JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Growth Inhibition of Prostate Cancer Cells by Epigallocatechin Gallate in the Presence of Cu^{2+}

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Green tea is an effective chemopreventive agent to human prostate cancer adenoma (PCA). Epigallocatechin gallate (EGCG) inhibited the growth of PCA cells and induced apoptosis. Cu^{2+} is a trace element necessary to our health. Many studies proved that bioactivity of EGCG is altered in the presence of Cu^{2+}. We investigated the effects of EGCG on PCA cells in the presence of Cu^{2+}. Also, we explored potential mechanisms via measurement of the relative chemiluminescence of growth medium for PCA cells. Chemiluminscence can be an indication of free radicals. Our test results showed that the addition of EGCG and Cu^{2+} to the growth medium decreased the relative viability of androgen-sensitive and androgen-insensitive human prostate cancer cells. However, the effects of EGCG on PCA cells depended on (1) the relative concentrations of added EGCG and Cu^{2+} and (2) their order of addition. Our results indicated that few free radicals may be generated in vitro. If so, free radicals generated intracellularly may be a major factor behind apoptosis and growth inhibition observed in the PCA cells. Thus, EGCG might exert its effects intracellularly.

KEYWORDS: Epigallocatechin gallate Cu^{2+}; PCA cells; chemiluminescence intensity; free radicals

INTRODUCTION

Prostate cancer adenoma (PCA) is the second leading cause of male mortality after lung cancer (*1*−*3*) worldwide. Some studies have found that tea extracts or catechins have antitumor effects in cells and animals (*4*−*6*). Epidemiological studies have shown that, compared to Western countries, the incidence of prostate cancer in Asian countries is relatively low (*7*−*8*). The reduced incidence of prostate cancer in Asian countries may be due in part to greater consumption of green tea. Epigallocatechin gallate (EGCG), is the most important bioactive component of green tea. Many of its biological activities have been reported, including antimutagenic, antibacterial, hypocholesterolemic, antioxidant, antitumor, and cancer preventive properties (*9*−*11*). Shutsung et al. showed that injections of EGCG rapidly reduced the size of human breast and prostate tumors in nude mice (*12*). Other studies found that EGCG induced apoptosis of prostate cancer cells and inhibited their growth (*13*−*14*). Meantime EGCG can inhibit activity of 5α-reductases, an important enzyme relative to prostate cancer (*15*).

Copper ion is an essential trace element for health. In turn, an imbalance in metabolism of copper ion could be an etiologic factor for prostate cancer. In the presence of copper ion, the bioactivity of EGCG is also altered, as demonstrated by in several reports (*16*−*17*). In the presence of copper ions, EGCG may be converted from a potential antioxidant into a pro-oxidant, and subsequently produce free radicals. Although the interactions of EGCG with copper ion have been investigated in several nonliving systems, mechanistic studies on cells have not been previously reported.

In this study, we have attempted to investigate effects of EGCG on the growth of androgen-sensitive and androgen-insensitive human prostate cancer cells in the presence of Cu^{2+}. A major objective has been to clarify the biochemical effects of EGCG and copper ion on the growth of PCA cells.

MATERIALS AND METHODS

Materials. Purified preparation of EGCG was obtained from Sigma (St. Louis. MO). The human PCA cells, namely PC-3 and LNCaP, were obtained from the Shanghai Institute of Cell Biology, Chinese Academy of Sciences. All other chemicals were of analytical grade.

Cell Culture. Both cell lines were cultured in F-12 medium (GIBCO, Invitrogen Corporation, Carlsbad, CA) supplemented with 10% fetal bovine serum and 100 U/mL penicillin−streptomycin. The cells were maintained at 37 °C and 5% CO_2 in a humid environment (Shellab, Cornelius, OR).

