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Flavonoids as Enhancers of X-Ray-induced Cell Damage in Hepatoma Cells

Johannes van Rijn[1] and Jaap van den Berg
Department of Radiotherapy, Academic Hospital Vrije Universiteit, Amsterdam, the Netherlands

ABSTRACT

The nuclear enzyme topoisomerase II, which is involved in replication, transcription, and probably repair of DNA, can be inhibited by a number of flavonoids. In conjunction with X-rays, three of these compounds were tested as to their effects on Reuber H35 hepatoma cells. In this combination, the isoflavone genistein, the flavone apigenin, and the flavonol quercetin caused an enhancement of radiation-induced cell death. This enhanced cytotoxicity was only observed when the flavonoids were applied following an irradiation treatment and is attributed to decreased repair of DNA radiation damage with a concomitant reduction of the rate of cell repopulation. Fractionated irradiations, given as five sequences of 3 Gy each over a period of 5 days, reduced the surviving cell population only by a factor of 20, whereas the continuous presence of genistein during radiation sequences resulted in a reduction of at least a factor of 10,000. Thus, these flavonoids not only seem to act as radiation enhancers but also exhibit potential antitumor activities.

INTRODUCTION

Topoisomerase II is a nuclear enzyme that transiently forms and then religates breakages in both DNA strands during replication, transcription, and other DNA-related processes (1). Some recent studies suggest that the topoisomerases are also involved in repair of radiation-induced DNA damage. Thus, the topoisomerase II inhibitor etoposide was shown to cause radiation sensitization in V79 lung fibroblasts and human melanoma cells (2, 3). Several bioflavonoids were also shown to act as inhibitors of topoisomerase II (4, 5). Of the ones chosen for the present study, the isoflavone genistein exhibits a variety of effects on cells, including inhibition of proliferation and induction of differentiation in tumor cells (6–9). It also increases radiation-induced delays of the G_2 subdivision in mouse zygotes (10). The structurally related flavone apigenin causes arrest in the G_2-M growth phase and leads to morphological differentiation in rat neuronal cells (11), whereas the flavonol quercetin exhibits antiproliferative properties in various cancer cells *in vitro* (12). The latter compound recently underwent a Phase I clinical trial (13). To our knowledge, the present study is the first report that examines the usefulness of flavonoids as modifying and enhancing agents of radiation-induced loss of cellular clonogenic capacities. The effects of the mentioned flavonoids were investigated on rat hepatoma cells treated with single X-ray doses, whereas the effects of genistein were also studied in combination with fractionated irradiations.

MATERIALS AND METHODS

Reuber H35 rat hepatoma cells were cultured in 25 cm^2 TC[2] flasks (NALGE NUNC Industries) in L15 medium supplemented with 10% fetal bovine serum (Life Technologies, Inc.). The cells were processed for the clonal assay at the end of the experimental procedures. After trypsinization and serial dilution, 500–20,000 cells were seeded in duplicate in TC flasks. After 10 days, the cell colonies were fixed with ethanol and stained with Giemsa solution. Colonies containing 50 or more cells were scored. The cloning efficiency of untreated H35 cells was at least 90%. Cultures for experiments contained approximately 500,000 cells in the logarithmic growth phase. Irradiation occurred with an orthoV (175 kV) X-ray beam at a dose rate of 50 cGy/min (Philips, Eindhoven, the Netherlands). Flavonoids (Sigma Chemical Co.) were dissolved in DMSO (10 mg in 0.1 ml DMSO) and directly diluted in culture medium to give a stock concentration of 240 μM. Control cultures were given the same amount of solvent, which did not affect clonogenicity.

