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The induction of apoptosis in proliferating human fibroblasts by oxygen radicals is associated with a p53- and p21^{WAF1CIP1} induction

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Abstract The role of reactive oxygen species (ROS) generated by hypoxanthine/xanthine oxidase (HX/XO) in the induction of apoptosis was studied in the human fibroblast cell line WI38. Apoptosis but not necrosis was observed in proliferating fibroblasts after 48 h incubation with 1 mM HX and 0.05 U/ml XO. Induction of apoptosis was hindered by catalase. Cell-cycle analysis revealed a reduction of cells in the S/G2 phase 24 and 48 h after stimulation, suggesting that ROS induce a G1 arrest in proliferating fibroblasts. This was supported by an accumulation of p53 and the cdk inhibitor p21$^{WAF1/CIP1}$. Since apoptosis was not inducible in senescent fibroblasts our data indicate that ROS mainly induces apoptosis in proliferating cells.

Key words: Fibroblast; Apoptosis; p53; Cell cycle; Proliferation; p21$^{WAF1/CIP1}$

1. Introduction

Recent work indicates that oxidative stress is required in many instances for execution of the apoptotic program [1], such as in response to γ-irradiation [2] and TNF [3]. Moreover, oxidative stress may be sufficient to induce apoptosis, as recent work has shown that agents that directly promote the production of reactive oxygen species, such as H_2O_2 [4,5] and menadione [6], can trigger apoptosis in diverse systems. Similarily, exogenous antioxidants have been reported to block apoptosis induced by tumor necrosis factor (TNF) [7], HIV [8], endotoxin [9], growth-factor withdrawal [10], and glucocorticoids [11].

On the other hand, several reports suggest that the primary mode of cell death induced by reactive oxygen species (ROS) is necrosis, not apoptosis. In chondrocyte cultures ROS, produced by the hypoxanthine/xanthine oxidase (HX/XO) system, induced necrosis but not apoptosis [12] and hypoxia did not affect the induction of apoptosis in lymphoma cell lines by either dexamethasone or by serum withdrawal [13].

In order to test the prediction that ROS is involved in apoptotic cell death, we stimulated a human lung fibroblast cell line (WI38) with ROS produced by the HX/XO system. Apoptosis was determined by DNA laddering and by means of TUNEL reaction. The up-regulation of p53 and p21, both involved in cell-cycle regulation and apoptotic signalling, as well as the induction of a G1 arrest, provide evidence that exogenously applied ROS induces apoptosis in human lung fibroblasts.

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2. Materials and methods

2.1. Cell culture

The human fibroblast cell line WI38 was obtained from Serva (Heidelberg, Germany). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) purchased from Serva (Heidelberg, Germany). The medium was supplemented with 10% fetal calf serum (FCS), penicillin/streptomycin and glutamine (Biochrom, Berlin, Germany). The cells were incubated at 37°C in 5% CO_2 atmosphere.

2.2. Culture conditions for stimulation assays

Cells (passage 26) were seeded in 10 cm Petri dishes at a density of 1×10^6 cells/7 ml. After 48 h cells were incubated in culture medium containing different concentrations of hypoxanthine (Sigma, Taufkirchen, Germany) and xanthine oxidase (Boehringer, Mannheim, Germany). Superoxide dismutase (Boehringer, from bovine erythrocytes, No. 567680) and catalase (Boehringer, from beef liver, No. 106810) were used in a concentration of 500 U/ml. For subsequent molecular analyses the cells were removed from the dishes by trypsination or in case of protein lysate preparation by mechanical scraping.

2.3. Determination of cell viability

For determination of the number of viable cells, cells were removed by trypsination and stained with trypan blue (Sigma, Taufkirchen, Germany) and the number of viable and dead cells were determined by light microscopy. All experiments were performed in quadruplicates.

