2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) inhibits apoptosis in cultured rat hepatocytes

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1. Introduction

In addition to proliferation, programmed cell death (apoptosis) is the major factor which contributes to the growth of a cell clone[1]. Clonal growth is a characteristic feature of phenotypically altered, preneoplastic hepatocytes in rodent liver[2]. These cells are thought to carry one or more critical but still undefined lesions in their genome resulting e.g. from the action of genotoxic liver carcinogens. Under the influence of liver tumour promoters the clonal expansion of preneoplastic liver foci is facilitated, an effect which apparently increases the probability for a further advance on their way to malignancy[3]. There is growing evidence that inhibition of apoptosis rather than stimulation of proliferation of preneoplastic hepatocytes represents the major mechanism of action of various liver tumour promoters[4].
The most potent tumour promoter in rodent liver is 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD[5,6]). In contrast to most other liver tumour promoters TCDD showed no or very restricted enhancement of hepatocellular proliferation in normal rat liver[7]. Similarly, TCDD failed to enhance DNA synthesis in normal rat hepatocytes in primary culture, although it slightly enhanced the proliferative response of the cells to epidermal growth factor[8]. Recently, a marked suppression of apoptosis in glutathione S-transferase-P-positive preneoplastic liver foci of TCDD-treated rats was found[9].
Therefore, we investigated the effect of TCDD on apoptosis in rat heptocytes cultures elicited by UV irradiation or the genotoxic aromatic amine 2-acetylaminofluorene (2-AAF). It was found that TCDD acts as a potent inhibitor of apoptosis induced by UV- or 2-AAF-treatment.

2. Materials and Methods

Male Wistar rats (Charles River, Kisslegg, Germany) were kept on tap water and a standard diet (Altromin, Lage, Germany). Hepatocytes were isolated as described[8], and seeded at a density of 100,000 cells/cm^2 in a 1:1 mixture of DMEM and Waymouth's MD 705/1 medium containing 10 mM HEPES, 50 nM dexamethasone, 5 µg each of insulin, transferrin, and selenous acid per ml (ITS premix; Collaborative Research, Lexington, MA), 10 % fetal calf serum, and 50 µg/ml gentamicin. After 3 h, medium was replaced by a 1:1 mixture of DMEM and Waymouth's MD 705/1 supplemented with 10 mM HEPES, 50 nM dexamethasone, 6.25 µg of each insulin, transferrin, selenous acid per ml, 1.25 mg/ml bovine serum albumin, 5.35 µg/ml linoleic acid (ITS$^+$ premix; Collaborative Research) and 50 µg/ml gentamicin. Then, apoptosis was initiated by addition of 2-AAF. After 12 h medium was replaced by fresh medium without 2-AAF, and the rate of apoptotic nuclei was determined at various timepoints using fluorescence microscopy as described by Oberhammer et al.[10]. For UV-treatment, cultures were irradiated 30 min prior to the second medium change. After the second medium change, TCDD dissolved in DMSO was added if indicated.
DNA fragmentation was analyzed after proteinase K digest of cell homogenates and sequential extraction with phenol, phenol/chloroform/isoamyl alcohol, and of chloroform/isoamyl

alcohol[11].The isolated DNA was analyzed by agarose gel electrophoresis.
For immunoprecipitation of p53 cells were treated with UV light, washed with ice-cold PBS, scraped off, incubated for 30 min in 500 µl extraction buffer (10 mM Tris-HCl, 140 mM NaCl, 3 mM $MgCl_2$, 0.5 % NP-40, pH 7.0) under repeated agitation, and centrifuged at 10,000 g. Equal amounts of protein were incubated overnight with 1-5 µg of primary antibody (monoclonal anti-p53; Dianova, Hamburg, Germany). The immunocomplex was then sedimented by centrifugation (10,000 g) at room temperature, washed twice in extraction buffer and once in PBS, dried, suspended in 50 µl Laemmli buffer, heated for 10 min at 65°C, and separated by PAGE. The proteins were electroblotted to a PVDF membrane (Milipore, Dreieich, Germany), and the antigen was visualized with a primary antibody (monoclonal anti-p53), and a secondary peroxidase-coupled anti-mouse IgG antibody using the Amersham ECL protocol.
For multiple comparisons (with an untreated control) the Dunnett test procedure was used applying SAS software (SAS, Cary, USA). A probability of error of 5 % was allowed for significance.

