Green Tea Extract (Epigallocatechin-3-Gallate) Reduces Efficacy of Radiotherapy on Prostate Cancer Cells

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OBJECTIVES To assess the influence of epigallocatechin-3-gallate (EGCG) on the efficacy of ionizing radiation on prostate cancer cells because of the increased use of dietary interventions, especially by patients with prostate cancer. Radiotherapy is used to treat localized prostate cancer. Some people consume green tea (EGCG) as a chemopreventive agent against prostate cancer. Green tea can act as an antioxidant and induce superoxide dismutase enzymes, which could scavenge the free oxygen radicals generated by radiotherapy.

METHODS Prostate cancer cell line DU145 cells were treated with EGCG or radiotherapy, or both. Cell death was assessed using trypan blue cell counting, and apoptosis was confirmed by assessing poly (adenosine phosphate ribose) polymerase cleavage. The antioxidant potential was assessed using Western immunoblotting for manganese superoxide dismutase and copper zinc superoxide dismutase enzymes. Radiotherapy was delivered using a linear accelerator. Cell cycle analysis was performed using flow cytometry.

RESULTS Radiotherapy at 3.5 Gy induced a 5.9-fold increase in apoptosis of DU145 cells. Subapoptotic doses of EGCG (1.5-7.5 μM) significantly reduced ionizing radiation-induced apoptosis ($P < .001$), with the inhibitory effect of EGCG on ionizing radiation being most effective when added 30 minutes before radiotherapy ($P < .001$). In addition, when radiotherapy and EGCG were used together, an approximate 1.5-fold increase in manganese superoxide dismutase levels was seen compared with the control and a 2-fold increase compared with radiotherapy alone.

CONCLUSIONS Radiotherapy is effective in inducing apoptosis in DU145 cells, but its effect was significantly reduced in the presence of EGCG, and this was associated with an increase in the induction of manganese superoxide dismutase. UROLOGY 78: 475.e15–475.e21, 2011. © 2011 Elsevier Inc.

Prostate cancer (CaP) is the second leading cause of cancer-related deaths among men in Western countries and thus represents a major and growing health problem. High-grade prostate intraepithelial neoplasia has been identified as a premalignant condition of CaP,[1] and it is known that some of these cases will progress to CaP within 1 year on repeated biopsy.[2,3] When organ-confined CaP develops, radical prostatectomy and radiotherapy are the primary therapeutic options. Radiotherapy is also used as palliative treatment of CaP. Once the CaP has spread to local and distant sites, hormonal therapy is used, but advanced CaP eventually becomes refractory to this treatment, and the disease recurs.

Lifestyle-related factors, in particular, a diet rich in fat, are considered the major contributors to CaP initiation and progression. Dietary components such as flavanols and theophenols have been proposed as chemopreventive agents in CaP. Chemopreventive agents might act by decreasing cell proliferation, inducing apoptosis and cell cycle arrest, and it has been suggested that they could act as antioxidants.[4,5] Dietary antioxidants have been defined by the Institute of Medicine of the U.S. National Academy of Sciences (Food and Nutrition Board 2000, available online at: http://www.nutrisci.wisc.edu/NS623/drivitcsum.pdf) as "substances in food that significantly decrease the adverse effect of reactive [oxygen] species (ROS) on normal physiological function in humans."

Oxidative modifications of the DNA bases occur naturally but are also induced by free oxygen radicals created by ionizing radiation (IR). The accumulation of ROS, including the superoxide free radical ($O2^-$), can damage

This study was supported by the Ralph Shackman Trust, Cambridge, UK, and University of Bristol, UK.

