCF-10A cells only an EGCg-resistant activity was observed (Fig. 1A).

Results similar to those observed with isolated plasma membrane vesicles were obtained as well with solubilized NADH oxidase preparations of NADH oxidase released from cultured cells by low pH treatment (Fig. 2). With BT-20 (Fig. 2A) and HeLa (Fig. 2B) preparations, activity was inhibited strongly by EGCg with an EC_{50} of between 1 and 10 nM. The released and solubilized NADH oxidase for the MCF-10A cells was largely unaffected by the EGCg (Fig. 2A). As with isolated plasma membrane vesicles, the specific activity of the released NADH oxidase preparations from BT-20 cells was greater (approximately twice) than that of the released preparations from MCF-10A cells. Following treatment with EGCg, the specific activity of the preparations from BT-20 cells was reduced to a level comparable to the specific activity of the preparations from MCF-10A cells. Thus, EGCg appears to inhibit specifically the drug-responsive (tNOX) NADH oxidase component of the tumorigenically transformed cell lines but not that of the constitutive (CNOX) NADH oxidase activity of the MCF-10A mammary epithelial line.

EGCg also inhibited the growth of the BT-20 mammary adenocarcinoma and HeLa cells in culture (Fig. 1C, D). Though not as strikingly as for the inhibition of NADH oxidase, EGCg did restrict the growth of the HeLa and BT-20 cells preferentially compared with that of MCF-10A (Fig. 1C, D). Growth of the MCF-10A mammary epithelial cells was unaffected by EGCg except at the very high concentration of 100 μM (Fig. 1C), whereas that of the tumorigenically transformed BT-20 and HeLa cells was 50% inhibited at about 5 μM (Fig. 1C, D).

Despite early growth inhibition of MCF-10A cells by EGCg, the cells recovered quickly and eventually grew normally (Fig. 3). This is in contrast to HeLa and BT-20 cells, which did not recover and died (Fig. 3).

When the cells treated with 10 or 50 μM EGCg were stained to reveal DAPI fluorescence after (but not before)