2.4. Western blot analysis

Cells were lysed by freezing and thawing the cells 3 times followed by sonication. Then the lysates were boiled in sodium dodecyl sulfate (SDS)–gel sample buffer for 5 min. Protein (20 μg) was electrophoretically resolved on denaturating 12% polyacrylamide gels. Proteins were transferred to nitrocellulose membranes (Schleicher and Schüll, Dassel, Germany) using a transblot apparatus (Phase, Lübeck, Germany). Non-specific interactions were blocked by pre-incubation of the membranes with a milk powder suspension (10% dry milk in PBS). After incubation of the membranes with monoclonal antibodies the binding of antibodies was detected using the ECL system (Amersham).

2.5. Immunprecipitation

Cell lysates were adjusted to a protein concentration of 1 mg/ml. The lysates were precleared twice by addition of 15 μl of protein A agarose (Oncogene Sciences, Cambridge, MA) to 1 ml of the lysate, incubation for 1 h at 4°C and centrifugation for 20 min at $13\,000\times g$. Then, lysates were incubated for 1 h with the monoclonal antibody at a concentration of 1 μg/ml, followed by an additional incubation with 20 μl of protein A agarose for 1 h. After centrifugation the pellet was washed twice with lysis buffer and once with 0.125 M Tris buffer. The pellet was resuspended in 50 μl of SDS–gel sample buffer and boiled for 5 min at 95°C. Samples were analysed according to the Western blot protocol.

2.6. RT-PCR of WAF1

For the molecular detection of WAF1 total RNA was isolated from the cells as described previously [14]. Reverse transcription was performed using the SUPERSCRIPT TM 2 Rnase H Reverse Transcriptase from Gibco (Berlin, Germany) according to the manufacturer's protocol. PCR was carried out using the primers 5′-CAGGATCCTGTGGGCGGATTAGGGCT-3′ and 5′-AGGATC-

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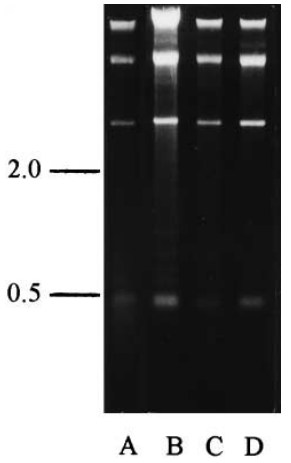

Fig. 1. DNA integrity analysis of WI38 cells treated with different concentrations of ROS. Cells were treated with medium only (Control, lane A), 1 mM HX/0.05 U/ml XO (lane B), 1 mM HX/0.05 U/ml XO+catalase (lane C), or 1 mM HX/0.05 U/ml XO+catalase/superoxide dismutase (lane D). DNA laddering was only observed in lane B, addition of catalase or catalase/superoxide dismutase inhibited DNA laddering (lanes C and D).

CATGTCAGAA-CCGGCTGG-3′, amplifying a 520 bp product, under the following conditions: 60 s at 94°C followed by 60 s 60°C and 60 s 72°C in a total of 30 cycles. The elongation was accomplished for a further 5 min at 72°C.

2.7. DNA extraction for nucleosomal ladder

Cells were digested in a solution of 5% Sarcosyl, 50 mM Tris, and 10 mM EDTA containing 20 U of proteinase K at 50°C with shaking for 12 h. Digestion was continued for 1 h after addition of 10 μg of Dnase-free RNAse. After digestion, DNA was extracted sequentially with phenol and phenol/chloroform/isoamyl alcohol and chloroform and then ethanol precipitated and resuspended in 10 mM Tris-HCl (pH 8.0)–1 mM EDTA. Electrophoresis was carried out on 10 μg from each DNA sample on a 1.0% agarose gel containing 0.1 μg/ml ethidium bromide and photographed under UV light.

2.8. Apoptosis assay

Apoptosis was determined by TUNEL reaction (TdT-mediated dUTP nick end labeling) using an in situ cell death detection kit (fluorescein) from Boehringer (Mannheim, Germany) according to the manufacturer's protocol. In all experiments cell viability was determined by trypan blue staining.