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Submitted: October 16, 2010, received (with revisions): March 16, 2011

doi:10.1016/j.urology.2011.03.031

membrane lipids, proteins, and DNA.[6] The exposure of cells to IR leads to the formation of ROS that are associated with radiation-induced cytotoxicity.[7] Leach et al[8] demonstrated increased ROS concentrations in DU145 cells after 1-10 Gy IR.[8] Normal cells have their own defense mechanisms to protect the cells from free oxygen radicals. Superoxide dismutase (SOD) is an antioxidant enzyme that catalyzes the conversion of the $O2^-$ into hydrogen peroxide and elemental oxygen and, as such, is considered an important antioxidant in aerobic cells and therefore would protect against the effects of radiation.[9] The first factor of enzymatic defense in the cell cytosol compartment is CuZnSOD and the mitochondrial factor is manganese superoxide dismutase (MnSOD).[10] In 2001, Salganik[11] suggested that excessive nutrient antioxidants could adversely affect cancer treatment by interfering with the initial induction of apoptosis by ROS. The level of antioxidant enzymes is lower in prostate adenocarcinoma than in benign prostate cells; however, a study by Baker et al[12] has shown metastatic lesions from primary CaP had higher levels of MnSOD and nuclear oxidative damage products than did primary tumors. In contrast to this, a study by Bostwick et al[13] of prostatic tissue sections showed no apparent differences among benign epithelium, prostatic intraepithelial neoplasia, and CaP. The level of SOD can be altered when the cells are exposed to oxidative stress and also by various dietary antioxidants.

Of all the dietary components, good evidence has shown that epigallocatechin-3-gallate (EGCG), the active component of green tea, exerts chemopreventive effects in the development and progression of CaP.[14] EGCG has been proposed to exert its chemopreventive effects at least in part by being an antioxidant[15] and scavenging the free oxygen radicals generated by radiotherapy to induce cell death. Neutralization of free radicals by dietary antioxidants could therefore potentially decrease the effectiveness of radiotherapy.

The American Cancer Society Work Group[16] has recommended the following advice with regards to this issue: "Unfortunately, this is one of the many critical questions without a good answer at this time. Therefore, it would be prudent to advise patients undergoing chemo- or radiotherapy not to exceed the upper intake limits of the Dietary Reference Intakes (Food and Nutrition Board 2000) for vitamin supplements and to avoid other nutritional supplements that contain antioxidant compounds." The growing popularity of healthy foods and complimentary therapies raises the general importance of these questions. However, unfortunately, the question remains unanswered for a number of cancers, including CaP. With the increased use of dietary interventions, especially by patients with CaP, in the present study, we considered the influence of EGCG on the efficacy of IR against CaP cells.

MATERIAL AND METHODS

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO), unless otherwise stated. The copper zinc superoxide dismutase (CuZnSOD) and MnSOD antibodies were purchased from Stressgen Biotechnologies, Ann Arbor MI).

Cell Lines

DU145 (androgen nonresponsive; purchased from American Type Culture Collection (Manassas, VA) CaP cells were grown in T25 flasks in a humidified 5% carbon dioxide atmosphere at 37°C using standard cell culture techniques. The cells were maintained in Roswell Park Memorial Institute 1640 cell culture media (Lonza, Basel, Switzerland) supplemented with 10% fetal bovine serum (GIBCO, Paisley, UK), penicillin (50 IU/mL, Britannia Pharmaceuticals, West Berkshire, UK), and streptomycin (50 μg/mL, Sigma-Aldrich) and 1% L-glutamine solution (2 mM, Sigma-Aldrich) growth media. The serum free media (SFM) was made by supplementing Dulbecco's Modified Eagle Medium F-12 tissue culture media with penicillin (50 IU/mL, Britannia Pharmaceuticals), streptomycin (50 μg/mL), 1% L-glutamine solution (2 mM), sodium bicarbonate (1 mg/mL), bovine serum albumin (0.2 mg/mL), and transferrin (0.01 mg/mL).

Measuring Cell Death and Apoptosis

Floating cells were collected and mixed with the adherent cells after trypsinization, and the resulting cell suspension was loaded onto a hemocytometer (1:1) with the trypan blue dye, which is taken up by dead cells. Both viable and dead cells were counted, from which both the percentage of dead cells and total cell number were calculated. Apoptosis was confirmed by assessing the induction of poly (adenosine phosphate ribose) polymerase (PARP) cleavage as described in the "Western Immunoblotting" section.