3. Results and Discussion

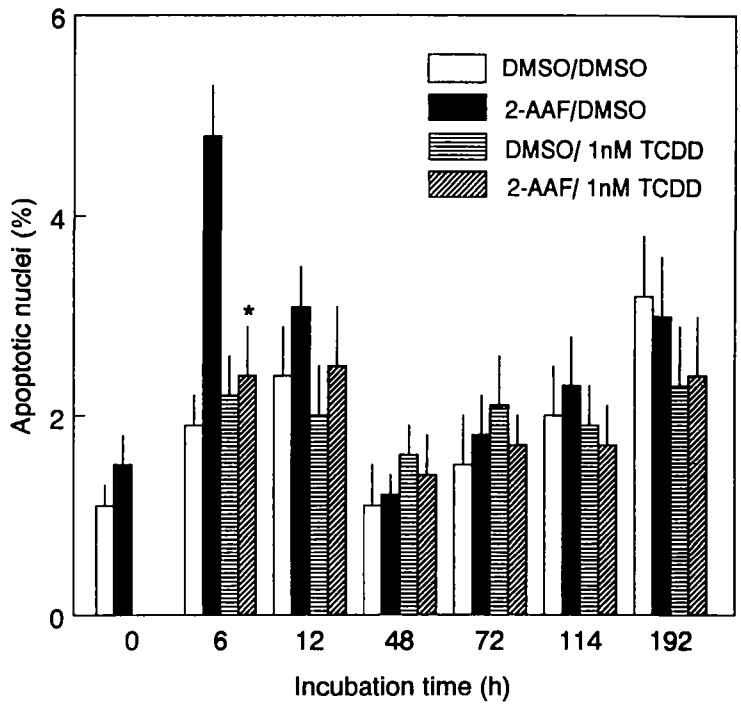


FIG. 1. Effects of TCDD on the frequency of apoptosis induced in rat hepatocytes in primary culture with 2-AAF. The percentage of apoptotic nuclei was determined in cells after treatment over 12 h with DMSO, medium change at '0 h', and addition of DMSO (DMSO/DMSO) or 1 nM TCDD (DMSO/1 nM TCDD) or after treatment over 12 h with 20 µM 2-AAF, medium change at '0 h', and addition of DMSO (2-AAF/DMSO) or 1 nM TCDD (2-AAF/1 nM TCDD). The bars represent means ± SD of four independent experiments comprising three countings of 10^3 nuclei each. The asterisk indicates a mean value significantly different ($p \leq 0.05$) from that of the 2-AAF/DMSO treatment.

When the carcinogenic aromatic amine 2-AAF was added to rat hepatocytes, a concentration-dependent increase in the percentage of apoptotic nuclei was obtained 18 h later i.e. 6 h after the second medium change. The incubation time of 12 h between the addition of 2-AAF and the second medium change was sufficient to metabolize 2-AAF completely, which was confirmed by HPLC analysis of the supernatants and cell extracts (not shown). ^{32}P-postlabeling studies revealed that overnight incubation of hepatocyte cultures with 50 µM 2-AAF led to the formation of DNA adducts. Higher 2-AAF concentrations than 50 µM resulted in overt cytotoxic effects monitored as leakage of lactate dehydrogenase into medium, and were not used in further experiments. UV irradiation also resulted in a dose-dependent significant increase in apoptotic nuclei which reached a maximum at 150 J/m^2.

In a subsequent series of experiments, the hypothesis was tested that treatment with TCDD modifies the apoptotic response of the culture. It was found that addition of 1 nM TCDD 12 h after 2-AAF-treatment almost completely abrogated the wave of apoptosis (Fig. 1). When the cultures were treated with a UV dose of 90 J/m^2, 1 nM TCDD also significantly reduced the incidence of apoptosis 6 and 12 h after medium change (Fig. 2).