RESULTS

Effects of the Flavonoids on Single-Dose Irradiated Cells. During the explorative phase of this study, exponentially growing cells were treated with various concentrations of genistein for various lengths of time either before, during, or following irradiation. Preincubation of the hepatoma cells with genistein for periods of up to 24 h did not affect the survival of irradiated cells (results not shown). However, treatment with genistein after irradiation caused an additional reduction in cell survival. To assess the effects of genistein, the compound was added 30 min before irradiation, and incubation was continued until 24 h, when the cells were plated to determine clonal survival. Irradiated cells were also plated immediately because delayed plating usually resulted in an increase of the surviving cell population due to cell division and repair of potential lethal damage.

Fig. 1 shows the results of irradiated cultures incubated with genistein for a total of 24 h. The enhancement of radiation

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[1] To whom requests for reprints should be addressed, at Department of Radiotherapy, Academic Hospital Vrije Universiteit, P.O. Box 7057, 1007 MB, Amsterdam, the Netherlands. Phone: (20) 4440414; Fax: (20) 4440410.

[2] The abbreviations used are: TC, tissue culture; PTK, protein tyrosine kinase.

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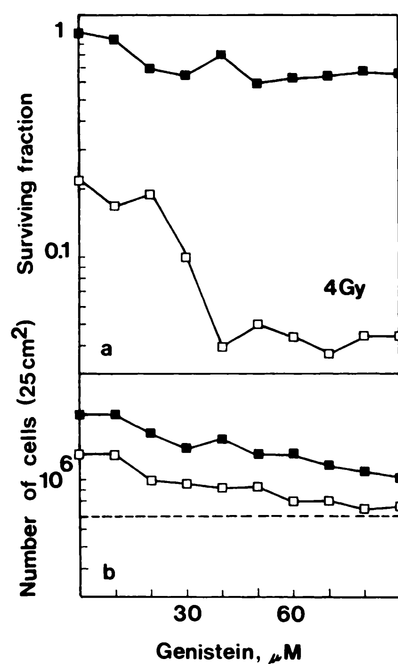

Fig. 1 Effect of genistein concentration on growth and survival of irradiated cells. Genistein (0–90 μM) was added 30 min before irradiation with 4 Gy X-rays, and the incubation was continued for 23.5 h followed by the clonal assay. *a*, cell survival; *b*, number of cells per TC flask; □, irradiated cells; ■, nonirradiated cells; - - - - -, number of cells at the time of irradiation.

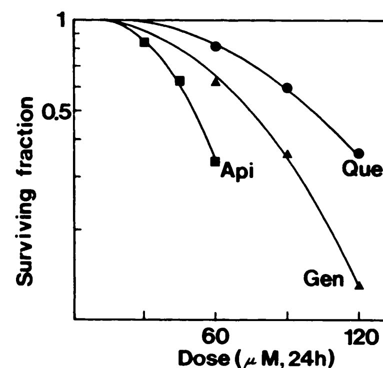

Fig. 2 Cytotoxicity of the flavonoids. Cells were exposed to various concentrations of genistein (▲), apigenin (■), or quercetin (●) for 24 h.

damage was evident from a concentration of 30 μM genistein (Fig. 1*a*). Furthermore, genistein inhibited cell proliferation in a dose-dependent manner that appears to be independent of the radiation-induced growth delay (Fig. 1*b*).

To test the efficiency of the X-rays, graded doses up to 8 Gy were applied to cells that were treated with 60, 90, or 120 μM genistein. In addition, we investigated the related flavonoids apigenin (60 μM) and quercetin (90 μM) likewise from 30 min before irradiation until 23.5 h following this treatment. The concentrations of the latter compounds were the optimum values providing the lowest cytotoxicity in coincidence with the highest degree of radiation enhancement. Genistein cytotoxicity (IC_{50} = ~75 μM) was intermediate to that of apigenin (IC_{50} = ~50 μM) and quercetin (IC_{50} = ~100 μM; Fig. 2). The three genistein concentrations caused a similar dose-dependent enhancement of radiation-induced cell death after correction for genistein toxicity (Fig. 3). The optimum dose of genistein therefore appears to be between 40 and 60 μM. The radiation enhancement by apigenin and quercetin was smaller than for genistein. The first two flavonoids showed a dose reduction factor at surviving fraction 0.1 of 1.5 as opposed to 2.0 for genistein. Drug treatment, either alone or in combination with a low dose of X-rays, almost completely prevented cell population growth (Fig. 3, *b, d,* and *f*).

