Bisphenol A diglycidyl ether induces apoptosis in tumour cells independently of peroxisome proliferator-activated receptor- γ , in caspase-dependent and -independent manners

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Peroxisome proliferator-activated receptors (PPARs) are nuclear transcription factors which are involved in many biological processes, such as regulation of cell differentiation, lipid metabolism, inflammation and cell death. PPARs consist of three families, PPAR- α , PPAR- δ and PPAR- γ . Bisphenol A diglycidyl ether (BADGE) has been described as a pure antagonist of PPAR- γ . However, recent data also revealed PPAR- γ -agonistic activities of BADGE. Here we show that BADGE kills trans-

formed cells by apoptosis and promotes the cytotoxic effects of tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) and indomethacin. The cytotoxic effect of BADGE does not require PPAR- γ expression and is mediated in caspase-dependent and caspase-independent manners.

Key words: cell death, colon cancer, Jurkat cell, nuclear hormone receptor.

INTRODUCTION

Peroxisome proliferator-activated receptors (PPARs) are members of the nuclear hormone receptor superfamily of ligand-activated transcription factors, which share homology with the receptors for retinoic acid, steroids and thyroid hormone [1]. PPARs form three families, PPAR- α , PPAR- δ and PPAR- γ . PPAR- γ exists in two isoforms, PPAR- γ 1 and PPAR- γ 2, which show different tissue distributions and functions. Both isoforms are derived from a single gene by utilization of different promoters and alternate splicing [2]. PPAR- γ 2 is expressed primarily in adipocytes. PPAR- γ 1 has been identified in a variety of tissues, including liver, spleen, intestine and adipose tissue [2,3]. PPAR- γ forms a heterodimer with the retinoid X receptor and activates target genes by binding to specific peroxisome proliferator-responsive elements (PPREs) located in the promoter regions of these genes [4]. PPAR- γ is involved primarily in the regulation of lipid metabolism, adipocyte differentiation and inflammation [5]. However, the widespread tissue distribution of PPAR- γ suggests functions in multiple biological actions. There is emerging evidence that PPAR ligands exhibit a potent anti-neoplastic activity in different tumours [5]. This anti-tumoural effect is mediated by induction of apoptosis, necrosis or inhibition of cell proliferation. Furthermore, we showed previously that the PPAR- γ agonist pioglitazone sensitizes tumour cells against tumour necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis [6]. Bisphenol A diglycidyl ether (BADGE) was identified as a PPAR- γ antagonist in 3T3-L1 and 3T3-F442A cells [7]. In contrast, Bishop-Bailey et al. [8] recently reported an agonistic activity of BADGE in an ECV304 cell line, where BADGE induced PPAR- γ activation and nuclear localization of the receptor and initiated apoptosis. In the present study, we show that BADGE induces apoptosis in different tumour cell lines in a PPAR- γ -independent manner and that

BADGE-induced apoptosis is mediated in caspase-dependent and caspase-independent manners. Furthermore, we demonstrate that BADGE sensitizes tumour cells against TRAIL- and indomethacin-induced apoptosis.

EXPERIMENTAL

Materials

BADGE was obtained from Tocris (Bristol, U.K.). FITC-labelled annexin-V (referred to as annexin-V-FITC) was purchased from Pharmingen (Heidelberg, Germany). Propidium iodide (PI) and valinomycin were from Sigma (Deisenhofen, Germany). JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolcarbocyanine iodide) was from Molecular Probes (Eugene, OR, U.S.A.). Anti-PPAR- γ and anti- β -actin antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). *N*-Benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (Z-VAD-fmk) and *N*-benzyloxycarbonyl-Leu-Glu-His-Asp-fluoromethylketone (Z-LEHD-fmk) were from Calbiochem (San Diego, CA, U.S.A.).

