Resistance to Tumor Necrosis Factor-α (TNF-α)-induced Apoptosis in Rat Hepatoma Cells Expressing TNF-α Is Linked to Low Antioxidant Enzyme Expression

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In order to study the mechanisms of resistance to tumor necrosis factor-α (TNF-α), we have constructed two stable transfectants producing TNF-α (Yv12-2 and Yv13-44) from the rat hepatoma H4IIE cell, which does not produce TNF-α. H4IIE cells were highly sensitive to apoptosis induced by TNF-α, whereas Yv2-12 and Yv13-44 cells were resistant. Manganese superoxide dismutase was not up-regulated in Yv2-12 and Yv13-44 cells and was unresponsive to induction by exogenous TNF-α and by H2O2 in H4IIE cells and in the transfectants. Catalase expression and activity were lower in Yv2-12 and Yv13-44 cells than in H4IIE cells; furthermore, the transfectants were more susceptible to H2O2. Treatment with exogenous TNF-α down-regulated catalase in H4IIE cells but not in Yv2-12 and Yv13-44 cells. Treatment of H4IIE cells with the catalase inhibitor 3-amino-1,2,4-triazole rendered them resistant to exogenous TNF-α. These data suggest a causal relationship between resistance to TNF-α and low catalase activity. Expression of copper and zinc containing superoxide dismutase was also decreased, whereas expression of glutathione peroxidase-1 was unchanged in Yv2-12 and Yv13-44 cells. Data from a microarray point to a down-regulation of genes in the resistant clones that code for antioxidative proteins and proteins involved in glutathione synthesis and function. We assume that a prooxidant signal linked to the down-regulation of antioxidant defense may be associated with resistance to apoptosis induced by TNF-α.

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1 The abbreviations used are: TNF-α, tumor necrosis factor-α; TNFR1, TNF receptor 1; TNFR2, TNF receptor 2; MnSOD, manganese superoxide dismutase; ROS, reactive oxygen species; ATX, 3-amino-1,2,4-triazole; GPx-1, glutathione peroxidase-1; CuZnSOD, copper and zinc-containing superoxide dismutase; ELISA, enzyme-linked immunosorbent assay.

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EXPERIMENTAL PROCEDURES

Cell Lines and Culture Conditions—H4IIE cells (a rat hepatoma cell line), transfectants derived from H4IIE cells, and L929 cells (a murine fibrosarcoma cell line) were grown in Dulbecco’s modified Eagle’s me-

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diurnum with z-glutamine containing 10% heat-inactivated fetal calf se-
rum, penicillin (100 units/ml), and streptomycin (100 μg/ml) at 37 °C in
a humidified atmosphere of 5% CO₂. For treatment experiments, me-
ium containing TNF-α (R&D, Wiesbaden, Germany), H₂O₂ (Sigma), or
3-amino-1,2,4-triazole (ATZ) (Sigma) was added and remained on the
cells for 24 h. Cell viability was tested by the neutral red assay accord-
ing to Borenfreund and Paumer (18).
Stable Transfection of TNF-α into H4IIE Cells—TNF-α cDNA was
obtained by RT-PCR from total rat hepatocyte RNA isolated with Triozol
(Invitrogen). Hepatocytes were prepared from male Wistar rats (250–300
g) by a modified method according to Lindl and Bauer 1989 (19).
The amplified product was purified and cloned into the pSPT18 vector.
Correct sequence was verified in randomly selected
pSPT18-TNF-α clones with vector-specific primers. For selection of tetracycline-respon-
sive transfectants, cells were transfected with 10 μg of pTet-ON or
pTet-OFF using calcium phosphate precipitation. Clones were selected
in the presence of 800 μg/ml G418 48 h after transfection for 2 weeks.
For establishing TNF-α overexpressing cells, the selected clones were
co-transfected with pTRE-TNF-α and pTK-Hyg (Clontech), a plasmid
that encodes hygromycin resistance. Clones were selected in the pres-
ence of 500 μg/ml hygromycin B 48 h after transfection, using calcium
phosphate precipitation. Isolates of clones Yv2-12 and Yv13-44 were then
tested for TNF-α expression by RT-PCR.