The enhancement effect of genistein in irradiated H35 cells is in contrast with the radiation protection by genistein in human B-lymphocyte precursors (14), which is attributed to its inhibition of PTKs. Therefore, in our protocol, we also tested the PTK inhibitor tyrphostin 51 and the tyrosine phosphatase inhibitor vanadate, which has the opposite effect of genistein in B-lymphocyte precursors. The selected concentrations of the two compounds inhibited the proliferation of H35 cells as effectively as genistein, alone and in combination with X-rays (Table 1). Tyrphostin 51 seemed to prevent the delayed plating recovery without causing any radiation sensitization. Vanadate caused an additional enhancement in combination with X-rays, which, however, does not agree with its putative antagonistic action to genistein.

Effects of Genistein on Cells Treated with Fractionated Doses of X-Rays. Genistein used at 60 μM did not lead to notable increases in cytotoxicity when used for longer periods than 24 h. To study the effects of genistein in combination with fractionated irradiations, cells were incubated continuously with 60 or 90 μM genistein and subjected to five doses of 3 Gy of X-rays given at 24-h intervals. The surviving cell fraction was assessed prior to and following every irradiation to determine recoveries in the preceding interval and the reduction of cell survival per radiation dose. The cytotoxicity of 60 μM genistein did not change significantly after the first 24-h period; the cloning efficiency remained at about 50% (Fig. 4*a*). At 90 μM, genistein became increasingly cytotoxic, leading to less than 3% survival after 96 h. Sixty μM genistein caused partial growth inhibition when compared with untreated cells, whereas 90 μM genistein led to complete cellular stasis (Fig. 4*b*).

Fractionated irradiation caused a progressive reduction of the cell proliferation (Fig. 4*c*), which turned into a regression in combination with genistein. Genistein also strongly reduced the number of surviving cells in the irradiated cultures (Fig. 4*d*). The initial cell number of approximately 500,000 cells per culture was reduced by only a factor of 20 following the X-ray doses, whereas in the presence of 60 or 90 μM genistein, the number of cells surviving irradiation was reduced by at least a factor of 10,000. With respect to the fraction of surviving cells (Fig. 4*e*), the (equivalent) dose reduction factor at surviving fraction 0.1 for 60 μM genistein is 2.3, which is comparable with the 2.0 for single-dose irradiation (Fig. 3).

X-rays caused a dose-dependent mitotic delay in cells, but the effect of 3 Gy was smaller than that of 24 h of incubation with 60 μM genistein (Fig. 4, *b* and *c*). This is demonstrated in