Radiotherapy

The cells were grown in T25 flasks for 24 hours in growth medium and then changed to SFM for an additional 24 hours. The cells were then dosed with EGCG at 3 hours, 1 hour, and 0.5 hour before radiotherapy by spiking the SFM with the required EGCG dose. The cells were then transported to the radiotherapy unit in polystyrene boxes to maintain the adequate temperature. The flasks were laid flat on the linear accelerator, and the top of the flask was covered with Scan Plas (tissue equivalent) in 1.5-cm thickness. Radiotherapy using a linear accelerator was given at a dose of 0.5-20 Gy at a focal source distance of 98 cm. A 35-cm × 35-cm field size was used for irradiation with a relative dose rate of 324 monitor units per minute for 3.5 Gy. Dosing with EGCG and RT was repeated every 24 hours for 72 hours. After which, the cells were counted using the trypan blue assay protocol.

Analysis of Cell Cycle Using Flow Cytometry

The distribution of DU145 throughout the cell cycle was assessed using flow cytometry at 72 hours after the last dose of IR, as described previously.[17] The cells were seeded at 0.2×10^6 cells/well in 6-well plates and dosed as reported in the previous section. For the analysis, trypsinized cells were fixed in 70% ethanol for a minimum of 24 hours before analysis by flow cytometry. The fixed cells were pelleted (6000 rpm, 5 minutes) and washed 3 times with phosphate-buffered saline (6000 rpm, 5 minutes). The supernatant was removed, and the cells were resuspended in reaction buffer (propidium iodide, 0.05 mg/mL; sodium citrate, 0.1%; RNase A, 0.02 mg/mL; and nonidet-40, 0.3%; pH 8.3) and incubated at 4°C for 30 minutes before

