Increased radiation sensitivity of an eosinophilic cell line following treatment with epigallocatechin-gallate, resveratrol and curcuma

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Abstract. Ionizing radiation is widely used in radiotherapy, in order to promote an apoptotic response in cancerous cells. Since the need to find new substances that would enhance the radiation-induced apoptosis in cancerous cells is great, we studied the effect of epigallocatechin-gallate (EGCG, a tea component), resveratrol (a wine component) and curcuma on cell proliferation and radiation-induced apoptosis in the human leukaemic cell line, EOL-1, derived from a patient with eosinophilic leukaemia. Cells were X-irradiated with 0, 2, 4, 6 or 8 Gy and cultured in the presence of EGCG, resveratrol or curcuma (concentrations ranging from 0 to 200 μM) for 1, 2 or 3 days of culture. Cell proliferation was measured using trypan blue exclusion. Apoptosis was evaluated using light microscopy (morphology study after May-Grünwald Giemsa staining) and flow cytometry (annexin-V staining). Irradiation alone induced a dose-related reduction in cell proliferation and the appearance of polyploid cells in EOL-1 cells. Additionally, EOL-1 cells underwent a dose-related increase of apoptosis which, from the second day on, was accompanied by a dose-related increase of necrosis. When cells were exposed to EGCG, resveratrol or curcuma alone, a decrease in cell proliferation was observed, beginning from 25 μM EGCG and 50 μM resveratrol and curcuma, while an increase in the percentage of apoptotic cells was noted from 50 μM EGCG, 100 μM resveratrol and curcuma in EOL-1 cells, after only one day of culture. Simultaneous exposure to X-irradiation and, EGCG, resveratrol or curcuma resulted in a synergistic decrease of cell proliferation as well as in a synergistic increase of apoptosis and necrosis. These results suggest that, depending on the concentration, EGCG, resveratrol and curcuma enhance radiation-induced apoptosis in the leukaemic cell line, EOL-1 (EGCG >resveratrol >curcuma). In order to further characterise the radiation-induced apoptosis of this leukaemic cell line, other flow cytometrical analyses are in progress.

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Introduction

Apoptosis is the most common form of eukaryotic cell death. It is a physiological suicide mechanism that preserves homeostasis, in which cell death naturally occurs during tissue turnover (1). In general, cells undergoing apoptosis display profound structural changes, including a rapid blebbing of the plasma membrane and nuclear disintegration. The nuclear collapse is associated with extensive damage to chromatin and DNA cleavage into oligonucleosomal-length DNA fragments after activation of calcium-dependent endogenous endonucleases (2). Apoptosis is essential in many physiological processes, including the embryonic development and the maturation of the immune system (3). It is currently the subject of intense research, partially because we now recognise that tumor cells are susceptible to death by apoptosis in response to drugs and/or radiation treatment. In the past few years, the interest for using apoptosis as a possible measure of radiosensitivity has increased substantially both with regard to the possibilities of using the extent of apoptosis as a biological dosimeter (4) and for estimating the radiosensitivity of cancer cells before radiotherapy (5-7). The status and level of expression of proteins that regulate apoptosis have even been proposed to serve as radiation exposure indicators or sensors.

Tea is the most popular and widely-consumed beverage in the world, after water, and is reported to possess beneficial health effects for humans (8). These beneficial effects are attributed to green tea polyphenols, mostly catechins, and have attracted considerable attention in recent years for preventing oxidative stress-related diseases, including cancer and cardiovascular and neurodegenerative diseases (9-12). Studies have verified that polyphenols in green tea are potent antioxidants, with the majority of beneficial effects elicited by epigallocatechin-3-gallate. Green tea contains several tea polyphenols, including epigallocatechin-3-gallate (EGCG), (-)-epigallocatechin (EGC), (-)-epicatechin-3-gallate (ECG),

(-)-epicatechin (EC), and (+)-catechin. The usual composition is 10-15% EGCG, 6-10% EGC, 2-3% ECG, and 2% EC, with EGCG being the main constituent. EGCG has been shown to possess antitumor promoting activity in several experimental models, which is attributed to its ability to block signal transduction pathways leading to carcinogenesis (13). In different studies, it was found that oral administration of EGCG or tea fractions inhibited the carcinogen-induced tumors of various organs in animals, including the duodenum, skin, liver, stomach, lung, esophagus and colon (8; 14-23). EGCG inhibits okadaic acid-induced TNF-alpha production and gene expression in BALB/3T3 cells (24). In studies on lung tumorigenesis induced by 4-(methylnitrosamino)-1-(3-pyridyl)-1-butadone, as well as skin tumorigenesis induced by chemicals and UV light in mice, EGCG preparations have been shown to inhibit carcinogenesis at the initiation, promotion and progression stages (16-19,25-27). Tea is one of the few agents known to inhibit carcinogenesis at the post-initiation stages. Nevertheless, the exact mechanisms underlying these activities are still elusive and mostly speculative. Antioxidant activities and interactions with certain enzymes or proteins implicated in cancer biology such as urokinase, ornithine decarboxylase, NADPH-cytochrome P450 reductase, protein kinase C, steroid 5 alpha reductase, DNA topoisomerase II, reverse transcriptase, TNF expression and more recently, nitric oxide synthase were postulated in several studies (24,28-34).

Resveratrol (*trans*-3,5,4'-trihydroxystilbene) is a major component of the polyphenols from grapes and red wine. Its synthesis is induced by stress conditions such as infection or trauma, UV irradiation, or exposure to ozone (35,36). Resveratrol is used for treatment of a wide variety of diseases including dermatitis, gonorrhea, fever, hyperlipidemia, arteriosclerosis, and inflammation (37). A number of studies have demonstrated the antioxidant effects of resveratrol (38) and its potential therapeutic applications in oxidative stress-related diseases such as cancer, arteriosclerosis, or the ischemic heart (39-43). Furthermore, it is capable of scavenging lipid hydroperoxyl free radicals, as well as hydroxyl and superoxide anion radicals.

Curcuma longa L. belongs to the family Zingiberaceae and has a long and distinguished human use in Eastern civilization. Its rhizome is used extensively for imparting color and flavor to foods. Turmeric, a powder from the dried rhizomes, is used for medicinal purposes and is reportedly used as an antiseptic, a cure for poisoning, to eliminate body waste products, for treating dyspepsia and respiratory disorders, as a cure for some skin diseases, including wound healing, and as a household remedy for treating sprains and swellings caused by injury (44). Curcuma (diferuloylmethane; 1,7-bis-(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione), which has been identified as the active component in turmeric, possesses antimicrobial, anticancer and topoisomerase-inhibitory activities, and has been reported to be an antioxidant as well as an anti-inflammatory agent (44-51). It has been demonstrated that topical application of curcuma inhibits benzo(α)pyrene-induced DNA adduct formation, and development of skin tumors as well as TPA[3]-induced epidermal DNA synthesis and tumor promotion in mouse skin (52-54). In addition, a tumoricidal activity of curcuma has been observed in a wide range of cell lines like NIH3T3, mouse sarcoma S180, human kidney cancer cell 293 (55), Chinese hamster ovary (47) and human basal cell carcinoma (56). Furthermore, curcuma has a strong inhibitory effect on cell proliferation in the HT-29 and HCT-15 human colon cancer cell lines (57). Importantly, dietary administration of curcuma during initiation and/or postinitiation periods significantly suppresses development of chemically-induced forestomach, duodenal and colon tumors in CF-1 mice (58); it also reduces formation of focal areas of dysplasia and aberrant crypt foci in the colon that are early preneoplastic lesions in rodents (46,53). Moreover, curcuma has been shown to induce apoptosis in numerous animal and human cells, although the cell death pathway depends very much on cell type (59-64). The pharmacological safety of curcuma is demonstrated by its consumption for centuries at up to 100 mg/day by people in certain countries (44). It has been found to reduce the number of cells with chromosomal aberrations, but protects normal cells from chromatid breaks due to exposure to γ-irradiation (65). However, the mechanisms underlying these diverse effects of curcuma are not fully understood. Among the possibilities, regulation of an array of cellular biochemical processes such as inhibition of nitric oxide synthase, receptor tyrosine kinase and protein kinase C (66-68) activities and the alteration of transcriptional factors c-*jun*/AP-1 and nuclear factor *K*B, p53 by curcuma have been suggested (69). Recently it has also been suggested that production of reactive oxygen intermediates may be the cause of tumor cell apoptosis as a result of curcuma treatment (59).

