Eur. J. Biochem. *254*, 38−43 (1998)

Blockage of apoptotic signaling of transforming growth factor-*β* in human hepatoma cells by carboxyfullerene

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(Received 26 January/16 March 1998) − EJB 98 0113/1

Transforming growth factor-*β* (TGF-*β*) has been shown to induce apoptosis in normal hepatocytes and hepatoma cells both *in vivo* and *in vitro*. However, the mechanism by which TGF-*β* induces apoptosis is not clear. The antiapoptotic activity of antioxidants including *N*-acetyl-L-cysteine (Ac-Cys), ascorbic acid and a novel free radical scavenger, carboxyfullerene (C_{60}) on TGF-*β*-treated human hepatoma Hep3B cells was examined. Only the water-soluble hexacarboxylic acid derivative of C_{60} was found to prevent TGF-*β*-induced apoptosis. Antiapoptotic activity of C_{60} correlated its ability to eliminate TGF-*β*-generated reactive oxygen species (ROSs). However, C_{60} did not interfere with TGF-*β*-activated PAI-1 promoter activity in the Hep3B cells. These results indicate that the signaling pathway of TGF-*β*-induced apoptosis may be related to the generation of ROSs and may be uncoupled from the TGF-*β*-activated gene promoter activity. Furthermore, the regioisomer of C_{60} with a C_3 symmetry was more potent in protecting cells from apoptosis than that with a D_3 symmetry, and the C_3 isomer had stronger interactions with lipid bilayers than the D_3 isomer. The spectroscopic analysis revealed that the C_3 isomer had stronger interactions with artificial lipid bilayers than the D_3 isomer. Therefore, our study indicates that C_{60} may interact with membrane to eliminate TGF-*β*-induced ROSs and to prevent apoptosis occur in human hepatoma cells.

Keywords: carboxyfullerene; transforming growth factor-*β*; apoptosis; reactive oxygen species.

Since its discovery, the pure carbon spheres of buckminsterfullerene (C_{60}) have generated great interest from many different branches of science and engineering. To investigate the chemical and physical characteristics of C_{60} (and its larger fullerenes), many novel properties of C_{60} were observed including its avid reactivity with free radicals [1]. Buckminsterfullerenes, for example, are capable of adding multiple radicals to each molecule. The addition of as many as 34 methyl radicals to a single C_{60} sphere has been reported, leading Krusic et al. [1] to characterize C_{60} as a 'radical sponge'. However, native C_{60} is soluble only in organic solvents. Dugan et al. [2] have evaluated the possibility that the potent innate anti-oxidant properties of C_{60} could be harnessed for use in biological systems by adding functional groups aimed at enhancing its water solubility. They recently reported neuroprotective effects of antioxidant polycarboxylated derivatives of C_{60} on cultured cortical neurons and *N*-methyl-D-aspartate (NMDA)-induced cell death [2]. How the water-soluble derivative of C_{60} protects cell from NMDA-induced death is not clear.

Transforming growth factor *β* (TGF-*β*) is a 25-kDa dimeric protein [3−6] with diverse biological activities. It stimulates or inhibits cell proliferation, depending on the cell types and the presence of other growth factors [7−11]. Recent studies have shown that TGF-*β* induces apoptosis in liver cells *in vitro* [12−17], and transgenic mice overexpressing TGF-*β* develop continuing apoptotic death of hepatocytes as well as hepatic fibrosis *in vivo* [18]. Exogenous administration of TGF-*β* to rodents also results in a significant increase in hepatic cell death [13, 15, 19]. These data strongly suggest that apoptosis induced by TGF-*β* may be involved in various hepatic lesions. However, despite the identification of TGF-*β* receptors and the genes involved in its downstream signaling pathway [20−27], the mechanism of TGF-*β*-induced apoptosis remains largely unknown.

