Apoptosis as a target for the tumor-promoting action of 2,3,7,8-TCDD

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Introduction

Inhibition of apoptosis is now recognized as a key mechanism of action of tumor-promoting agents (1,2). 2,3,7,8-TCDD (TCDD) acts as the most potent tumor-promoting agent in rodent hepatocarcinogenesis (3,4). In rat liver, it was shown to suppress the programmed cell death (apoptosis) of preneoplastic hepatocellular clones (foci) thus facilitating their growth and further progression in the course of carcinogenesis (5).

In rodent hepatocytes in primary culture, TCDD suppressed apoptosis (6) induced by treatment with UV-light or with the chemical carcinogen 2-acetylaminofluorene (2-AAF). This effect coincided with a suppression of the increase in p53, a tumor suppressor playing a key role in apoptosis, usually observable after treatment with 2-AAF or UV-light.

Materials and Methods

Rat hepatocytes were prepared and cultured as described (6). TCDD was added in DMSO, and controls received DMSO only. For initiation of apoptosis, cells were treated with UV light as described (6) and medium was replaced by fresh medium after 30 min. After 10 h, the morphological determination of apoptotic nuclei was performed as described (6). For preparation of cell extracts, a modification of the method by Fritsche et al. (7) was used. Aliquots of extract containing 400 µg protein were incubated with 4 µl monoclonal anti-p53-antibodies (Dianova) on ice for 12 h. After centrifugation at 12,000 g and 4°C over 10 min, the immunoprecipitate was washed twice with ice-cold PBS, and was air-dried. The pellet was dissolved in 20 µl Laemmli buffer and analyzed by PAGE and western blotting. After washing twice in PBS containing 0.05% Tween 20, the membrane was blocked with 5% dry milk powder in PBS/Tween 20, and then incubated with anti-p53 antibodies in a dilution of 1:2,500 in PBS/Tween 20. After repeated washing in PBS/Tween 20, the membrane was then incubated with horesradish peroxidase-coupled anti-mouse IgG antibodies in a dilution of 1:10,000 in PBS/Tween 20. Then, the immunoreaction was visualized using the enhanced chemiluminescence technique (Amersham).

7-Ethoxyresorufin O-deethylase (EROD) activity was determined fluorimetrically (8).

For phosphorylation experiments in intact hepatocytes, cells were seeded at a density of 6×10^4

cells/cm^2 on 50 mm collagen-coated culture dishes. After 15 h medium was removed, the cultures were washed with 1 ml sterile PBS, and 2 ml phosphate-free DMEM substituted with 100 µCi [^{32}P]-orthophosphate/ml were added. After 2 h, medium was replaced, and cultures were treated with DMSO or TCDD/DMSO, respectively. After 6 h cells were harvested, transfered into Eppendorf cups, and p53 was extracted and immunoprecipitated as described above. After SDS-PAGE, the gels were dried and radioactivity was visualized.

For phosphorylation experiments in cell homogenates, hepatocytes were harvested 15 h after plating, and lysed in 500 µl extraction buffer for 30 min. After centrifugation at 12,000 g and 4°C for 10 min, the supernatant (lysate) was removed and kept at -80°C. After slow thawing, the lysate was divided into four equal portions of 80-100 µl (600-800 µg protein) each which were transfered to 1.5 ml Eppendorf cups. After adding extraction buffer to a total volume of 400 µl, 15 µl Protein G PLUS/Protein A-Agarose (Calbiochem) were added to each cup. For precipitation of c-*src*, 10 µl monoclonal anti-v-*scr* antibodies (Ab-1; Calbiochem; 100 µg/ml) were added. Then the cups were transfered to 50 ml vials filled with ice, and agitated at 4°C for 90 min. After removal of the immunoprecipitate by centrifugation at 10,000 g for 1 min, the supernatants were transfered to fresh Eppendorf cups, pre-incubated at 30°C for 5 min, and treated with 1 nM TCDD (dissolved in DMSO) or with DMSO only. Then, 0.37 - 0.56 MBq [^{32}P]-γ-ATP were added to each cup, and incubation was continued at 30°C for 10 min. The cups were transfered on ice, and 15 µl Protein G PLUS/Protein A-Agarose were added to each cup. For immunoprecipitation of p53, 10 µl anti-p53 antibodies and 15 µl of Protein A Agarose were added. The cups were incubated over 90 min under agitation in lead containers filled with ice. The immunoprecipitates were sedimented by centrifugation at 10,000 g over 1 min, washed twice in ice-cold PBS, and analyzed by SDS-PAGE as described above. The gels were dried, and radioactivity was visualized using a phosphoimager.