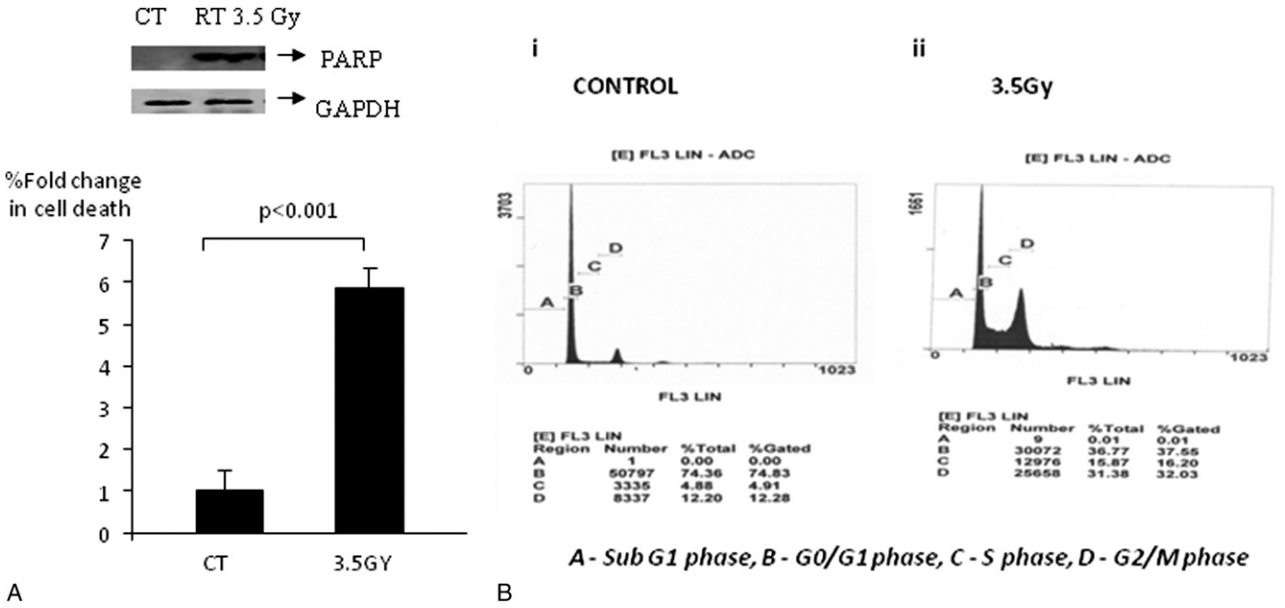

Figure 1. Response of DU145cells to radiotherapy. DU145 cells were exposed to radiotherapy at a dose of 3.5 Gy for 3 consecutive days. Viability of DU145 cells 72 hours after radiation measured using trypan blue dye exclusion assay. **(A)** Graph showing mean ± standard error of mean for 3 experiments, each performed in triplicate. Apoptosis confirmed by assessing PARP abundance. **(Insert)** Representative Western blot from experiments repeated 3 times of lysates from cells treated with radiotherapy for PARP, with anti-glyceraldehyde-3-phosphate dehydrogenase used as loading control. **(B)** Cell cycle analysis. Cells processed 72 hours after last dose of radiotherapy, and cell cycle analysis performed using flow cytometry. Graphs show G_2/M phase cell cycle arrest induced by radiotherapy compared with control.

measurement using a FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ). The data were analyzed using the Cell Quest Software package (BD Biosciences).

Western Immunoblotting

The cells were lysed (1 mL containing 10 μM Tris-HCl, 5 μM ethylenediaminetetraacetic acid, 50 μM NaCl, 30 μM sodium pyrophosphate, 50 μM sodium fluoride, 100 μM sodium orthovanadate, 1% Triton, and 1 μM phenylmethylsulfonyl fluoride, pH 7.6) and loaded according to the protein concentration of lysates as determined using a BCA Protein Assay Kit (Pierce, Thermo Fisher Scientific, Waltham, MA), separated on an 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel and transferred to Hybond N+ nylon membranes (Amersham, Bucks, UK), as previously reported.[18] Nonspecific binding sites on the nitrocellulose membranes were blocked overnight with 5% milk in Tris-buffered saline/2% Tween for probing with the anti-p85 cleaved subunit of PARP (1:750, Promega, Southampton, UK), anti-glyceraldehyde-3-phosphate dehydrogenase (1:1000, Chemicon, Hampshire, UK). Nonspecific-binding sites on the nitrocellulose membranes were blocked overnight with 5% bovine serum albumin in Tris-buffered saline/2% Tween for probing with MnSOD (1:3500, Stressgen Biotechnologies) and CuZnSOD (1:3500, Stressgen Biotechnologies). After the removal of excess unbound antibody, appropriate secondary antibodies conjugated to peroxidase in the same blocking agent used for the primary antibody were added for 1 hour. Binding of the peroxidase was visualized by enhanced chemiluminescence according to the manufacturer's instructions. Chemiluminescence was detected using the ChemiDoc-IT Imaging System (UVP, Bio-Rad, Hertfordshire, UK) and analyzed using Vision Works ls Analysis Software (UVP, Upland, CA).

Statistical Analysis

The data were analyzed using Microsoft Excel, version 5.0a (Redmond, WA) and analysis of variance followed by the least-significant difference post hoc test. A statistically significant difference was considered present at $P < .05$.

RESULTS

Effect of Radiotherapy on DU145 Cells

After the dose responses and time course experiments in response to IR (data not shown), we selected 3.5 Gy IR for 3 consecutive days for all subsequent experiments. This dose was close to the therapeutic dose of IR for the treatment of patients with CaP and induced an approximate 5.9-fold increase in cell death compared with the untreated cells (Fig. 1A). We confirmed this was apoptotic by showing an increase in the abundance of PARP cleavage (Fig. 1A, insert). Using flow cytometry, we also showed that, relative to the control cells, this dose of IR significantly increased the percentage of cells in the G_2/M phase, concomitant with a decrease in the number of cells in the G_0/G_1 phase (Fig. 1B), consistent with these cells being sensitive to radiation-induced cell death in the late G_2 phase of the cell cycle, as has been demonstrated previously.[19]

Effect of EGCG on Radiotherapy-Induced Apoptosis

Having established a 50% lethal dose of IR for this cell line, we next treated the cells with or without increasing subapoptotic doses of EGCG (1.5-7.5 μM; as determined