In the study reported here, we examined the anti-apoptotic activity of a few antioxidants on TGF-*β*-treated human hepatoma Hep3B cells and found only the water-soluble hexacarboxylic acid derivative of carboxyfullerene (C_{60}) could block TGF-*β*-induced apoptosis. These C_{60} derivatives not only protected cells from TGF-*β*-induced apoptosis but also abolished TGF-*β*-generated reactive oxygen species (ROSs). Nevertheless, it did not interfere with TGF-*β*-activated plasminogen activator inhibitor-1 (PAI-1) promoter activity.

MATERIALS AND METHODS

Cell culture. The human hepatoma Hep3B cell line was maintained at 37 °C in Dulbecco's modified Eagle's medium (DMEM, Gibco) containing 10% fetal calf serum (Gibco) and antibiotics in a humidified atmosphere of 5% CO_2.

Chemicals. Recombinant human TGF-*β*1 (Austral Biologicals) was stored at −80 °C in 10 mM HCl and 5 mg/ml bovine

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Abbreviations. TGF-*β*, transforming growth factor *β*1; C_{60}, carboxyfullerene; ROSs, reactive oxygen species; PAI-1, plasminogen activator inhibitor-1; DMEM, Dulbecco's modified Eagle's medium; PySOPtd-Etn, L-phosphatidylethanolamine-*N*-(1-pyrene sulfonyl) sodium; Ac-Cys, *N*-acetyl-L-cysteine; NMDA, *N*-methyl-D-aspartate.