This report examines the usefulness of epigallocatechin gallate (EGCG), resveratrol and curcuma as modifying and enhancing agents of radiation-induced apoptosis. The effects of EGCG, resveratrol and curcuma were investigated on the EOL-1 cell line treated with a single X-ray dose (2 to 8 Gy). To test our hypothesis, we chose growth inhibitory activity and induction of apoptosis as parameters of synergistic effects between EGCG, resveratrol, curcuma and radiation. Here we present evidence that cotreatment with EGCG, resveratrol or curcuma and irradiation synergistically induced apoptosis of EOL-1 cells. These results strongly indicate that these three components enhance the killing of cancer cells induced by irradiation.

Materials and methods

Chemicals. EGCG, resveratrol, curcuma and propidium iodide (PI) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). EGCG, resveratrol and curcuma were of highest purity available (>98%) and were used without further purification. Stock of EGCG (1 mM), resveratrol or curcuma were made in water (for EGCG) or methanol (for resveratrol and curcuma) and protected from light. Concentrations from 0 to 200 μM were obtained by diluting stock solution of EGCG, resveratrol or curcuma in Dulbecco's modified Eagle's medium (DMEM). DMEM, foetal bovine serum (FBS), streptomycin, penicillin, phosphate buffer saline (PBS) were purchased from Invitrogen Life Technologies (Grand Island, NY, USA). May-Grünwald and Giemsa stock

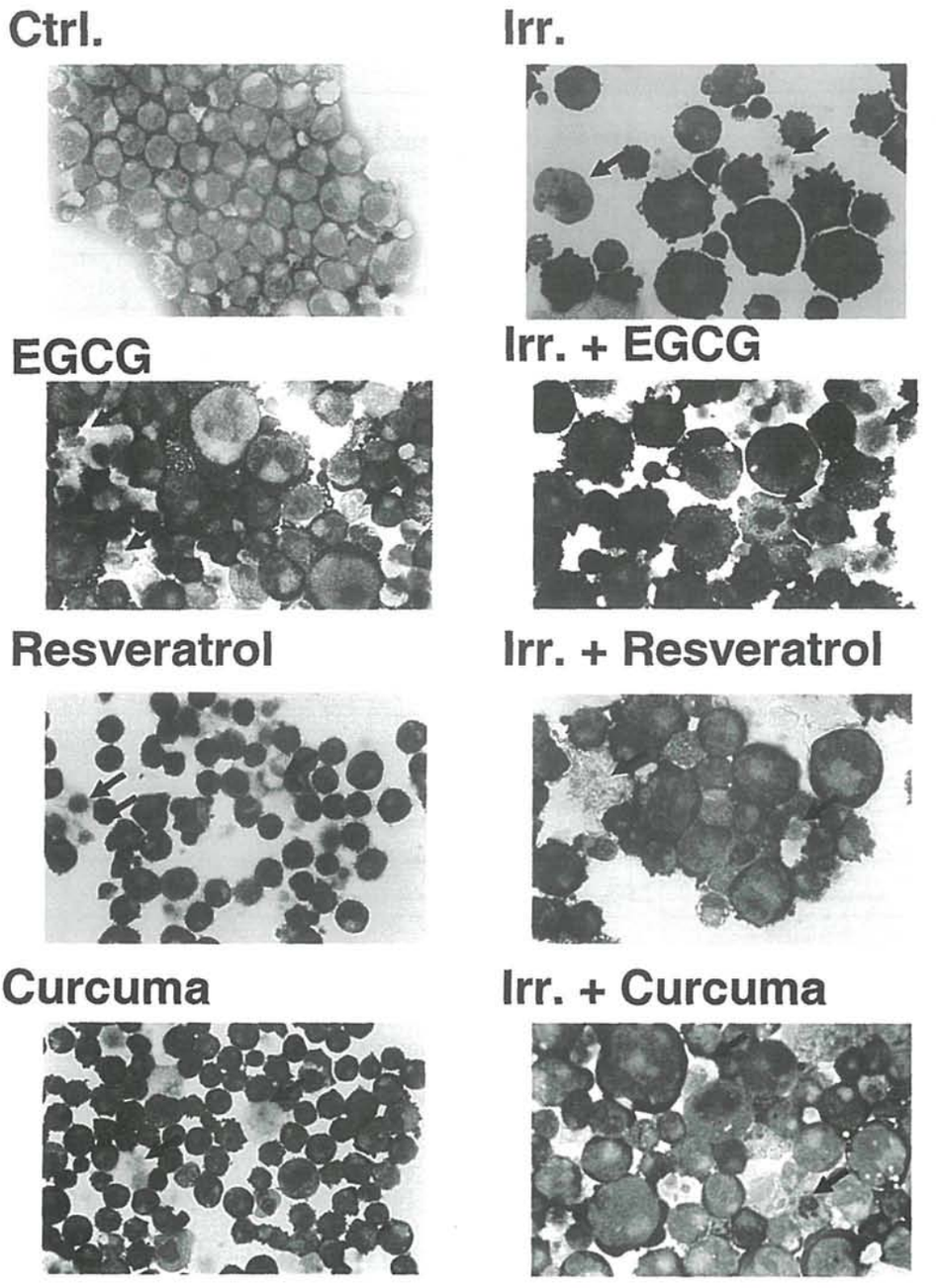


Figure 1. Morphology of a 3 day-culture of EOL-1 cells untreated, after treatment with EGCG, resveratrol or curcuma, after irradiation or both (EGCG, resveratrol or curcuma and irradiation). The concentrations of EGCG, resveratrol and curcuma represented were 200 µM. The doses of irradiation were 4 Gy. Cells were stained with May-Grünwald Giemsa. Representative slides for independent experiments are shown. The arrows show cell rounding and chromosome condensation in the early stage of apoptosis followed by degeneration. Endoreduplication is visible in irradiated cells with multilobular nuclei.

solutions were provided by Merck-Belgolabo (Overijse, Belgium). Solutions were prepared in water from a 'Milli-Q' system (Millipore).

Culture conditions. EOL-1 cells (derived from eosinophilic leukaemia) were obtained from American Type Culture Collection (ATCC, Rockville, MD, USA) and maintained in DMEM in 175 cm^3 flasks from Falcon (Becton-Dickinson, Franklin Lakes, NJ, USA) at 37˚C in a humidified incubator containing 5% CO_2/air. The DMEM medium was supplemented with 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin. Cells were sub-cultured at 1:3 ratio for routine maintenance and experiments were performed twice a week. Exponentially growing EOL-1 cells (growing in suspension) were plated at 10^5 cells/cm^2/ml just before adding EGCG, resveratrol or curcuma. Fresh medium (5 ml/well) containing different concentrations of EGCG, resveratrol or curcuma (from 0 to 200 µM) was added 1 h before the irradiation (0 to 8 Gy) and the culture of the cell line was continued for 1, 2 or 3 days.

