Prevention of toxin-induced cytoskeletal disruption and apoptotic liver cell death by the grapefruit flavonoid, naringin

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Abstract

The protein phosphatase-inhibitory algal toxins, okadaic acid and microcystin-LR, induced overphosphorylation of keratin and disruption of the keratin cytoskeleton in freshly isolated rat hepatocytes. In hepatocyte cultures, the toxins elicited DNA fragmentation and apoptotic cell death within 24 h. All these toxin effects could be prevented by the grapefruit flavonoid, naringin. The cytoprotective effect of naringin was apparently limited to normal hepatocytes, since the toxin-induced apoptosis of hepatoma cells, rat or human, was not prevented by the flavonoid. *Cell Death and Differentiation* (2000) **7,** 739 – 746.

Keywords: apoptosis; cytoprotectant; flow cytometry; hepatocyte; hepatoma; hepatotoxin

Abbreviations: DMEM, Dulbecco's modified Eagle's medium; EDTA, ethylenediamine tetraacetic acid; EGTA, ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid; K8, keratin 8; K18, keratin 18; PBS, phosphate-buffered saline; PBS-T, PBS with 0.1% Tween-80; SDS, sodium dodecyl sulfate; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP-digoxigenin nick end-labeling

Introduction

Toxins produced by marine and freshwater algae represent a significant environmental hazard. Okadaic acid, synthesized by dinoflagellates and concentrated in mussels, is a major causative agent of diarrhetic shellfish poisoning,[1,2] and microcystins, made by blue-green algae which sometimes infest drinking water supplies, are strong hepatotoxins that have caused sickness and death in both humans and animals.[3–6] In isolated rat hepatocytes, algal toxins have been shown to induce a disruption of various cytoskeletal elements[7–10] as well as to elicit apoptotic cell death.[11]

Since the algal toxins are protein phosphatase inhibitors which cause overphosphorylation of cellular proteins,[1,12] some of their biochemical effects can be antagonized by protein kinase inhibitors. For example, the inhibition of hepatocytic autophagy and endocytosis by okadaic acid can be antagonized by protein kinase-inhibitory flavonoids as well as by specific inhibitors of Ca^{2+}/calmodulin-dependent protein kinase II.[13–15] Furthermore, the flavonoid, PD-98059, a potent inhibitor of MAP kinase kinases,[16] was recently found to prevent the okadaic acid-induced death of cultured rat neurons,[17] suggesting a potential cytoprotective role for this class of compounds. Here we show that another flavonoid, naringin (the bitter-tasting component of grapefruit), can protect rat hepatocytes against toxin-induced overphosphorylation and disruption of the keratin cytoskeletal network, as well as against toxin-induced apoptotic cell death. Interestingly, the protective effect of naringin may be confined to normal hepatocytes, since the toxin-induced apoptosis of human or rat hepatoma cells was unaffected by the flavonoid.
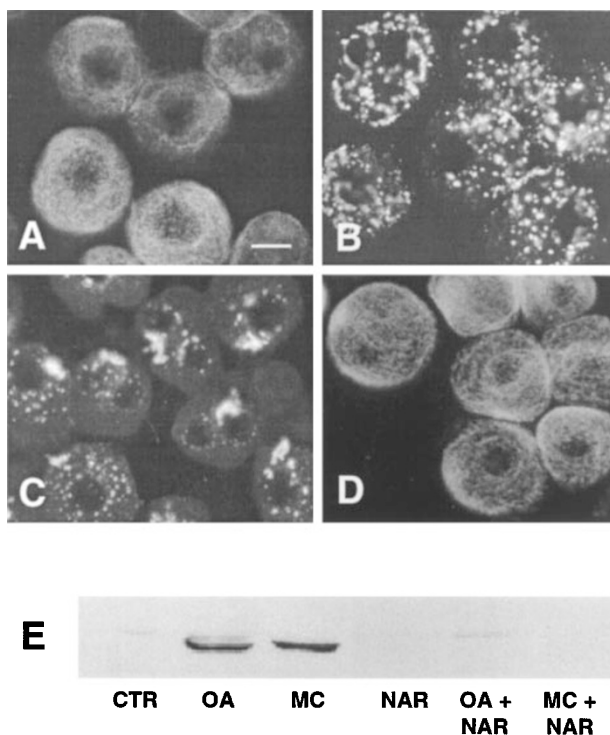
Results and discussion

Prevention of toxin-induced cytoskeletal changes by naringin

Both okadaic acid and microcystin-LR induced a disruption of the keratin intermediate filament organization in freshly isolated rat hepatocytes, as revealed by fluorescence microscopy of keratin immunostained cells (Figure 1). Whereas control cells displayed a diffuse filamentous keratin network (Figure 1A), more than 90% of cells treated for 1 h with okadaic acid (30 nM; Figure 1B) or microcystin (1 μM; Figure 1C) had their keratin network collapse into numerous small spherical aggregates. The presence of naringin (100 μM) completely prevented these toxin-induced changes (Figure 1D).

