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Activation of Caspases in Lethal Experimental Hepatitis and Prevention by Acute Phase Proteins[1]

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Lethal hepatitis can be induced by an agonistic anti-Fas Ab in normal mice or by TNF in mice sensitized to D-(+)-galactosamine or actinomycin D. In all three models, we found that apoptosis of hepatocytes is an early and necessary step to cause lethality. In the three models, we observed activation of the major executioner caspases-3 and -7. Two acute-phase proteins, α_1-acid glycoprotein and α_1-antitrypsin, differentially prevent lethality: α_1-acid glycoprotein protects in both TNF models and not in the anti-Fas model, while α_1-antitrypsin confers protection in the TNF/D-(+)-galactosamine model only. The protection is inversely correlated with activation of caspase-3 and caspase-7. The data suggest that activation of caspase-3 and -7 is essential in the in vivo induction of apoptosis leading to lethal hepatitis and that acute phase proteins are powerful inhibitors of apoptosis and caspase activation. Furthermore, Bcl-2 transgenic mice, expressing Bcl-2 specifically in hepatocytes, are protected against a lethal challenge with anti-Fas or with TNF/D-(+)-galactosamine, but not against TNF/actinomycin D. The acute-phase proteins might constitute an inducible anti-apoptotic protective system, which in pathology or disturbed homeostasis prevents excessive apoptosis. ***The Journal of Immunology,*** **1999, 163: 5235–5241.**

Hepatitis, either virally or chemically induced, is correlated with increased serum TNF levels (1). As shown by us and others, TNF, when given in combination with D-(+)-galactosamine (GalN)[4] or actinomycin D (ActD), induces lethal hepatitis in mice (2–5), a process that is mediated by TNF-RI (5). Triggering of CD95/Fas, another member of the TNF-R superfamily, also leads to lethal hepatitis (5, 6). Evidence exists that, in contrast to the CD95/Fas model, in the TNF models a simultaneous inflammatory component plays an essential role (7). In all three models, apoptosis of hepatocytes was observed. During recent years, cysteinyl aspartate-specific proteases or caspases have been found to be implicated in the process of apoptosis. These caspases mediate cell death by cleaving essential substrates (8). Caspases operating in the apoptotic process can be divided in two groups, the initiator caspases and the executioner caspases. Initiator caspases are those caspases involved in the activation of the executioner caspases that cleave specific death substrates such as poly(ADP-ribose) polymerase, DNA polymerase kinase, and ICAD, the inhibitor of the caspase-activated DNase (9). Caspase-3 (previously known as CPP32, Yama, or apopain) and caspase-7 (previously known as Mch3, CMH-1, or ICE-LAP3) are two main executioner caspases, and both caspases are activated during apoptosis. Recently, two different pathways of activation of executioner caspases have been reported in the case of Fas-mediated apoptosis, depending on the cell type (10). During Fas-mediated type I apoptosis, there is an efficient recruitment of procaspase-8 in the so-called death-inducing signaling complex (11). Procaspase-8 is activated by oligomerization (12), and consequently caspase-8 (previously known as Mch5, MACH, or FLICE) directly proteolyses procaspase-3 and -7 (13). Release of cytochrome *c* from the mitochondria is observed, but is not essential for induction of apoptosis (10). During type II apoptosis, for unknown reasons minor procaspase-8 recruitment occurs and low levels of caspase-8 are activated (10). Cytochrome *c* released from the mitochondria is required for amplification of the caspase cascade. Cytochrome *c* binds to Apaf-1 and ATP and activates procaspase-9, which in turn activates procaspase-3 and -7 (14). In type II apoptosis, cytochrome *c* release is absolutely required to activate downstream caspases. Inhibition of cytochrome *c* release by Bcl-2 prevents type II apoptosis.

In this paper, we intended to study the activation of caspases in three experimental models of apoptosis, viz. TNF/GalN, TNF/ActD, and anti-Fas, all three resembling acute viral hepatitis (15–18). We and others (3–6) have demonstrated that these models cause massive apoptosis of hepatocytes of mice in vivo, followed by the release of liver transaminases. We could also show a differential inhibition of apoptosis by acute-phase proteins in the three models (5). We show here that apoptosis is an early event, associated with activation of caspase-3 and -7, and that pretreatment with the acute-phase proteins α_1-acid glycoprotein (α_1-AGP) and α_1-antitrypsin (α_1-AT) prevents caspase activation and lethality. Furthermore, transgenic overexpression of Bcl-2 protected differentially in the three models, which suggests either different mechanisms of induction of apoptosis or involvement of other tissues in inducing lethality in the three models.