**TNF-α Detection**—Total TNF-α concentration in the culture medium
was determined by commercially available rat TNF-α ELISA according
to the manufacturer’s recommendations (Endogen, Boston, MA). This
kit is sensitive to 5 pg/ml TNF-α and does not cross-react with TNF-β,
interleukin-1, or interleukin-6. Biologically active TNF-α was quanti-
fied by the L929 assay as described previously (20). One unit of TNF-α
was defined as the reciprocal of the dilution at which 50% L929 cyto-
toxicity was observed.

**Catalase Activity**—Catalase activity was determined by measuring
the initial rate of decay of H₂O₂ absorbance at 240 nm, using an
extinction coefficient of 43.6 M⁻¹ cm⁻¹. Prior to the catalase assay,
hemoglobin was removed by incubation with ethanol and Triton X-100 according
to Cohen et al. (21). Each assay mixture consisted of a cell sample (150
μg of protein/ml) and H₂O₂ at an initial concentration of 19 μM H₂O₂ in
50 μM phosphate buffer (pH 7.0).

**RNA Isolation and Northern Blot Analysis**—Total RNA was isolated
from cells using Trizol (Invitrogen). Total RNA (5–10 μg) was resolved
by electrophoresis in a 1.5% agarose, 2.2% formaldehyde gel. RNA was transferred to
nylon membrane (Amersham Biosciences) as described by Sambrook et al. (22). Purified cDNAs were labeled with [α³²P]dCTP (111 TBq/mmol; Hartmann Analytic, Braunschweig, Germany) by ran-
dom hexamer priming (Roche Diagnostics). Blots were prehybridized
and hybridized with cDNAs as described previously (23) and then
exposed to X-ray film (Kodak XAR-5) for 3 days with an intensifying
screen at −80 °C. 18 S RNA served as internal loading control. Auto-
radiographs were analyzed by densitometric scanning using the Quan-
ty One system from Bio-Rad.

**Microarray**—Gene expression was analyzed using Atlas Rat Toxicol-
ogy Array II (Clontech). Total RNA was extracted from TNF-α-trans-
fected and parental H4IIE cells using Trizol (Invitrogen). The sample
was treated with RNAse-free DNase I for 60 min, extracted using
phenol-chloroform, precipitated with ethanol, and then resuspended in
assay buffer (50 mM 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid, 100
mM NaCl, 0.1% Tween 20, pH 8.0) and incubated with 1:500–1:1000
dilution of antibodies against MnSOD (Upstate, Milton Keynes,
UK) and catalase (Calbiochem). Blots were then probed with the ap-
propriate horseradish peroxidase-conjugated secondary antibody.
Bound antibody was visualized using enhanced chemiluminescence
reagent (Roche Diagnostics).

**Analysis of DNA Fragmentation**—After incubation with TNF-α or
H₂O₂ for 24 h, 5 × 10⁶ cells were harvested. Cell pellets were resus-
pected in lysis buffer (20 mM Tris, 5 mM EDTA, 0.1 mM NaCl, 0.5% SDS,
RNase A 10 μg/ml, pH 8.0) and incubated for 1 h at 37 °C. DNA was
extracted with an equal volume of phenol-chloroform:isoamyl alcohol
(25:24:1) and precipitated with 0.7 volume isopropanol and 0.1 volume
3 M NaCl. The pellets were resuspended in 50 μl of Tris/EDTA buffer,
and DNA was electrophoresed using a 2% agarose gel at 70 V for 3–4 h.
The DNA bands were visualized by ethidium bromide staining and
photographed under UV light using a transilluminator.

**Anti-histone ELISA**—Quantitative measurements of apoptosis was
performed using an ELISA that quantifies DNA fragmentation by
measuring cytoplasmic histone-DNA fragments (Cell Death ELISA;
Roche Diagnostics). Cells were incubated with TNF-α or H₂O₂ as
described above; apoptosis was assessed 24 h later according to the
manufacturer’s recommendations.