Irradiation. Irradiation of the cells was performed with a Pantak HF420 RX machine operating at 250 kV, 15 mA, 1 mm Cu filtration and at a dose rate of 0.375 Gy/min. Dosimetry

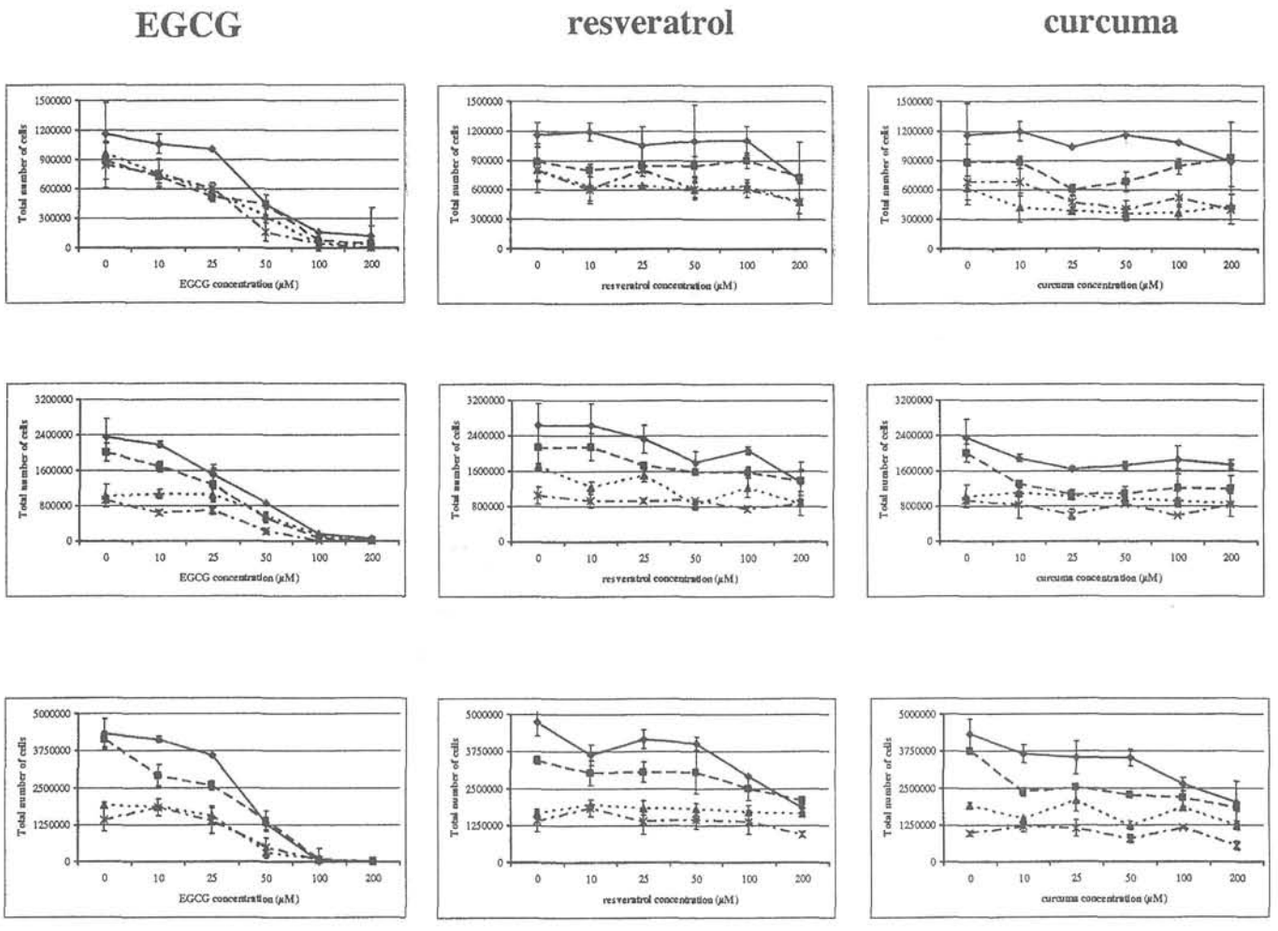


Figure 2. Growth inhibition of EOL-1 cells. Cells were treated with EGCG, resveratrol or curcuma at various concentrations: control, 10, 25, 50, 100 or 200 μM and unirradiated (♦) or irradiated with 2 (■), 4 (▲), 6 (x) Gy for 1, 2 or 3 days. The number of viable cells was counted, based on trypan blue dye exclusion. The means for three independent experiments ± SD are shown for days 1, 2 and 3.

was performed on a regular basis with an 0.6 cm^3 ionisation chamber (NE 2571), which was connected to a dosemeter (Farmer dosemeter 2570). The chamber was placed in parallel to the irradiated cell flasks. Dose homogeneity was evaluated as being <1.5%. Control cultures were not irradiated, but otherwise treated like the irradiated cultures.

Growth inhibition study. After 1, 2 or 3 days, numbers of viable cells were counted by the trypan blue dye exclusion test. For that, cells from each well were incubated with 0.2% trypan blue dye in 1 ml PBS for 10 min at room temperature. The number of viable cells and cells with dye-permeable plasma membrane, designated as dead, were estimated by the trypan blue exclusion test at time 0 (start of the incubation in the presence of EGCG, resveratrol and curcuma) as well as 24 (day 1), 48 (day 2) and 72 (day 3) hours for all 3 cell lines. At least 100 cells were counted in the Bürker haemocytometer at x100 microscope magnification for each sample. A Zeiss phase contrast microscope was used to visualise the cells (Nikon, Diaphot 300). The total number of cells (cell growth) was expressed in absolute numbers.

Microscopy. Briefly, for morphological observations, cells were first centrifuged on cytospine slides. Then, they were fixed with methanol absolute (5 min), stained with May-Grünwald (5 min in a mixing solution of 50% May-Grünwald and 50% buffer pH 6.2) and then with Giemsa (5 min in 16.6% of Giemsa and 83.4% of buffer pH 6.7). Buffer pH 6.2 consists of 1.9% (v/v) of Na_2HPO_4 ($6x10^{-2}$ M) and 8.1% of KH_2PO_4 ($6x10^{-2}$ M). Buffer pH 6.7 consists of 3.6 % Na_2HPO_4 ($6x10^{-2}$ M) and 6.4% KH_2PO_4 ($6x10^{-2}$ M). Cells were then rinsed in buffer pH 6.7 for 15 sec and in distilled water for 30 sec and dried. Cells were mounted with a glass coverslip with DePeX (EMS, Fort Washington, PA, USA) and analysed under a Zeiss microscope (Axioskop) and pictures were taken at x400 magnification.

Annexin-V and PI staining. For annexin-V and PI stainings, cells were collected and washed in PBS (with 1% FBS) and then stained according to the Annexin V-FITC kit (Immuno-Source, Halle-Voerdsel, Belgium) following the instructions of the manufacturer. Cells were then immediately analysed by flow cytometry.

Flow cytometry. Forward scatter, side scatter, green (annexin-V) and red (PI) fluorescences were measured using a flow cytometer equipped with an argon ion laser with an excitation wavelength at 488 nm. A threshold on the forward

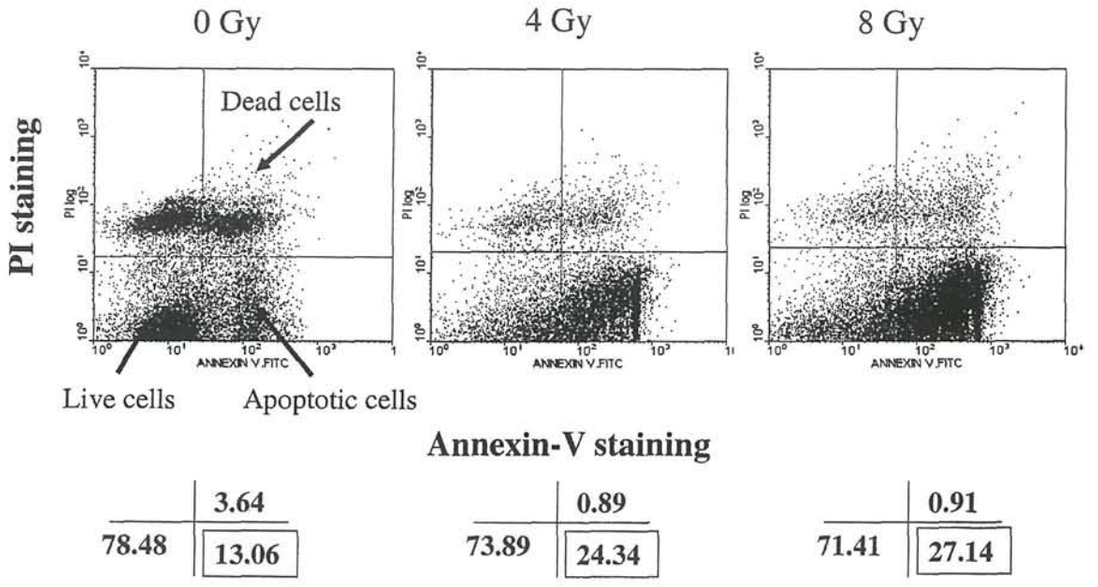


Figure 3. Flow cytometry analysis of annexin-V/PI stainings of EOL-1 after 3 days of culture. Cells were untreated or irradiated with 4 or 8 Gy. Annexin-V and PI stainings were performed as indicated in Materials and methods. Representative dot-plots for three independent experiments are shown.

scatter was used in order to not take into account the cell particles. A gate was made on the forward scatter and on the side scatter and the green and red fluorescences of the cells inside the gate were measured. At least 10,000 cells (twice) were analysed per condition in triplicates.