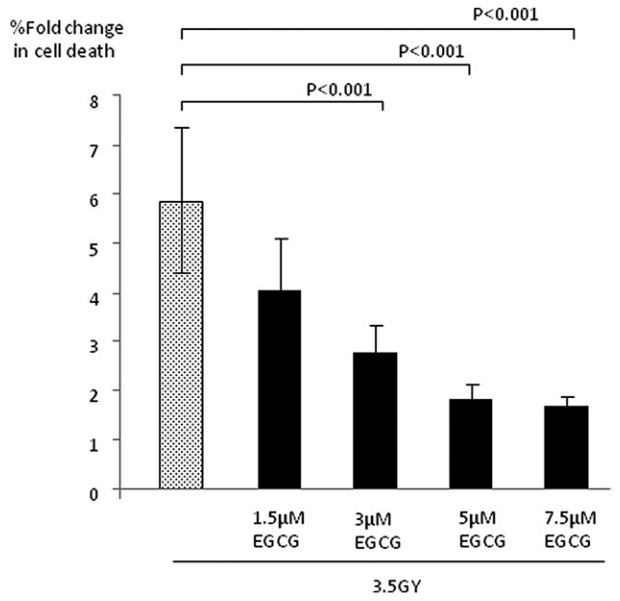

Figure 2. Effect of EGCG (1.5-7.5 μM) on radiotherapy-induced apoptosis. DU145 cells dosed with increasing doses of EGCG (1.5-7.5 μM) 0.5 hour before 3.5 Gy of radiotherapy for 3 consecutive days. Apoptosis measured 72 hours from last dose of IR using trypan blue dye exclusion assay. Graph shows mean ± standard error of mean of 3 experiments, each performed in triplicate.

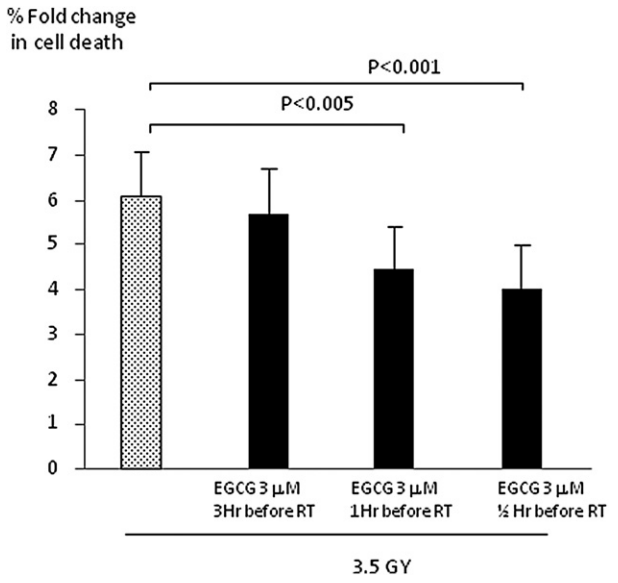

Figure 3. Effect of EGCG administered at different points on radiotherapy-induced apoptosis. DU145 cells were dosed with green tea (3 μM) at different intervals (3 hours to 0.5 hour) before radiotherapy (3.5 Gy) for 3 consecutive days. Cell death assessed using trypan blue dye exclusion assay. Graphs represent mean ± standard error of mean of 3 experiments, each performed in triplicate.

previously[20]), 30 minutes before radiotherapy with 3.5 Gy. We initially selected 30 minutes because EGCG in solution has a short half-life of 30-90 minutes.[15,21] Radiotherapy for 3 consecutive days induced a significant 5.9-fold increase in apoptosis in DU145 cells relative to the SFM control (Fig. 2). EGCG at subapoptotic doses (1.5-7.5 μM) did not induce cell death alone, and when EGCG was combined with IR, the x-fold change in IR-induced apoptosis relative to the respective EGCG control was dose-dependently reduced ($P < .001$; Fig. 2).

This established that pre-exposure of DU145 cells to subapoptotic doses of EGCG reduced IR-induced apoptosis. We investigated whether this was time dependent by adding EGCG at different points, ranging from 3 hours to 30 minutes before IR. Figure 3 shows that there was a significant ($P < .005$) 6-fold induction of apoptosis by IR alone. This induction of apoptosis by IR was reduced significantly in the presence of EGCG (3 μM), with the inhibitory effect of EGCG on IR most effective when added 30 minutes before IR ($P < .001$), and the effect was nonsignificant when added 3 hours before IR.