**Caspase Assay**—Determination of caspase activity was carried out in
96-well plates using 50 μg of protein for each measurement. Colorimet-
ric substrates for caspases were purchased from Calbiochem. Activity of
caspase 3, 8, and 9 was detected by measuring the proteolytic cleavage
of the substrates in assay buffer (50 mM 4′-2-hydroxyethyl)piperazine-
1-ethanesulfonic acid, 100 mM NaCl, 0.1% 3-[3-(chlamidompropyl)dim-
ethylammonio]-1-propanesulfonic acid, 10 mM dithiothreitol, 100 μM
EDTA, pH 7.4) using the absorbance of released p-nitro-
aniline at 405 nm.

**Statistical Analyses**—All data were analyzed using one-way analysis
of variance, followed by least significant difference post hoc analysis
to determine statistical significance. p values <0.05 were considered sta-
tistically significant.

**RESULTS**

Stable transfection of H4IIE cells with TNF-α cDNA resulted in
the emergence of two clones (Yv2-12 and Yv13-44) that
constitutively expressed TNF-α to a different extent. These
clones were used for further experimentation. The concentra-
tion of total TNF-α protein and biologically active TNF-α in
the medium was one order of magnitude higher in Yv2-12
cells than in Yv13-44 cells. The wild type cells (wtH4IIE cells) did
not produce TNF-α. No difference in cell proliferation and
morphology was observed between wtH4IIE cells and
transfectants.

WtH4IIE cells, but not Yv2-12 and Yv13-44 cells, were
highly susceptible to the cytotoxic action of exogenous TNF-α at
a concentration of 25 ng/ml. Yv13-44 showed a slight loss of
viability at 50 ng/ml (Fig. 1), and Yv2-12 was completely re-
sistant to TNF-α up to 500 ng/ml. Apoptosis as detected by
oligonucleosomal DNA fragmentation (Fig. 2A) and by an anti-
histone ELISA (Fig. 2B) was elicited by treatment with exoge-
nous TNF-α in wtH4IIE cells but not in Yv2-12 and Yv13-44
cells. In wtH4IIE cells, but not in Yv2–12 and Yv13–44 cells, a slight increase in p53 and bax expression was found, although bcl-2 expression was not altered. Activation of caspase 3 (Fig. 3A), caspase 8 (Fig. 3B), and caspase 9 (Fig. 3C) upon treatment with exogenous TNF-α was found in wtH4IIE cells but not in Yv2–12 cells, whereas Yv13–44 cells displayed intermediate activation of caspase 3 but not of caspase 8 and 9.

TNF receptor expression was measured in order to exclude the possibility that resistance to TNF-α in the transfectants is because of a loss of TNFR1 and/or TNFR2 expression. The wtH4IIE cells as well as the two TNF-α-resistant clones expressed the TNFR1, whereas none of them expressed the TNFR2. TNFR1 functionality was not lost, as shown by the induction of NF-κB activation by exogenous TNF-α in the resistant clones (data not shown).

Because formation of ROS is assumed to be involved in TNF-α cytotoxicity, TNF-α resistance might be caused by the up-regulation of antioxidant enzymes. An increase of MnSOD expression has been shown to occur in other TNF-α-resistant cell types (10, 11). It might, therefore, be assumed that this adaptation process also occurred in Yv2–12 and Yv13–44 cells. Fig. 4 shows that neither the MnSOD mRNA level nor the MnSOD protein level in Yv2–12 and Yv13–44 cells differed from those in wtH4IIE cells. MnSOD induction could not be obtained in either wtH4IIE cells or Yv2–12 and Yv13–44 cells by exogenous TNF-α.

To provide a possible explanation for the TNF-α resistance of the clones, we examined whether catalase and GPx-1 expression is increased in Yv2–12 and Yv13–44 cells. Fig. 5 demonstrates that no difference in GPx-1 mRNA expression was found between wild type cells and TNF-resistant cells and that contrary to expectations catalase mRNA was even lower in the TNF-α-resistant clones than in wtH4IIE cells (Fig. 5B). Catalase protein expression and catalase enzyme activity were also decreased in the transfectants (Fig. 5, C and D). Treatment with exogenous TNF-α down-regulated catalase expression and enzyme activity in wtH4IIE cells but not in the TNF-α-resistant clones (Fig. 6). A slight induction of GPx-1 by exogenous TNF-α could be achieved in wtH4IIE cells but not in Yv2–12 and Yv13–44 cells.