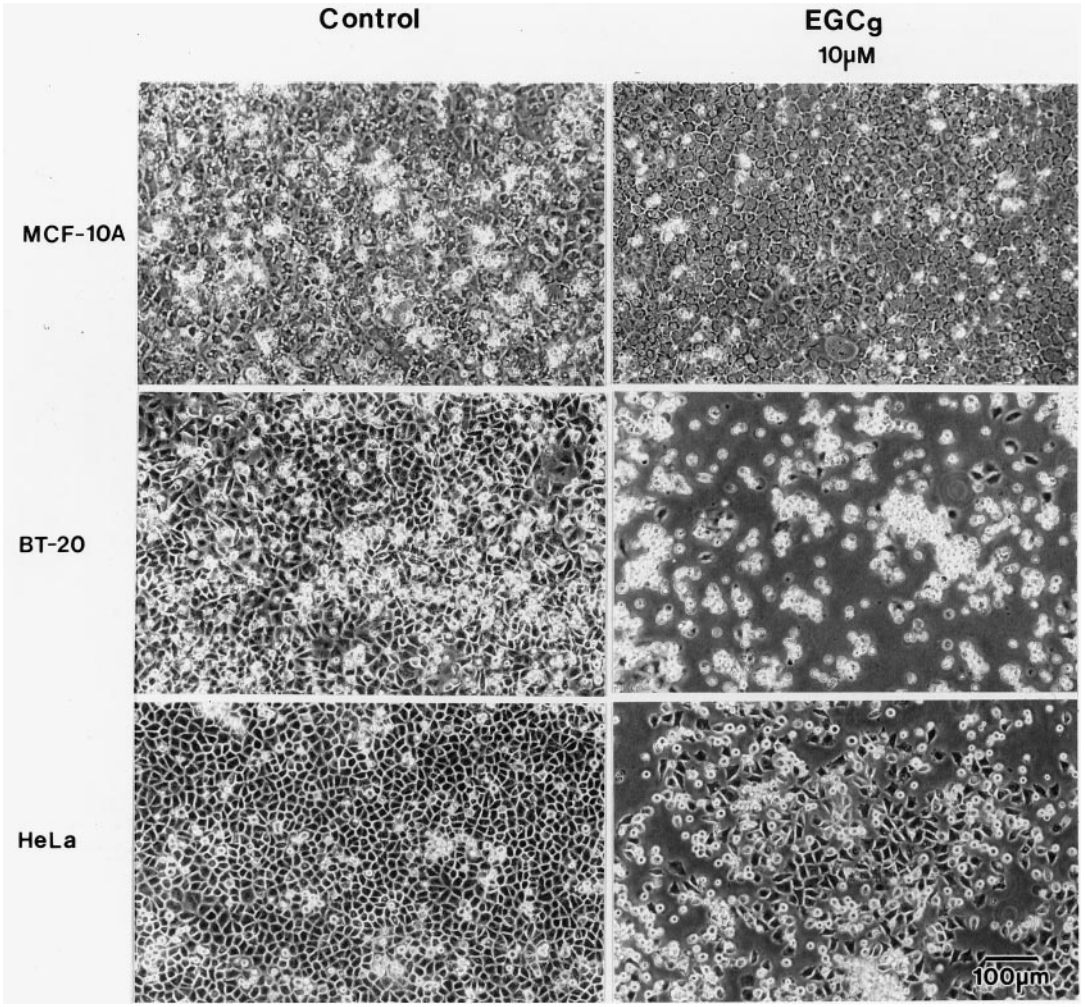

FIG. 3. Photomicrographs of MCF-10A mammary epithelial (non-cancer), BT-20 mammary adenocarcinoma, and HeLa cells treated for 96 hr with 10 μM EGCg added at t = 0. The BT-20 and HeLa cells stopped growing and died, whereas the MCF-10 cells recovered fully.

72 hr of growth inhibition, a very large percentage of the treated cells showed nuclear DNA with the condensed and fragmented appearance characteristic of apoptotic cells. The appearance is illustrated in Fig. 4 for cells continuously treated for 96 hr with 10 or 50 μM EGCg.

Measurements of the diameters of treated HeLa and BT-20 cells taken directly from printed micrographs revealed that, on average, the cells treated with 5–50 μM EGCg exhibited volumes ≈50% those of untreated cells. At 1 μM EGCg, there was no response of any of the cell lines at 72 hr despite the fact that this EGCg concentration inhibited the tNOX activity of isolated plasma membranes. The possibility was considered that the combination of a reversible inhibition and rapid uptake or loss of EGCg (including oxidation and polymerization) from the culture media containing cells might result in an overall lack of growth inhibition at 1 μM EGCg after 3 days. With repeated additions of 100 nM EGCg (every 2 hr during the day), growth was inhibited during the day but recovered during the night (Fig. 5). Since the growth rate recovered completely during the night, there was no apoptosis as a result. However, when the concentration of EGCg was increased to 1 μM provided twice daily, growth was inhibited, and the resultant cells were smaller. Cell diameters were reduced on average by about 25% and cell volume by 50% by the twice-daily 1 μM EGCg addition beginning on day 1. Cell number also was reduced by about 25% with both HeLa and BT-20 cells by the 1 μM EGCg provided twice daily, whereas with the non-cancer MCF-10A cells, growth rate and cell diameters were still unaffected or were increased slightly.

The activity of both the solubilized and partially purified NADH oxidase released from cells by low pH treatment (Fig. 6) and that found normally in sera pooled from cancer patients [29] were inhibited as well by tea infusions (Table 1). Both black tea and green tea infusions were effective in proportion to their content of EGCg. Green tea infusions were approximately 10 times more effective than those of black tea and contained approximately 10 times more EGCg. When expressed on an EGCg basis, the EC_{50} was