Immunoblotting with a site-specific antibody which recognized phosphoserine 33 on keratin 18[22] showed that this site became overphosphorylated in cells treated with okadaic acid or microcystin (Figure 1E). The keratin phosphorylation was more sensitive to okadaic acid than to microcystin, probably relating to the fact that okadaic acid is internalized more directly by hepatocytes than microcystin,[14,23] the latter apparently requiring uptake by bile acid transporters.[24,25] The high potency of okadaic acid would suggest a type 2A protein phosphatase as the enzyme being inhibited by the toxin; the type 1 phosphatases being several orders of magnitude less sensitive to okadaic acid.[1]

Naringin completely prevented the keratin overphosphorylations induced by either okadaic acid or microcystin (Figure 1E). This flavonoid has no direct effect on either basal or okadaic acid-suppressed protein phosphatase 2A activity under cell-free conditions (our unpublished results),

strand breaks, two of the hallmarks of apoptosis, were detected by a flow-cytometric TUNEL method.[20] Hepatocytes differ from most other cell types in that the majority of their nuclei are polyploid,[33] but the stoichiometric staining of DNA with Hoechst 33258 readily resolved normal nuclei (R1 gate) of diploid (2N), tetraploid (4N) or octoploid (8N) DNA content as well as some 2N/4N aggregates in the 6N position (Figure 2A). In control hepatocyte cultures, only 5–10% of the nuclei were recorded as apoptotic (R2 gate), i.e., with fragmented DNA (subdiploid DNA content) or DNA strand breaks (high FITC immunofluorescence). In contrast, cultures treated with 30 nM okadaic acid for 24 h had 30–40% apoptotic nuclei (Figure 2B). The apoptosis was verified by the ability of okadaic acid to induce extensive internucleosomal DNA cleavage in the hepatocytes, giving rise to a 'DNA ladder' in agarose gels (Figure 2C). Furthermore, a nonspecific inhibitor of apoptotic caspases, zVAD·fmk,[34] was able to suppress the apoptosis completely (results not shown).

To check that the toxin-induced apoptosis led to cell death, and not just to DNA damage, okadaic acid-treated hepatocyte cultures were examined in the light microscope, using morphological evaluation and staining with trypan blue to distinguish intact cells from dead and dying cells. As shown in Figure 2D, okadaic acid caused extensive, dose-dependent hepatocyte death. At 100 nM okadaic acid, nearly all cells were dead after 24 h of treatment. Most of the dead cells detached from the culture substratum. High doses of microcystin had similar lethal effects (results not shown).

The toxin-induced morphological changes associated with the apoptotic death of cultured hepatocytes, such as surface blebbing, cell rounding and detachment, and nuclear fragmentation as detected by Hoechst staining, were completely preventable by inclusion of naringin in the culture medium (Figure 3A–H). The cells remained attached to the culture dish, retaining their normal flattened shape and round, intact nuclei. The ability of naringin to suppress toxin-induced apoptosis was most clearly illustrated by TUNEL analysis: whereas 300 nM microcystin induced almost 50% apoptosis in 24 h, no effect of the toxin was observed in the presence of naringin (Figure 3I). Similarly, the dose-dependent apoptosis induced by okadaic acid was strongly suppressed by naringin (Figure 3J), as was okadaic acid-induced cell death (Table 1). A number of other flavonoids, tested at concentrations up to 300 μM (genistein, apiin, rhoifolin, neohesperidin) failed to antagonize okadaic acid-induced apoptosis (results not shown). The protective effect of naringin is thus unlikely to reflect the general antioxidant properties of flavonoids, in which regard naringin is among the least active.[35,36] A protein kinase-inhibitory effect of naringin would seem more probable, in analogy with the ability of PD-98059, a flavonoidic MAP kinase inhibitor,[16] to prevent okadaic acid-induced death of cultured rat neurons.[17] PD-98059, incidentally, had no cytoprotective effect on hepatocytes (results not shown).