Cell culture

Jurkat (human T-cell lymphoma) cells were cultured in RPMI 1640 medium supplemented with 10 % fetal bovine serum and 1 % penicillin/streptomycin. HCT-116 (human epithelial colorectal carcinoma) cells were maintained in McCoy's 5A medium containing 10 % fetal bovine serum and 1 % penicillin/streptomycin. BADGE was dissolved in DMSO and added to the cell culture at a final concentration of 5–100 μ M. Control cultures received the same amount of DMSO, which was less than 0.5 % (v/v) of the culture medium.

Abbreviations used: BADGE, bisphenol A diglycidyl ether; PPAR, peroxisome proliferator-activated receptor; TRAIL, tumour necrosis factor-related apoptosis-inducing ligand; JC-1, 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolcarbocyanine iodide; PI, propidium iodide; RT-PCR, reverse transcriptase PCR; Z-VAD-fmk, *N*-Benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone; Z-LEHD-fmk, *N*-benzyloxycarbonyl-Leu-Glu-His-Asp-fluoromethylketone.

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Apoptosis assays

Cell death was determined using annexin-V-FITC and PI. For annexin-V-FITC staining, cells were treated with BADGE alone or in combination with other substances. Following this, they were harvested, washed with PBS (pH 7.4), centrifuged and resuspended in binding buffer (10 mM Hepes, pH 7.4/140 mM NaCl/2.5 mM CaCl_2), followed by incubation for 15 min in the dark at room temperature. FITC fluorescence intensity was determined by flow cytometry using a FACScan flow cytometer. For PI staining, after treatment of cells they were harvested, washed and resuspended in PBS (pH 7.4) containing 2 $\mu\text{g}/\text{ml}$ PI. PI fluorescence intensity was determined by flow cytometry as above. Data analysis was performed using Cell Quest software (Becton-Dickinson).

Analysis of mitochondrial membrane potential

Jurkat cells were incubated for 6 h in the aforementioned RPMI 1640 culture medium at 37 °C in the absence and presence of different BADGE concentrations. Control samples were incubated for 10 min at 37 °C with the K^+ ionophore valinomycin (1 μM). Then cells were incubated with JC-1 (final concentration 10 $\mu\text{g}/\text{ml}$) for 10 min at room temperature in the dark. After washing with PBS, cells were resuspended in 250 μl of PBS and analysed by flow cytometry using a FACScan flow cytometer. The photomultiplier values of the fluorescence light detectors FL-1 and FL-2 were set as described by Cossarizza et al. [9]. Data were acquired and analysed using Cell Quest software.

Reverse transcriptase PCR (RT-PCR)

Total RNA (1 μg) was reverse transcribed using the One Step RT-PCR kit from Qiagen according to the manufacturer's instructions. To identify PPAR- γ mRNA the following primers were used [10]: forward primer, 5'-TCTCTCCGTAATGGAAACC-3'; reverse primer, 5'-GCATTATGAGACATCCCCAC-3'. To identify β -actin mRNA the following primers were used: forward primer, 5'-AGGCCAACCAGCGAGAAGATGACC-3'; reverse primer, 5'-GAAGTCCAGGGCGACGTAGCAC-3'. The reaction conditions were as follows: initial denaturation at 95 °C for 2 min, and 40 cycles of amplification (95 °C for 45 s, 55 °C for 50 s and 72 °C for 50 s) followed for a final extension for 10 min at 72 °C [10]. The obtained PCR products were separated on a 1% (w/v) agarose gel containing ethidium bromide.

Western-blot analysis

Total proteins were extracted from Jurkat and HT-29 cells and protein concentrations determined using a protein assay from Bio-Rad according to the manufacturer's protocol. Proteins (50 μg) were resolved by SDS/PAGE (12.5% gel) and electrotransferred on to a nitrocellulose membrane (pore size, 0.2 μm). Immunodetection was carried out using anti-PPAR- γ and anti- β -actin antibodies in 50 mM Tris/HCl, pH 7.4/150 mM NaCl/0.1% Tween 20, followed by horseradish peroxidase-conjugated secondary antibodies. Detection was performed using an ECL kit (Amersham Bioscience).