Growth Suppression by MTT Assay. EGCG was dissolved in double-distilled water. Cells were seeded on a 96-well plate for 48 h and then incubated with different concentrations of $CuSO_4$ or EGCG for 24 h. (The concentration of $CuSO_4$ in F-12 medium was 0.00064 μM, which was not considered further with regard to the concentrations of chemicals added (i.e., up to 640μM $CuSO_4$ and up to 500 μM EGCG). A relatively wide scale of metal ion and ligand concentration was used, an approach used in other reports as well (*18*−*20*)). Thereafter, 20 μL of 5 mg/mL MTT (3-[4,5-dimethythiazol-2-yl]-2,5-

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10.1021/jf035057u CCC: $27.50 © 2004 American Chemical Society
Published on Web 01/15/2004

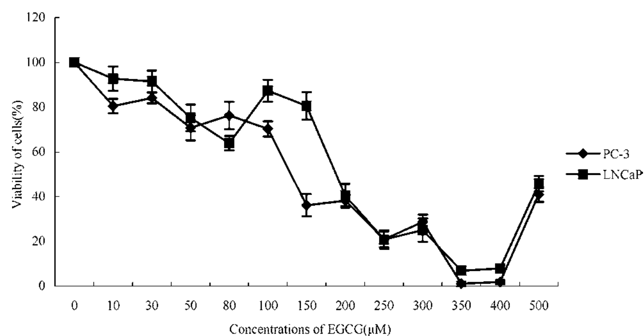

Figure 1. Cytotoxicity of EGCG on PC-3 and LNCaP PCA cell by MTT assay ($n = 7$).

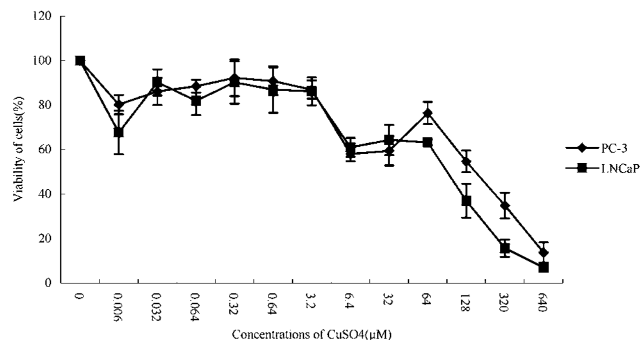

Figure 2. Cytotoxicity of Cu^{2+} on PC-3 and LNCaP PCA cell by MTT assay ($n = 7$).

diphenyltetrazolium bromide) was added, and the cells were incubated for 4 h (to allow the formation of formazan precipitate, which subsequently was dissolved in dimethyl sulfoxide). The absorbance in each well was then measured with a microplate reader (Thermal Lab system, Finland) at 490 nm. In addition, we investigated the interaction between EGCG and $CuSO_4$. The cells were incubated with EGCG for 30min; thereafter, Cu^{2+} was added to the cell. In other experiments, the order of the addition of EGCG and $CuSO_4$ was reversed. Cells were incubated for 24 h, and cell viability was determined with the MTT assay.

Estimation of Free Radical Activity with a Chemiluminescence Analyzer. To control F-12 medium at 37 °C and 5% CO_2 in a humid environment, different concentrations of $CuSO_4$ or EGCG were added for 0, 12, and 24 h. The chemiluminescence intensity was determined with a chemiluminescence analyzer (Lumat LB 9507, EG&G Berthold, Germany). Chemiluminescence intensity of F-12 medium containing mixture of EGCG with $CuSO_4$ was investigated as well. Following the addition of various concentrations of EGCG to F-12 medium, $CuSO_4$ was then added at one of several different concentrations. In another set of experiments, the order of addition of EGCG and $CuSO_4$ was reversed.

RESULT AND DISCUSSION

Viability of PCA Cells. *Effects of EGCG and $CuSO_4$ on Viability of PC-3 and LNCaP PCA cell.* MTT assay demonstrated that EGCG or $CuSO_4$ treatment of androgen-insensitive PC-3 and androgen-sensitive LNCaP PCA cells resulted in a maximum loss of cell viability at 24 h (data was not shown). EGCG (10−400 μM) and $CuSO_4$ (0.0064−640 μM) treatments caused dose-dependent increases in the inhibition of cell growth, respectively, in both PC-3 and LNCaP PCA cells, as compared to untreated controls. When the concentration of EGCG reached 500 μM, the viability of PC-3 and LNCaP PCA cells was increased (**Figures 1** and **2**). EGCG and Cu^{2+} inhibited the growth of PCA cells, and EGCG had antitumor effects at certain concentrations, results that comport with another report (*21*). On the other hand, at high doses (>500 μM), EGCG accelerated the proliferation of PCA cells.