Statistical analysis. Results were expressed as mean ± standard deviation (SD). All experiments were performed in triplicates. The Student's t-test was performed to compare growth inhibition and induction of apoptosis by EGCG, resveratrol or curcuma and irradiation.

Results

Effect on cell morphology of EGCG, resveratrol or curcuma coupled with irradiation. Exponentially growing cells were treated with various concentrations of EGCG, resveratrol or curcuma for various lengths of time (1, 2 or 3 days) and X-irradiated with doses of 2, 4, 6 and 8 Gy. Fig. 1 shows May-Grünwald Giemsa images of EOL-1 cells treated with EGCG, resveratrol, curcuma and/or irradiated. In control cultures, EOL-1 cells contained a single nucleus rounded in shape.

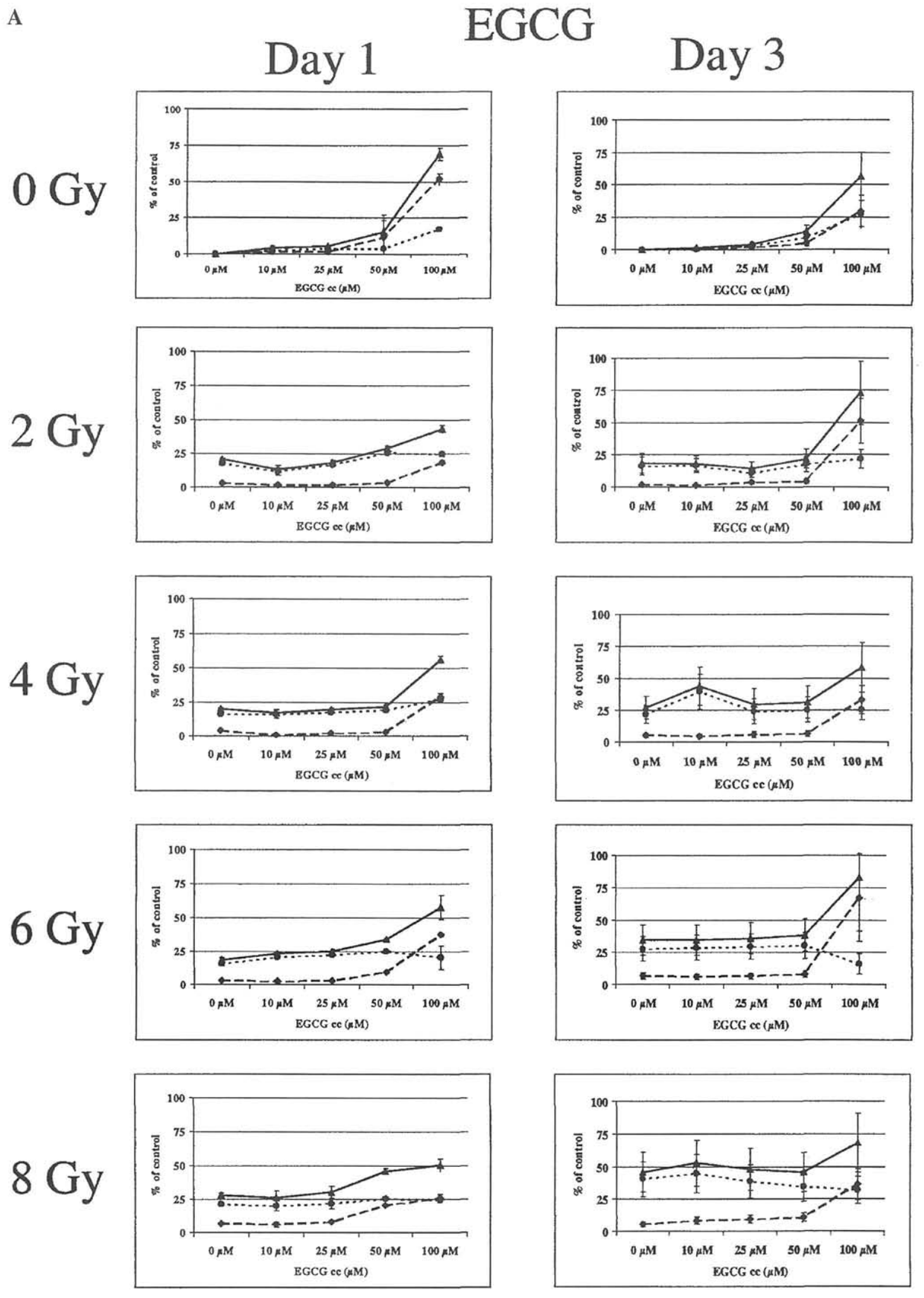
In comparison with control conditions, the treatment of EOL-1 cells with high concentration of EGCG, resveratrol or curcuma induced an increasing number of apoptotic cells with the presence of cell debris. Phenotypically, apoptosis is characterised by cell shrinkage, chromatin compaction, nuclear blebbing and collapse of the nucleus into small intact fragments (apoptotic bodies). The microscopic appearance of apoptotic bodies is quite diverse; most are round or roughly oval in shape. Apoptotic bodies vary in size, but they are a little smaller than the parent cells. Some apoptotic cells contain pyknotic chromatin, and some are devoid of a nuclear component.

After exposure to irradiation, EOL-1 cells still contained a single nucleus but larger than that of control cells. A higher number of nuclei displaying aberrant morphology, including invagination of the nuclear membrane, progressive lobulation and eventually micronucleation could be noted in irradiated cultures in comparison with control ones.

The number of apoptotic cells was even increased in samples treated with both agents (EGCG, resveratrol or curcuma at high concentrations and irradiation). Although typical apoptotic bodies were not often visible, the majority of cells had fragmented chromatin. Concomitant irradiation and EGCG, resveratrol or curcuma treatment thus induced even more apoptosis than EGCG, resveratrol, curcuma or irradiation alone.

Enhancement of cell growth inhibition by EGCG, resveratrol or curcuma coupled with irradiation. Fig. 2 shows the relative numbers of viable cells, as measured by the trypan blue exclusion of EOL-1 cultures irradiated with doses from 2 to 8 Gy and/or incubated with various concentrations of EGCG, resveratrol or curcuma for 1, 2 or 3 days.

In unirradiated EOL-1 cultures, EGCG or resveratrol treatment significantly inhibited cell proliferation at 200 μM. This observation could be made after already the first day of culture. The second and third day of culture, a significant decrease of cell proliferation could also be observed from 25 μM EGCG or 50 μM resveratrol. Interestingly, high concentrations of EGCG almost completely prevented cell population growth of EOL-1. In EOL-1 cultures treated with curcuma, no difference could be observed in the cell proliferation after one day of culture even at high concentrations. However, the curcuma treatment inhibited cell proliferation with significant results from 50 μM onwards from day 2 and 3. In general, the proliferation of cells was inhibited as the concentrations of EGCG, resveratrol or curcuma were increased. In conclusion, concerning the effect of EGCG, resveratrol or curcuma alone, EOL-1 cells seemed to be more sensitive to EGCG than resveratrol. The sensitivity to curcuma was even lower than the one to resveratrol.



