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Reactive oxygen species mediates the apoptosis induced by transforming growth factor β_2 in human lens epithelial cells

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Abstract

Transforming growth factor β_2 (TGF-β_2), a growth regulator of human lens epithelial cells (HLECs), also regulates the death of these cells. Dose-response analysis showed that the TGF-β_2 concentration needed to induce HLECs death (100 pg/ml) was 10 times that needed to inhibit growth in these cells (10 pg/ml). TGF-β_2-induced apoptosis in HLECs was preceded by an induction of reactive oxygen species (ROS) and a decrease in glutathione in the intracellular content, indicating that this factor induces oxidative stress in HLECs. Studies performed to analyze the levels of c-*fos* mRNA, a gene whose expression is modulated by the redox state, demonstrated that only high, apoptotic concentrations of TGF-β_2 (100 pg/ml) produced an increase in the mRNA levels of this gene, the level of induction being similar to that found when cells were incubated in the presence of hydrogen peroxide. Finally, the cell death induced by TGF-β_2 in HLECs was partially blocked by radical scavengers, which decreased the percentage of apoptotic cells, whereas these agents did not modify the growth-inhibitory effect elicited by TGF-β_2 in these cells. The results presented in this paper provide evidence for the involvement of an oxidative process in the apoptosis elicited by TGF-β_2 in HLECs.

Keywords: Transforming growth factor β_2; Reactive oxygen species; Apoptosis; Human lens epithelial cell

Lethal cellular programs that lead to apoptosis may be triggered by a variety of exogenous and environmental stimuli. Transforming growth factor-β (TGF-β) constitutes one of these apoptotic factors for certain types of cells [1]. In the eye, TGF-β is one of the most important ligands involved in the regulation of cell behavior in ocular tissues in the physiological or pathological processes of development or tissue repair, although various other growth factors are also involved [2]. As a multifunctional growth factor, it is able to regulate cell proliferation, differentiation, morphogenesis, and cell death. It has been reported that TGF-β can induce apoptosis and reduce the level of Bcl-2, a protooncogene that is unique among cellular genes in its ability to block apoptotic death by activating cellular antioxidant defenses in human lens epithelial cells (HLECs), as has been demonstrated *in vitro* [3,4]. Furthermore, TGF-β has also been reported to induce apoptotic cell death in lens epithelial explants and in various rodent models [5]. However, the mechanisms by which TGF-β induces cell death are still only partly understood.

The mechanisms by which TGF-β exerts its effects have been extensively studied. At this stage, it is especially the question of the signaling pathways from the receptor to the nuclei which remains to be elucidated. Some reports have related the activity of TGF-β to the production of hydrogen peroxide [6,7]. Reactive oxygen species (ROS) intermediates are, in general, considered to be cytotoxic and are implicated in the progression of cancer, inflammation, radiation injury, and aging [8,9]. Peroxides and highly reactive free radicals can also trigger cell death, and although some investigators believe that oxidation is merely another metabolic disturbance that leads cells to respond to external stimuli, others propose a more central role for

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reactive oxygen species in cell death. Studies have previously demonstrated that the lens *in vitro* is highly susceptible to damage by ROS, as evidenced by the loss of transparency and decrements in the active transport of Na^+–K^+-ATPase dependent cation pump. GSH and ATP also decrease. Other changes are protein insolubilization and the generation of lipid peroxides [10].

In the light of all these observations, the aim of our work has been to evaluate the possible implications of reactive oxygen intermediates in the mechanisms by which TGF-β_2 induces apoptotic death in the lens. Our experimental model uses the human lens epithelial cell line HLE B-3. Research has shown that these cells are able to carry out both proliferation and differentiation processes simultaneously [11]. This is a useful model in which to study the possible role of TGF-β in the regulation of HLEC apoptosis and the role of oxygen radicals in its molecular mechanism of action.