RESULTS

To elucidate the biological action of BADGE, we investigated the effect of this substance on PPAR- γ -positive colon carcinoma

cells (HCT-116). For this, we incubated HCT-116 cells with or without BADGE at various concentrations for 24 h. Previously, it has been demonstrated that indomethacin induces apoptosis in colon carcinoma cells [11]. Additionally, we showed that PPAR- γ agonists sensitize colon carcinoma cells against proapoptotic stimuli [6]. We therefore also tested whether BADGE would be able to sensitize HCT-116 cells against indomethacin-induced apoptosis. As shown in Figure 1, BADGE increased apoptosis in HCT-116 cells in a concentration-dependent manner. Interestingly, indomethacin-induced apoptosis was enhanced dramatically by BADGE.

To investigate whether PPAR- γ expression is required for the proapoptotic and sensitizing effect of BADGE we incubated PPAR- γ -negative Jurkat cells without or with BADGE in the absence and presence of indomethacin. By RT-PCR and Western-blot analysis we were able to demonstrate the expression of PPAR- γ in colon carcinoma cells but not in Jurkat cells (Figure 2). Incubation of cells with BADGE or indomethacin for up to 12 h did not result in the appearance of bands in the RT-PCR (results not shown). As observed in our experiments with HCT-116 cells, BADGE induced cell death in Jurkat cells in a concentration-dependent manner and sensitized these cells against indomethacin-induced apoptosis (Figure 3).

To test whether BADGE-induced cell death is caused by apoptosis or necrosis, Jurkat cells incubated for 6 h with or without BADGE (100 μM) were stained with FITC-labelled annexin-V or PI. As shown in Figure 4, incubation with BADGE resulted in phosphatidylserine externalization, indicating that cell death was induced by apoptosis. At this time point of early apoptosis, PI staining of BADGE-treated cells was negative. Furthermore, in the forward and side light-scatter detectors, cells appeared smaller and more granulated, thus meeting the morphological criteria typical for apoptotic cells.

Having seen that BADGE sensitizes tumour cells against indomethacin-induced cell death, we were interested to test whether BADGE would also promote TRAIL-induced apoptosis. For this, we preincubated Jurkat cells for 2 h with 50 μM BADGE before incubation of cells with TRAIL (200 ng/ml) for 24 h. TRAIL exerted only a weak proapoptotic effect on Jurkat cells (Figure 5). In combination with BADGE, the cytotoxic effect of TRAIL was increased dramatically.

Figure 1 Proapoptotic effect of BADGE in HCT-116 cells

Cells were preincubated for 2 h without and with BADGE. After addition of indomethacin incubation was continued for 22 h. The percentage of apoptotic cells was determined by flow cytometry as described in the Experimental section. Data presented are means $\pm$ S.E.M. of the percentages of apoptotic cells pooled from two experiments. Control, cells were treated with vehicle alone; B, BADGE; In, indomethacin; B + In, BADGE and indomethacin.

Figure 2 Determination of PPAR- γ expression in colon cancer and Jurkat cells

(**A**) RT-PCR of PPAR- γ using mRNA from HCT-116 and Jurkat cells. mRNA was reverse-transcribed and PPAR- γ and β -actin sequences were amplified by PCR. (**B**) Western blot of PPAR- γ protein from HCT-116 and Jurkat cells. Cell lysate was fractionated by SDS/PAGE (12.5% gel; 50 μ g of protein/lane), blotted and probed for PPAR- γ and β -actin as described in the Experimental section.

Figure 3 Proapoptotic effect of BADGE in Jurkat cells

Cells were preincubated for 2 h without and with BADGE (B). After addition of indomethacin (In) incubation was continued for 22 h. The percentage of apoptotic cells was determined by flow cytometry as described in the Experimental section. Data presented are means $\pm$ S.E.M. of the percentages of apoptotic cells pooled from two experiments. Control, cells were treated with vehicle alone.