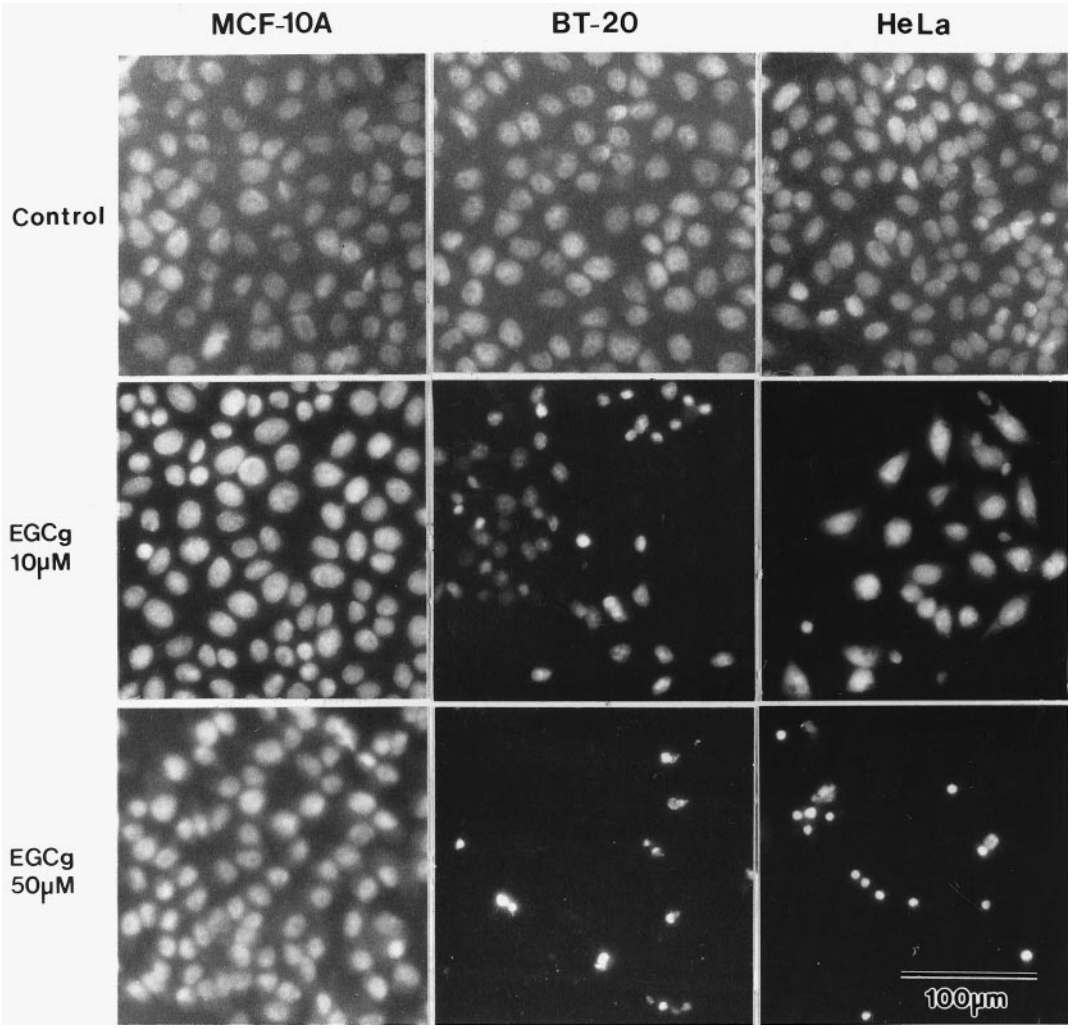

FIG. 4. Photomicrographs of MCF-10A, BT-20, and HeLa cells stained with DAPI [21] to show condensed chromatin after 96 hr in the presence of 10 or 50 μM EGCg, characteristic of apoptosis for BT-20 and HeLa but not for MCF-10A cells. Cells were grown on coverslips in the absence (upper panel) or presence (lower 2 panels) of 10 or 50 μM EGCg and then fixed. Nuclear DNA was stained with DAPI and analyzed with a fluorescence microscope.

equivalent to 2 μM EGCg (1 μg/mL) for both green and black tea infusions as summarized in Table 1.

DISCUSSION

Our laboratory has identified an NADH oxidase activity of the plasma membrane in rat liver [1–3], keratinocytes [6], and plant stems [30]. The NADH oxidase of plasma membranes of tumorigenically transformed cell lines subsequently was shown to exhibit a second form of the activity that responded to a small number of antitumor or putative antitumor substances including an antitumor sulfonylurea [10] and the vanilloid quinone site inhibitor capsaicin [9]. Plasma membranes from cell lines not tumorigenically transformed did not respond to these drugs, nor was the growth of cell lines not tumorigenically transformed affected by these drugs. With all tissues and cell lines examined thus far, stimulations or inhibitions of the activity have correlated closely with inhibitions or stimulations of growth [31–33]. Hydroquinones (e.g., reduced coenzyme Q) of the plasma membrane have been identified as being among the physiological electron donors for the activity [34].