Disruption of the hepatocellular cytoskeleton has been postulated to be a direct cause of death in microcystin-

Figure 1 Prevention, by naringin, of toxin-induced phosphorylation and disruption of the hepatocytic keratin cytoskeleton. Freshly isolated rat hepatocytes were incubated for 1 h at 37°C with the additions indicated. (**A–D**) The cells were sedimented onto glass slides, fixed in 100% methanol and processed for indirect immunofluorescence staining of keratin, using a monoclonal antibody against K8 as described in Materials and Methods. (**A**) No additions (control); (**B**) Okadaic acid, 30 nM; (**C**) Microcystin-LR, 1 μM; (**D**) Okadaic acid+naringin, 100 μM (cells treated with microcystin plus naringin, or with naringin alone, likewise looked like control cells). (**E**) The cells were lysed in an SDS-containing buffer, and their proteins separated by electrophoresis in 10% SDS-polyacrylamide gels. Immunoblotting was performed using a mouse monoclonal antibody against K18-pSer33. Lane 1, no additions (control); lane 2, okadaic acid, 30 nM; lane 3, microcystin-LR, 1 μM; lane 4, naringin, 100 μM; lane 5, okadaic acid+naringin; lane 6, microcystin-LR+naringin

suggesting that it inhibits the phosphorylation of keratin rather than promoting its dephosphorylation. Many other flavonoids have been shown to be effective and specific protein kinase inhibitors.[26–28] A number of protein kinases have been shown, under cell-free conditions, to be able to phosphorylate keratins 8 and 18; these include MAP kinase, the cyclin-dependent kinase Cdc2, S6 kinase, AMP-activated protein kinase, Ca^{2+}/calmodulin-dependent protein kinase II, protein kinase C and protein kinase A.[22,29–31] The putative naringin-sensitive kinase has not yet been identified, but at least none of the latter three kinases seem to be directly affected by the flavonoid (our unpublished results).

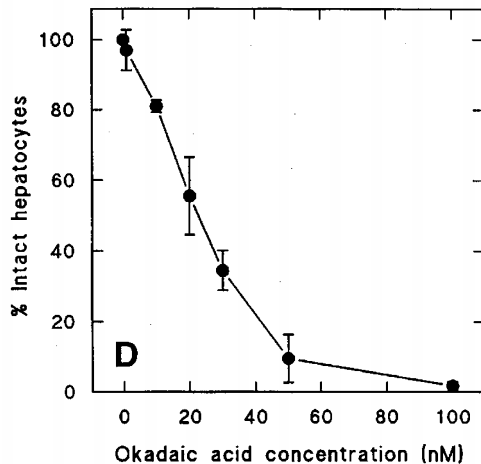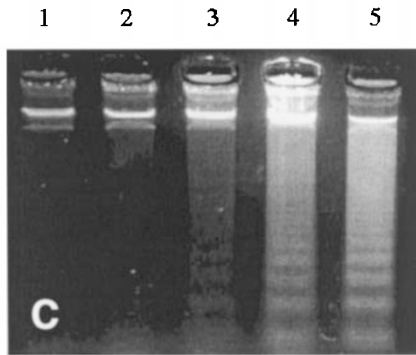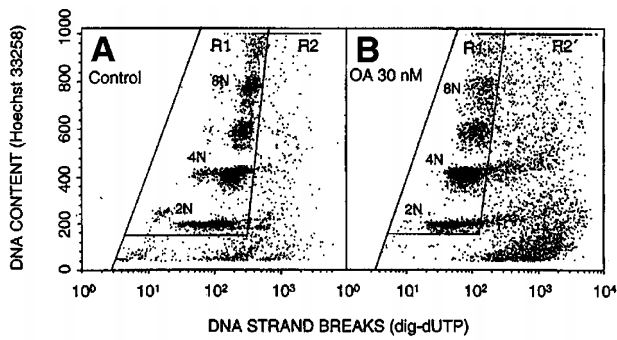## Protection, by naringin, of normal rat hepatocytes against toxin-induced apoptotic cell death

To investigate whether the toxin-antagonistic effect of naringin might include a prevention of toxin-induced apoptotic cell death,[11] short-term cultures of rat hepatocytes were established.[32] DNA fragmentation and DNA