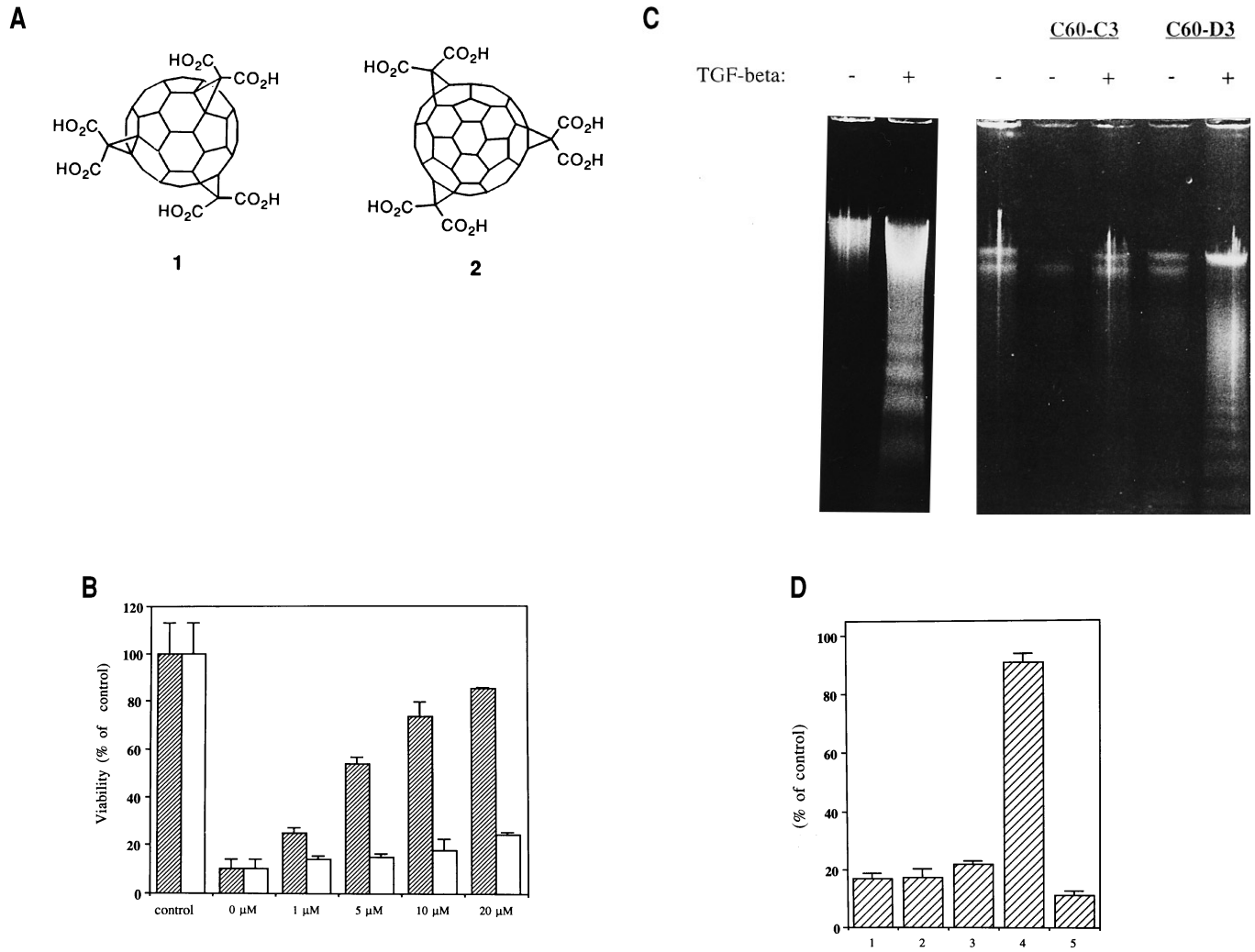

Fig. 1. Protective effect of carboxyfullerene on TGF-β-induced apoptosis. (A) Structure of two regioisomers of water-soluble hexacarboxylic acid derivatives of C_{60}, $C_{60}[C(COOH)_2]_3$ (compounds 1 and 2 are in C_3 and D_3 symmetry, respectively). (B) The TGF-β-induced apoptosis in the Hep3B cells is inhibited by C_3, but not by D_3 in a dose-dependent manner. Cells were treated with TGF-β (120 pM) for 30 min, then different concentrations of C_3 (hatched) or D_3 (unfilled) were added as indicated. The viable cell number was determined after 48 h using the trypan-blue-exclusion method. (C) C_3, but not D_3, prevents the internucleosomal DNA fragmentation in TGF-β-treated Hep3B cells. Cells were treated with 120 pM TGF-β for 24 h in the presence of C_3 (20 μM) or D_3 (20 μM), then scraped into lysis buffer and electrophoresis as described in Materials and Methods. (D) Viability of TGF-β-treated Hep3B cells in the presence of small molecular antioxidants. Viable cell numbers of TGF-β-treated (120 pM, 48 h) cells (lane 1) or cells treated with TGF-β in the presence of 1 mM ascorbic acid (lane 2), 20 mM Ac-Cys (lane 3), 20 μM C_3 (lane 4) and 20 μM D_3 (lane 5) cells. All experiments were performed at least three times, in duplicate, in each experiment. The bars indicate the average values ± SEM from duplicates in a representative experiment.

serum albumin. Two regioisomers of C_{60}, C_3 and D_3, were synthesized and purified following the method of Lamparth and Hirsch [28, 29]. The purity of these compounds were verified in NMR and ultraviolet/visible spectral analyses. The fluorescent probe 2′,7′-dichlorofluoresceine diacetate and 8-hydroxypyrene-1,3,6-trisulfonic acid trisodium were from Molecular Probes. 1,2-Diphytanoyl-*sn*-glycero-3-phosphocholine and L-phosphatidylethanolamine-*N*-(1-pyrene sulfonyl) sodium (PySOPtdEtn) were from Avanti Polar Lipids.

Cell treatment. The cells were plated on 24-well cell-culture plates, at a density of 8×10^4 cells/well, and allowed to attach for 24 h. The cells were washed with 136.7 mM NaCl, 2.7 mM KCl, 10.1 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4 ($NaCl/P_i$) and changed to DMEM without serum. After 48 h, cells were treated with or without TGF-β1 for 48 h. Following the treatment, the cells were trypsinized and harvested. The number of viable cells was counted by the trypan-blue-exclusion method.

Cell transfection. Hep3B cells were transfected by a modified calcium phosphate coprecipitation procedure [30], typically using 10 μg p800Luc and 5 μg pCMV-βGAL DNA. After transfection, cells were recovered for 16 h in DMEM with 10% fetal calf serum, then treated with or without 120 pM TGF-β1 in DMEM with 10% fetal calf serum. After 24 h, the cells were harvested and lysed in reporter lysis buffer, and the cell lysates were assayed for luciferase and β-galactosidase activities. The luciferase assay was carried out using assay reagents from Analytic Luminescence Laboratory [31] and luminometer monolight 2010. β-Galactosidase was assayed in an assay buffer [31a] and the activity measured at 420 nm in a spectrophotometer. The luciferase activity, which reflects the promoter activity of PAI-1, was normalized to β-galactosidase to account for the transfection efficiency.