In the present report, we demonstrated that the activity of the cell surface NADH oxidase of human mammary adenocarcinoma cells and HeLa cells (human cervical carcinoma derivation) were inhibited by EGCg, a catechin present in high concentrations in green tea and thought to be one of the principal compounds responsible for the anticancer (chemopreventative) activities attributed to green tea [12]. In contrast to the NADH oxidase of the plasma membranes of the carcinoma cell lines, the NADH oxidase of plasma membranes from mammary epithelial cells was not inhibited.

Studies with animals [17, 19–21] and with cells in culture [12, 22], together with human studies [13–18], have indicated anticancer effects for tea, especially in chemopre-

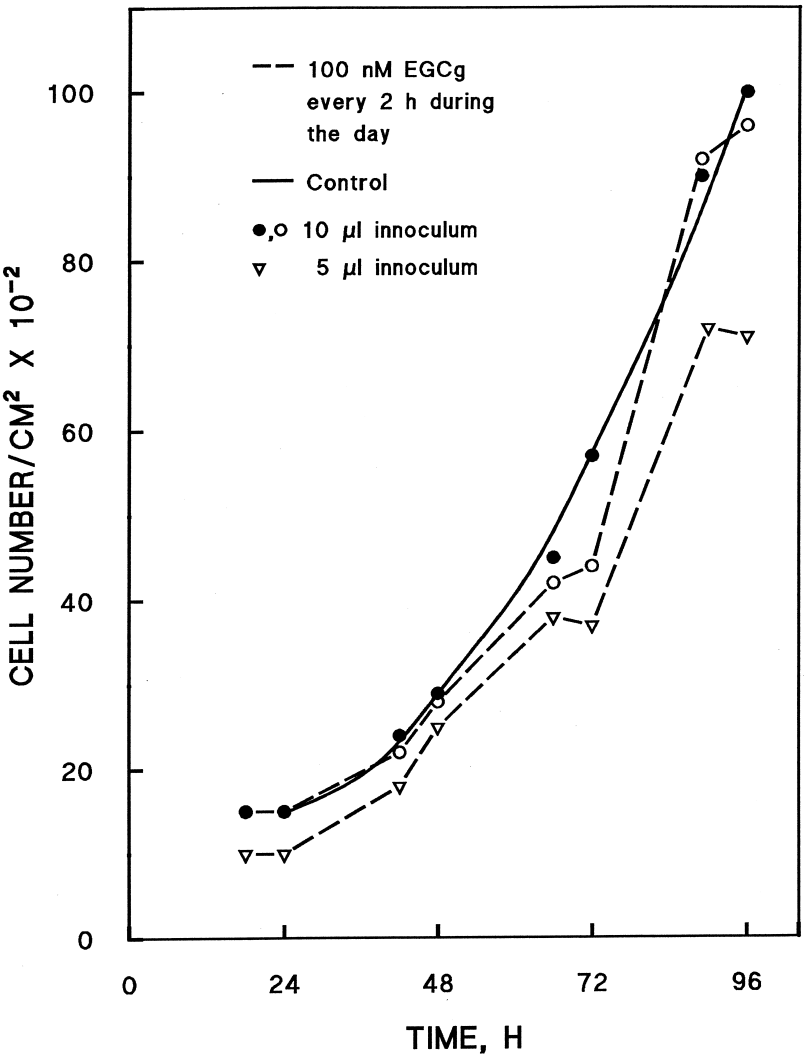

FIG. 5. Growth of HeLa cells (increase in cell number) with time of culture. Key: Solid symbols, solid line: No addition. Open symbols, dashed lines: EGCg (100 nM) was added every 2 hr during the day (8:00 a.m. until 5:00 p.m.). Two different levels of innoculum 10 μL = 15 cells/mm^2 (circles) or 5 μL = 8 cells/mm^2 (triangles), were compared.

vention. In general, these effects have been attributed to the major tea catechin [12, 28]. Other antioxidant catechins and polyphenols present in tea have less marked anticancer or chemopreventative properties. The activity is considered to be related in some measure to regulation of cell cycle progression and induction of p53-dependent apoptosis [35].