To test the conclusion from Figs. 5 and 6 that low catalase activity is associated with TNF-α resistance, cells were treated with the catalase inhibitor ATZ. Fig. 7 shows that in wtH4IIE cells ATZ treatment did, indeed, markedly lower the sensitivity
to TNF-α. No influence of ATZ treatment on TNF-α sensitivity was observed in the Yv2–12 and Yv13–44 cells.

To test the initial hypothesis that TNF-α-resistant cells also display resistance toward H₂O₂, cytotoxicity of H₂O₂ was compared in wtH4IIE cells and in Yv2–12 and Yv13–44 cells. Fig. 8A shows that Yv2–12 and Yv13–44 cells are even more susceptible toward H₂O₂ than wtH4IIE cells. In Fig. 8, B and C, the induction of apoptosis by H₂O₂ as detected by oligonucleosomal DNA fragmentation and by an anti-histone ELISA was compared in wtH4IIE cells versus Yv2–12 and Yv13–44 cells. The transfectants were considerably more sensitive to apoptosis induced by H₂O₂ than the wtH4IIE cells.

MnSOD expression as well as catalase expression can be increased by H₂O₂ in a number of cells (22, 24–27). In our experiments, similar to the results with exogenous TNF-α, both in the wild type cell and the TNF-α-resistant cells, MnSOD is unresponsive to H₂O₂ treatment. Catalase mRNA expression and enzyme activity could be increased by H₂O₂ treatment both in wtH4IIE cells and in Yv2–12 and Yv13–44 cells.

A DNA microarray comparing wtH4IIE cells and Yv2–12 cells was performed to support the hypothesis that TNF-α resistance is paralleled by a shift toward a more pro-oxidative state and to identify additional genes differentially regulated in the resistant cells. Of 450 genes, 8 were up-regulated and 31 were down-regulated in the Yv2–12 cells by a factor of at least 2. Preliminary evaluation of the microarray data indeed points to a down-regulation of genes for antioxidant enzymes and of genes involved in glutathione synthesis and in detoxifying phase II metabolism (Table I). At present, the down-regulation of copper and zinc containing superoxide dismutase (CuZnSOD) indicated by the microarray has been verified by Northern blotting in the Yv2–12 and Yv13–44 cells. Moreover, down-regulation of CuZnSOD was obtained in wtH4IIE cells, but not in Yv2–12 and Yv13–44 cells, upon treatment with exogenous TNF-α.

**DISCUSSION**

With the experiments reported here, we have collected evidence that resistance to TNF-α-induced apoptosis can be associated with a down-regulation of antioxidant proteins. For this,
we have constructed two TNF-α-producing stable transfectants from a wild type rat hepatoma cell that does not express TNF-α. The wtH4IIE cell is highly sensitive to TNF-α-induced apoptosis, whereas the transfectants are resistant to TNF-α-induced apoptosis.

TNF-α induces apoptosis via the death receptor pathway, and down-regulation of TNF-α receptors could theoretically be the mechanism of TNF-α resistance. This is, however, not the main mechanism in our transfectants because (a) both the wild type cell and the transfectants express the TNFR1 to a similar extent, (b) both the wild type cell and the transfectants do not express the TNFR2 at all, and (c) NF-κB activation by TNF-α, assumed to require TNF-R1 functionality, can be induced in the transfectants. In vivo, Sass et al. (28) also did not find a down-regulation of TNFR1 in TNF-α-resistant mice.