DNA fragmentation. Fragmented DNA was analyzed by electrophoresis as described by Smith et al. [32]. The cells were plated on a 90-mm dish at a density of 2×10^6 cells/dish.

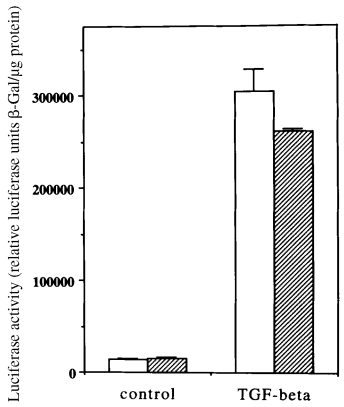

Fig. 2. PAI-1 induction of TGF-β in Hep3B cells is not affected by C_3. After transfecting with plasmid p800Luc, cells were treated with 120 pM TGF-β for 24 h in the absence (unfilled) or presence (hatched) of C_3 (20 μM). Preparation of cell lysates and the luciferase activity assay were performed as described in Materials and Methods. The experiment was performed at least three times, in duplicate, in each experiment. The bars indicate the average values ± SEM from duplicates in a representative experiment.

Following the treatment described, cell pellets were resuspended in 20 μl lysis buffer containing 10 mM EDTA, 50 mM Tris/HCl, pH 8.0, 0.5% (mass/vol.) sodium lauryl sarkosinate and 0.5 mg/ml RNase A, then incubated at 50 °C for 1 h. 10 μl proteinase K (0.5 mg/ml) was added to each sample and incubation at 50 °C for a further 1 h. Samples were heated to 70 °C, and 10 μl 10 mM EDTA, pH 8.0, containing 1% (mass/vol.) low-gelling-temperature agarose, 0.25% (mass/vol.) bromophenol blue and 40% (mass/vol.) sucrose was mixed with each sample before loading into the dry wells of a 2% (mass/vol.) agarose gel containing 0.1 μg/ml ethidium bromide. Electrophoresis was conducted at 50 V in 0.5×Tris/borate/EDTA buffer (45 mM Tris/borate, 1 mM EDTA, pH 8.0) until the marker dye had migrated 3−4 cm.

Measurement of intracellular reactive oxygen species by flow cytometry. The oxidation-sensitive fluorescent probe 2′,7′-dichlorofluoresceine diacetate was used to measure intracellular ROSs. Cells were incubated under different experimental conditions, detached by trypsinization, and incubated in 5 μM 2′,7′-dichlorofluoresceine diacetate. The cellular fluorescence intensity was measured after 60 min oxidation by 2′,7′-dichlorofluoresceine diacetate in a Facscan flow cytometer (Becton Dickinson). For each analysis, 10 000 events were recorded.

Liposome preparation and fluorescence quenching assay. In a typical experiment, the wall of a test tube was coated by 1.1 mg 1,2-diphytanoyl-*sn*-glycero-3-phosphocholine and a pyrene-labelled lipid, L-phosphatidylethanolamine-*N*-(1-pyrene sulfonyl) sodium (5 mol/100 mol) in chloroform and blown dry by N_2 purging. 10 ml Hepes, pH 7.3, was added to the test tube, and the solution sonicated (Heat System, model XL2020, 50 W) by a 15-s on/15-s off cycle for 12 min. During sonication, the liposome-forming solution was continuously purged with N_2 gas to minimize oxidation of lipids. Liposome formed in such a procedure has an average diameter of approximately 100 nm, as measured by transmission electron microscopic measurements. In the fluorescence quenching experiments, the excitation wavelengths are 406 nm for 8-hydroxypyrene-1,3,6-trisulfonic acid trisodium (l_{EM} = 511.1 nm), 347 nm for the pyrene-labelled lipid, PySOPtdEtn (l_{EM} = 380.5 nm). The Stern-Volmer equation [33, 34] expresses the fluorescence intensity ratio (I_0/I) as a function of the quencher concentration [Q],