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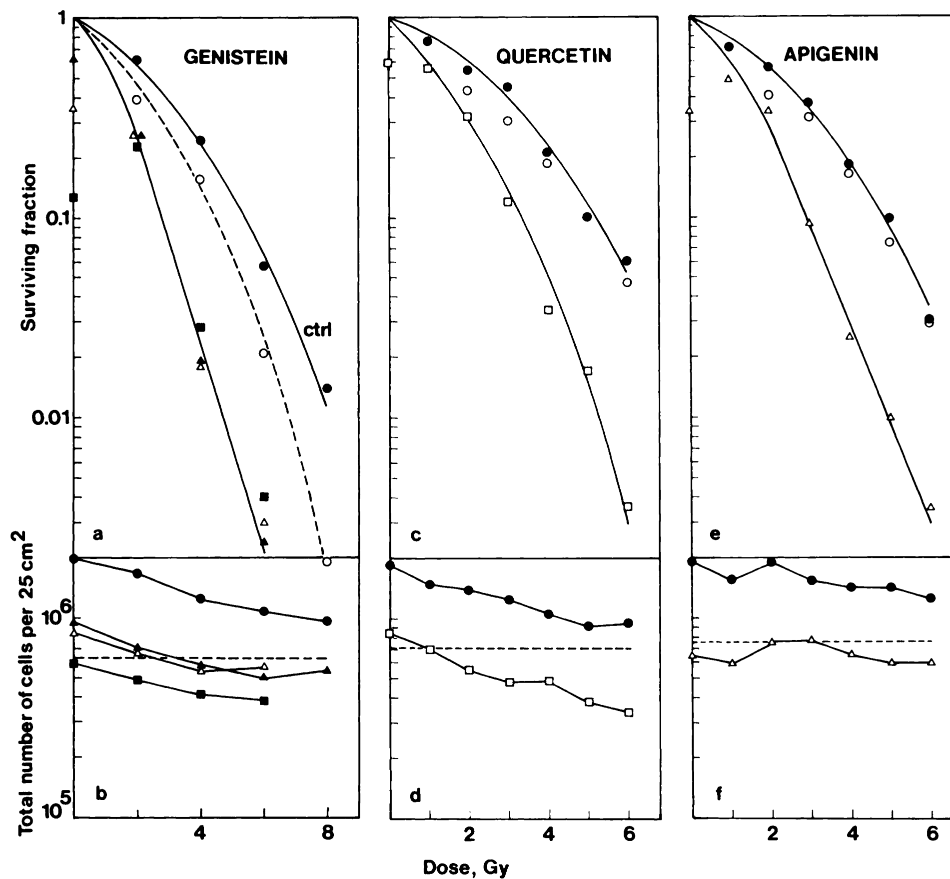

Fig. 3 Comparison of the effects of genistein, quercetin, and apigenin on irradiated cells. Flavonoids were added 30 min prior to X-rays, and the incubation was continued for 23.5 h. *a* and *b*: ▲, 60 μM genistein; △, 90 μM genistein; ■, 120 μM genistein. *c* and *d*: □, 90 μM quercetin. *e* and *f*, △, 60 μM apigenin. *a*, *c*, and *e*, fraction of surviving cells; curves are normalized for drug toxicity (marked on the ordinate). Control-irradiated cells were plated both immediately (○) and after 23.5 h (●). *b*, *d*, and *f*, number of cells per TC flask; - - - - -, number of cells at the time of irradiation.

Table 1 Effect of tyrphostine and vanadate on proliferation and survival of irradiated cells

The PTK inhibitor tyrphostin 51 (100 μM) and the tyrosine phosphatase inhibitor vanadate (100 μM) were compared with genistein (60 μM) for their effect on irradiated cells. Cells were exposed to the various compounds from 30 min prior to irradiation with 5 Gy X-rays until 23.5 h after irradiation. Irradiated cells without drugs were plated with or without 24 h delay. None of the compounds caused more than 20% reduction of the cloning efficiency in 24 h. Survival data were corrected for drug toxicity.

	Surviving fraction		Proliferation factor (0–24 h)	
	0 h	24 h	No X-rays	5 Gy
Control	1.0	1.0	3.5	
5 Gy	0.08	0.12		1.6
Tyrphostin 51		0.08	1.7	1.0
Vanadate		0.05	1.6	0.9
Genistein		0.02	2.2	1.1

Table 2, which includes the results of a duplicate experiment. Assuming that cell loss was negligible during the first interval of fractionated irradiation, the increases in cell number reflect the proliferation rates of the cultures. On the average, untreated cultures increased their cell number by a factor of 3.4 during the 24-h interval. This factor was reduced to 2.65 after irradiation with 3 Gy when all cells were included or to 3.1 when only the surviving cells were considered. This apparent preferential recovery of surviving cells after irradiation was not observed in the presence of genistein (growth factor, 1.3).