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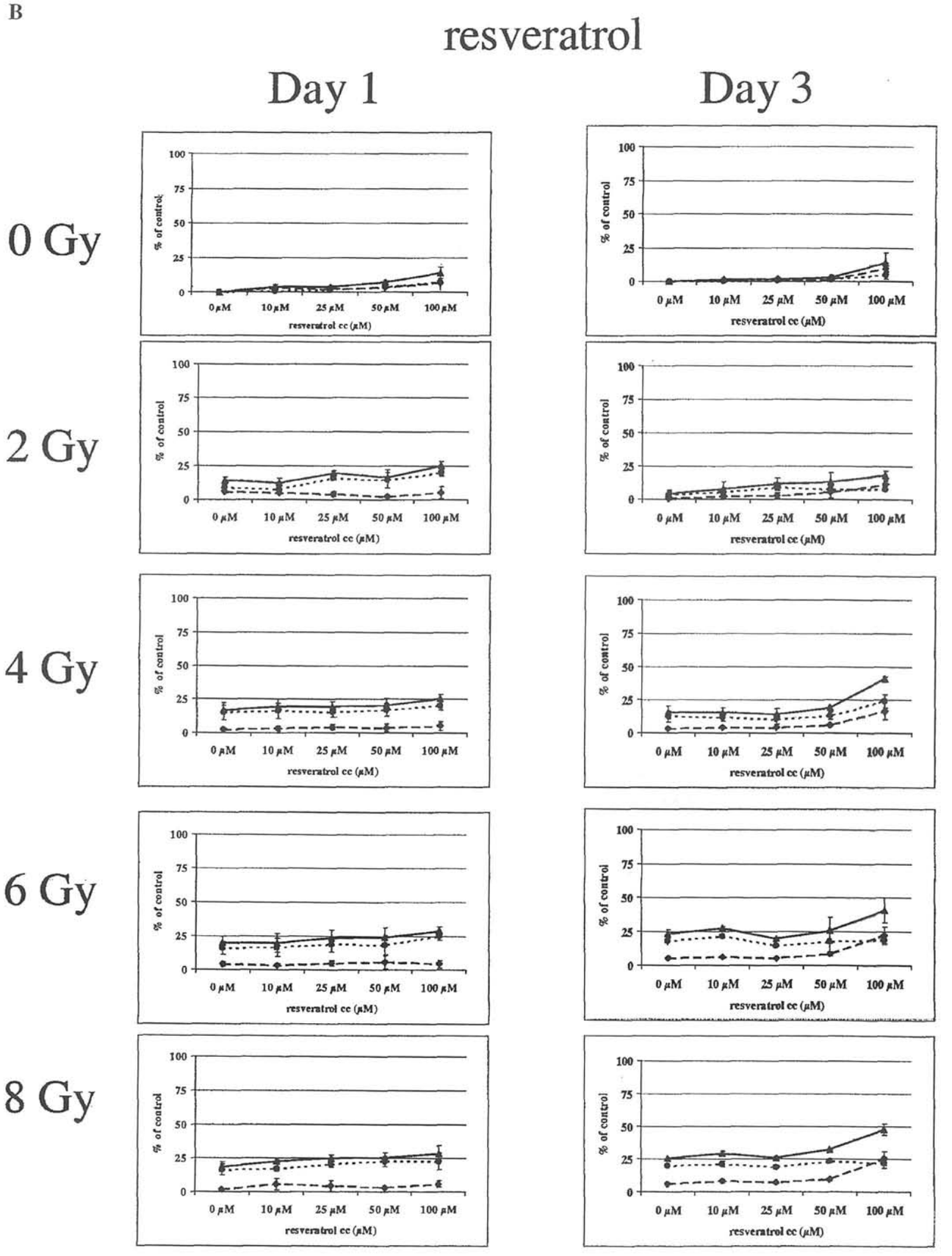

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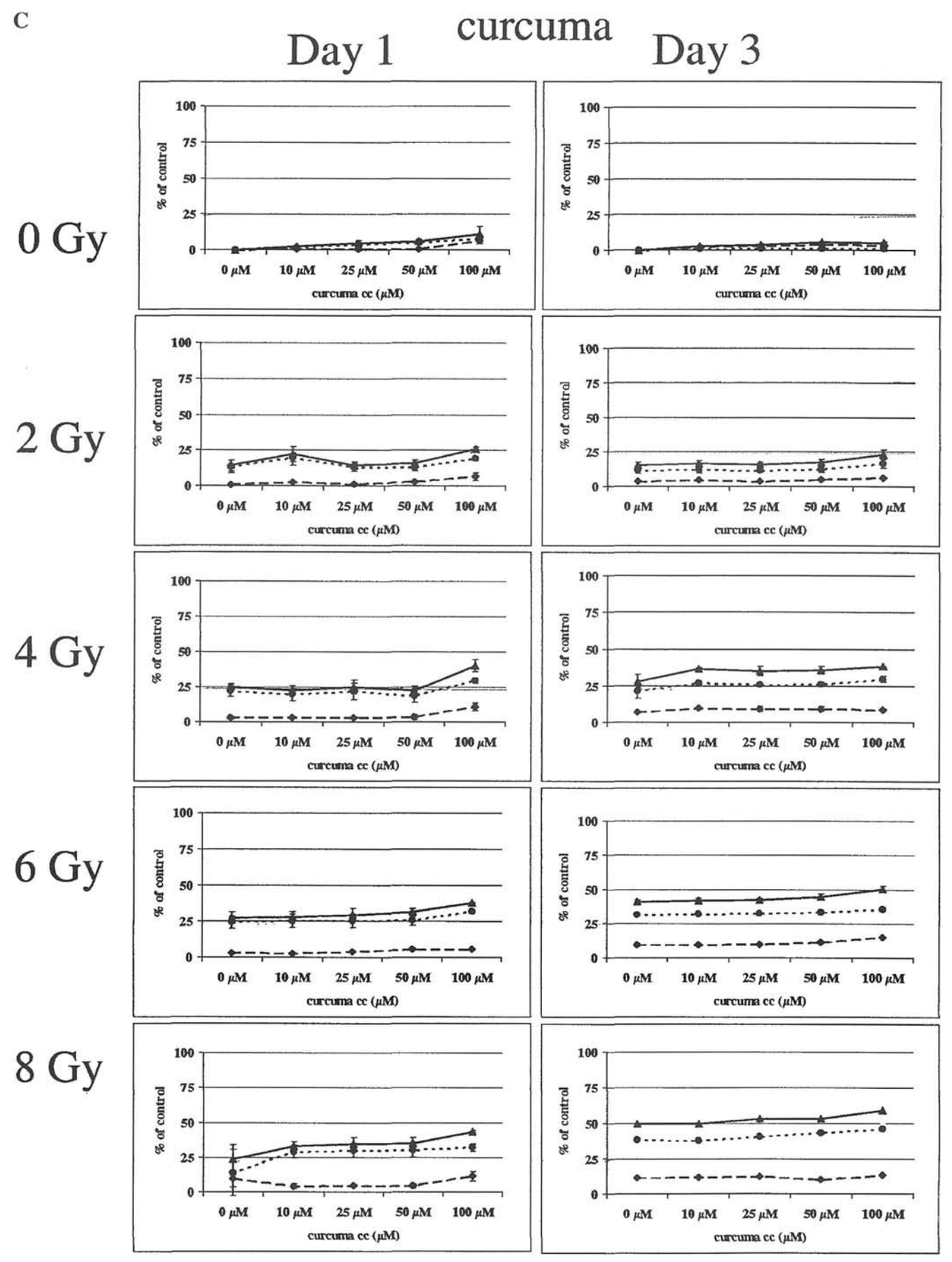
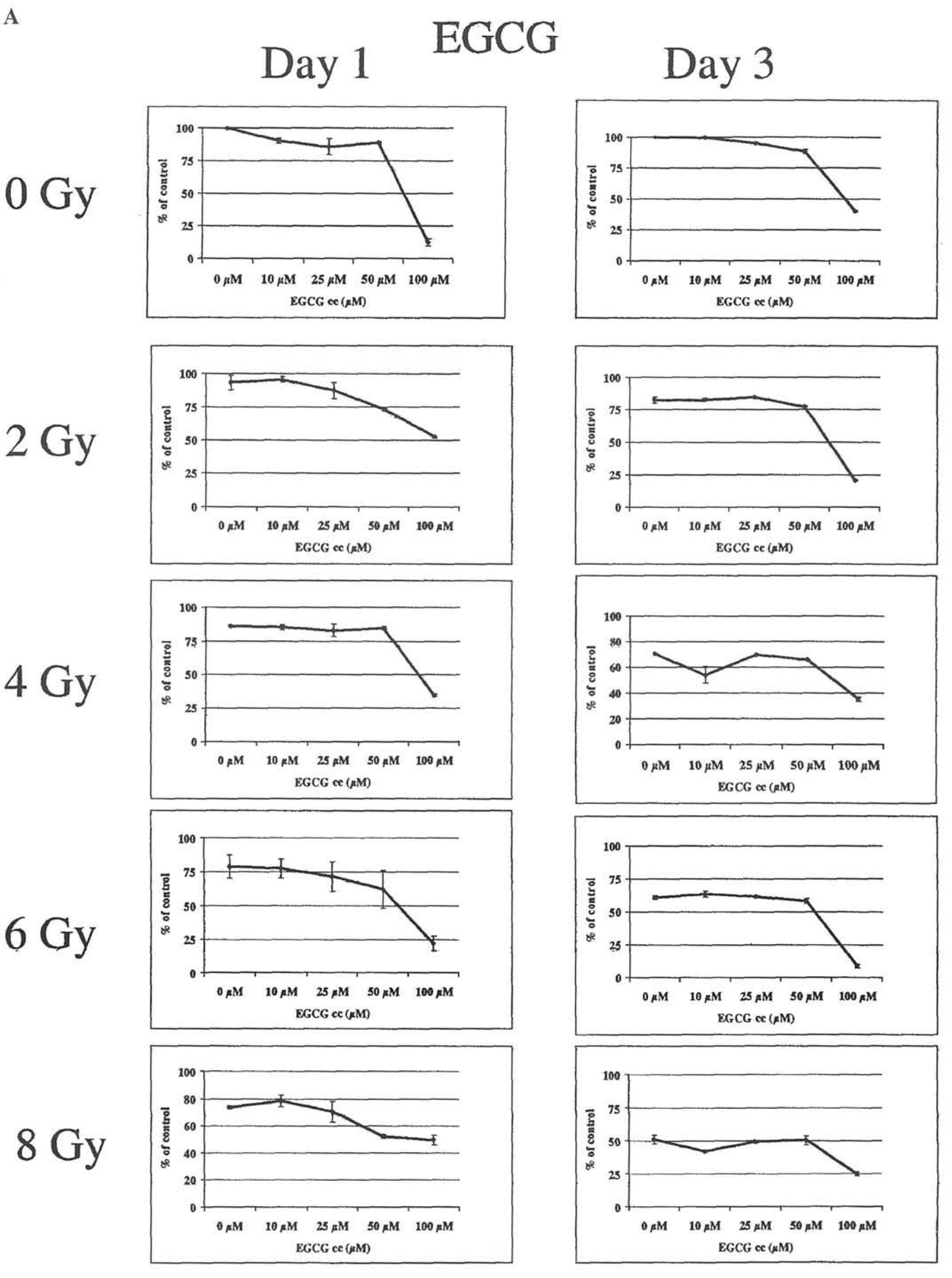