$$I_0/I = 1 + k_q t[Q] , \quad (1)$$

where I_0 and I are the fluorescence intensities in the absence and presence of quenchers, and t is the fluorescence lifetime in the absence of quenchers. By comparing the slope ($k_q t$) of the Stern-Volmer plot, one can obtain the relative magnitudes of quenching rates for two different quenchers. Partition function measurements indicate that both C_3 and D_3 stay in the aqueous and or-

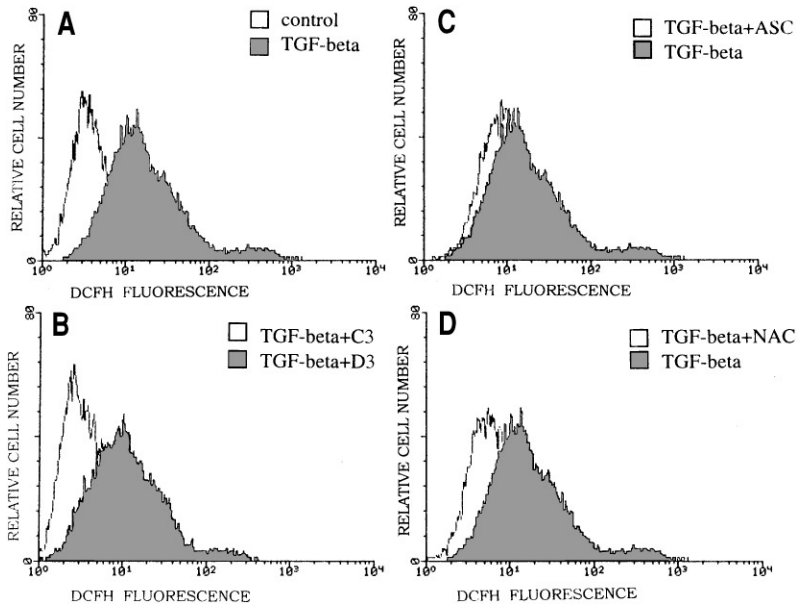

Fig. 3. C_{60} but not other antioxidants can block TGF-β-induced intracellular ROSs. Cells were treated with different agents as indicated, then detached and incubated for 60 min, with 5 μM oxidation-sensitive fluorescent probe (2′,7′-dichlorofluoresceine diacetate). The fluorescence intensity was measured by flow cytometry. (A) Histogram of control Hep3B cells (white area) or TGF-β-treated (120 pM, 12 h) cells (shadowed area). (B) Histogram of TGF-β-treated Hep3B cells with 20 μM C_3 (white area) or 20 μM D_3 (shadowed area). (C) Histogram of TGF-β-treated Hep3B cells with (white area) or without (shadowed area) 1 mM ascorbic acid (ASC). (D) Histogram of TGF-β-treated Hep3B cells with (white area) or without (shadowed area) 20 mM Ac-Cys.

ganic interface region, respectively. Therefore, in the lipid-attach pyrene system, both C_3 and D_3 can only quench those pyrene probes on the outer liposome surface. This means that pyrene probes in the inner liposome surface (approximately 50% of the total) will never be quenched and only contribute its fluorescence intensities as background. Therefore, $(0.5 \cdot I_0)$ was subtracted from the fluorescence intensities I_0 and I. Thus, $(I_0 - 0.5 \cdot I_0)/(I - 0.5 \cdot I_0)$ was plotted as a function of the C_3 and D_3 concentrations for the lipid attached pyrene system (Fig. 4) whereas I_0/I was plotted as the quencher concentration for the water-soluble pyrene (8-hydroxypyrene-1,3,6-trisulfonic acid trisodium) system, since all fluorescent molecules can be accessed by quenchers.