In our studies, not only did EGCg inhibit the NADH oxidase of plasma membrane vesicles from cancer cells and not that of normal cells, but also cell growth exhibited a parallel response to the substance. In other studies not reported here, growth of non-cancerous Chinese hamster ovary cells also resisted inhibition by EGCg. Growth of HeLa cells was inhibited almost completely by EGCg, whereas growth of the MCF-10A mammary epithelial cells was much less affected by EGCg. Yet, a single addition of a higher concentration of EGCg was required to inhibit growth of cells in culture than to inhibit NADH oxidase in isolated plasma membranes or purified NOX preparations. As illustrated by Fig. 5, this appears to be the result of rapid uptake and loss of EGCg from culture medium containing cells at nanomolar and submicromolar concentrations of EGCg. Also, the transient growth inhibitions achieved by even repeated additions of 100 nM EGCg did not result in apoptosis. Indications are that NOX activity and growth must be inhibited continuously for approximately 72 hr or more to induce apoptosis. Even a modest 30% decrease in cell diameter might exert little or no effect in slowing of cell multiplication. It would appear that a nearly complete inhibition of the cell volume increase following division would be required to block cell proliferation. For example, with repeated additions (twice daily) at 1 μM EGCg, the EGCg concentration achieving complete inhibition of tNOX in BT-20 cells, growth inhibition and apoptosis in BT-20 cells were achieved. Growth and apoptosis of MCF-10A mammary epithelial cells were still unaffected even with repeated additions of 1 μM EGCg.

Under conditions where growth of HeLa cells was blocked by EGCg, nuclei exhibited patterns of fluorescence characteristic of apoptosis [27]. The apoptotic change was also indicated by cell size measurements and overall morphological changes. Thus, the cyanide-resistant NADH oxidase of the plasma membrane appears to represent an enzymatic activity whose inhibition by EGCg may correlate