Figure 2 Okadaic acid-induced DNA fragmentation and apoptotic cell death of cultured rat hepatocytes. (**A – B**) Rat hepatocytes were cultured for 24 h at 37°C in the absence (**A**) or presence (**B**) of 30 nM okadaic acid. Nuclei were isolated by a citrate-detergent method, and DNA strand breaks end-labeled *in situ* with digoxigenin-dUTP using terminal transferase (TUNEL method). The nuclei were stained with anti-digoxigenin-FITC (to detect DNA strand breaks) and Hoechst 33258 (to quantify DNA), and analysed by two-parametric flow cytometry. The analysis gates were set to discriminate normal nuclei of various ploidies (gate R1) from apoptotic nuclei with DNA loss and/or DNA strand breaks (gate R2). (**C**) DNA, extracted from hepatocytes cultured for 24 h at various okadaic acid concentrations, was subjected to electrophoresis in a 1.4% agarose gel. The gel was stained with SYBR™Green and photographed under ultraviolet illumination. Lane 1, control; lane 2, 1 nM okadaic acid; lane 3, 10 nM okadaic acid; lane 4, 30 nM okadaic acid; lane 5, 100 nM okadaic acid. (**D**) Hepatocyte cultures, incubated for 24 h at the concentration of okadaic indicated, were aspirated to remove detached, dead cells (uniformly staining with trypan blue). The monolayers were then fixed in 70% ethanol, or examined directly after addition of trypan blue. The number of attached cells in

treated rodents, the consequent weakening of cellular mechanical strength and cell-to-cell contacts resulting in hepatic hemorrhage.[8,37] The relationship between the cytoskeleton and apoptosis is less clear: like other cytoskeletal proteins, keratins are frequently cleaved secondarily to the activation of apoptotic proteinases (caspases),[38,39] but a role for keratins in anti-apoptotic signaling has also been postulated.[40] Whether the disruption of the keratin network plays a causal role in the toxin-induced apoptosis of cultured hepatocytes remains to be shown, but at least the parallel effects of naringin on apoptosis and cytokeratin alterations strongly suggest the existence of a common regulatory element.

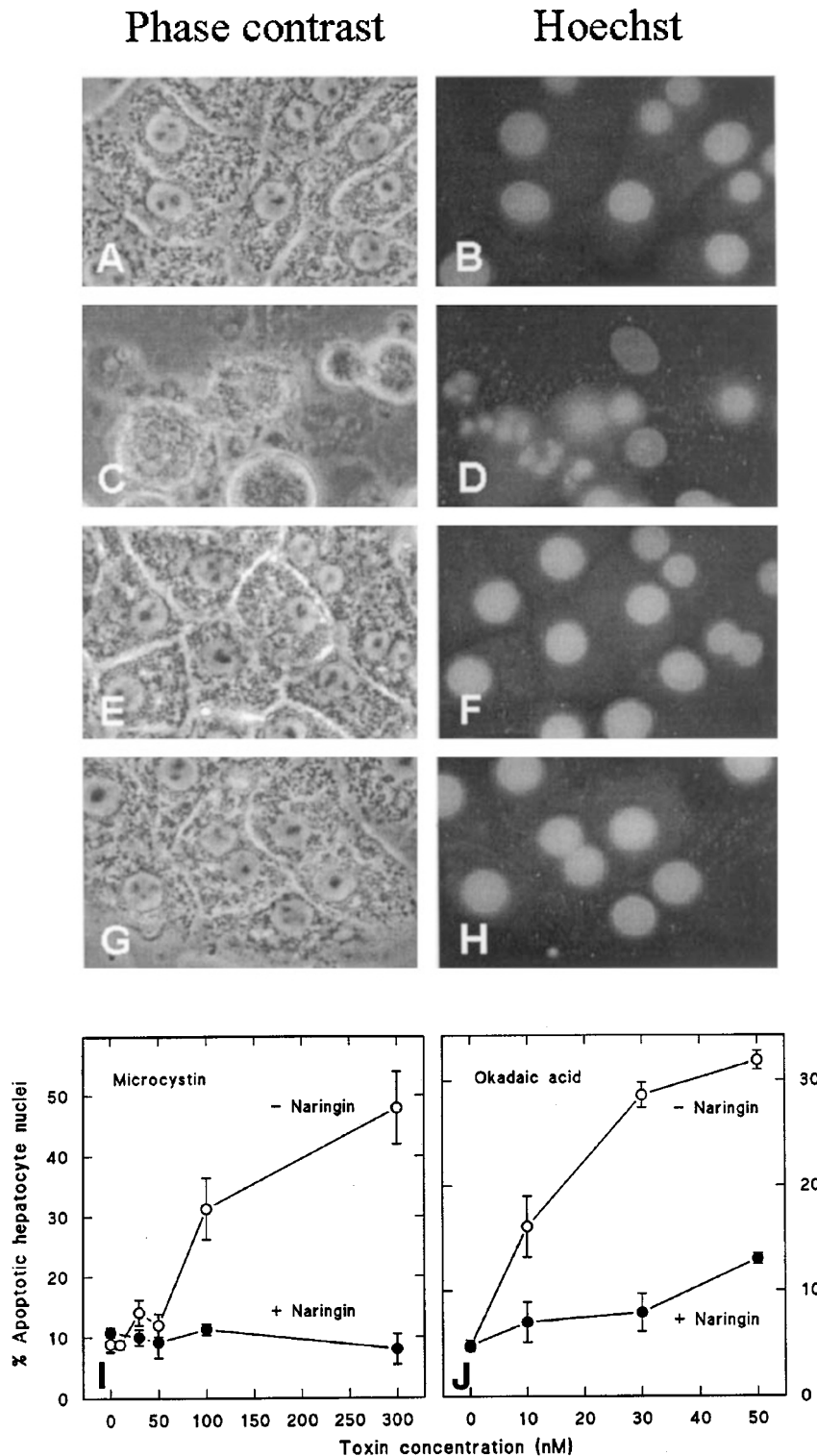## Inability of naringin to protect hepatoma cells against toxin-induced apoptosis