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[4] Abbreviations used in this paper: GalN, galactosamine; α_1-AGP, α_1-acid glycoprotein; α_1-AT, α_1-antitrypsin; Ac-DEVD-amc, acetyl-Asp(OMe)-Glu(OMe)-Val-Asp(OMe)-aminomethylcoumarin; ActD, actinomycin D; ALT, alanine aminotransferase; HEK, human embryonic kidney.

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Materials and Methods

Animals

Female C57BL/6 mice were obtained from Iffa Credo (Saint Germain-sur-l'Arbresle, France) and were used at the age of 8–10 wk. Heterozygous Bcl-2 transgenic mice were generated as follows. A fragment containing the coding sequence of a human Bcl-2 cDNA, flanked by a rabbit β-globin intron and the rabbit β-globin polyadenylation signal (19), was inserted downstream of the human α_1-AT promoter. Transgenic mice were generated as described previously (20) using (C57BL/6 $\times$ DBA2)F_1 eggs. The highest expressor line, as judged by Western blotting of liver extracts, was used for experiments. Heterozygous Bcl-2 transgenic mice were crossed with C57BL/6 animals. Transgenic offspring were identified by PCR and crossed again with C57BL/6 to obtain 50% transgenic and 50% wild-type mice. Male and female transgenic and nontransgenic mice were used at the age of 7–12 wk. The animals were housed in a temperature-controlled, air-conditioned room with 12-h light/dark cycles and received water and food ad libitum.

Reagents

Recombinant murine TNF-α (TNF) was produced in *Escherichia coli* and purified to homogeneity in the first author's laboratory. TNF had a sp. act. of 2.6×10^8 IU/mg and an endotoxin contamination of 0.07 ng/mg of protein. Endotoxin levels were assessed by a chromogenic *Limulus* amebocyte lysate assay (Coatest; Chromogenix, Stockholm, Sweden). Bovine α_1-AGP, human α_1-AT, ActD, and GalN were obtained from Sigma (St. Louis, MO). Monoclonal hamster anti-mouse Fas Ab Jo2 (IgG) (6) was purchased from PharMingen (San Diego, CA) and had an endotoxin level of 0.05 ng/mg protein according to the manufacturer.

Injections and blood collections

Cytokines and chemicals were dissolved in endotoxin-free PBS before use. Intraperitoneal and i.v. injections were conducted in volumes of 0.5 and 0.2 ml, respectively. Blood was taken from the retro-orbital plexus under light ether anesthesia and was allowed to clot for 30 min at 37°C and 1 h at 4°C, followed by centrifugation at 16,000 $\times$ *g*. Serum was stored at −20°C.

Determination of serum alanyl aminotransferase (ALT) and body temperature measurement

The ALT content was determined using an enzymatic/colorimetric kit (Sigma). Rectal body temperatures were measured with an electronic thermometer (model 2001; Comark Electronics, Littlehampton, U.K.).

Liver homogenate preparation

Livers were cut to small pieces, washed three times with glycerol buffer (10% glycerol, 5 mM EDTA, 10 mM Tris/HCl, pH 7.4, 200 mM NaCl), and homogenized with a tissue grinder (Wheaton Scientific, Millville, NJ) in the same buffer, supplemented with 1 mM PMSF, 0.3 mM aprotinin, 1 mM leupeptin, and 1 mM oxidized glutathione. All steps were performed on ice. Samples were frozen immediately at −20°C.

Assays for apoptosis

Liver homogenates were centrifuged for 20 min at 13,000 $\times$ *g*, and supernatant was stored at 4°C. Apoptosis was quantified by immunochemical determination of histon-complexed DNA fragments in a microtiter plate (21). Briefly, plates were coated with an Ab directed against histon H2B. After blocking, homogenates were added and a biotinylated detection Ab specific for the nucleosome subparticle of histones H2A, H2B, and DNA (22) was administered. Detection was performed with alkaline phosphatase-conjugated streptavidin (Sanvertech, Boechout, Belgium) and substrate (Sigma). Livers from PBS-treated animals were taken at 100%.