DISCUSSION

Postirradiation applications of the flavonoids genistein, apigenin, or quercetin to hepatoma cells enhanced cell death. The reduction of cell survival appears to be due to multiple effects exerted by these compounds.

Genistein inhibits both platelet-derived growth factor- and epidermal growth factor-induced mitogenesis and initiation of DNA synthesis in quiescent rat liver cells and in mouse fibroblasts (15). These effects were thought to be related to inhibition of PTKs (16), although the kinetics of inhibition of genistein and some other flavonoids on these PTKs do not closely correlate with their inhibition of cell proliferation (17). In a study with human B lymphocyte precursors, genistein-treated cells express resistance to ionizing radiation with respect to apoptosis and

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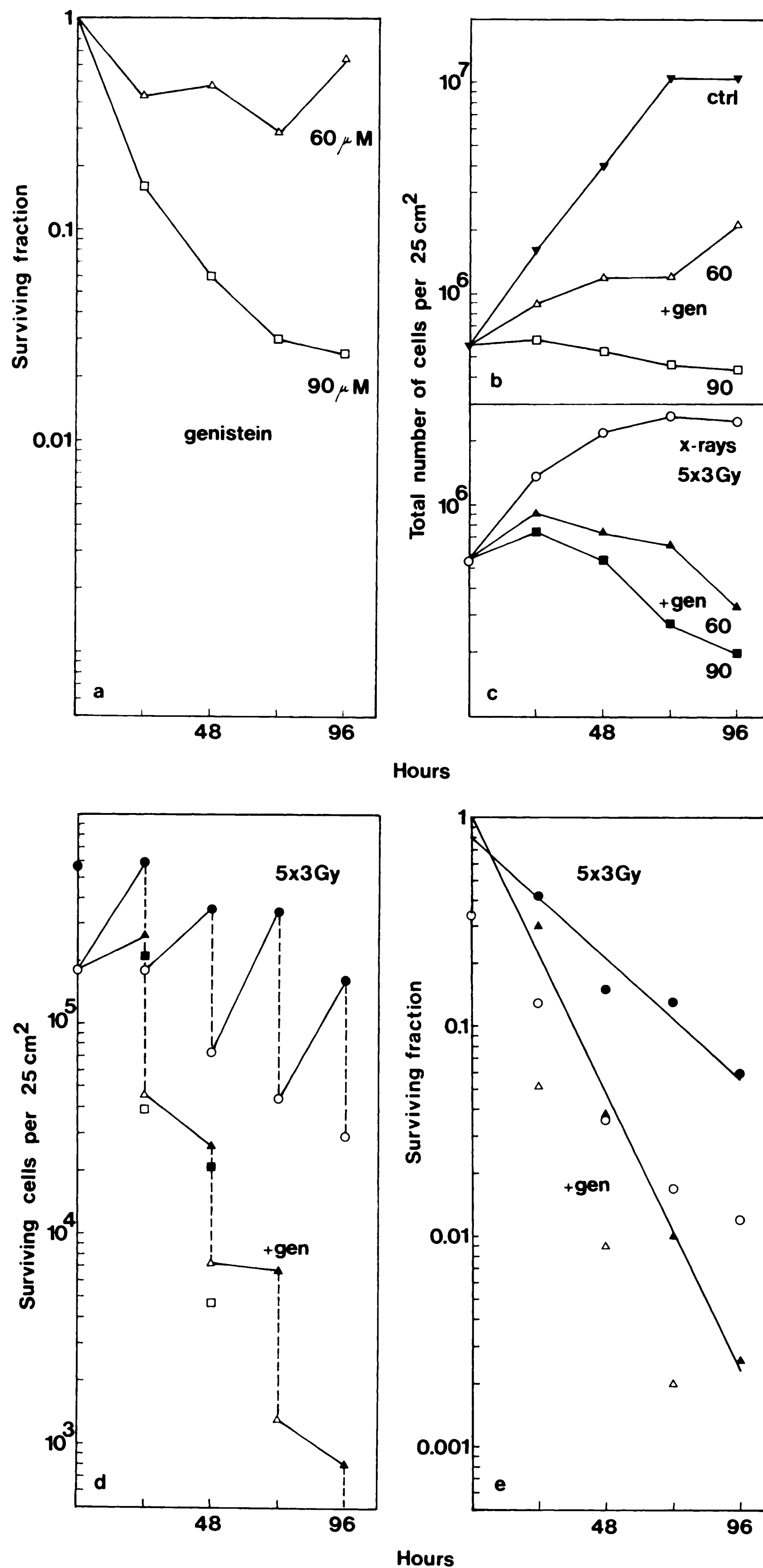