Several hepatoma cell lines (the Morris $7800C_1$ rat hepatoma cells and the human hepatoma cell lines Hep3B and C3A) were found to be as susceptible to okadaic acid-induced apoptotic cell death as were normal rat hepatocytes. Figure 4A – D shows that the toxin caused extensive nuclear fragmentation in Hep3B cells, and Figure 4E demonstrates internucleosomal DNA cleavage ('DNA ladder') in okadaic acid-treated Morris $7800C_1$ cells. In contrast to okadaic acid, microcystin had no effect on hepatoma cells at any concentration (results not shown), possibly because hepatoma cells tend to lack the bile acid transporters required for cellular microcystin uptake.[25]

Dose-dependent apoptosis was well demonstrated by the TUNEL method both in Morris $7800C_1$ cells (Figure 4F) and in Hep3B cells (Figure 4G). Surprisingly, naringin, at 100 μM, did not antagonize okadaic acid in any of the hepatoma cell lines (including the C3A cells, not shown here). Higher naringin concentrations (up to 1 mM) were equally ineffective, and the use of alternative culture media (including the medium used for hepatocytes) did not alter the results. Naringin sensitivity may thus, like bile acid transport, be a property easily lost in hepatoma cells.

Okadaic acid reduced hepatoma cell viability in very much the same way that it affected normal hepatocytes, and after 24 h with 30 nM okadaic acid the fraction of intact cells was reduced to about 30% both in hepatocyte cultures and in cultures of Morris $7800C_1$ hepatoma cells (Table 1). In contrast to its protection of normal hepatocytes, naringin did not detectably preserve the viability of the hepatoma cells. It is thus clear that this flavonoid cytoprotectant prevents the death of normal rat liver cells, but not the death of cancer cells derived from the same tissue.

The cytoprotective effect of naringin may be tissue-specific: it did not prevent the okadaic acid-induced death

Phase contrast   Hoechst

Figure 3 Naringin protects normal rat hepatocytes against toxin-induced apoptotic death. (**A** – **H**) Hepatocytes were cultured for 24 h under the following conditions: (**A**,**B**) control; (**C**,**D**) okadaic acid, 30 nM; (**E**,**F**) naringin, 100 μM; (**G**,**H**) naringin+okadaic acid. The cells were stained supravitally with Hoechst 33258 (2.5 μg/ml); 10 min later the brightly blue-fluorescent nuclei of live cells were examined in the fluorescence microscope (right) and compared with the cellular morphology as seen under phase contrast (left). $\times$ 540. (**I** – **J**) Hepatocytes were cultured for 24 h with microcystin-LR (**I**) or okadaic acid (**J**) at the concentration indicated, in the absence ($\bigcirc$) or presence ($\bullet$) of naringin, 100 μM. Nuclei were isolated by a citrate-detergent technique, labeled according to the TUNEL method, and analyzed by two-parametric flow cytometry. Apoptotic nuclei have been scored as per cent of the total number analyzed (10 000). Each value is the mean $\pm$ S.E. or range of 2 – 5 experiments

Table 1 Selective protection, by naringin, against okadaic acid-induced cell death in normal hepatocytes vs hepatoma cells

	% Intact cells	
Treatment	Normal hepatocytes	Hepatoma cells
None (control)	100 (3)	100 (3)
Naringin (100 μM)	85 ± 3 (3)	92 ± 7 (3)
Okadaic acid (30 nM)	30 ± 4 (3)*	27 ± 2 (4)*
Okadaic acid+naringin	75 ± 5 (3)†	34 ± 7 (4)