2.9. Proliferation assay

The proliferation was determined by BrdU incorporation and subsequent BrdU detection using a commercially available BrdU labeling and detection kit (Boehringer, Mannheim, Germany). The cells were processed according to the manufacturer's protocol.

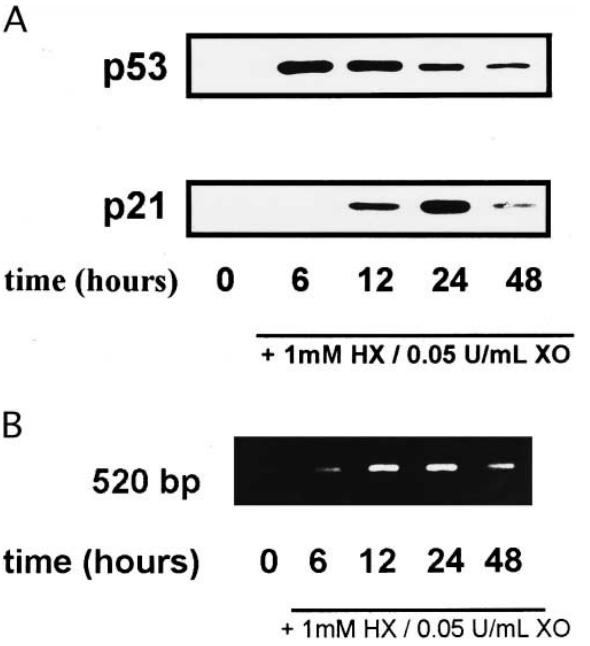

Fig. 2. A: Immunoprecipitation of p53 and Western blot analysis of $p21^{WAF1/CIP1}$ from WI38 cells treated with 1 mM HX/0.05 U/ml XO. p53 protein increased reaching a maximum 12 h after stimulation. In RT-PCR an increase of WAF1/CIP1-RNA was detectable 6 h after stimulation (B). Transcription of p21WAF1/CIP1 showed an increase 12 h after stimulation reaching a maximum 24 h after stimulation. Results were reproducible in two other experiments.

3. Results

3.1. Induction of apoptosis in WI38 cells is dependent on oxygen radical concentration

We have recently shown that the HX/XO system constantly releases ROS over a time period of 1 h [15] with a release of approximately 30–40 nmol/ml/min using a concentration of 1 mM HX and 0.05 U/ml XO. In order to determine the effects of ROS, WI38 cells were incubated with 1 mM HX/0.05 U XO. Apoptosis was determined by means of the TUNEL reaction. At a concentration of 1 mM HX/0.05 U XO apoptotic changes were observed in 40% of the cells after 48 h (Table 1 and Fig. 1). In the presence of superoxide dismutase the apoptotic changes were not inhibited, whereas catalase alone or catalase plus superoxide dismutase were able to inhibit apoptosis. In all experiments trypan blue staining revealed that the number of dead cells did not differ sig-

Table 1
Portion of dead cells and apoptotic cells in WI38 cell cultures stimulated with different concentrations of ROS

Culture conditions	Dead cells after 24 h (%)	Dead cells after 48 h (%)	Apoptotic fraction after 24 h (%)	Apoptotic fraction after 48 h (%)
Control (medium)	4.5 (1.8)	6.9 (1.7)	5.5 (2)	6.4 (2.1)
+1 mM HX/0.05 U/ml XO	26.1 (4.9)*	44.6 (5.1)*	19.5 (4.4)*	40.3 (6.3)*
+1 mM HX/0.05 U/ml XO+CAT	8.6 (2.9)**	7.1 (3.8)**	7.2 (3.1)**	7.4 (3.5)**
+1 mM HX/0.05 U/ml XO+SOD	23.6 (7.6)	45.4 (11.2)	18.9 (5.7)	42.6 (9.8)
+1 mM HX/0.05 U/ml XO+CAT+SOD	8.2 (2.1)	7.9 (3.6)	6.9 (3.3)**	7.5 (4.7)**

Cell viability was determined by Trypan blue staining; apoptosis was determined by TUNEL reaction and subsequent FACS analysis.
CAT: catalase (500 U/ml); SOD: superoxide dismutase (500 U/ml).
All experiments were performed in quadruplicate; values in parentheses represent standard deviation.
*$P < 0.001$ as compared to control; **$P < 0.001$ as compared to 1 mM HX/0.05 U/ml XO.