RESULTS

The anti-apoptotic action of carboxyfullerene on TGF-*β*-induced apoptosis. Two regioisomers of water-soluble hexacarboxylic acid derivatives of C_{60}, $C_{60}(C(COOH)_2)_3$ (1 and 2; Fig. 1A) were synthesized [28, 29]. Chemically, these two isomers have identical abilities to capture $^{\cdot}OH$ and $O_2^{-\cdot}$ free radicals *in vitro* [2], but the methano bridges in compounds 1 and 2 are in C_3 and D_3 symmetry, respectively. When C_3 and D_3 were added separately to cultured Hep3B cells previously exposed to TGF-*β*, C_3 protected 90% of the cells from apoptosis at a concentration of 20 μM, whereas D_3 had much less protective activity (Fig. 1B). Furthermore, only C_3 but not D_3 prevented the most typical indicator of cell apoptosis, DNA fragmentation in the TGF-*β*-treated Hep3B cells (Fig. 1C). The anti-apoptotic activity of other water-soluble antioxidants was also examined; neither ascorbic acid nor Ac-Cys protected cells from TGF-*β*-induced apoptosis (Fig. 1D).

The anti-apoptotic action of carboxyfullerene does not interfere with the signaling pathway of TGF-*β*. Since TGF-*β* has also been shown to activate the promoter activity of the PAI-1 gene in a variety of cell types [35], Hep3B cells were transiently transfected with pLuc800, which contains the luciferase reporter gene driven by the PAI-1 promoter, and examined whether C_3 affected the TGF-*β*-activated PAI-1 promoter activity. The result (Fig. 2) shows that while TGF-*β* increased the level of luciferase activity in Hep3B cells around 15-fold, the addition of C_3 (20 μM) had no effect on the stimulation.

Flow cytometric analysis of intracellular reactive oxygen species produced by TGF-*β*. To address whether C_{60} protects cells from TGF-*β*-induced apoptosis by scavenging free radicals, the oxidation-sensitive fluorescent probe 2′,7′-dichlorofluoresceine diacetate [36] was used to examine the generation of ROSs in TGF-*β*-treated cells in a flow cytometer. When the Hep3B cells were treated with TGF-*β* for 12 h, dichlorofluoresceine diacetate fluorescence was significantly increased in these cells (Fig. 3A). However, TGF-*β*-induced dichlorofluoresceine were completely abolished by addition of C_3 but not by addition of D_3 (Fig. 3B). Similarly, two other antioxidants, ascorbic acid and Ac-Cys, did not exhibit a significant effect on the TGF-*β*-induced dichlorofluoresceine shift (Fig. 3C).

Interaction of carboxyfullerene with an artificial lipid membrane. C_{60} has been shown to be incorporated into an artificial lipid membrane [37]. In order to understand why C_3 was more potent than D_3 in protecting cells from ROSs and apoptosis, Stern-Volmer fluorescence quenching [33] was used to examine the interactions of C_3 or D_3 with the lipid membrane. The fluorescence quenching of a water-soluble pyrene (8-hydroxypyrene-1,3,6-trisulfonic acid trisodium) by both C_3 and D_3 were nearly identical (Fig. 4). Assuming that the lifetime of the singlet-excited pyrene is in the proximity of 530 ns [38], the quenching rate constants can be estimated from the slopes of the plot as 2.4×10^{10} M^{-1} s^{-1} and 9.5×10^{9} M^{-1} s^{-1} for C_3 and D_3, respectively. When the pyrene chromophore was labelled to the head of a phosphatidylethanolamine lipid, the fluorescence quenching rates became much faster, i.e. 3.0×10^{11} M^{-1} s^{-1} and 1.0×10^{11} M^{-1} s^{-1} for C_3 and D_3, respectively (Fig. 4). These quenching rates are far beyond the diffusion-controlled rates, indicating that both C_3 and D_3 isomers have strong interactions with the lipid bilayer phase. The threefold larger quenching rate indicates that the C_3 isomer of carboxyfullerene has stronger interactions with lipid bilayers than the D_3 isomer.