In order to learn more about the mechanisms involved in BADGE-induced apoptosis, we treated Jurkat cells with or without BADGE in the absence and presence of the cell-

Figure 4 Externalization of phosphatidylserine in Jurkat cells by BADGE

Cells were incubated for 6 h without or with BADGE. Then cell aliquots were stained with annexin-V-FITC (**A**) or PI (**B**) and analysed by FACScan as described in the Experimental section. The experiment was repeated twice with identical results. Data presented are means $\pm$ S.E.M. Control, cells were incubated with vehicle alone.

Figure 5 BADGE promotes TRAIL-induced apoptosis

Cells were preincubated for 2 h without and with BADGE (50 μ M). After addition of TRAIL (200 ng/ml) incubation was continued for 22 h. The percentage of apoptotic cells was determined by flow cytometry as described in the Experimental section. Data presented are means $\pm$ S.E.M. from two experiments.

permeable broad-range caspase inhibitor Z-VAD-fmk (which inhibits caspases 1, 3, 4 and 7 irreversibly). Control samples were incubated with a combination of cycloheximide (1 μ g/ml) and

Figure 6 Z-VAD-fmk inhibits BADGE-induced apoptosis

Jurkat cells were preincubated for 1 h without and with Z-VAD-fmk (VAD; 20 µM). After addition of BADGE (B) and a combination of cycloheximide (1 µg/ml) and TRAIL (200 ng/ml; Cyclo + TRAIL), incubation was continued for 6 h. The percentage of apoptotic cells was determined by flow cytometry as described in the Experimental section. Data presented are means $\pm$ S.E.M. from two experiments. Control, cells were treated with vehicle alone.

TRAIL (200 ng/ml), which are known to induce apoptosis in a caspase-dependent manner. Both BADGE- and cycloheximide/TRAIL-induced cell death was blocked by the caspase inhibitor, indicating that induction of apoptosis is caspase-dependent in both cases (Figure 6).

To characterize the apoptotic pathway further we investigated whether BADGE was able to alter the mitochondrial membrane potential, which would result in the release of proapoptotic mediators and lead finally to an activation of caspase-3. For this, we incubated Jurkat cells for 6 h with and without increasing concentrations of BADGE. Control samples were treated with the K⁺ ionophore valinomycin. After staining with JC-1, mito-

Figure 8 Z-LEHD-fmk inhibits BADGE-induced apoptosis

Jurkat cells were preincubated for 1 h without and with Z-LEHD-fmk (C91; 20 µM). After addition of BADGE (B) incubation was continued for 6 h. The percentage of apoptotic cells was determined by flow cytometry as described in the Experimental section. Data presented are means $\pm$ S.E.M. from two experiments. Control, cells were treated with vehicle alone.

chondrial potential was analysed by flow cytometry. As shown in Figure 7, BADGE depolarized the mitochondrial membranes in a concentration-dependent manner, suggesting that BADGE activates the mitochondrial apoptotic pathway. In accordance with this assumption, we found that preincubation with the cell-permeable caspase-9 inhibitor Z-LEHD-fmk blocked BADGE-induced apoptosis in Jurkat cells (Figure 8).

As mentioned above, BADGE-induced early apoptosis could be blocked by caspase-3 and -9 inhibitors. Interestingly, BADGE-induced late apoptosis could not be inhibited by these inhibitors, suggesting that BADGE utilizes both caspase-dependent and -independent pathways to induce cell death (Figure 9).

Figure 7 BADGE depolarizes mitochondrial membrane potential in Jurkat cells

Jurkat cells were incubated for 6 h in the absence and presence of different BADGE concentrations. Control samples were exposed for 10 min at 37 °C to valinomycin (1 µM). Then cells were incubated with JC-1 (10 µg/ml) for 10 min at room temperature. Percentages of cells with low mitochondrial membrane potential ($\Delta\Psi$) were determined by flow cytometry as described in the Experimental section. Data presented in the histogram are means $\pm$ S.E.M. from two experiments. FL2-H, fluorescence light detector 2 height, showing the height of the fluorescence light peak in the histogram.