Internucleosomal DNA cleavage was studied by gel electrophoresis. DNA was prepared from 500 μl of centrifuged liver homogenate. The proteins were removed with a phenol/chloroform/isoamyl alcohol (50/48/2) and chloroform/isoamyl alcohol (24/1) extraction. DNA was precipitated overnight with 1/10 volume 5 M ammonium acetate and 9/10 volume ethanol (96%) at −20°C. DNA was resolved in Tris/EDTA, and RNA was removed by RNase H treatment. Samples were analyzed on 1.8% agarose gel and stained with ethidium bromide.

Western blotting

For Western blotting, 200 μg total protein was loaded on a 15% SDS-polyacrylamide gel. After electrophoretic separation and blotting to a nitrocellulose membrane, the different caspase fragments were detected using polyclonal Abs raised against recombinant murine caspases (23) and developed by enhanced chemiluminescence (Amersham Pharmacia Biotech, Rainham, U.K.).

Table I. *Induction of apoptosis and release of ALT as a function of time in several models of hepatitis in mice (*n = *2 for all groups)*

	Time After Challenge (h)	DNA Fragmentation[a]	ALT (U/L)[b]
TNF/GalN[c]	1	162	89
	2	182	111
	4	996	155
	6	1024	5106
	7	1167	4884
TNF/ActD[d]	1	98	122
	2	92	133
	4	445	1110
	5	725	3774
Anti-Fas[e]	0.5	97	155
	1	206	178
	2	824	577
	3	902	3996
TNF[f]	3	104	46
	6	130	120
	9	122	1195
GalN[g]	2	90	88
	6	110	110
ActD[h]	2	80	80
	7	80	65

[a] PBS-treated mice were taken as 100% DNA fragmentation.
[b] The value for PBS-treated mice was 133 U/L.
[c] TNF/GalN challenge: 0.5 μg TNF + 20 mg GalN i.p. (LD_{100}).
[d] TNF/ActD challenge: 0.5 μg TNF + 20 μg ActD i.p. (LD_{100}).
[e] Anti-Fas challenge: 15 μg i.v. (LD_{100}).
[f] TNF challenge: 20 μg i.v. (LD_{100}).
[g] GalN challenge: 20 mg i.p.
[h] ActD challenge: 20 μg i.p.

Fluorogenic substrate assay for caspase activity

Caspase-like activities were determined by incubation of liver homogenate (containing 25 μg of total protein) with 50 μM of the fluorogenic substrate acetyl-Asp(OMe)-Glu(OMe)-Val-Asp(OMe)-aminomethylcoumarin (Ac-DEVD-amc) (Peptide Institute, Osaka, Japan) in 200 μl cell-free system buffer containing 10 mM HEPES, pH 7.4, 220 mM mannitol, 68 mM sucrose, 2 mM NaCl, 2.5 mM KH_2PO_4, 0.5 mM EGTA, 2 mM $MgCl_2$, 5 mM pyruvate, 0.1 mM PMSF, and 1 mM DTT. The release of fluorescent amc was measured for 1 h at 2-min intervals by fluorometry (Cytofluor; PerSeptive Biosystems, Cambridge, MA). Data are expressed as the maximal rate of increase in fluorescence per min (ΔF_{max}/min).

Statistics

Significant differences in DNA fragmentation, serum ALT, and body temperature were calculated using a two-tailed *t* test. Significant differences in final survival time were calculated using Fisher's exact test.

Results

Induction of apoptosis and transaminase release

We previously reported the conditions needed to induce lethal hepatitis using TNF/GalN, TNF/ActD, or anti-Fas (2, 5); lymphotoxin, in combination with GalN, also induced lethal hepatitis, similar to TNF/GalN (our unpublished observations). Here, we studied the induction of apoptosis and release of liver-specific enzymes as a function of time after the administration of a lethal dose (LD_{100}) of TNF/GalN, TNF/ActD, TNF, or anti-Fas. As demonstrated in Table I, in all three models of lethal hepatitis, apoptosis is detected by the presence of histon-complexed DNA fragments in the liver homogenates, before the release of ALT. Both TNF models have comparable kinetics of events; however, induction of apoptosis in anti-Fas-treated mice appears much faster. In these models, apparently necrosis is a secondary phenomenon, as ALT release follows apoptosis. TNF alone, even in very high doses, was unable to