Effect of EGCG and Radiotherapy on Abundance of SOD Enzymes

Next, we assessed the level of MnSOD and CuZnSOD in DU145 cells exposed to IR with or without pre-exposure to EGCG. A significant reduction was found in the level of MnSOD with IR ($P < .05$; Fig. 4). Overall, MnSOD abundance was not significantly affected by EGCG alone at any point. However, when IR and EGCG were used in combination, an approximate 1.5-fold increase was seen in the MnSOD levels compared with the control and a 2-fold increase compared with IR alone. In contrast, we found that the levels of CuZnSOD in the cells treated with IR and EGCG alone or in combination were unaffected (data not shown).

COMMENT

Radiotherapy is an effective modality of treatment against CaP. The use of antioxidants such as green tea at treatment with IR is still controversial, because it might interfere with the induction of apoptosis induced by IR by scavenging free radicals. In the present study, we used physiologically relevant levels, which are achievable in the plasma of humans after EGCG consumption. The plasma EGCG concentration after regular green tea consumption is usually less than 1 μM, and the levels of EGCG we used in the present study were close to physiologic levels. Greater plasma levels of EGCG can be obtained after consumption of pharmacologic doses of EGCG supplements.[22-26] We also used low-dose IR using a linear accelerator, which again equates closely to the therapeutic dose used for treatment of CaP. Our results have shown that pre-exposure of EGCG at subapoptotic doses combined with IR significantly reduced the apoptotic effect of low-dose IR. This effect was also observed with oral cancer cells. EGCG, at concentrations that could be physiologically achievable in saliva, protected these cells against radiotherapy.[27]

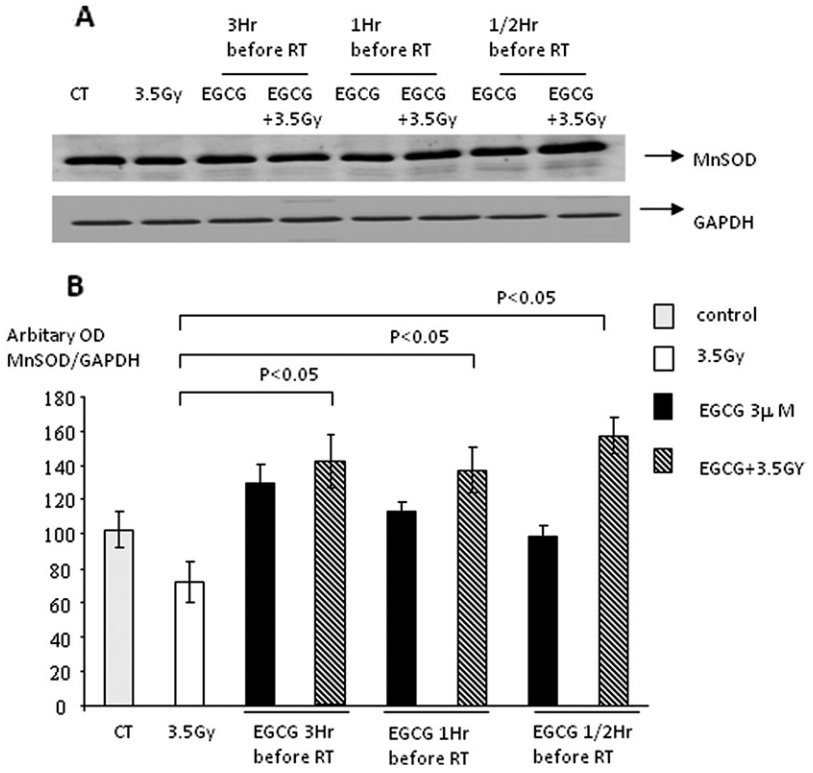

Figure 4. Western blot analysis of DU145 for MnSOD. **(A)** Cells pre-exposed to 3 μM of EGCG at 3 hours, 1 hour, and 0.5 hour before IR for 3 consecutive days. Cell lysates were prepared 72 hours after last dose of IR and probed for MnSOD and anti-glyceraldehyde-3-phosphate dehydrogenase. **(B)** Graph of mean optical density measurements ± standard error of mean for 3 repeat Western immunoblots showing x-fold induction of MnSOD matched to anti-glyceraldehyde-3-phosphate dehydrogenase.