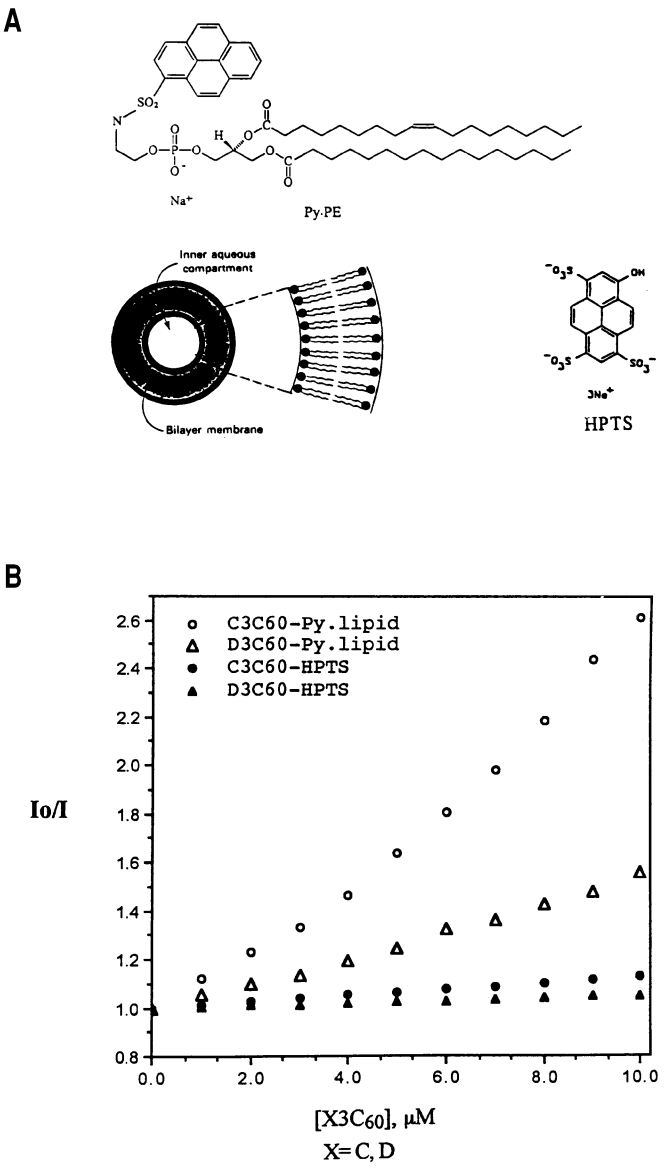

Fig. 4. C_3 interacts better than D_3 with lipid membranes. (A) Structure of reactive compounds PySOPtdEtn, 8-hydroxypyrene-1,3,6-trisulfonic acid trisodium and artificial lipid bilayer. (B) Fluorescence intensity ratio (I_0/I) as a function of the quencher (C_3 or D_3) concentration. The bottom two traces (filled circle and triangle) are the fluorescence quenching of a water-soluble pyrene (8-hydroxypyrene-1,3,6-trisulfonic acid trisodium) by the C_3 and D_3 isomers, respectively. The upper two traces (open circle and triangle) are from the fluorescence quenching of a lipid-bound pyrene (PyPtdEtn) by the C_3 and D_3 isomers, respectively. All solutions contain 10 mM Hepes, pH 7.3, and 0.16 M NaCl.

DISCUSSION

We and others have previously reported that TGF-β induces apoptosis in both cultured primary hepatocytes and hepatoma cells [12−17]. The involvement of caspase family proteases in this apoptotic processes was also reported recently [40]. However, the mechanism of TGF-β-induced apoptosis in human hepatoma cells is still not clear. TGF-β has been shown to reduce the amounts of antioxidative enzymes in rat hepatocytes [39] and to induce production of hydrogen peroxide [41, 42] and reactive oxygen intermediates [43] *in vivo*. These observations indicate that TGF-β-induced apoptosis may be mediated through the generation of ROSs in the cells.