Effects of EGCG on the viability of PCA cells in the presence of Cu^{2+}. Following the incorporation of 30 μM EGCG into the cell, which was performed after first adding one of several concentrations of Cu^{2+}, the viability of LNCaP PCA cell was significantly increased when the concentrations of added Cu^{2+} were 0.0064, 64, and 320 μM (**Figure 3A**). At these concentrations, EGCG did not protect the PC-3 PCA cell from the Cu^{2+}-ion-induced damage. At higher doses of added EGCG (150−200 μM), this tea constituent accelerated the Cu^{2+}-ion-induced damage even more (**Figure 3B**). Thus, EGCG generally inhibited the growth of the PC-3 PCA cell in the presence of copper ion. However, when certain concentrations of Cu^{2+} were added beforehand, the effects of subsequent additions of EGCG at varied concentrations and on different PCA cell lines were markedly different. At low doses, EGCG decreased the viability of LNCaP cells. At concentrations of EGCG approaching 300 μM, the viability of LNCaP cell was sharply increased (**Figure 3C**). In contrast to its effects on LNCaP cells, EGCG increased the viability of PC-3 cell regardless of the concentration (**Figure 3C**).

The Effects of EGCG on Viability of PCA Cell when Cu^{2+} was Added Last. The aforementioned cell lines were treated with different concentrations of EGCG for 30 min, after which 32 μM Cu^{2+} was added; the viability of LNCaP PCA cell decreased (**Figure 4A**). The subsequent addition of Cu^{2+} could not accelerate the EGCG-induced damage unless the concentration of EGCG exceeded 250 μM (**Figure 4A**). In other words, addition of copper ion accelerated the EGCG-induced damage of cells. The sensitivity of two PCA cell lines was markedly different, however. In the presence of certain concentrations of EGCG, the cytotoxicity of Cu^{2+} was decreased (**Figure 4B**). We infer that Cu^{2+} reacts with EGCG possibly by chelation, and the effects of the metal ion on the cells are subsequently decreased.

Determination of Free Radicals. *Changes in the Chemiluminescence Intensity of F-12 Medium Following the Addition of EGCG and Cu^{2+}.* The chemiluminescence intensity of F-12 medium containing $CuSO_4$ was dose-dependent at 0 and 24 h. At 12 h however, the chemiluminescence intensity was the same regardless of the concentration of $CuSO_4$ used. In addition, the chemiluminescence intensity of F-12 medium, at different concentrations of $CuSO_4$, was maximal at 12 h (**Figure 5**). The effect of EGCG on chemiluminescence intensity of F-12 medium was dose-dependent yet time-independent (**Figure 6**). EGCG induced oxidative stress at high doses. This result was consistent with those obtained with MTT assay. In the absence of EGCG or an equivalent chemical, the auto-oxidation of Cu^{2+} in this system may not readily occur.

Effects of EGCG on the Chemiluminescence Intensity of F-12 Medium, in the Presence of Cu^{2+}. When 200 μM EGCG was added last, the chemiluminescence intensity of F-12 medium was sharply increased. In addition, the changes in chemiluminescence intensity were maximal at 0 h. We infer that the added copper ion was oxidized, and in turn, free radicals were produced. The incorporation of 30 μM EGCG, after prior addition of varied concentrations of Cu^{2+}, caused the chemiluminescence intensity of F-12 medium to decrease at 0 and 12 h; conversely, the chemiluminescence intensity increased at 24 h. EGCG promoted increases in the chemiluminescence intensity of F-12 medium only slightly at 0 h if 32 μM $CuSO_4$ had been added first. The chemiluminescence intensity of F-12 medium at 12 and 24 h were the same, however, and did not change even if EGCG was added at different concentrations