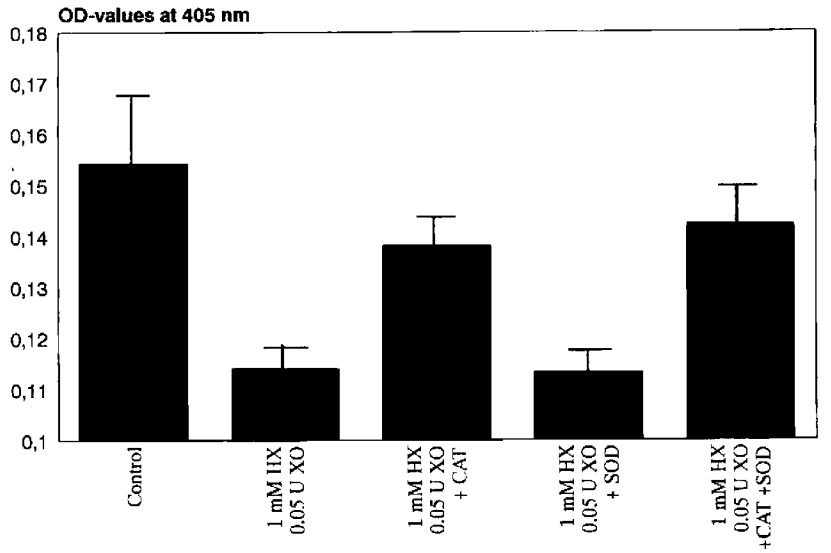

Fig. 3. Proliferation analysis of WI38 by BrdU incorporation analysis. Experiments were performed 8 times. Proliferation was markedly inhibited by addition of 1 mM HX/0.05 U/ml XO ($P < 0.001$ as compared to control). These effects were reversed by addition of catalase or catalase +superoxide dismutase ($P < 0.001$ as compared to 1 mM HX/0.05 U/ml XO). Superoxide dismutase alone did not inhibit the proliferation arrest. Higher concentrations of ROS totally inhibited cell proliferation, whereas lower concentrations of ROS did not inhibit cell proliferation ($P < 0.0001$ and not significant, respectively).

nificantly from the number of apoptotic cells, which implies that the majority of cell death was due to apoptotic and not necrotic changes (Table 1).

3.2. *ROS induce p53 and p21 (WAF1) in human fibroblasts*

Using 1 mM HX and 0.05 U/ml XO immunoprecipitation of p53 revealed an increase of p53 protein in WI38 cells reaching a maximum 12 h after stimulation followed by a decrease at 24 h and 48 h (Fig. 2A). In RT-PCR an increase of WAF1-mRNA was detectable 6 h after stimulation (Fig. 2B). Transcription of p21 protein was shown by Western blotting showing an increase of p21 12 h after stimulation reaching maximum levels at 24 h after stimulation (Fig. 2A). Addition of catalase markedly reduced the induction of p53 and p21.