Materials and methods

Cell culture and treatment. Human lens epithelial cell line HLE B-3 was bought from ATCC and maintained as described previously [12]. In short, the cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY, USA). The cells were grown to confluence at 37 °C in a humidified atmosphere containing 5% CO_2, washed with Ca^{2+}/Mg^{2+}-free PBS (PBS-CMF), and dissociated with trypsin–EDTA solution (0.05% trypsin–0.02% ethylenediamine tetra acetic acid solution; Gibco, Grand Island, NY, USA). Following passage, the cells were seeded in a 60-mm culture dish (Falcon; Becton–Dickinson, Oxnard, CA, USA) and allowed to reach 75–80% confluence prior to the recombined human TGF-β_2 (R&D Systems, Oxon, UK) treatment. TGF-β_2 was stored in PBS containing 2 mg/ml bovine serum albumin (BSA), and added into the serum-free medium to the final concentration. The medium was changed every 24 h, and the appropriate agents were added to the fresh medium. At the indicated time points, the cells were collected for real time-PCR or ROS analysis. Cells were used at the same number of passages for all experiments.

Analysis of cell proliferation by MTT assay. The cells were plated in 96-well plates at a density of 1×10^4 cells/well for 16–20 h. Then the cells were treated with different concentrations of TGF-β_2 and radical scavengers for the indicated hours in serum-free medium. After treatment, the cells were washed twice with phosphate-buffered saline (PBS), as described previously [13]. The medium was replaced and treated according to the manufacturer's instructions (ADCC 30–1010 K). The intensity was measured at 570 nm using a plate reader (Molecular Dynamics, OPTImax) for enzyme-linked immunosorbent assays. The relative rate of proliferation was calculated by dividing the absorbance of treated cells by that of the untreated control (incubated in serum-free medium) in each experiment.

Analysis of cells apoptosis by TUNEL assay. Cell death was analyzed using the *in situ* cell death detection kit-fluorescein (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions. In short, cells cultured for 24 h after treatment in a 24-well chamber slide were fixed in freshly prepared 4% paraformaldehyde solution at room temperature, and permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate solution on ice. Nonspecific antigens were blocked in 2% BSA for 30 min at room temperature. TUNEL (TdTdUTP terminal nick-end labeling) reaction mixture (50 ml) was added to each sample and incubated in a humidified chamber for 60 min at 37 °C in the dark. A negative control was always included in each batch, and the label solution (i.e., without terminal transferase) was applied instead of the TUNEL reaction mixture. Six independent specimens were used in each count. The percentage of TUNEL-positive cells in all of the HLECs was calculated using Mac SCOPE and analyzed using unpaired Student's *t*-test.

Measurement of intracellular ROS. At the end of treatment, cultures were washed twice with Earle's solution, and incubated at 37 °C with 50 μmol/L 29,79-dichlorofluoresceine diacetate (DCFH-DA, Sigma Chemical Co.) which reacts with intracellular free radicals to form the fluorescent dye DCFH. After 30 min, DCFH-DA solution was discarded and the cells were washed three times. DCFH fluorescence (excitation, 485 nm and emission, 525 nm) was measured in a fluorescence multiwell plate reader.

Glutathione determination. HLECs, incubated in the absence or presence of TGF-β_2, were washed twice with phosphate-buffered saline, scraped, and pelleted at 4 °C. The cell pellet was sonicated in 1 ml of 50 mM MES. After centrifugation at 10,000*g* for 15 min at 4 °C, the supernatant was removed and stored on ice. An equal volume of the MPA reagent (1 g/ml metaphosphoric acid, Aldrich) was added to the sample and mixed on a vortex mixer. The mixture was laid at room temperature for 5 min and centrifuged at 4000*g* for 2 min. Then the supernatant was carefully collected without disturbing the precipitate. Fifty microliters of TEAM reagent per ml of the supernatant was added and mixed immediately. The sample at this point was ready for assay of total GSH and GSSG using the Glutathione Assay Kit (Cayman Chemical Company, USA). The glutathione disulfide (GSSG) content was determined after derivatization of the above extract with 2-vinylpyridine. Ten microliters of the 1 M 2-vinylpyridine solution was added per ml of sample. The samples were measured the absorbance in the wells at 405 nm using a plate reader at 25 min. The values of total GSH or GSSG for each sample were determined using linear regression to calculate the values obtained from a standard curve and expressed as nmol per mg of protein.