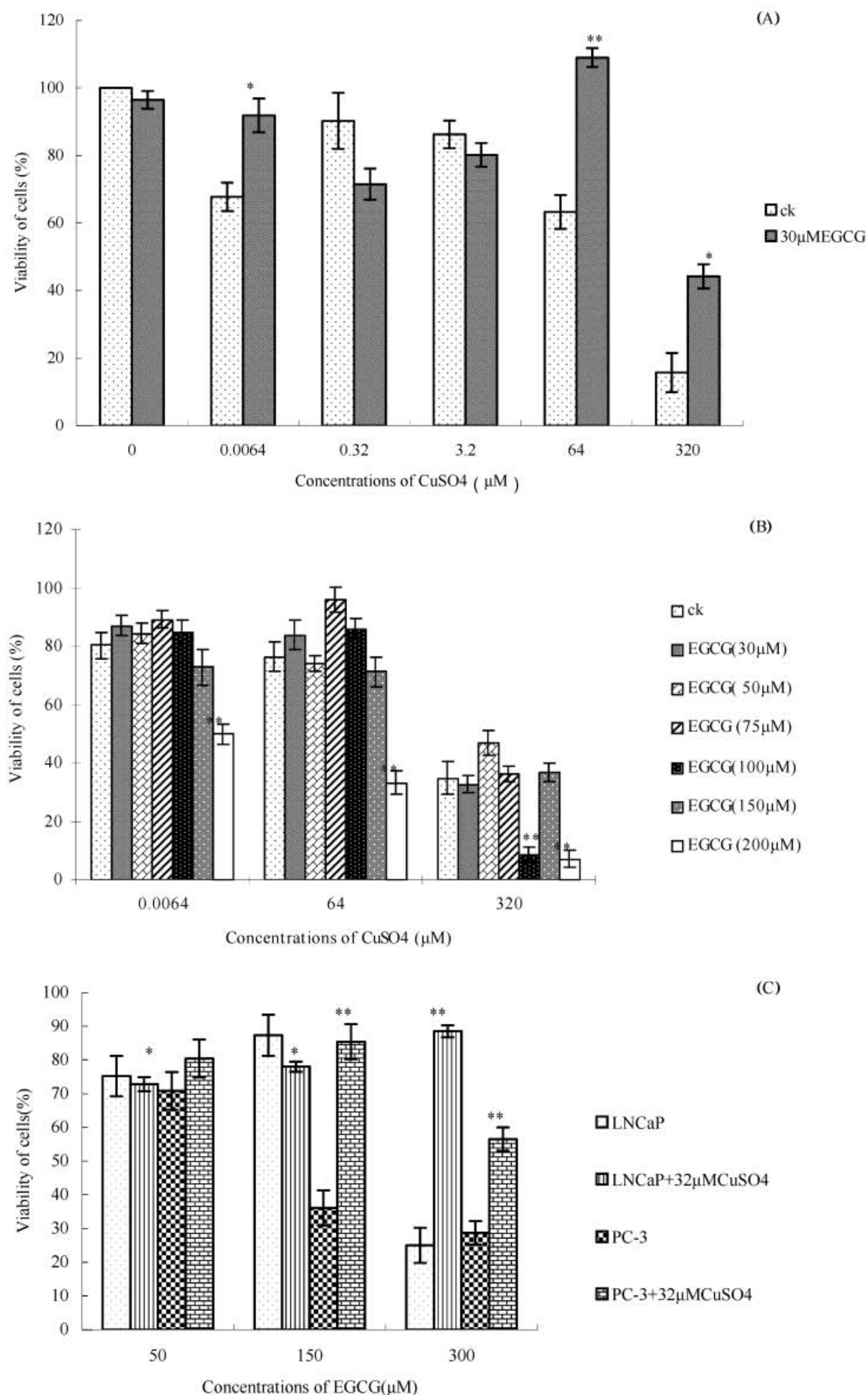

Figure 3. Effects of EGCG on viability of LNCaP (A,C) and PC-3 PCA cell (B,C) in the presence of Cu^{2+}. A, B, added different concentrations of Cu^{2+}, then mixed with certain concentrations of EGCG; C, added certain concentrations of Cu^{2+}, then mixed with different concentrations of EGCG. (*, $p < 0.05$, **, $p < 0.01$).

(**Figure 7**). The chelation of copper ion by EGCG might account for many of our experimental findings.

The Effects of EGCG on Chemiluminescence Intensity in F-12 Medium when Cu^{2+} Was Added Last. At 0 h, 32 μM Cu^{2+} increased the chemiluminescence intensity of F-12 medium in the presence of different concentrations of EGCG. However, at 12 and 24 h, the chemiluminescence intensity was decreased and minimal at 12 h. At 0 h, EGCG appeared to act as a pro-oxidant in the presence of copper ion. When 30 μM EGCG had been added first, the subsequent addition of $CuSO_4$ slightly decreased the chemiluminescence intensity of F-12 medium at 0 and 12 h. However, at 24 h, the presence of copper ion slightly increased the chemiluminescence intensity of F-12 medium. Thus, the increased chemiluminescence intensity of F-12 medium was time-dependent. The effects of copper ion on EGCG were very different when 200 μM EGCG was added