A mitochondrial component is involved in TNF-α-induced cytotoxicity (13, 14). This has supported the assumption that fortification of mitochondrial defense against oxidative stress by up-regulating MnSOD could be a mechanism of TNF-α resistance. In contrast to our initial expectations, up-regulation of MnSOD expression did not occur in our TNF-α-producing transfectants and thus could be excluded as a mechanism of TNF-α resistance in this cell type. MnSOD is expressed to the same extent in the wtH4IIE cell and in the TNF-α-resistant clones derived from it and is unresponsive to induction by exogenous TNF-α. Induction of the enzyme in wtH4IIE cells and in the TNF-α-resistant clones could not be achieved with H2O2 either. In contrast, MnSOD can be induced by TNF-α (29, 30) and H2O2 (23) in primary rat hepatocytes. The reason for the intrinsic inability of the H4IIE cell and its descendents to up-regulate MnSOD has not yet been elucidated.

It might have been expected that the expression of other antioxidant enzymes in the TNF-α-resistant cells was increased as a substitute for the lack of up-regulation of MnSOD. However, gene expression analysis revealed that in the transfectants none of the antioxidant enzymes examined is up-regulated, whereas two antioxidant enzymes, catalase and CuZnSOD, are even down-regulated. This expression pattern is

![Figure 7](image_url) **FIG. 7.** Effect of ATZ, an inhibitor of catalase, on cell viability and caspase activity after treatment with TNF-α. H4IIE, Yv2–12, and Yv13–44 cells were treated with 20 mM ATZ, 15 ng/ml TNF-α (TNF), or 20 mM ATZ + 15 ng/ml TNF-α for 24 h. A, cell viability was assessed by neutral red assay. Data are means ± S.E. (n = 4); *p < 0.05 versus TNF-treated H4IIE cells. The activities of caspase-3 (B) and caspase-8 (C) were measured as described under “Experimental Procedures.” Data are means ± S.E. (n = 3); *p < 0.05 versus TNF-treated H4IIE cells.

![Figure 8](image_url) **FIG. 8.** Overexpression of TNF-α increases sensitivity to H2O2-induced cytotoxicity. A, cells were treated with various concentrations of H2O2 for 24 h, and cell viability was determined by neutral red assay. Data are means ± S.E. (n = 4–5); *p < 0.05 versus H4IIE at each concentration. B, cells were treated with various concentrations of H2O2 for 24 h, and DNA fragmentation was assessed by electrophoresis. C, oligonucleosomal DNA fragmentation after treatment with H2O2 was assessed by an anti-histone ELISA. Results are triplicates of two independent experiments.
mimicked by TNF-α treatment of the TNF-α-sensitive wtH4IIE cell: Exogenous TNF-α leads to a decrease of catalase and CuZnSOD expression in these cells. As a consequence of low catalase activity, the sensitivity of the transfectants to H₂O₂ was increased. A decrease in catalase activity (31) and catalase mRNA (32) upon TNF-α treatment has also been observed in vivo. The finding that exposure to endogenous TNF-α in the TNF-α-resistant clones and to exogenous TNF-α in the TNF-α-sensitive wild type cell leads to a decrease in catalase expression and activity suggests that the down-regulation of this antioxidant enzyme is not merely an epiphenomenon of the transfection process. Additionally, we performed a pharmacological experiment that strongly indicates a causal relationship between low catalase activity and TNF-α resistance: Exposure of TNF-α-sensitive wtH4IIE cells to the catalase inhibitor ATZ renders these cells partially resistant to exogenous TNF-α.

We conclude from these findings that a pro-oxidant signal linked to down-regulation of antioxidant defense contributes to the TNF-α-resistant state. This signal may transfer information via mediator concentrations of ROS too low to exert cytotoxic effects. The conclusion that a shift of the pro-oxidant-antioxidant balance of the cell has occurred in the TNF-α-resistant clones is further supported by the preliminary results from a cDNA microarray analysis that suggest the down-regulation of a number of proteins with antioxidative properties in the Yv₂–12 cell, including thioredoxin-2 and metallothionein-1 as well as a number of enzymes involved in glutathione supply and function.