Results and Discussion

In rat hepatocytes in primary culture, apoptosis initiated by UV-irradiation was suppressed with 1 nM TCDD (Fig. 1). TCDD treatment of intact cells led to hyperphosphorylation of p53 in a concentration-dependent manner (Fig. 2). A similar effect on p53 phosphorylation was recently reported for the tumor promoter ocadaic acid (9). The concentration-response relationships of p53 phosphorylation and of Ah receptor-regulated induction of CYP1A1-catalyzed 7-ethoxyresorufin O-deethylase (EROD) activity were found to be identical (not shown) suggesting a role of AhR activation in TCDD-stimulated p53 phosphorylation. Furthermore, immunoprecipitation of c-*src*, a tyrosine kinase previously found to be activated upon AhR activation (10), strongly attenutated the effect of TCDD on p53 phosphorylation in cell homogenates (Fig. 3).

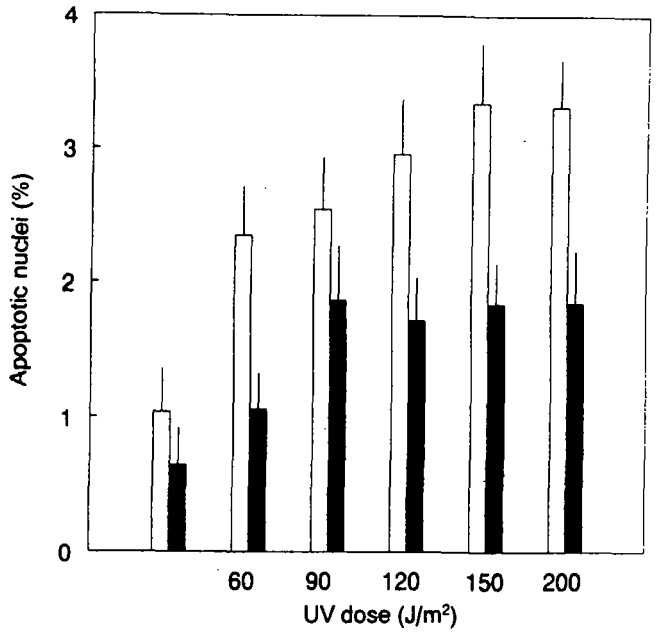

Fig. 1. Effect of TCDD (1 nM) treatment (closed bars) of UV-initiated apoptosis in rat hepatocytes, 10 h after irradiation, in comparison to DMSO-treated controls (open bars). Data represent means ± S.D.from four independent experiments.

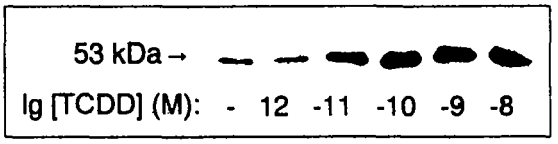

Fig. 2. Effect of TCDD (1 nM for 6 h) on phosphorylation of p53 in rat hepatocytes. The pattern is representative for three independent experiments.

In conclusion, TCDD is able to suppress apoptosis in hepatocytes initiated by treatment with UV light. In cultured hepatocytes, this effect coincides with a suppression of the p53 response probably mediated by AhR-dependent *c-src* activation and subsequent hyperphosphorylation of p53. The role of this type of hyperphosphorylation for p53 functions requires further investigation. Other mediators of apoptosis may also be subject to hyperphosphorylation by activated c-*src*.

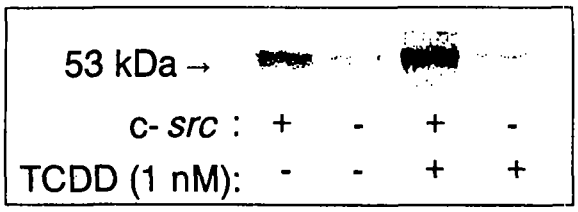

Fig. 3. Effect of TCDD (1 nM for 15 min) on phosphorylation of p53 in rat liver homogenate, without (+) or with (-) preceeding immunopercipitation of c-*src*. The pattern is representative for three independent experiments.

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References

1. Bursch W, Lauer B, Timmermann-Trosiener I, Barthel G, Schuppler J and Schulte-Hermann, R; *Carcinogenesis* **1984**, 5, 43.
2. Wright S, Zhong J and Larrick J; *FASEB J.* **1994**, 8, 654.
3. Pitot HC, Goldsworthy T, Campbell HA and Poland A; *Cancer Res.* **1980**, 40, 3616.
4. Schrenk D, Buchmann A, Dietz K, Lipp H-P, Brunner H, Sirma H, Münzel P, Hagenmaier H, Gebhardt R and Bock KW; *Carcinogenesis* **1994**, 15, 509.
5. Stinchcombe S, Buchmann A, Bock KW and Schwarz M; *Carcinogenesis* **1995**, 16, 1271.
6. Wörner W and Schrenk D; *Cancer Res.***1996**, 56, 1272.
7. Fritsche M, Haessler C and Brandner G; *Oncogene* **1993**, 8, 307.
8. Burke D and Mayer R; *Drug Metab. Dispos* **1974**, 2, 583.
9. Yatsunami J, Komori A, Ohta T, Suganuma M and Fujiki H; *Cancer Res.***1993**, 53, 239.
10. Enan E and Matsumura F; *Biochem. Pharmacol.* **1996**, 52, 1599.