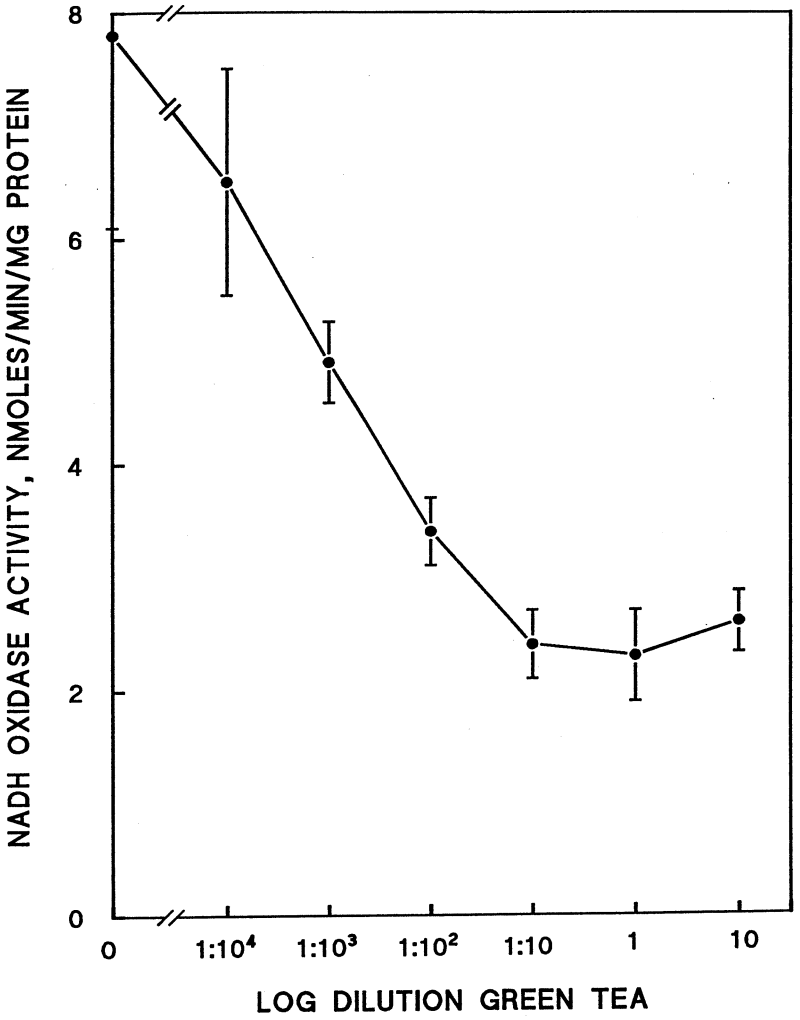

FIG. 6. Inhibition of partially purified tNOX from HeLa cells by green tea infusions. The EC_{50} for inhibition of the enzymatic activity was at a tea dilution of about 1:1000. The preparations contained an activity resistant to inhibition as well, so that the inhibition by the tea infusions was not complete, and further inhibition by green tea was not observed above a dilution of about 1:10. Results are averages of duplicate determinations in each of three separate experiments (N = 6) ± SD among experiments (N = 3).

with an inhibition of growth and with subsequent induction of apoptosis in susceptible cancer cell lines.

During the initiation of apoptosis, different proapoptotic and antiapoptotic proteins are up- or down-regulated, thus promoting the activation of caspases, chromatin condensation, and DNA fragmentation [36]. Apoptosis may be initiated by various actions as diverse as DNA damage, growth factor withdrawal, or radical generation or as a result of receptor occupancy. We have no information on how inhibition of NOX activity might induce apoptosis despite the observation that compounds that result in prolonged inhibition of the NOX form associated with cancer cells do result in the induction of apoptosis. A role of redox systems both at the cell surface and in the cytosol in concert with effects on mitogen-activated kinases has been proposed and may be relevant to our observations [37].

TABLE 1. Inhibition of tNOX activity by tea infusions and by (−)-epigallocatechin-3-gallate (EGCg), the major tea polyphenol (catechin) of green tea

Source	EC_{50}	EGCg μg/mL
Black tea	1:10 to 1:100	1
Green tea	1:1000	1
(−)-Epigallocatechin-3-gallate (EGCg)	2 μM	1

Sera pooled from patients with cancer, which contain a circulating form of tNOX [29], were utilized as a convenient source of drug-responsive NOX. The EGCg content was determined as described [28]. Results were repeated 3 to 5 times with different sources and preparations of both black and green tea and with consistent findings. For all three sources, the EGCg content at the EC_{50} was ca. 1 μg/mL, suggesting that EGCg content was primarily responsible for the inhibition of tNOX activity by both black and green tea infusions. Similar results with green tea infusions were obtained with the solubilized enzyme preparations from HeLa cells (Fig. 6).

Tea is one of the few agents that seem to inhibit cancer formation at all stages of development: initiation, promotion, and progression [38]. It may not be accurate to think of tea simply as a good source of antioxidants. This report proposes a mechanism whereby tea polyphenols may exert at least some of their anti-cancer effects through a non-antioxidant mechanism. In previous studies, inhibition of the enzyme tNOX, specifically expressed on the surface of cancer cells, could be correlated with inhibition of cancer cell division and growth and induction of apoptosis [9]. Here, we show that EGCg, a tea catechin, also is a potent specific inhibitor of tNOX. Our findings now provide a non-antioxidant mechanism that may help explain the high degree of specificity of inhibition of the proliferation

of several cancer cell lines by EGCg that has been observed previously [12, 22, 39].

This work was supported in part by awards to D.M.M. from the Phi Beta Psi Sorority and to D.M.M. and D.J.M. from Pharmanex.