Small antioxidants, including Ac-Cys, reduced-form glutathione, and ascorbic acid have been shown to protect cells from apoptosis under several experimental conditions [44, 45]. However, these antioxidants do not show notable anti-apoptotic activity or block TGF-β-induced intracellular peroxide production in TGF-β-treated Hep3B cells (Figs 1 and 3). One possible explanation may be that the hepatoma cells have a higher consumption or efflux rate of water-soluble antioxidants. Therefore, it becomes difficult to accumulate enough antioxidants inside the cells. In contrast, the water-soluble C_{60} derivatives effectively suppress TGF-β-triggered apoptotic processes and block TGF-β-induced intracellular peroxide production. C_{60} is an excellent electron acceptor and a highly reactive molecule towards free radicals [1]. Our finding further substantiates the notion that free radicals are crucial for the TGF-β-induced apoptosis. Furthermore, Hsu et al. [45a] have found that C_{60} can prevent ceramide-triggered, but not Fas-triggered apoptosis in Jurkat cells, suggests that C_{60} is a useful tool to study the involvement of ROSs in different apoptotic processes.

The observation that the C_3 derivative selectively inhibits TGF-β-induced apoptosis is surprising. Recently, the C_3 isomer was also reported to be more potent than the D_3 isomer in protecting neurons from NMDA-induced cell death [2]. One possible explanation for such selectivity is that the C_3 isomer has a bipolar structure with all hydrophilic moieties on one side and a hydrophobic portion on the other side of the fullerene cage, whereas the D_3 isomer has hydrophilic moieties around the equator. The high hydrophobic moment of C_3 may facilitate its interaction with the cell membrane and its efficient capture of free radicals. This notion is supported by our fluorescence-quenching results.

The fluorescence-quenching behaviors of the water-soluble pyrene (8-hydroxypyrene-1,3,6-trisulfonic acid trisodium) by both C_3 and D_3 are very similar, with quenching rate constants of $2.4\times10^{10}\ M^{-1}\ s^{-1}$ and $9.5\times10^{9}\ M^{-1}\ s^{-1}$, respectively. These values indicate that the fluorescence quenching of the water-soluble pyrene by either C_{60} isomer is essentially diffusion controlled. However, the fluorescence quenching rates become $3.0\times10^{11}\ M^{-1}\ s^{-1}$ and $1.0\times10^{11}\ M^{-1}\ s^{-1}$ for C_3 and D_3, respectively, when the pyrene chromophore is labelled to the head of a phosphatidylethanolamine lipid and incorporated into liposomes. Assuming that the quenching rate on the liposome surface remained diffusion controlled, and that the observed quenching rate was largely due to an increase of the quencher concentration, one obtains 30-fold and 10-fold higher quencher concentrations on the liposome surface than in the bulk solution phase for the C_3 and D_3 isomers, respectively. This would mean that the surface concentration of the C_3 isomer on the liposomes is approximately threefold higher than that of the D_3 isomer, suggesting that the C_3 isomer has better interactions with lipid membranes than the D_3 isomer.

In addition to its antiproliferative effect, TGF-β regulates the expression of a variety of genes including those for extracellular matrix proteins and cell cycle regulators [46]. The induction of PAI-1 expression is often used as a biochemical marker for TGF-β responsiveness. In the present study, it is shown that addition of the free radical scavenger C_{60} blocks the TGF-β-induced intracellular peroxide production and apoptosis without interfering with the signal which activates PAI-1 promoter activity. These results further support the hypothesis that TGF-β generates at least two distinct signaling pathways in the cell, one leading to generation of ROSs that may be related to apoptosis and the other leading to the activation of gene expression.

The selectivity of the stereoisomer of carboxyfullerene to block TGF-β-induced ROSs and apoptosis not only confirms previous reports suggesting ROSs as important mediators of TGF-β-induced apoptosis, but also offers a novel reagent to dissect the cascade pathway of death signals, such as release of cytochrome *c*, leading to the sequential activation of different caspases triggered by ROSs under TGF-β treatment in human hepatoma cells.

The studies presented here have been supported by National Science Council Grant NSC 86-2316-B-075-002-M29 and Department of Health Grant DOH 86-HR-507, Taiwan, Republic of China.

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