RNA isolation and real time RT-PCR. Total cellular RNA was extracted from 2×10^7 HLECs, using TRIzol reagent and then digested with DNase I (Life Technologies). Purified RNA was resuspended in 20 μl of RNAse-free water. Three microliters of RNA was then used as template for cDNA synthesis in the presence of 1 μl of M-MLV reverse transcriptase (200 U) (Progema), 4 μl of First Strand buffer 5× (Promega), oligd(T) 2.5 μmol/L, 1 μl of dNTP 10 mM (Roche) and 5 μl of RNAse-free water. After incubation for 60 min at 42 °C, the reverse transcriptase was inactivated at 72 °C for 10 min.

cDNAs were amplified by real time RT-PCR, using the Applied Biosystems FTC 2000 sequence detection system. The reaction mixture consisted (40 μl) of 4 μl cDNA, 35.1 μl SYBR Green PCR mix (biocore Hangzhou), 0.5 μl 5 U *Taq* DNA polymerase, 0.3 μl 20 pmol/μl each primer (invitrogen). The cDNA was denatured by heating to 94 °C for 3 min. The template was amplified by 40 rounds of PCR (denaturation at 94 °C for 15 s, annealing at 60 °C for 20 s, extension at 72 °C for 30 s), collection light at 60 °C. The primer set for c-*fos* was 5′-TGA TAC ACT CCA AGC GGA GA-3′ and 5′-GTC AGA TCA AGG GAA GCC AC-3′; GAPDH was 5′-GTC GGT GTG AAC GGA TTT-3′ and 5′-ACT CCA CGA CGT ACT CAG C-3′.

Statistics. Data are expressed as the means ± SE from a minimum of three independent experiments, unless otherwise indicated. Statistical analysis was performed with Student's *t*-test using SAS statistical analysis software, with $^{**}P < 0.05$ as the criterion of significance.

Results

TGF-β_2-induced cell growth inhibition and apoptotic death in HLECs

To examine the effect of TGF-β_2 on cell growth, HLECs were treated with 10–1000 pg/ml TGF-β_2 for 12–48 h. TGF-β_2 inhibited cell proliferation in both a time and concentration dependent manner. Maximum effect was obtained at a dose of 100 pg/ml, where less than 50% of the cells died. Moreover, TGF-β_2 was able to induce changes in cell morphology. Twelve hours after the addition of the factor (100 pg/ml), cells lose cell contacts and

migrate on the plate, and after 24 h in the presence of TGF-β_2, cellular blebbing and detachment of degenerated HLECs were always observed.

To assess if HLEC death involved the process of apoptosis, TUNEL assay was performed. In normal incubated HELCs, TUNEL-positive cells were hardly detected. Cells that had been incubated in the presence of TGF-β_2 (100 pg/ml) for different time periods revealed that TUNEL-positive cells gradually increased significantly ($P < 0.05$) and peaked on the 48 h. On the other hand, cells incubated at the lower concentration TGF-β_2 (10 pg/ml) revealed TUNEL-positive cells increased negligibly.

Analysis of intracellular reactive oxygen species produced by TGF-β_2

We next examined the generation of reactive oxygen species in HLECs that were treated with TGF-β_2. For this, we used DCFH-DA, an oxidation-sensitive fluorescent probe [14,15]. When cells were incubated in the presence of 10 pg/ml TGF-β_2, DCFH fluorescence increased slightly with no statistical significance. However, when cells were treated with higher doses of this factor (100 pg/ml), they showed a significant increase in DCFH fluorescence (Fig. 1). This effect was time-dependent, with a maximum at 2 h. After this time point, reactive oxygen species decreased, returning to control values at 24 h (Fig. 1).

Effect of TGF-β_2 on glutathione intracellular levels in HLECs

Since the oxidative stress and the presence of peroxides produce changes in the GSSG/GSH ratio and in the glutathione concentration [16], and TGF-β has been reported to induce glutathione depletion [17]. We have measured these parameters in HLECs incubated in the absence or presence of TGF-β_2 (100 pg/ml) (Fig. 2). In the results, TGF-β_2 produced an increase in the GSSG/GSH ratio. Simultaneously, a decrease in the glutathione intracellular concentration was always observed.