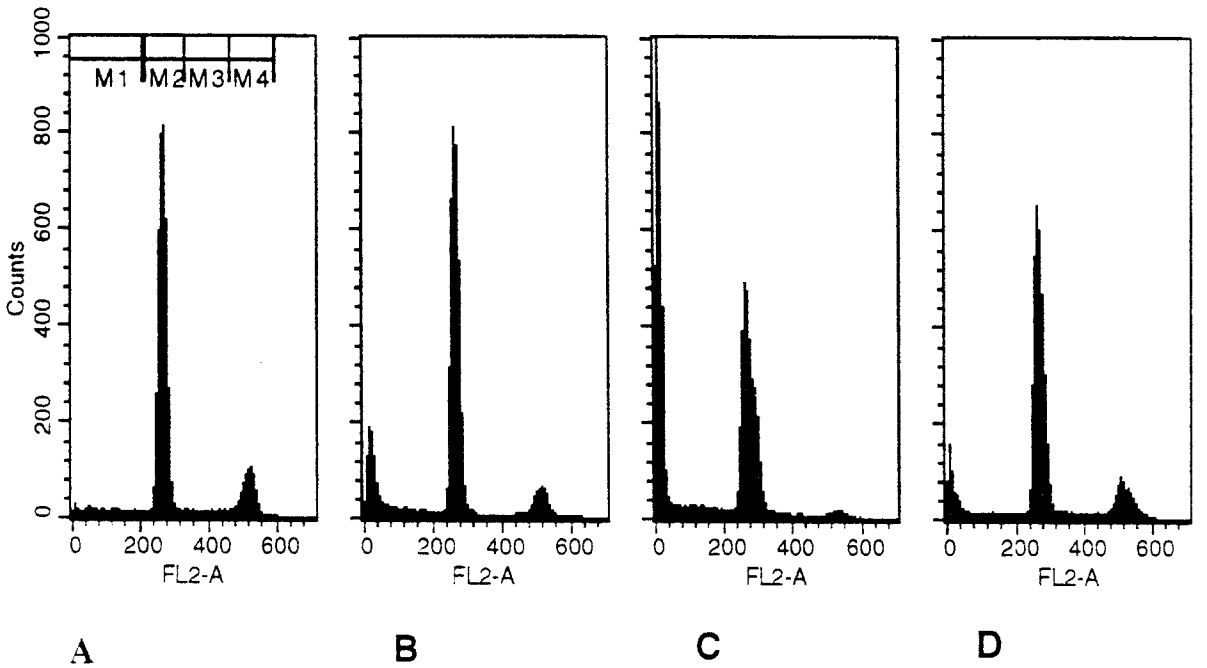

Fig. 4. Cell-cycle analysis of WI38 (passage 26): medium (control, A), 1 mM HX/0.05 U/ml XO 24 h (B) and 48 h (C) after stimulation and 48 h after stimulation with 1 mM HX/0.05 U/ml XO+catalase+superoxide dismutase (D). M1: apoptotic fraction, M2: G0/G1 fraction, M3: S fraction, M4: G2/M fraction. During 48 h the apoptotic fraction increased to 45%, whereas the S/G2/M fraction decreased from 20.5% to 2.8%. The G0/G1 fraction was only marginally affected (decrease from 72% to 59%) indicating a G1 arrest. The ROS-mediated effects were blockable by addition of catalase and superoxide dismutase. The results were reproducible in two other independent experiments.