*$P < 0.001$ vs control; †$P < 0.05$ vs okadaic acid. Normal rat hepatocytes or Morris 7800C_1 rat hepatoma cells were kept in culture for 24 h before the addition of naringin (100 μM) or okadaic acid (30 nM; 30 min later) as indicated. After another 24 h, the cultures were fixed in 70% ethanol, and the number of adherent cells with normal morphology was counted in 25 microscope fields and expressed as per cent of the number of cells in control dishes. Each value is the mean $\pm$ S.E. of the number of independent experiments given in parentheses

of cultured rat neurons,[17] nor could it preserve the cytoskeletal integrity and viability of isolated rat colonocytes undergoing detachment-induced apoptosis.[41] Conversely, the synthetic flavonoid, PD-98059, protected neurons but not hepatocytes. The possibility of developing tissue-specific cytoprotective flavonoids is intriguing, since such agents might conceivably be of interest not only for protection against environmental toxins, but also for protection of normal tissues against degenerative changes occurring under various pathological conditions. There should be no shortage of candidates among the thousands of structurally different flavonoids present within the plant kingdom.[42]

Materials and Methods

Materials

Dulbecco's modified Eagles medium (DMEM), Ham's F-10 and F-12 media, penicillin/streptomycin, fungizone, garamycin, genistein, microcystin-LR, anti-I-PPLO, calf and horse serum were all purchased from Gibco BRL (Paisley, Scotland, UK). Digoxigenin-11-dUTP, terminal transferase and anti-digoxigenin-fluorescein Fab fragments were obtained from Boehringer Mannheim GmbH (Germany). Monoclonal mouse antibody against rat keratin 8 (K8) was a generous gift from Prof. Normand Marceau (University of Laval, Quebec, Canada). The monoclonal phosphokeratin antibody against K18-pSer33 (Clone IB4) was purchased from Clontech (Palo Alto, CA, USA); this antibody is no longer commercially available. Okadaic acid was obtained from Moana Bioproducts Inc. (Hawaii, USA) or Alexis Biochemicals (Läufelfingen, Switzerland); PD-98059 from New England Biolabs (Beverly, MA, USA); apiin and neohesperidin from Extrasynthase SA (Lyon, France). Naringin, rhoifolin and all other biochemicals were obtained from Sigma Chem. Co. (St. Louis, MO, USA). The Morris 7800C_1 hepatoma cell line was kindly provided by Dr. Øystein Spydevold (Institute for Medical Biochemistry, University of Oslo, Norway); other cell lines (Hep3B, C3A) were purchased from the American Type Culture Collection (Manassas, VA, USA).
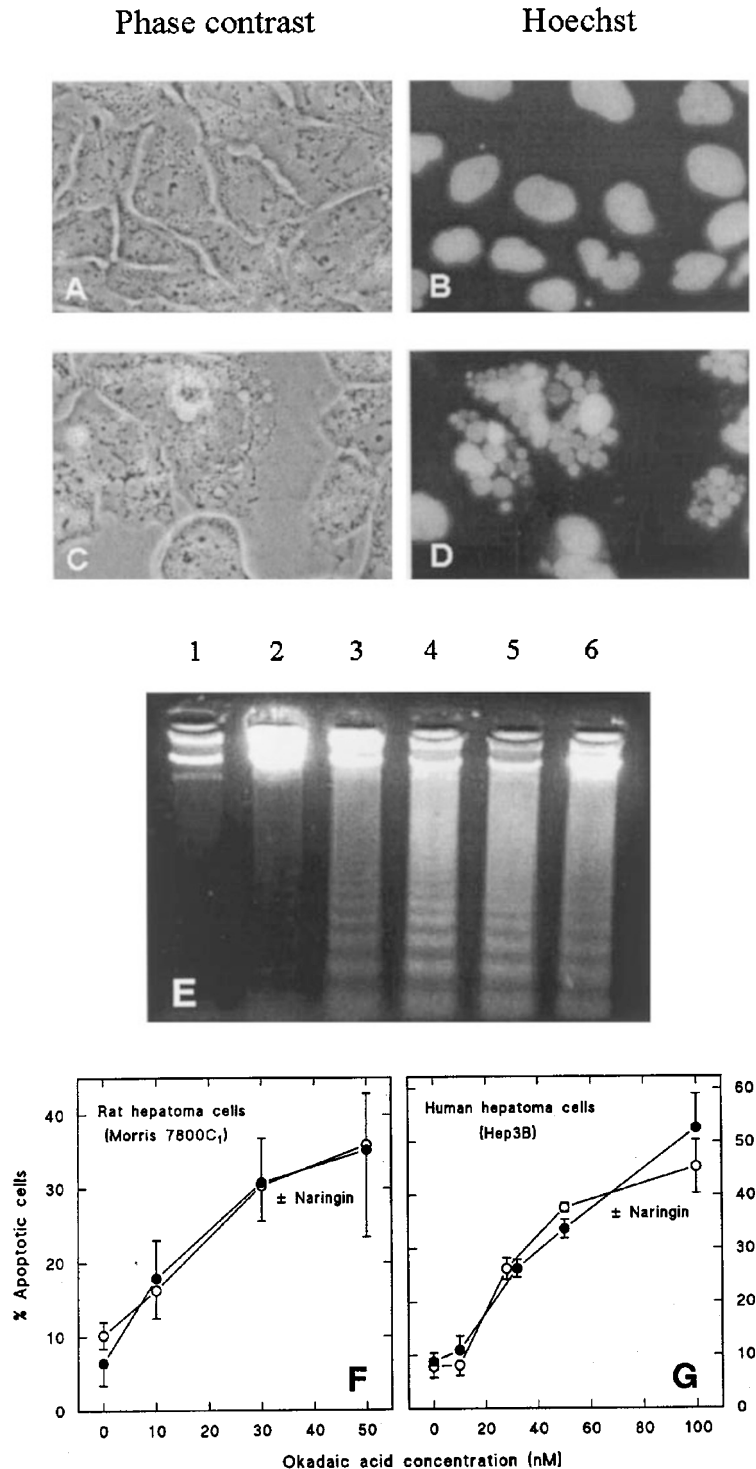
Cell culture