References

1. Brightman AO, Wang J, Miu RK-M, Sun IL, Barr R, Crane FL and Morré DJ, A growth factor- and hormone-stimulated NADH oxidase from rat liver plasma membrane. *Biochim Biophys Acta* **1105:** 109–117, 1992.
2. Morré DJ and Brightman AO, NADH oxidase of plasma membranes. *J Bioenerg Biomemb* **23:** 469–489, 1991.
3. Morré DJ and Crane FL, NADH oxidase. In: *Oxidoreduction at the Plasma Membrane: Relation to Growth and Transport*. Vol. I. Animals (Eds. Crane FL, Morré DJ and Löw H), pp. 67–84. CRC, Boca Raton, FL, 1990.
4. Morré DJ, Crane FL, Eriksson LC, Löw H and Morré DM, NADH oxidase of liver plasma membrane stimulated by diferric transferrin and neoplastic transformation induced by the carcinogen 2-acetylaminofluorene. *Biochim Biophys Acta* **1057:** 140–156, 1991.
5. Bruno M, Brightman AO, Lawrence J, Werderitsh D, Morré DM and Morré DJ, Stimulation of NADH oxidase activity from rat liver plasma membranes by growth factors and hormones is decreased or absent with hepatoma plasma membranes. *Biochem J* **284:** 625–628, 1992.
6. Morré DJ, Morré DM, Paulik M, Batova A, Broome A-M, Pirisi L and Creek KE, Retinoic acid and calcitriol inhibition of growth and NADH oxidase of normal and immortalized human keratinocytes. *Biochim Biophys Acta* **1134:** 217–222, 1992.
7. Morré DJ, Brightman A, Wang J, Barr R and Crane FL, Roles for plasma membrane redox systems in cell growth. In: *Plasma Membrane Oxidoreductases in Control of Animal and Plant Growth. Proceedings of the NATO Advanced Research Workshop* (Eds. Crane FL, Löw H and Morré DJ), pp. 45–55. Alan R. Liss, New York, 1988.
8. Sun IL, Sun EE, Crane FL, Morré DJ, Lindgren A and Löw H, Requirement for coenzyme Q in plasma membrane electron transport. *Proc Natl Acad Sci USA* **89:** 11126–11130, 1992.
9. Morré DJ, Chueh P-J and Morré DM, Capsaicin inhibits preferentially the NADH oxidase and growth of transformed cells in culture. *Proc Natl Acad Sci USA* **92:** 1831–1835, 1995.
10. Morré DJ, Wu L-Y and Morré DM, Inhibition of NADH oxidase activity and growth of HeLa cells by the antitumor sulfonylurea, *N*-(4-methylphenylsulfonyl)-*N′*-(4-chlorophenyl)urea and response to epidermal growth factor. *Biochim Biophys Acta* **1355:** 114–120, 1997.
11. Weisburger JH, Tea and health: A historical perspective. *Cancer Lett* **114:** 315–317, 1997.
12. Chen ZP, Schell JB, Ho CT and Chen KY, Green tea epigallocatechin gallate shows a pronounced growth inhibitory effect on cancerous cells but not on their normal counterparts. *Cancer Lett* **129:** 173–179, 1998.
13. Blot WJ, Chow WH and McLaughlin JK, Tea and cancer: A review of the epidemiological evidence. *Eur J Cancer Prev* **5:** 425–438, 1996.
14. Kohlmeier L, Weterings KG, Steck S and Kok FJ, Tea and cancer prevention: An evaluation of the epidemiologic literature. *Nutr Cancer* **27:** 1–13, 1997.
15. Yang CS and Wang ZY, Tea and cancer. *J Natl Cancer Inst* **85:** 1038–1049, 1993.
16. Fujiki H, Suganuma M, Okabe S, Komori A, Sueoka E, Sueoka N, Kozu T and Sakai Y, Japanese green tea as a cancer preventive in humans. *Nutr Rev* **54:** S67–70, 1996.
17. Mitscher LA, Jung M, Shankel D, Dou J-H, Steele L and Pillai SP, Chemoprotection: A review of the potential therapeutic antioxidant properties of green tea (*Camellia sinensis*) and certain of its constituents. *Med Res Rev* **17:** 327–365, 1997.
18. Bushman JL, Green tea and cancer in humans: A review of the literature. *Nutr Cancer* **31:** 151–159, 1998.
19. Fujiki H, Suganuma M, Okabe S, Sueoka N, Komori A, Sueoka E, Kozu T, Tada Y, Suga K, Imai K and Nakachi K, Cancer inhibition by green tea. *Mutat Res* **402:** 307–310, 1998.