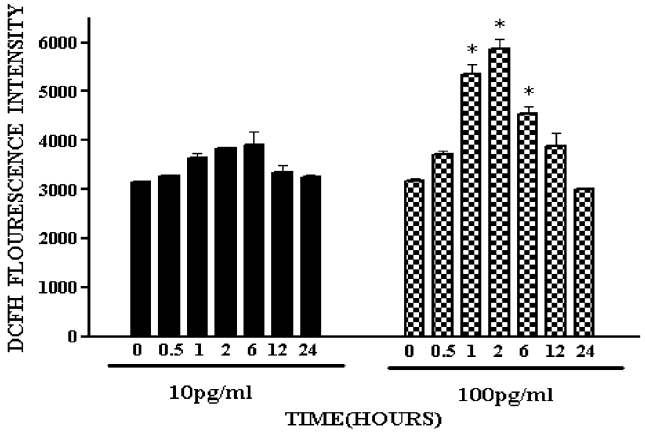

Fig. 1. TGF-β_2 induction of intracellular reactive oxygen species content. After culture in the absence or presence of TGF-β_2, HLECs were incubated for 30 min with 50 μmol/L of an oxidation-sensitive fluorescent probe (DCFH-DA), measuring the fluorescence intensity by a fluorescence multiwell plate reader. Cells were incubated for different time periods (0.5, 1, 2, 6, 12, and 24 h) in the presence of TGF-β_2 (10 and 100 pg/ml), and the production of reactive oxygen species was analyzed as described previously. Data are expressed as a percentage of DCFH fluorescence with respect to control value and are the means ± SE from three independent experiments ($^*P < 0.05$, compared with control group).

Effect of TGF-β_2 effect on c-fos mRNA Levels

Evidence has shown reactive oxygen intermediates play an important role in the control of gene expression. When HLECs were incubated in the presence of 10 pg/ml and 100 pg/ml concentrations of TGF-β_2 for 12 h, the oxidative stress induced in the cells produced an increase in the c-*fos* mRNA level. It is important to point out that a significant increase was only observed when high concentrations of TGF-β_2 (100 pg/ml) were used ($P < 0.05$), but not when cells were incubated in the presence of low, non-apoptotic concentrations (10 pg/ml). Moreover, this effect on c-*fos* was similar to that found when cells were incubated in the presence of 500 μM hydrogen peroxide.

Effect of radical scavengers on both the production of ROS and induction of apoptosis by TGF-β_2 in HLECs

In order to determine whether the increase in reactive oxygen species is related to the HLEC death, we next

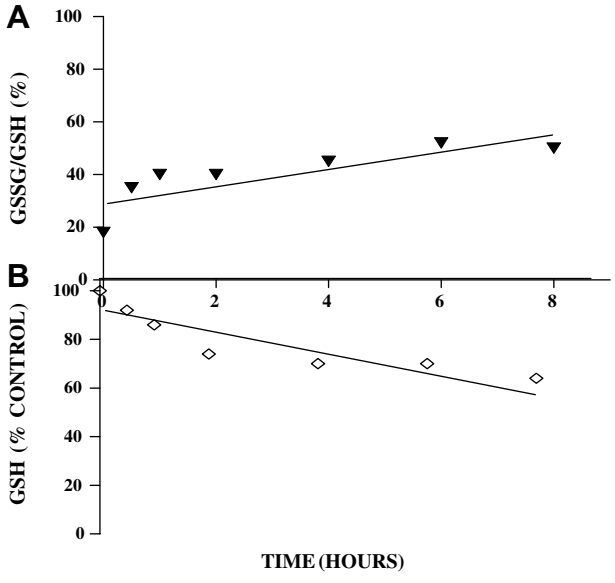

Fig. 2. Effect of TGF-β_2 on glutathione intracellular levels in HLECs. After incubating HLECs in the absence or presence of 100 pg/ml TGF-β_2, cells were collected, and cellular glutathione was extracted and determined spectrophotometrically as described under Materials and methods. The means of triplicate dishes of a representative experiment are shown (A). The GSSG/GSH ratio (%) in TGF-β_2-treated cells is presented as a function of time in the presence of the factor. Control cells (in the absence of TGF-β_2) did not show changes in this ratio (data not shown). (B) In this same experiment, the glutathione intracellular concentration is presented versus the time of TGF-β_2 treatment. In this case, data are calculated, at each time point, as the percentage with respect to control (untreated) cells.