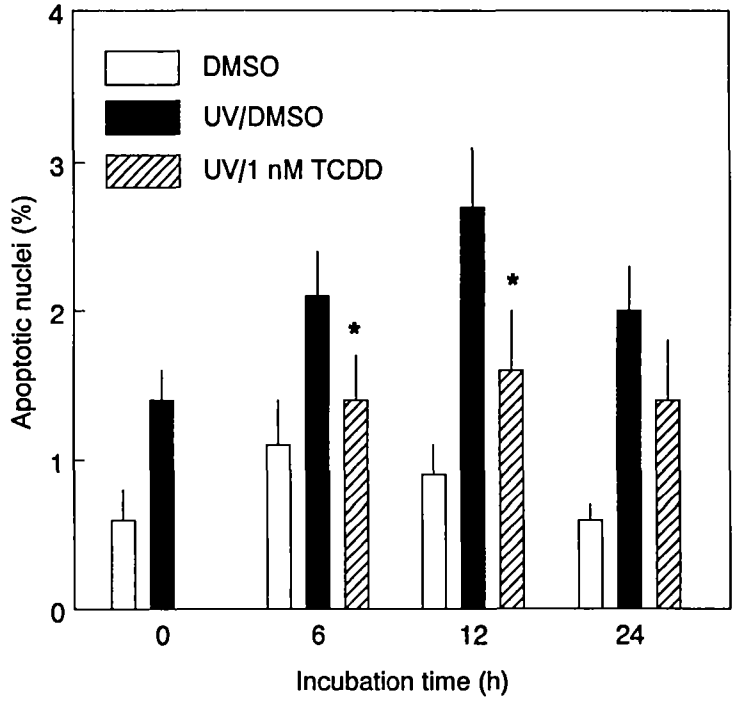
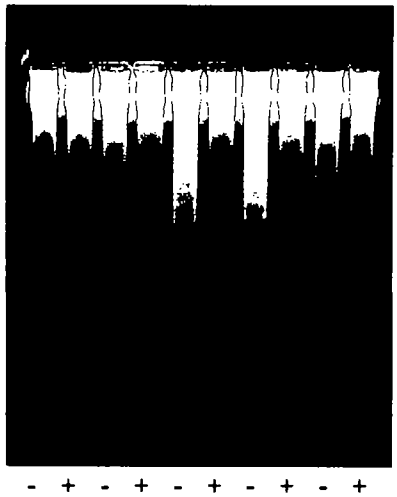


FIG. 2. Effects of TCDD on the frequency of apoptosis induced in rat hepatocytes in primary culture with UV light. Cells were treated with UV light (90 J/m^2), and the percentage of apoptotic nuclei was determined after 30 min ('0 h') and at later timepoints as indicated. Directly after irradiation, DMSO (UV/DMSO or 1 nM TCDD (UV/1 nM TCDD) were added. Controls were treated with DMSO only (DMSO). The bars represent means ± SD of four independent experiments comprising three countings of 10^3 nuclei each. The asterisks indicate mean values significantly different ($p \leq 0.05$) from that of the UV/DMSO treatment.

In addition to the morphological detection of apoptotic nuclei, internucleosomal fragmentation of DNA ('laddering') was analyzed after UV treatment. At the high density used, a certain

amount of background fragmentation was observed (Fig. 3). 24 h after irradiation with 90 or 120 J/m^2 a marked increase in fragments was obtained. When 1 nM TCDD was added 30 min after irradiation, the increase in DNA fragmentation was almost completely blocked. Interestingly, addition of 1 nM TCDD without prior UV treatment resulted in a slight increase in DNA fragmentation.



TCDD (1 nM):	-	+	-	+	-	+	-	+	-	+
UV (J/m^2):	0	0	60	60	90	90	120	120	150	150

FIG. 3. Effects of TCDD on internucleosomal DNA degradation in rat hepatocytes in primary culture. Hepatocyte cultures were treated with UV doses (upper line) of 0, 60, 90, 120, and 150 J/m^2 as indicated, and (lower line) DMSO (-) or 1 nM TCDD (+) was added. After 24 h cells were harvested, DNA was isolated and analyzed by agarose gel electrophoresis. Each lane contained 30 µg of DNA. DNA fragments were visualized with ethidium bromide/fluorescence.

Immunoprecipitation of the tumour suppressor gene product p53 from total cell homogenates revealed a dose-dependent increase 24 h after UV treatment (Fig. 4). In agreement with the DNA fragmentation experiment, addition of 1 nM TCDD 30 min after irradiation almost completely abrogated the UV-dependent increase in p53. At the zero and 90 J/m^2 level, however, 1 nM TCDD resulted in a slight increase in p53.

TCDD (1 nM):	-	+	-	+	-	+	-	+
UV (J/m^2):	0	0	90	90	120	120	150	150

FIG. 4. Western blot of immunoprecipitated p53 from homogenates of rat hepatocyte cultures 24 h after treatment with various UV doses as indicated. 30 min after irradiation, DMSO (-) or 1 nM TCDD (+) was added. P53 was immunoprecipitated from equal amounts of homogenized cellular protein using monoclonal anti-p53-antibodies. Representative blot of three blots.

In conclusion our results demonstrate that the liver tumour promoter TCDD significantly suppresses apoptosis induced in rat hepatocytes by treatment with 2-AAF or UV light. The findings suggest that prevention of apoptosis initiated by DNA damage is a major mechanism of

action of TCDD during liver tumour promotion. Furthermore, our findings indicate that TCDD suppresses the increase in p53 after apoptogenic UV treatment. Further work is necessary to investigate the underlying mechanism i.e. the influence of TCDD on the half-life, phosphorylation state and transcriptional activity of p53.

4. References

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