Figure 4. EGCG (A), resveratrol (B) or curcuma (C) and irradiation-induced apoptosis and necrosis of EOL-1 cells as determined by the percentage of cells in the apoptotic and necrotic fractions after staining with annexin-V and PI by flow cytometry as indicated in Materials and methods. The cells were treated for 1 or 3 days with different concentrations of EGCG, resveratrol or curcuma (ranging from 0 to 200 μM), unirradiated or irradiated with 2, 4, 6 or 8 Gy. Apoptotic cells (●), necrotic cells (♦), and the total of the two populations (▲) are represented in the graphs.



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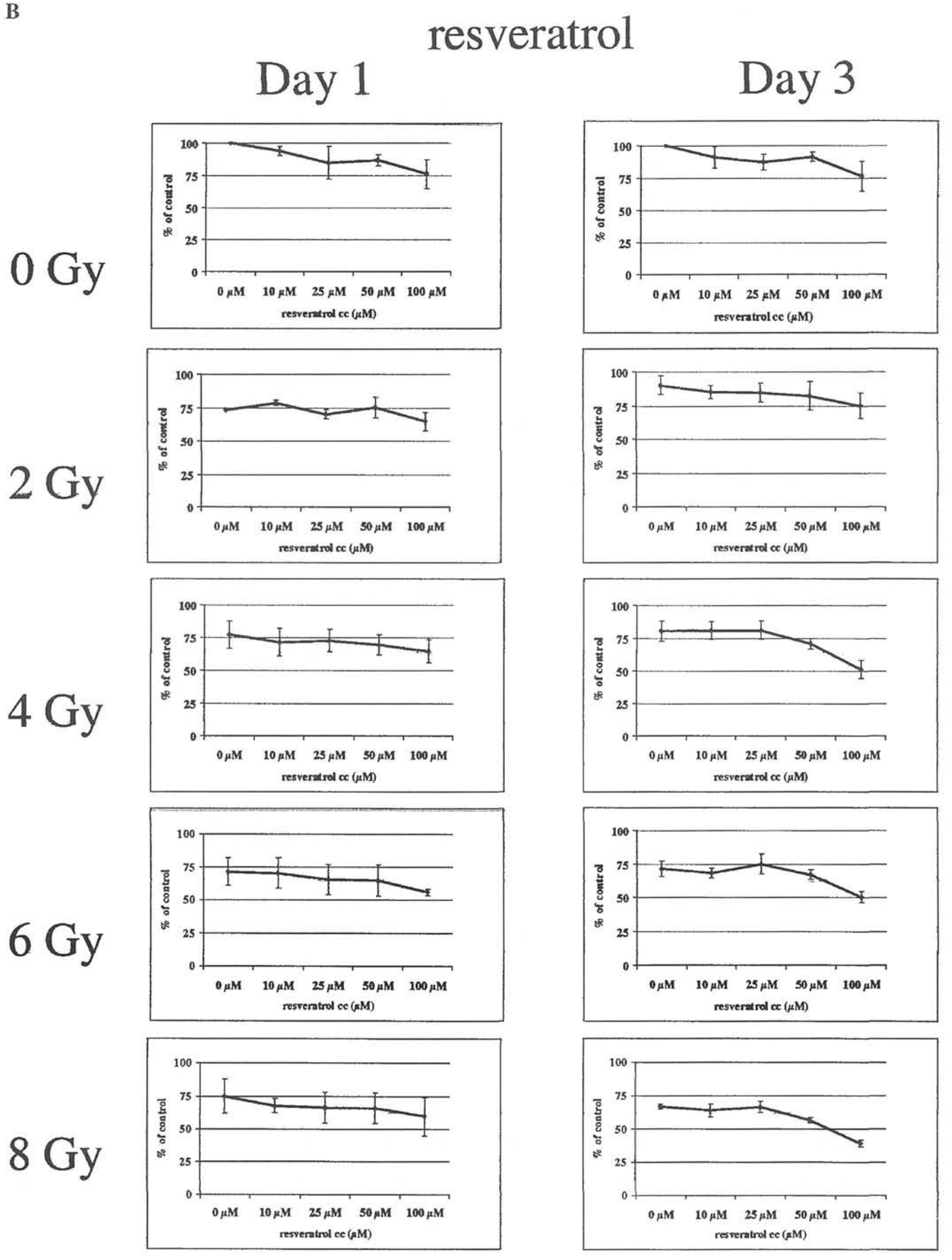