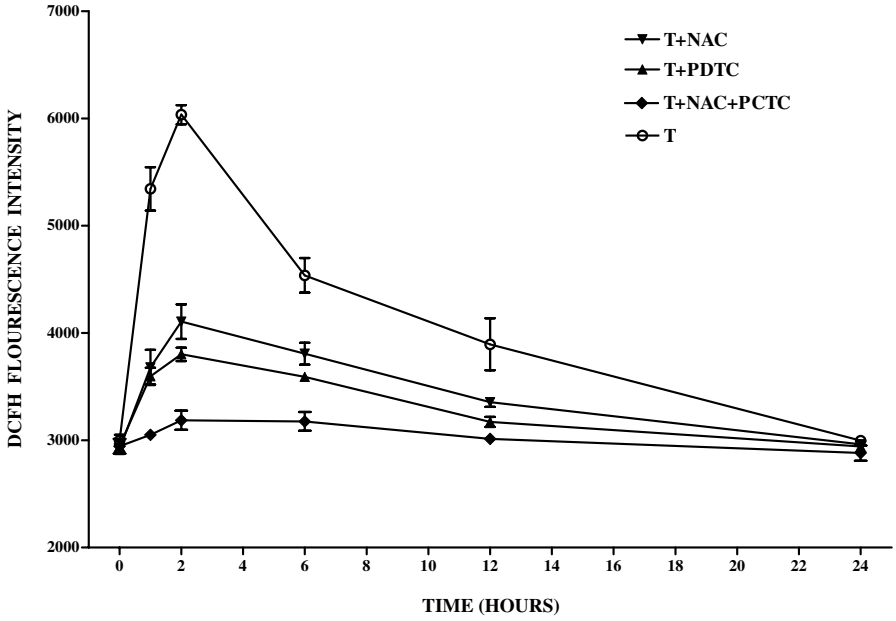

Fig. 3. Effect of radical scavengers on TGF-β_2-induced ROS production. Cells were treated with 100 pg/ml TGF-β_2 alone or in the presence of different radical scavengers: 50 μM PDTC and 20 mM NAC added 30 min after the TGF-β_2 treatment. After the indicated time, cellular reactive oxygen species was analyzed as described in Materials and method. Data are expressed as a percentage of DCFH fluorescence with respect to the control value and are means ± SE from three independent experiments ($^{*}P < 0.05$, compared with control group).

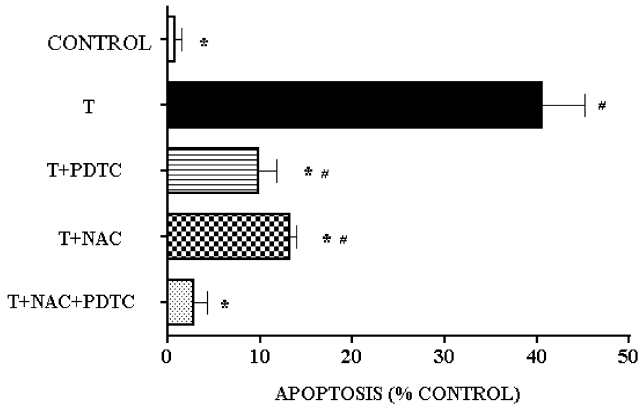

Fig. 4. Effect of radical scavengers on TGF-β_2-induced apoptosis. Cells were left untreated or were treated with 100 pg/ml TGF-β_2 alone or in the presence of different radical scavengers: 50 μM PDTC and 20 mM NAC were added 30 min after the TGF-β_2 treatment. Following a 24 h treatment period, cells were fixed for TUNEL assay as described under Materials and methods. Results are expressed as a percentage of control and are means ± SE of triplicate determinations from three experiments ($^{*}P < 0.05$, compared with TGF-β_2 group, $^{\#}P < 0.05$, compared with the control group).

examined the effects of radical scavengers, i.e., *N*-acetyl-Lcysteine (NAC) and pyrrolidine carbodithioic acid (PDTC) (Sigma Chemical Co.) on the apoptosis induced by TGF-β_2 in the cells. We incubated the cells in the presence of different concentrations of these factors in order to choose efficient but not cytotoxic doses (data not shown). A summary of the results is shown in Figs. 3 and 4. Both NAC and PDTC were able to decrease the production of ROS induced by TGF-β_2 (Fig. 3). A similar effect can be observed in the cell death experiment. Combinations of these agents were able to completely prevent the induction of cell death by TGF-β_2 (Fig. 4).