Fig. 4 Effect of genistein on cells treated with fractionated irradiation. Cell cultures were given five fractions of 3 Gy X-rays at 24-h intervals in the continuous presence of genistein, which was renewed daily. *a*, effect of 60 (△) or 90 (□) µM genistein on cell survival. *b*, proliferation of untreated cells (▼) and the effect of 60 (△) or 90 (□) µM genistein. *c*, proliferation of irradiated cells (○) and the effect of 60 (▲) or 90 (■) µM genistein. *d*, effect of fractionated irradiation and genistein on the surviving cell population. *e*, effect of 60 µM genistein on the surviving fraction of irradiated cells. *d* and *e*, after each irradiation, cultures of cells were put in the clonal assay (□, ○, and △) and again after 24 h prior to the next radiation dose (■, ●, and ▲). Cells were irradiated alone (○ and ●) or in the presence of 60 (△, and ▲) or 90 (□ and ■) µM genistein. Data are corrected for drug toxicity according to *a*. In *e*, curves are given only for the delayed plating (▲ and ●) using exponential regression.

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Table 2 Effect of X-rays and genistein on cell proliferation

Cell proliferation is shown during the 24-h period following 3 Gy X-rays in the presence or absence of 60 μM genistein. Data are drawn from Fig. 4 and from a similar fractionation experiment.

	Proliferation factor (0–24h)	
	Control	Genistein
Control cells	3.4 (3.0–3.8)	1.95 (1.6–2.3)
Irradiated cells	2.65 (2.5–2.8)	1.8 (1.7–1.9)
Surviving irradiated cells	3.1 (3.2–3.0)	1.3 (1.5–1.1)

proliferation inhibition, whereas vanadate, a tyrosine phosphatase inhibitor, has the opposite effect (14). In contrast with this earlier study (14), genistein enhanced the radiation response in H35 cells. Furthermore, treatment with the PTK inhibitor tyrphostin 51 led to inhibition of cell growth but did not affect any radiation sensitization. These results suggest that PTK does not play a role in the radiation-induced cell death in H35 cells. Most likely, the responses to ionizing radiation are different for B-lymphocyte precursors and the hepatoma cells. Irradiated lymphoid cells undergo interphase death, which is expressed as apoptosis, whereas radiation-induced cell death in H35 cells appears to be mainly of the mitotic type. Genistein was also shown to inhibit Taxol-induced apoptosis in human leukemic cells and in some other studies (18).

Genistein can block the cell cycle in the G_2-M phase, which is possibly related to inhibition of topoisomerase II (8, 10, 19). When irradiated cells are treated with genistein, a higher frequency of DNA-protein cross links is found than after irradiation alone (4). The topoisomerase II inhibitor etoposide was shown to cause radiation sensitization in V79 cells (2) and in human melanoma cells (3). These results suggest an involvement of topoisomerases in the repair of sublethal or potentially lethal damage as caused by irradiation.

We have shown that in hepatoma cells, three different flavonoids caused an enhancement of radiation toxicity and that genistein in conjunction with a regime of fractionated radiation did not allow recovery of the surviving cell population during the intervals. It is therefore suggested that genistein and the other flavonoids prevent repopulation, as well as repair of radiation damage, possibly by inhibition of topoisomerase II activity.

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