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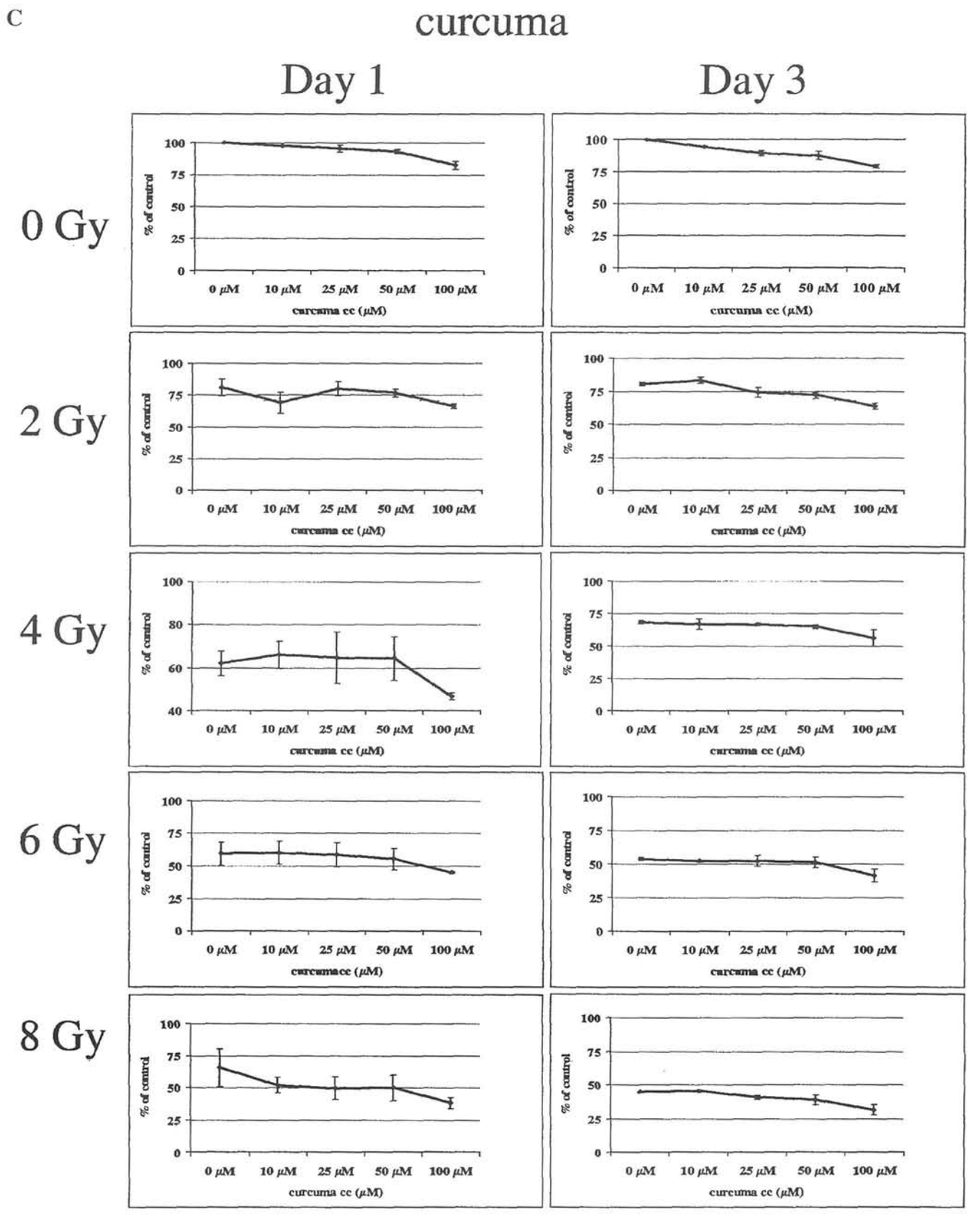

Figure 5. Percentage of EOL-1 living cells. For 1 or 3 days, cells were untreated, treated with EGCG (A), resveratrol (B) or curcuma (C) at various concentrations from 0 to 200 mM and/or irradiated with 2, 4, 6 or 8 Gy.

With regard to the effect of irradiation alone on EOL-1 cells, a significant effect on the inhibition of growth could be observed with a dose of 4 Gy onwards at day 1. EOL-1 cells seemed to respond in a dose-dependent manner to X-irradiation. The extent of growth inhibition seen in the examined cell line appeared to reflect its radiation sensitivity showing that EOL-1 was moderately radiation resistant.

To test the combined efficiency of the X-rays and EGCG, resveratrol or curcuma, graded doses up to 8 Gy were applied to cells that were treated with 10, 25, 50, 100 or 200 μM EGCG, resveratrol or curcuma. For a dose of 4 Gy, a significant decrease of cell proliferation was observed for a combined X-irradiation treatment and 25, 50 or 25 μM for EGCG, resveratrol or curcuma, respectively.

Changes in membrane asymmetry induced by EGCG, resveratrol or curcuma coupled with irradiation. The significant growth inhibitory activity of EGCG, resveratrol or curcuma and irradiation led us to investigate whether part of the effect was a result of programmed cell death (or apoptosis) induction. Apoptosis leads to the translocation of phosphatidylserine (PS) from the inner of the cell membrane to the outside. It is possible to detect PS by using FITC-labelled Annexin V, which is a Ca^{++} dependent phospholipid-binding protein. By combining staining of Annexin-FITC with propidium iodide (PI), we can obtain a profile where live cells are negative for both dyes, dead cells are positive for both, while apoptotic cells are positive only for Annexin-FITC.

Fig. 3 shows representative dot-plots (annexin-V versus PI) of EOL-1 cells 3 days after irradiation. Whereas only a few apoptotic and necrotic cells are present in control conditions (0 Gy), the percentage of apoptotic cells increases with the dose of irradiation (24 and 27% with 4 and 8 Gy, respectively).

Fig. 4 (A, B and C) summarises the percentage of cells present in the fractions corresponding to apoptotic (annexin V positive cells) and necrotic (annexin V and PI positive) cells after 1 or 3 days of culture of EOL-1 cells treated with EGCG (Fig. 4A), resveratrol (Fig. 4B) or curcuma (Fig. 4C) at various concentrations and/or X-irradiated with 2, 4, 6 or 8 Gy. Fig. 5 (A, C and C) summarises the percentage of cells present in the fraction corresponding to the living cells (annexin V and PI negative) cells after 1 or 3 days of culture of EOL-1 cells treated with EGCG (Fig. 5A), resveratrol (Fig. 5B) or curcuma (Fig. 5C) at various concentrations and/or X-irradiated with 2, 4, 6 or 8 Gy.

In unirradiated cells, the percentage of apoptotic cells was proportional to the concentration of EGCG or resveratrol with a significant increase in comparison with control conditions from 50 μM EGCG and 100 μM resveratrol onwards. The increase of apoptotic and necrotic cells was correlated to a decrease of living cells. EGCG induced more apoptosis and necrosis than resveratrol for the same concentration. Furthermore, resveratrol induced more apoptosis and necrosis than curcuma. These results corroborated fairly well the results obtained concerning cell proliferation.

In irradiated cells, apoptosis and necrosis are proportionally enhanced in function of the dose of irradiation with a significant increase of apoptotic and necrotic cells starting from 2 Gy onwards.

Concomitant EGCG, resveratrol or curcuma and irradiation treatment were stronger inducers of apoptosis than irradiation or EGCG, resveratrol or curcuma treatment alone. This could be explained by the fact that relatively more cells did not survive after irradiation and treatment with EGCG, resveratrol or curcuma than after irradiation alone. The extent of apoptosis seen in the EOL-1 correlated fairly well with the results of cell morphology and cell proliferation.

Discussion

Mechanisms that suppress tumorigenesis often involve modulation of signal transduction pathways, leading to alterations in gene expression, arrest of cell cycle progression or apoptosis. Apoptosis is a mode of cell death used by multicellular organisms to eradicate cells in diverse physiological and pathological settings. Several studies have demonstrated that apoptosis may be involved in cell death induced by chemotherapeutic agents, including cisplatin, camptothecin, etoposide, etc. There is accumulating evidence that the efficiency of anti-tumor agents is related to the intrinsic propensity of the target tumor cells to respond to these agents by apoptosis (70). Recent evidence also shows that suppression of apoptosis by tumor-promoting agents in pre-neoplastic cells is thought to be an important mechanism in tumor promotion (71). In this context, it is noteworthy that apoptosis-inducing ability seems to have become a primary factor in considering the efficiency of chemopreventive agents. Furthermore, the concept that cancer can be prevented or certain diet-derived substances can postpone its onset is currently eliciting considerable interest. EGCG, resveratrol and curcuma have been demonstrated to inhibit tumor initiation induced by various carcinogens. However, the molecular events of EGCG, resveratrol and curcuma actions have not been studied in detail. The aim of this study was thus to identify the events which may ultimately initiate the apoptotic cascade leading to cancer cell death as a result of concomitant EGCG, resveratrol or curcuma and, irradiation treatment. From these results we can conclude that EGCG, resveratrol and curcuma enhance growth inhibition as well as apoptosis in combination with irradiation in a human cancer cell line.