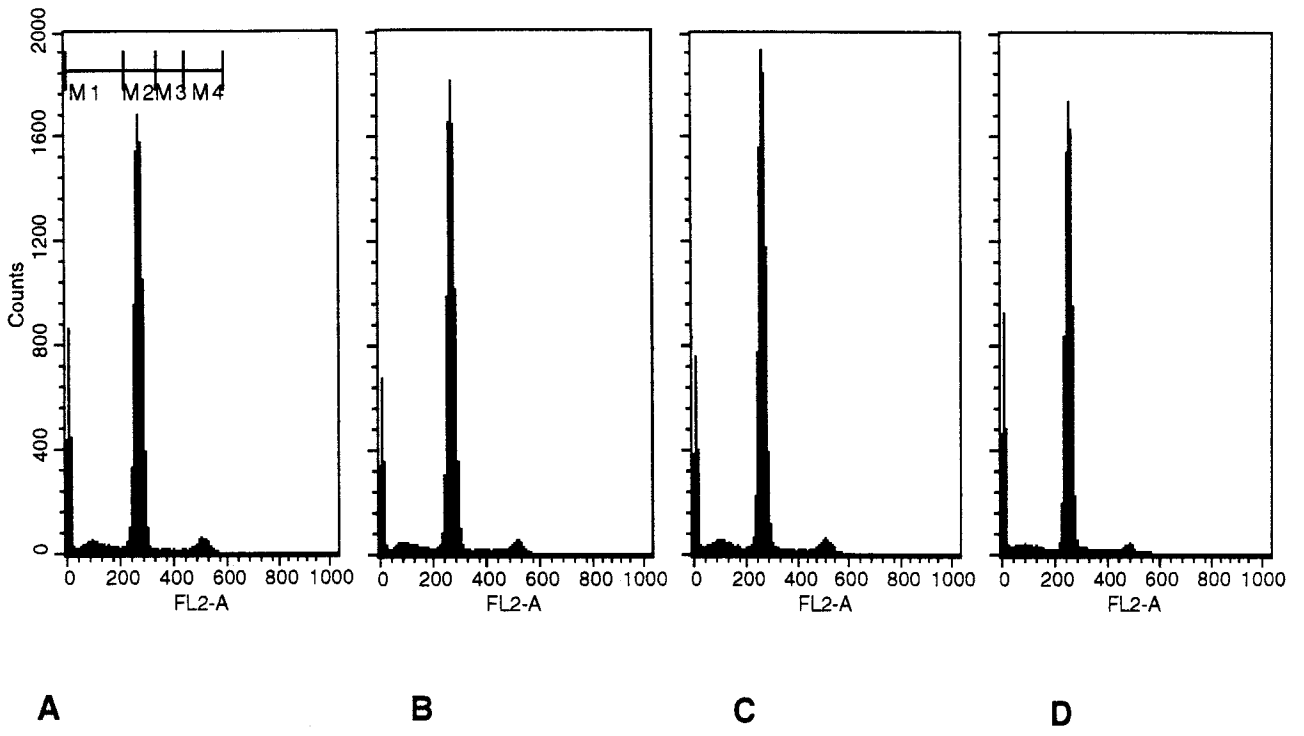

Fig. 5. Cell-cycle analysis of WI38 (passage 46): medium (control, A), 1 mM HX/0.05 U/ml XO 24 h (B) and 48 h (C) after stimulation and 48 h after stimulation with 1 mM HX/0.05 U/ml XO+catalase+superoxide dismutase (D). M1: apoptotic fraction, M2: G0/G1 fraction, M3: S fraction, M4: G2/M fraction. During 48 h the apoptotic fraction remained constant between 10% and 14%. Similarly the S/G2/M fraction and the G0/G1 fraction remained constant (between 4.2–6.1% and 81.5–82.9%, respectively). The results were reproducible in two other independent experiments.

3.3. ROS induces a temporary G1 arrest in human fibroblasts

Determination of proliferation by BrdU incorporation revealed that proliferation was markedly inhibited by the generation of ROS at 1 mM HX/0.05 U/ml XO (Fig. 3, $P < 0.001$ as compared to negative control). Addition of catalase inhibited these effects ($P < 0.001$ as compared to 1 mM HX/0.05 U/ml XO), whereas superoxide dismutase did not influence the proliferation arrest induced by ROS (Fig. 3). In cell-cycle analysis a reduction of the cells in the S/G2 phase was observed 24 h and 48 h after stimulation (1 mM HX/0.05 U/ml XO) whereas the G1 fraction remained constant, suggesting that cells were hindered to enter S-phase (Fig. 4). These changes were inhibited by addition of catalase.

3.4. ROS does not induce apoptosis in senescent fibroblasts

In order to evaluate whether ROS mainly induces apoptosis in proliferating cells, especially in S/G2 phase, WI38 from passage 46, which only has a small capacity to proliferate, was incubated with HX and XO. At a concentration of 1 mM HX and 0.05 U/ml XO no apoptosis was observed. Trypan blue staining confirmed that also no necrosis had occurred since no significant differences in cell viability were observed (viable cells at 24 h: Control: 93.3%, 1 mM HX/0.05 U/ml XO: 91.2%, viable cells at 48 h: Control: 91.8%, 1 mM HX/0.05 U/ml XO: 89.7% viable cells) Even cell cycle or p53 protein expression was not affected by ROS, indicating that ROS mainly acts in proliferating cells (S/G2) (Fig. 5).