Figure 9 BADGE-induced late apoptosis is not inhibited by caspase inhibitors

Jurkat cells were preincubated for 1 h without and with Z-VAD-fmk (VAD; 20 µM) and Z-LEHD-fmk (C91; 20 µM). After addition of BADGE (B) incubation was continued for 23 h. The percentage of apoptotic cells was determined by flow cytometry as described in the Experimental section. Data presented are means $\pm$ S.E.M. from two experiments. Control, cells were treated with vehicle alone.

DISCUSSION

Recently, BADGE, a synthetic compound that is used in the production of industrial plastics, was identified as a pure antagonist for the nuclear hormone receptor PPAR- γ , with micromolar affinity [7]. In this study, BADGE antagonized the ability of PPAR- γ agonists to activate the transcriptional and adipogenic effects of PPAR- γ . BADGE also blocked the ability of adipogenic cell lines to undergo hormone-mediated cell differentiation. In contrast, another report showed that BADGE acts as a PPAR- γ agonist in an immortalized human umbilical vein endothelial cell line (ECV304) [8]. In this cell line BADGE induced PPAR- γ activation and nuclear localization of the receptor and initiated cell death. Previously, we found that PPAR- γ agonists cause apoptosis in colon carcinoma cell lines and are able to sensitize tumour cells against other proapoptotic stimuli [6]. As a putative PPAR- γ agonist, BADGE should exert similar effects on colon carcinoma cells. To test this hypothesis, we treated HCT-116 cells with BADGE in the absence and presence of indomethacin, which is known to be cytotoxic for colon cancer cells. BADGE potently induced apoptosis in HCT-116 cells in a concentration-dependent manner. Furthermore, BADGE sensitized these cells against indomethacin-induced apoptosis.

Whereas regulation of lipid metabolism and adipocyte differentiation by PPAR- γ agonists are most probably mediated via PPAR- γ , PPAR- γ expression is not essential for PPAR- γ ligands to exert anti-inflammatory effects in macrophages [12] and anti-proliferative effects in mouse embryonic stem cells [13]. To investigate whether PPAR- γ expression is required for the proapoptotic and sensitizing effect of BADGE, we incubated PPAR- γ -negative Jurkat cells with a range of BADGE concentrations in the absence and presence of indomethacin. Surprisingly, as observed in experiments with HCT-116 cells, BADGE strongly induced cell death in Jurkat cells and sensitized these cells against indomethacin-caused apoptosis. This suggests that BADGE is able to induce cell death independently from PPAR- γ expression. Previously, we reported that the PPAR- γ agonist pioglitazone increases basal apoptosis cells and promotes TRAIL-induced cytotoxicity in Jurkat cells [6]. Since T-cells have been reported to express PPAR- γ we, unfortunately, did not test whether the Jurkat cells used for our experiments also expressed this tran-

scription factor. Based on our findings reported here that these cells do not express PPAR- γ it seems very likely that pioglitazone, like BADGE, initiates cell death independently of PPAR- γ .

An early event during apoptosis is the externalization of phosphatidylserine, which is normally confined totally to the inner leaflet of the plasma membrane. Annexin-V is a member of a protein family that binds to aminophospholipids. Annexin-V binds specifically to phosphatidylserine in a calcium-dependent manner [14,15]. The utility of annexin-V as a probe to monitor changes in the distribution of phosphatidylserine in the plasma membrane during apoptosis has been demonstrated by several groups [16–18]. In order to test whether BADGE causes cell death by apoptosis or necrosis, Jurkat cells were treated with BADGE and stained with annexin-V-FITC or PI. We found that BADGE induced phosphatidylserine externalization with positive annexin-V-FITC staining, whereas cells remained PI-negative. Furthermore, in the forward and sideward scatter, cells exhibited increased granulation and reduced size, which are typical signs for apoptotic cells. These data indicate that BADGE induces cell death by apoptosis.