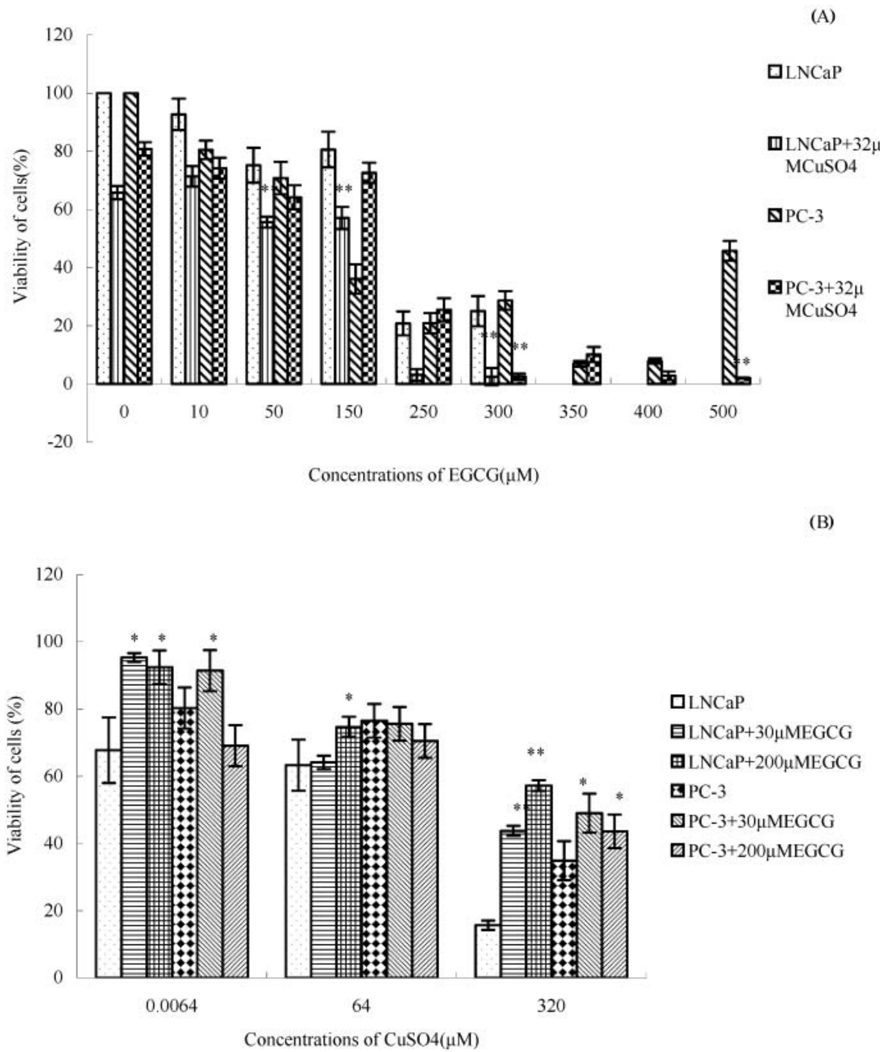

Figure 4. Cytotoxicity of EGCG on LNCaP and PC-3 PCA cell when Cu^{2+} was added last. A, added different concentrations of EGCG, then mixed with certain concentrations of Cu^{2+}; B, added certain concentrations of EGCG, then mixed with different concentrations of Cu^{2+}. (*, $p < 0.05$, **, $p < 0.01$).

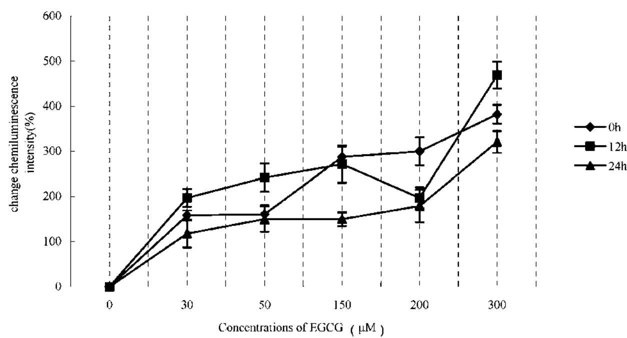

Figure 5. Dose-independent and time-independent chemiluminescence intensity of F-12 medium added different concentrations of EGCG. ($n = 5$).