4. Discussion

Oxidative stress resulting from toxic effects of ROS plays an important role in the pathogenesis of a variety of diseases and important biological processes [16–19]. Toxic effects of reactive oxygen metabolites, including the superoxide anion, the H_2O_2 and the hydroxyl radical involve chemical alterations in proteins, lipids, carbohydrates and nucleic acids [20–23]. Oxidants are able to produce DNA damage [24] that can cause a specific cellular response whereby the protein product of the tumor suppressor gene p53 accumulates to high levels [25]. This increase seems to be directly responsible for the arrest of cells in the G1 phase of the cell cycle or, in case of severe DNA damage, p53 is supposed to cause apoptosis [26]. To examine the effects of ROS on the cellular and molecular response of human fibroblasts concerning the p53-dependent induction of apoptosis we incubated human fibroblasts with ROS generated by the HX/XO system which has been studied extensively [15,27–30] and results in both, the univalent and divalent reduction of O_2 resulting in the formation of O_2^- and H_2O_2, respectively. In the presence of redox-active transition metals such as iron or copper the hydroxyl radical is generated via the Fenton-reaction. H_2O_2 also seems to form the hydroxyl radical ($HO^{\cdot}$) intracellularly which appears to be the most likely free radical responsible for DNA damage [28]. The hypothesis of the clastogenic properties of H_2O_2 is also supported by the finding that superoxide dismutase did not protect from XO-induced apoptosis and that catalase, which eliminates H_2O_2 to O_2 and water, protected cells from apoptosis.

Our studies suggest that ROS induces apoptosis rather than necrosis in human fibroblasts. As a consequence of DNA damage we were able to demonstrate an increase of the protein product of the tumor suppressor gene p53 reaching a

maximum 12 h after stimulation with 1 mM HX/0.05 U/ml XO. p53 is able to bind in a sequence specific manner to DNA and activates the transcription of other genes [31,32]. The cdk inhibitor WAF1/CIP1 represents such a gene and its expression is induced by p53 in the cellular response to DNA damage [12,33]. Consequently the accumulation of p53 was followed by an increase of $p21^{WAF1/CIP1}$ and resulted in a reduced proliferation rate of cells, which was due to a G1 arrest, as demonstrated by cell-cycle analysis and apoptosis. Both these effects were blocked by the addition of catalase. Fibroblasts from passage 46, having almost no capacity to proliferate, were not influenced by ROS to undergo apoptosis. Also no changes in cell cycle or p53 expression were observed. This suggests that ROS acts via a p53 increase in the G1 phase of proliferating cells.

Thus far the induction of apoptosis by reactive oxygen metabolites is controversially discussed. In human chondrocytes it has been shown that ROS generated by HX/XO induced necrosis of the cells, whereas nitric oxide induces apoptosis [1]. On the other hand, oxidative stress has been suggested to play a role as a common mediator of apoptosis [34] and some independent observations in diverse systems support the hypothesis for a role of oxidative mechanisms in the induction of apoptosis [35,36]. In human tumors it has been shown that hypoxic regions with high radical generation correlate with apoptotic changes [37]. A further hint for this hypothesis is the apoptosis inhibitor molecule bcl-2. The antioxidant activity of bcl-2 strongly implicates an involvement of ROS in the induction of programmed cell death [38].

In conclusion our results suggest that ROS is able to induce apoptosis in proliferating human fibroblasts, acting in the G1 phase of the cell cycle and increasing the p53 and $p21^{WAF1/CIP1}$ protein levels. Further studies will be required to clarify whether p53 is directly associated by redox processes involving the site-specific DNA-binding capacity [39] or whether p53 is indirectly increased by other factors, which are induced by oxidative stress. A possible candidate could be the nuclear factor kappa B which is known to be induced via free radical reactions and on its part activates as a transcription factor p53 [40].

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