TRAIL is a newly identified member of the tumour necrosis factor superfamily of proteins, sharing high amino acid sequence homology with the cytotoxic protein Fas ligand [19]. One exciting property of TRAIL is its ability to selectively kill tumour cells leaving non-transformed cells unharmed [20]. The cytotoxic effect of TRAIL can be observed in tumour cell lines derived from different tissues; however, there are many tumour cell lines which, like non-transformed cells, are resistant to TRAIL. Previous reports showed that chemotherapeutic agents can promote TRAIL-induced apoptosis in different tumour cell lines [21,22]. Additionally, we found that the PPAR- γ agonist pioglitazone sensitizes transformed cells against TRAIL-induced cell death [6]. In the present study, we demonstrate that BADGE not only induces apoptosis but also sensitizes tumour cells against cytotoxic agents like TRAIL and indomethacin. Previously, it has been shown that functional nuclear factor κ B protects cells against apoptosis induced by death receptors; cleavage of nuclear factor κ B subunits by caspase-3 sensitized cells against Fas-induced cell death [23]. One mechanism by which BADGE might sensitize cells against apoptotic stimuli may be mediated by such a process. On the other hand, down-regulation of survival genes might also play a role. Clearly, more work has to be done to elucidate precisely the exact mechanisms of sensitization. Based on the structure of BADGE it might be possible to develop compounds that are suitable for future anti-neoplastic therapies.

Apoptosis finally results in the fragmentation of genomic DNA, which is dependent on the activation of caspase-3 [24]. Caspase-3 is a member of the family of cysteine proteases which, after activation, form cascade-cleaving death substrates that cleave after asparagine residues. Our data show that BADGE-induced apoptosis was inhibited by the caspase-3 inhibitor Z-VAD-fmk indicating a caspase-dependent signal transduction pathway.

The caspase cascade is activated by two main pathways. One pathway is activated by death receptors like Fas, tumour necrosis factor receptor 1 and TRAIL receptors 1 and 2, which interact with the initiator caspase-8. The other pathway is mediated by the mitochondrial release of cytochrome *c* and recruitment of initiator caspase-9. The lipophilic cation JC-1 is a fluorescent probe that is used to assess changes in the mitochondrial membrane potential in intact cells [9,25]. JC-1 exists in a monomeric form emitting at 527 nm after excitation at 490 nm. In the presence of high membrane potential, JC-1 forms J-aggregates that are associated with a large shift in emission (to 590 nm). Experiments in which cells were incubated for 6 h

without and with BADGE followed by JC-1 staining showed that BADGE caused a shift in JC-1 fluorescence intensity in a concentration-dependent manner as a consequence of depolarization of the mitochondrial membrane potential. This suggests that BADGE initiates cell death via the mitochondrial apoptotic pathway. This assumption is supported by the finding that BADGE-induced apoptosis is inhibited by the caspase-9 inhibitor Z-LEHD-fmk.

BADGE-induced apoptosis is a caspase-dependent process. However, this is only true for the early stage of apoptosis since late apoptosis, determined after prolonged exposure of cells to BADGE, could not be inhibited by caspase-3 and -9 inhibitors. There is emerging evidence that caspases are not required for all aspects of apoptosis. Thus apoptosis caused by disialo-ganglioside 3, staurosporine, etoposide (VP-16), dexamethasone and many other proapoptotic stimuli was not blocked by Z-VAD-fmk [26]. Cellular components that might be involved in the execution of cell death initiated by the aforementioned substances are oxygen radicals or factors released from mitochondria, like cytochrome *c* and apoptosis-inducing factor. Current investigations in our laboratory are underway to elucidate the mechanisms involved in BADGE-induced late apoptosis.

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