In contrast to these results the inhibitory effect of TGF-β_2 on HLEC growth was not modified in the presence of radical scavengers. Thus, when these cells were incubated in the presence of bFGF (10 ng/ml) (R&D Systems, Oxon, UK) and TGF-β_2 (10 and 100 pg/ml), the bFGF-induced cell proliferation was completely blocked by TGF-β_2, regardless of whether NAC or PDTC was present. These results seem to indicate that the oxidative stress induced by TGF-β_2 does not preclude its well understood inhibitory effect on HLEC growth.

Discussion

In the realm of ocular disease, apoptosis has been implicated in a number of pathologies including glaucoma, retinitis pigmentosa, retinoblastoma, retinal ischemia, diabetic retinopathy, and cataract [18]. Several studies of the human lens have suggested a connection between apoptosis and cataractogenesis [19,20].

In many models of apoptosis, cells are induced to die as a result of changes in environmental stimuli, such as growth factors. TGF-β constitutes one of these apoptotic factors for certain types of cells, including the HLECs. It is one of the best known physiological inhibitors of epithelial cell proliferation [21]. Altered intraocular fluid concentrations of TGF-β have been reported in the context of various ocular conditions and pathologies [22]. A growing body of evidence has suggested that the cataractogenic properties of TGF-β in particular implicates its ability to induce apoptosis in HLECs [23]. It has also been reported

that expression of TGF-β was significantly increased in anterior polar type cataracts compared to nuclear type cataracts. TGF-β_2 is expressed at much higher levels than the other TGF-β isoforms in the aqueous humor that bathes the lens tissue [24] as well as in the vitreous [25]. The results presented here demonstrate that TGF-β_2 can act as a modulator of both growth and apoptosis of HLECs. Furthermore, dose-response analysis showed that the TGF-β_2 concentration needed to induce HLEC death (100 pg/ml) was 10 times that needed to inhibit growth in these cells (10 pg/ml).

TGF-β_2-induced apoptosis in HLECs is preceded by an enhancement in reactive oxygen species production, an increase in the GSSG/GSH ratio, and a decrease in the glutathione intracellular content. Reduced glutathione (GSH) functions intracellularly to reduce numerous oxidizing compounds, including reactive oxygen species. It has been proposed that efflux of GSSG from the cell then occurs in order to preserve the cellular normal redox state so that a depletion in the glutathione levels is regularly observed [26]. Thus, an increase in the reactive oxygen species production and a decrease in the glutathione concentrations indicate that TGF-β_2 induces oxidative stress in HLECs. However, at present it is not clear if TGF-β_2-induced peroxide production induces growth inhibition, apoptosis, or both. The results presented in this paper clearly relate TGF-β_2-induced reactive oxygen species production to HLEC death. First, low concentrations of TGF-β_2 (10 pg/ml) which are sufficient to completely block HLEC growth do not induce reactive oxygen intermediate production in these cells (Fig. 1). Second, studies performed to analyze c-*fos* expression, a gene modulated by redox state, demonstrate that only high, apoptotic concentrations of TGF-β_2 (100 pg/ml) effect an increase in its mRNA levels. Finally, TGF-β_2-induced cell death in HLECs may be either partially blocked by single radical scavengers (Fig. 4) or totally blocked by combinations of both. However, these agents do not preclude the TGF-β_2 growth inhibitory effect in these cells. These results strongly suggest that TGF-β_2 induces apoptosis in HLECs through the generation of reactive oxygen intermediates.

The results presented in this paper provide evidence for the involvement of an oxidative process in the apoptosis elicited by TGF-β_2 in HLECs. Further work will be necessary to completely determine the molecular mechanism by which high concentrations of TGF-β_2 induce oxygen radical production and apoptosis, whereas lower concentrations of this factor, sufficient to inhibit cell growth, do not.

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