20. Liao S, Umekita Y, Guo J, Kokontis JM and Hiipakka RA, Growth inhibition and regression of human prostate and breast tumors in athymic mice by tea epigallocatechin gallate. *Cancer Lett* **96:** 239–243, 1995.
21. Stoner GD and Mukhtar H, Polyphenols as cancer chemopreventive agents. *J Cell Biochem* **22** (Suppl.)**:** 169–180, 1995.
22. Ahmad N, Feyes DK, Nieminen AL, Agarwal R and Mukhtar H, Green tea constituent epigallocatechin-3-gallate and induction of apoptosis and cell cycle arrest in human carcinoma cells. *J Natl Cancer Inst* **89:** 1881–1886, 1997.
23. Morré DJ, Isolation of Golgi apparatus, *Methods Enzymol* **22**: 130–148, 1971.
24. Morré DJ and Morré DM, Mammalian plasma membranes by aqueous two-phase partition. *Biotechniques* **7:** 946–958, 1989.
25. del Castillo-Olivares A, Yantiri F, Chueh P-J, Wang S, Sweeting M, Sedlak D, Morré DM, Burgess J and Morré DJ, A drug-responsive and protease-resistant peripheral NADH oxidase complex from the surface of HeLa S cells. *Arch Biochem Biophys* **358:** 125–140, 1998.
26. Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner RH, Provenzano EK, Fujimoto EK, Goeke NM, Olson BJ and Klenk DC, Measurement of protein using bicinchoninic acid. *Anal Biochem* **150:** 76–85, 1985.
27. Wolvetang EJ, Johnson KL, Krauer K, Ralph SJ and Linnane AW, Mitochondrial respiratory chain inhibitors induce apoptosis. *FEBS Lett* **339:** 40–44, 1994.
28. Katiyar SK, Agarwal R, Wang ZY, Bhatia AK and Mukhtar H, (−)-Epigallocatechin-3-gallate in *Camellia sinensis* leaves from Himalayan region of Sikkim: Inhibitory effects against biochemical events and tumor initiation in Sencar mouse skin. *Nutr Cancer* **18:** 73–83, 1992.
29. Morré DJ, Caldwell S, Mayorga A, Wu L-Y and Morré DM, NADH oxidase activity from sera altered by capsaicin is widely distributed among cancer patients. *Arch Biochem Biophys* **342:** 224–230, 1997.
30. Brightman AO, Barr R, Crane FL and Morré DJ, Auxin-stimulated NADH oxidase purified from plasma membrane of soybean. *Plant Physiol* **86:** 1264–1269, 1988.
31. Morré DJ, Crane FL, Barr R, Penel C and Wu L-Y, Inhibition of plasma membrane redox activities and elongation growth of soybean. *Physiol Plant* **72:** 236–240, 1988.
32. Morré DJ, Brightman AO, Wu L-Y, Barr R, Leak B and Crane FL, Role of plasma membrane redox activities in elongation growth in plants. *Physiol Plant* **73:** 187–193, 1988.
33. Morré DJ, NADH oxidase: A multifunctional ectoprotein of the eukaryotic cell surface. In: *Plasma Membrane Redox Systems and Their Role in Biological Stress and Disease* (Eds. Asard H, Bérczi A and Caubergs RJ), pp. 121–156. Kluwer, Dordrecht, 1998.
34. Kishi T, Morré DM and Morré DJ, The plasma membrane NADH oxidase of HeLa cells has hydroquinone oxidase activity. *Biochim Biophys Acta* **1412:** 66–67, 1999.
35. Katdare M, Osborne MP and Telang NT, Inhibition of aberrant proliferation and induction of apoptosis in pre-

neoplastic human mammary epithelial cells by natural phytochemicals. *Oncol Rep* **5:** 311–315, 1998.
36. Hale AJ, Smith CA, Sutherland LC, Stoneman VE, Longthorne VL, Culhane AC and Williams GT, Apoptosis, Molecular regulation of cell death. *Eur J Biochem* **236:** 1–26, 1996.
37. Sandu KB, Callsen D and Brüne B, Protection against nitric oxide-induced apoptosis in rat mesangial cells demands mitogen-activated protein kinases and reduced glutathione. *Mol Pharmacol* **56:** 744–751, 1999.
38. Dreosti IE, Bioactive ingredients: Antioxidants and polyphenols in tea. *Nutr Rev* **54:** S51–S58, 1996.
39. Pascha AG, Butler R and Young CY, Induction of apoptosis in prostate cancer cell lines by the green tea component, (−)-epigallocatechin-3-gallate. *Cancer Lett* **120:** 1–7, 1998.