One mechanism by which EGCG could reduce the effect of IR on apoptosis is by acting as an antioxidant and altering the level of SOD enzymes. Cytoplasmic and mitochondrial SOD play an important part in neutralizing the free oxygen radicals responsible for oxidative injury induced by external stresses, such as carcinogens, radiation, infection, or chemotherapy.[18,28,29] The levels of antioxidant enzymes are subject to change when exposed to various oxidative stresses.[30] A number of animal studies have shown that antioxidant enzyme activity in the presence of oxidative stress is prevented after administration of tea and tea polyphenols. Infection-associated decreases in erythrocyte SOD activity in mice infected with *Mycobacterium tuberculosis* were attenuated when fed with green tea extract.[28] Park et al[29] has shown that green tea polyphenols can act as biologic antioxidants in protecting osteoblasts from the oxidative stress induced by hydrogen peroxide. Providing rats with green tea extract in their drinking water has also been shown to attenuate ethanol-associated decreases in the serum and liver levels of SOD.[31]

A study by Vucic et al[19] showed that treatment with gamma-IR at supratherapeutic doses for 24, 48, and 72 hours in DU145 cells stimulated both CuZnSOD and MnSOD protein expression in a time-dependent manner, by approximately 3-3.5-fold[19] and culminated in resistance to IR. We found that the level of MnSOD was not affected by EGCG alone but was significantly decreased in the presence of a physiologic, apoptotic dose of IR. The combination of EGCG with IR reduced the ability of IR to decrease the MnSOD levels, and this correlated with a reduction in IR-induced apoptosis. DU145 cells are notoriously radioresistant and have high basal levels of MnSOD, as our Western blots confirmed. MnSOD is also known to be overexpressed in cancer cells.[32] We observed that therapeutic doses of gamma-IR resulted in a decrease in levels of MnSOD; however, exposure to EGCG effectively prevented this decrease. The efficacy of the radiotherapy might well have been due to a direct effect of EGCG, potentially as a direct antioxidant. The data, however, suggest that potential alternative mechanisms exist for the effect. EGCG reduced the radiation-induced suppression of MnSOD and preserved the antioxidant effects of this enzyme. This could have also contributed to the resistance to radiotherapy. Also, effects on other antioxidant pathways not examined have clearly not been ruled out.

We found no effect of either IR or EGCG on the abundance of CuZnSOD at any point examined. CuZnSOD is believed to play a key role in the first line of antioxidant defense; however, the major defense mechanism is achieved through induction of MnSOD. This was demonstrated in MnSOD knockout mice, which did not survive past 3 weeks of age. In contrast, CuZnSOD

knockout mice appeared normal showing that MnSOD is essential for life.[33] Studies of various human tissues have also shown that the abundance of MnSOD was roughly one half as large as that of CuZnSOD.[34] The differential levels of SOD enzymes in our study might be because CuZnSOD is expressed transiently in the early phase of IR and that MnSOD maintains a sustained effect even 72 hours after the last IR dose.[35] The differential expression of SOD enzymes has also been seen in tracheobronchial epithelial cells, in which MnSOD mRNA was selectively induced in vitro[30] and also in rat pulmonary epithelial cells.[36]

The present preliminary study using DU145 cells has shown that EGCG reduces IR-induced apoptosis concomitant with an increase in the abundance of MnSOD enzymes. EGCG could potentially scavenge the free oxygen radicals (ROS) produced by IR and thus reduce the effect of ROS available for the induction of apoptosis. The protection from cell death was most effective when the cells were exposed to EGCG for 30 minutes before IR but was not apparent when exposed for 3 hours. Our results, however, add to the evidence that dietary antioxidants could interfere with IR. Studies using other cell lines, animal studies, and clinical trials are warranted to ensure that patients can be appropriately advised about green tea consumption when undergoing radiotherapy for CaP.

Acknowledgment. To the staff of the radiotherapy department in Bristol Hematology and Oncology Centre, UK, with gratitude, for their help with the radiotherapy techniques and use of the machine.

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