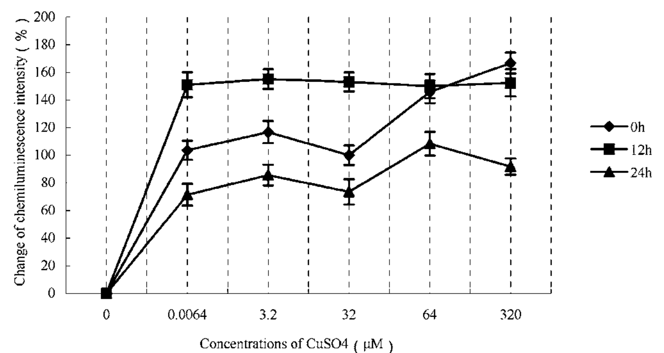

Figure 6. Dose-independent and time-independent chemiluminescence intensity of F-12 medium added different concentrations of Cu^{2+}. ($n = 5$).

first. At 12 h, the chemiluminescence intensity of F-12 medium was decreased slightly. At 0 and 24 h, chemiluminescence intensity of F-12 medium was very different when concentration of $CuSO_4$ was changed. The change in chemiluminescence intensity appears to be dependent on the relative ratio of EGCG and copper ion, which is consistent with the MTT assay results.

EGCG is reported to act as pro-oxidant in certain nonliving systems, especially in the presence of Cu^{2+} (*22*−*23*) ; free radicals were also detected in related studies (*21*) . In turn, we have investigated the interaction of EGCG and Cu^{2+} at pH values (7.2−7.4) that PCA cells encounter in vitro. With changing of concentrations and orders of EGCG and Cu^{2+}, their interaction was altered. We infer that, depending on the particular conditions, EGCG may act as a pro-oxidant, antioxidant, and—in the presence of copper ion—as a metal chelator (*24*) . In some studies, the induction of apoptosis by EGCG might be related to on the formation of reactive oxygen species. With the MTT assay, we found that EGCG and copper ion inhibited the growth of PCA cell lines. In this study, the

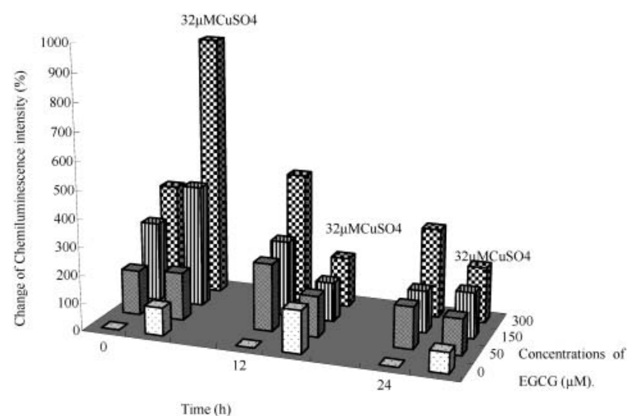

Figure 7. Effects of EGCG on chemiluminescence intensity of F-12 medium with different concentrations of EGCG in the presence of 32 μM Cu^{2+}. ($n = 5$).

chemiluminescence intensity in the medium might correspond to the relative viability of the PCA cell line. On the other hand, the results of our chemiluminescence assays provided little evidence that significant quantities of free radicals had been produced in the (extracellular) medium, a finding which seems to be at odds with that reported by others in nonliving systems (*21*) . The F-12 Medium used in this study is a complex system. In turn, EGCG and copper might interact with additional metal ions and ligands of system, respectively, which could also inhibit the production of free radicals. In view of the multidentate structure of EGCG, one cannot rule out the formation of high molecular weight (insoluble EGCG chelates of Cu^{2+} a priori; if such structures were formed in vivo, the local concentration of the metal ion complexes (e.g., precipitated on cell surfaces) could be integral to the unknown modes of action of these chemicals. One can anticipate an array of metal−ligand complexes may be formed in vivo, some of which may undergo various degrees of oxidation and/or polymerization. We conclude that extracellular free radicals were not the main factor behind the inhibition of PCA cell growth apoptosis; if so, EGCG and its derivatives and complexes might have intracellular actions in our experiments.

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Received for review September 16, 2003. Revised manuscript received December 4, 2003. Accepted December 10, 2003.

JF035057U