Irradiation. Concerning the effects of ionising radiation on EOL-1 cell, the EOL-1 cell line appeared to have a moderate radiation sensitivity in comparison with other cell lines that we study in our laboratory (data not shown). As regards the occurrence of endoreduplication in EOL-1 irradiated cells, it seems that cells were actually accomplishing a new cycle without mitosis but consisting of new S and Gap phases. Such an endoreduplication process is different from situations in which multiple initiations occur within a given S phase or which consists in reoccurring S-phases (72). Endoreduplication from G2 phase has been reported in various cell lines exposed to such external factors as DNA-damaging agents (73), the Simian virus 40 (74) or protein kinase inhibitors (75-77). However, the exact mechanism of the process remains highly speculative. The active mitotic cyclin B1/Cdk1 complex is known to play a direct role in preventing DNA replication by inhibiting the assembly of 'pre-replication centers' (78-81). The specific genetic background of the EOL-1 cell line may have influenced the magnitude of the endoreduplication process, due to the lack of expression of the p53 tumor suppressor protein, a natural transcriptional activator of the p21 gene (82-83). The inhibitory role of the cyclin-dependent kinases inhibitor

$p21^{WAF1/CIP1}$ on endoreduplication in G2 is well substantiated in cells where $p21^{WAF1/CIP1}$ is either inhibited or overexpressed and exposed to G2-blocking agents (73,74,84,85).

EGCG. In recent years, evidence has been accumulated showing that tea has anti-cancer properties. Intense interest in green tea as a cancer preventive in humans has increased for six main reasons: (a) wide range of target organs in rodent carcinogenesis experiments (86); (b) growth inhibition of various human cancer cell lines (87-89); (c) inhibition of lung metastasis in mice (87); (d) wide distribution of $[^3H]EGCG^4$ in various organs of mice (90); (e) cancer-preventive results with humans from a prospective cohort study; and (f) no severe adverse effects with green tea tablets in humans (90). Although the epidemiological data are lacking, the experimental studies have consistently demonstrated that drinking of tea, either black or green tea, substantially inhibited carcinogen- or UV irradiation-induced animal tumors including skin, lung, stomach, colon and breast cancer (8,14-23). In spite of exciting results in animal studies, the mechanisms of anticarcinogenic action of tea have remained largely unclear. Some studies have attributed the inhitory effects of green or black tea on UV-induced carcinogenesis to the antioxidant and free radical-scavenging activities of the teas, since numerous antioxidants such as ascorbic acid (91), alpha-tocopherol (92), beta-caroten (93), selenium (94), butylated hydroxytoluene (95) and a mixture of dietary antioxidants (96), have been reported to inhibit UV-induced skin carcinogenesis and that several polyphenolic substances in green and black teas have been shown to have antioxidant activities (97). Concerning EGCG in particular, a recent study has shown that it possesses antioxidant activity higher than chain-breaking antioxidants such as vitamin E and vitamin C (9). Unlike other chain-breaking antioxidants, such as vitamin E and butylated hydroxytoluene, EGCG has been shown to be an equally or better effective antioxidant against both hydrophilic and hydrophobic free radicals (10). Furthermore, EGCG can cross blood-brain barrier and penetrate the cell membrane with partition reaction reaching equilibrium (10,98). Thus, it could be suggested that EGCG not only inhibits the release and accumulation of reactive oxygen species at the affected site, but may also interfere with their cellular generation to promote cell viability. In our study, we showed that EGCG was able to induce a synergical effect with irradiation on the inhibition of cell growth and induction of apoptosis.

Resveratrol. Flavonoids constitute the main polyphenolic compounds in red wine and there are two major classes: antocyanins and flavanols. In addition, wine contains non-flavonoids, particularly stilbenes (resveratrol and its glycosides) (99). Resveratrol has been the subject of much study since grapes and red wine are probably the most important foodstuffs containing this substance (99). Resveratol is an antioxidant. To be considered as an antioxidant, a polyphenol must satisfy two basic conditions: first, when present at a low concentration relative to the substrate to be oxidised, it can delay or prevent auto-oxidation or free radical-mediated oxidation; secondly, the resulting free radical formed after scavenging must be stable upon further oxidation (100). Resveratrol has been reported to have cancer chemopreventive properties. It inhibits cellular events associated with tumor initiation, promotion and progression. The mechanisms by which resveratrol exerts its antitumor effects are still unclear but may include: (1) inhibition of ribonucleotide reductase (101), DNA polymerase (102), protein kinase C (103) or cyclooxygenase-2 activities; (2) inhibition of reactive oxygen species (ROS)-mediated carcinogenesis; (3) inhibition of cell division; and (iv) apoptotic cell death activation (105-108). In this study, resveratrol was able to inhibit cell proliferation and induce apoptosis of EOL-1 cells. Furthermore, it showed synergical effect when applied together with irradiation.

Curcuma. Curcuma is the major compound in turmeric responsible for imparting the bright orange-yellow color. Turmeric powders and pastes have found many uses in cooking and the food industry and have been used in folkloric remedies. Thus the evaluation of the constituents of *C. longa* for its medicinal and biological properties is very important. In recent years, evidence has been accumulated showing that curcuma has anti-cancer properties. The activities of curcuma reported here partially support the anecdotal claims and the folkloric uses of turmeric or concoctions containing turmeric for treating various ailments. However, additional *in vivo* studies with curcuma in humans are required to substantiate such claims. Curcuma can diffuse easily into the cytosol, triggering specific apoptotic events. Due to its unique physicochemical property curcuma passes easily through the plasma membrane and evokes membrane perturbation by affecting the membrane lipid bilayer. Curcuma may also cause structural and functional changes in cellular membrane integrity leading to the flipping of phosphatidylserine to the outer cell surface, which participates in the free radical release (59). Bhaumik *et al* also showed that the treatment of cells with curcuma led to the activation of caspase-3-like proteases, which trigger the downstream cascade of apoptotic events (59). However, there are also reports showing that curcuma treatment was not always associated with programmed cell death (109-111) and sometimes it even protected cells against apoptosis (112-116). Thus, cell propensity to undergo curcuma-induced apoptosis can differ dramatically, depending on the cell type (117). Moreover, curcuma inhibits several mediators and enzymes involved in cell mitogenic signal transduction pathways (55), activator protein 1 and nuclear factor kB activation (119). Hanif *et al* (57) provided evidence that curcuma inhibits cell proliferation and induces cell cycle changes in the colonic adenocarcinoma cell lines, HT-29 and HCT-15. Finally, dietary curcuma significantly inhibits phospholipase A2 in colonic mucosa and tumors leading to the release of arachidonic acid from phospholipids, alters cyclooxygenase and lipoxygenase activities, and modifies PGE2 levels (46). Concerning the effect of curcuma on the cell cycle, curcuma has been shown to reduce selectively the expression of some cell cycle related proteins such as proliferating cell nuclear antigen (PCNA), cdk1 and cyclin E, but not cdk2 and cyclin D1. PCNA and cdk1 functions at S, G1, G2 phase, respectively, suggesting that curcuma might interfere with the cell-cycle progression, and that this may be

mediated through reducing the function or level of some specific cell-cycle regulators. In our study, no significant effect of curcuma could be observed on the cell cycle. However, curcuma induced apoptosis.

With regard to the effect of EGCG, resveratrol or curcuma alone, we have shown that in the EOL-1 cell line, each caused an enhancement of radiation toxicity. It is therefore suggested that these components prevent repopulation, as well as repair of radiation damage. Our study demonstrated a mechanism of action that may in part explain the observed health benefits related to tea, wine or curcuma consumption. Our findings open the door to future studies examining the pharmacological potential of EGCG, resveratrol and curcuma in health and disease. Over the years, cancer therapy has witnessed many exciting developments, but cure of cancer still remains as complex as the disease itself, since the mechanisms of tumor killing are still not fully realised. Identification of new substances leading to tumor cell death as well as increasing radiation sensitivity of the cells may be of immense help to selectively induce apoptosis in cancer cells. Knowledge acquired from this study will therefore lead us one step towards that goal.

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