Normal, primary hepatocytes were isolated from male Wistar rats (200–250 g) by two-step collagenase perfusion[18] and cultured at 37°C on a substratum of adsorbed collagen at a cell density of $8.5 \cdot 10^4$ cells/cm^2. The culture medium was a 1:1 mixture of DMEM and Ham's F-12, supplemented with L-glutamine (2 mM), epidermal growth factor (5 nM), insulin (67 nM), dexamethasone (26 nM) and garamycin (3 μM). The 7800C_1 Morris rat hepatoma cells were plated at a density of $1.5 \cdot 10^4$ cells/cm^2 in Ham's F-10 supplemented with 10% horse serum, 3% calf serum, penicillin (50 U/ml), streptomycin (50 μg/ml), fungizone (2.5 μg/ml) and anti-I-PPLO (50 μg/ml). Hep3B and C3A human hepatoma cells were cultured in DMEM with 10% fetal calf serum, at a plating density of $1.3 \cdot 10^4$ cells/cm^2. After an attachment period of 2.5–3 h, the medium was changed, and incubation continued for another 24 or 48 h.

Immunofluorescence microscopy

Cells incubated for 1 h at 37°C were washed three times (1600 rev/min for 5 min at 4°C) and resuspended in suspension buffer to obtain a cell density of about $0.3 \cdot 10^6$ cells/ml. The cells were then sedimented (750 rev/min for 5 min) onto microscope slides using a Cytospin cytocentrifuge (Shandon Scientific Ltd., Cheshire, UK) before being fixed in 100% methanol for 10 min at −20°C and washed three times with phosphate-buffered saline (PBS; pH 7.5). For visualization of keratin intermediate filaments, cells were overlaid for 30 min with a monoclonal mouse antibody against rat K8 diluted 1:1000 in PBS. The cells were then washed three times in PBS and incubated with a fluorescein-conjugated anti-mouse antibody diluted 1:40 in PBS for 30 min in the dark. Finally the cells were washed three times in PBS and mounted in Mowiol (Hoechst, Frankfurt, Germany). Antibody incubations were performed in a humid chamber at room temperature. The cells were examined in a Nikon Optiphot microscope and photographed with a Kodak TMAX 400 film.

Gel electrophoresis and immunoblotting

Samples for immunoblotting were lysed in sodium dodecyl sulfate (SDS) buffer (20 mM Tris-HCl, pH 7.2, 5 mM EGTA, 10 mM sodium pyrophosphate, 0.4% SDS, 1 mM phenylmethylsulfonylfluoride, 10 μg/ml leupeptin, 10 μg/ml antipain). Whole cell extracts were diluted 1:1 in double strength SDS-gel-loading buffer (single-strength SDS-gel-loading buffer; 50 mM Tris-HCl; pH 6.8, 1 M mercaptoethanol, 2% SDS, 0.1% bromophenol blue, 10% glycerol) and separated by 10% SDS-polyacrylamide gel electrophoresis.[19] The separated proteins were transferred to nitrocellulose paper using a semi-dry transfer unit (Bio-Rad Laboratories) and blocked by an overnight incubation at 4°C with 5% dry milk in PBS. The membranes were then washed three times for 5 min in PBS with 0.1% Tween-20 (PBS-T) and incubated for 2 h at room temperature with a phosphospecific antibody against keratin 18 (K18-pSer33), diluted 1:30 in PBS-T. After washing three times for 5 min in PBS-T, the membranes were incubated for 30 min at room temperature with anti-mouse-horseradish peroxidase, diluted 1:2000 in PBS-T. The membranes were then washed twice in PBS-T and once in PBS before they were finally visualized by chemiluminescence using the ECL Western Blotting Detection Kit (Amersham).