The conclusion that low activity of antioxidant enzymes may be involved in TNF-α resistance is at a first glance not consistent with two interpretations brought forward in the literature. First, it is commonly assumed that the antioxidant enzyme MnSOD, induced via the NF-κB survival pathway, is the main protective principle against TNF-α-induced cytotoxicity (8). In our cells, MnSOD cannot contribute to protection against TNF-α because the enzyme is intrinsically unresponsive to induction. Even in cells in which MnSOD is induced, protection may not be linked to the antioxidant character of the enzyme. The reaction product is H₂O₂, and the antioxidant potential of the MnSOD reaction can only be realized by subsequent reduction of H₂O₂ to H₂O by glutathione peroxidases or catalase. Siemankowski et al. (33) have reported that in MCF-7 cells TNF-α induces MnSOD without any change in the expression of other antioxidant enzymes, including GPx-1 and catalase. This may indicate that the mechanism by which MnSOD protects against TNF-α could be pro-oxidative in nature because the steady state level of H₂O₂ increases. It has been concluded from experiments in cell lines exposing HeLa cells that H₂O₂ produced via MnSOD induction creates an oxidative injury leading to mitochondrial degeneration, proliferation arrest, and resistance to TNF-α-induced cytotoxicity (34). On the other hand, the down-regulation of the cytoplasmic CuZnSOD observed in the present work, not only in the TNF-α-resistant clones but also in the TNF-α-treated wild type H4IIE cells, is not consistent with this view because the resulting pattern of SOD activities would decrease H₂O₂ delivery to GPx and catalase. We assume that a coordinated response of H₂O₂-delivering and H₂O₂-consuming enzymes to TNF-α exposure is required for the maintenance of a well-dosed pro-oxidant signal to the TNF-α-dependent signaling cascade. Secondly, in the mouse, transcriptional and/or translational inhibitors sensitize cells and animals to the toxic action of TNF-α (35). This suggests that the de novo synthesis of protective proteins is needed for TNF-α resistance and down-regulation (e.g. of catalase) as a protective mechanism does not seem to fit in this picture. However, catalase is known to be in part regulated post-translationally via the degradation of the catalase mRNA by a protein (36). If in the presence of a transcriptional or translational inhibitor (a situation linked to high TNF-α sensitivity) this protein is no longer formed, this would result in an apparent up-regulation of catalase while uninhibited de novo synthesis of this regulatory protein would be required to achieve a state of TNF-α resistance.

In conclusion, we have shown that in TNF-α-resistant cell lines, although they are induced in the resistant animals (28), by introduction of a randomized hybrid ribozyme library into MCF-7 cells, a number of genes functional in TNF-α-induced apoptosis were recently identified (42). It may be worthwhile testing whether these genes are down-regulated in a TNF-α-resistant cell. In conclusion, we have shown that in TNF-α-resistant transfectants derived from a TNF-α-sensitive rat hepatoma cell line a down-regulation of certain antioxidant proteins occurs in the absence of any increase in the expression of the other antioxidant proteins examined. We suggest that a pro-oxidant signal

### Table I

| GenBank™ no. | Gene name                          | Function | H4IIE/2–12* |
|-------------|-----------------------------------|----------|-------------|
| J03752      | Glutathione S-transferase (GST-12)| Metabolism| 3 ↓        |
| J02992      | Glutathione S-transferase (GST α) | Metabolism| 4 ↓        |
| X02904      | Glutathione S-transferase (GST γ) | Metabolism| 3 ↓        |
| J05181      | γ glutamylcysteine synthetase      | Metabolism| 2 ↓        |
| M10161      | CuZnSOD                           | Antioxidative enzyme| 2 ↓ |
| M11670      | Catalase                          | Antioxidative enzyme| 2.5 ↓     |
| U73525      | Thioredoxin-2                     | Antioxidative enzyme| 2.5 ↓     |
| J00750      | Metallothionein 1                 | Antioxidative enzyme| 5 ↓        |
| X02918      | Protein disulfide isomerase       | Stress response protein| 3 ↓        |

Normalized data were obtained by dividing each spot intensity by the mean of the spot intensity on the filter. Data are represented as mean of ratio (H4IIE/Yv₂–12) from two separate array experiments.
linked to the down-regulation of antioxidant defense could be associated with the TNF-α-resistant state.

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