Phase contrast   Hoechst

Figure 4 Lack of protective effect of naringin against toxin-induced apoptosis in rat and human hepatoma cells. (**A** – **D**) Hep3B human hepatoma cells were cultured for 24 h in the absence (**A,B**) or presence (**C,D**) of 30 nM okadaic acid, then stained with the fluorescent vital stain Hoechst 33258 (2.5 μg/ml). Left, phase contrast; right, fluorescent microscopy. Notice extensive nuclear fragmentation in okadaic acid-treated cells (**D**). ×590. (**E**) Morris $7800C_1$ rat hepatoma cells were cultured for 24 h with up to 100 μM okadaic acid. DNA was extracted and subjected to electrophoresis in a 1.4% agarose gel, stained with SYBR^{TM}Green, and photographed under ultraviolet illumination. Lane 1, control; lane 2, okadaic acid, 1 nM; lane 3, okadaic acid, 10 nM; lane 4, okadaic acid, 15 nM; lane 5, okadaic acid, 30 nM; lane 6, okadaic acid, 100 nM. (**F** – **G**) Morris $7800C_1$ rat hepatoma cells (**F**), or Hep3B human hepatoma cells (**G**) were cultured for 24 h with okadaic acid at the concentration indicated, in the absence (○) or presence (●) of naringin, 100 μM. The cells were dislodged by trypsinization, labeled according to the TUNEL method, and analyzed by flow cytometry. Apoptotic cells are scored as per cent of the total number analyzed (10 000). Each value is the mean ± S.E of 3 – 5 experiments

Measurement of apoptosis by DNA labeling (TUNEL)

Whole hepatoma cells, or nuclei from cultured hepatocytes (which cannot be dissociated into single cells), prepared by a citrate-detergent method, were fixed in 1% paraformaldehyde for 15 min on ice and permeabilized in 70% ethanol at -20°C. DNA strand breaks were end-labeled by the TUNEL method, using incubation with terminal transferase to incorporate digoxigenin-11-dUTP, which was then stained with anti-digoxigenin-FITC.[20] Staining with Hoechst 33258 was used to detect total DNA and nuclear fragmentation. Cells or nuclei were analyzed by two-parametric flow cytometry in a FACScan flow cytometer (Becton Dickinson), using the gate settings shown in Figure 1; cells, nuclei with DNA strand breaks (right-hand part of cytogram) or fragmented DNA (lower part) were collectively defined as apoptotic, and scored as the percentage of total.

Agarose gel analysis of DNA fragmentation

Cell samples were harvested from the culture dishes, and apoptotic DNA fragments isolated according to Hermann et al.[21] Briefly, cells were washed once in PBS and pelleted by centrifugation before being lysed with lysis buffer (1% NP-40 in 20 mM EDTA, 50 mM Tris-HCl, pH 7.5; 60 μl/cell sample) and recentrifuged for 5 min at $1600 \times g$. The supernatants were collected and the extraction was repeated with the same amount of lysis buffer. The supernatants were then brought to 1% SDS and treated with RNase A (final concentration 5 mg/ml) for $1-2$ h in a water bath at 37°C followed by proteinase K (final concentration 2.5 mg/ml) for at least 2 h at 37°C. Finally, 0.5 vol 10 M ammonium acetate was added and the DNA precipitated with 2.5 vol ethanol at -20°C overnight. The resuspended nuclei were subjected to the same treatment as the supernatants, and were used as controls of the complete recovery of apoptotic DNA fragments. The DNA fragments were separated in a 1.4% agarose gel stained with SYBR^{TM}Green and photographed under ultraviolet illumination.

Cellular viability

Cellular viability was measured by examining the cultures in the microscope, scoring the adherent cells (in 25 microscopical fields per culture dish) as morphologically normal (intact) or grossly abnormal (dead or dying; verified by trypan blue staining). Detached cells were always dead. The number of intact cells in toxin-treated cultures was expressed as per cent of the number in comparable control cultures from the same experiment. To assess alterations in nuclear morphology, the cell cultures were stained supravitally with Hoechst-33258 (10 min at 37°C, 24 h after seeding), and the brightly blue-fluorescent nuclei were examined in live cells in the fluorescence microscope.

Acknowledgments

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