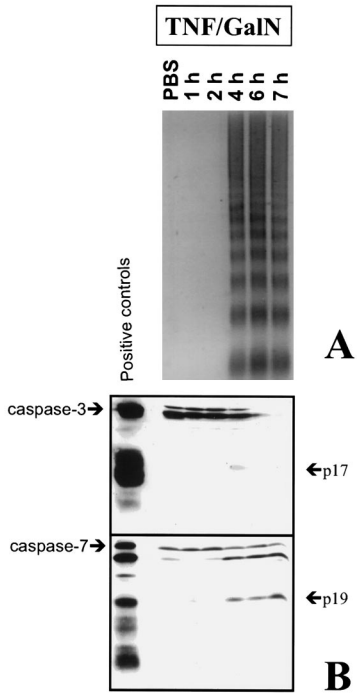

FIGURE 1. DNA laddering and activation of caspases occurs from 4 h after TNF/GalN challenge. Mice were injected i.p. with 0.5 μg TNF + 20 mg GalN. Then, 1, 2, 4, 6, or 7 h after challenge, livers were excised and homogenized. *A*, Agarose gel (1.8%) from DNA extracted from liver samples after ethidium bromide staining. *B*, Western blot of liver homogenates for caspase-3 and -7. Positive controls are caspase-3- and caspase-7-transfected HEK cells. Samples represent pooled homogenates of two mice. Shown is a representative example of at least three independent experiments.

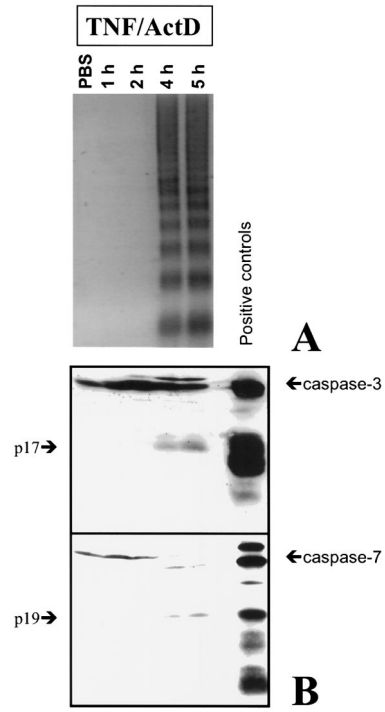

FIGURE 2. DNA laddering and activation of caspases occurs from 4 h after TNF/ActD challenge. Mice were injected i.p. with 0.3 μg TNF + 20 μg ActD. Then, 1, 2, 4, or 5 h after challenge, livers were excised and homogenized. *A*, Agarose gel (1.8%) from DNA extracted from liver homogenates after ethidium bromide staining. *B*, Western blot of liver homogenates tested for caspase-3 and -7. Positive controls are caspase-3- and caspase-7-transfected HEK cells. Samples represent pooled homogenates of two mice. Shown is a representative example of at least three independent experiments.

induce apoptosis, although a clear ALT increase was observed. Neither GalN nor ActD alone had any effect on the parameters examined (Table I).

In vivo correlation between induction of apoptosis and activation of caspases

To verify the induction of apoptosis during lethal hepatitis, mice were treated with a lethal dose of TNF/GalN, TNF/ActD, or anti-Fas, livers were collected at several time points, and the extracted DNA was analyzed on agarose gels to visualize DNA ladders, indicative of apoptosis. We found that DNA ladders appear rather suddenly and coinciding with the immunochemical detection of DNA fragmentation (Figs. 1–3 and Table I). By Western blotting and immunodetection with specific antisera, we found that activation of procaspase-3 as well as procaspase-7 coincided with the appearance of DNA ladder patterns. TNF, GalN, or ActD alone had no effect (results not shown). The samples in which caspase-3 and caspase-7 fragments were detected cleaved fluorogenic Ac-DEVD-amc but not acetyl-Tyr-Val-Ala-Asp-aminomethylcoumarin, which confirms that the detected caspase-3 and caspase-7 fragments represent active caspases (Fig. 4).

Inhibitory effects of protective acute-phase proteins

We described earlier that α_1-AGP protects mice against lethality induced by TNF/GalN or TNF/ActD but not against anti-Fas and that α_1-AT protects only against TNF/GalN (5, 24). In the experiments of Fig. 4, we studied the effect of a protective dose of these acute-phase proteins on induction of apoptosis, release of transaminases, and caspase activation in the three hepatotoxicity models. We found that protection against lethality correlates with the inhibition of apoptosis, as measured by DNA fragmentation, and secondary necrosis, as determined by ALT release. Using Ac-DEVD-amc as a substrate for caspase-3 and caspase-7 (25, 26), we could also demonstrate a correlation between inhibition of apoptosis and reduction of Ac-DEVD-amc cleavage activity in the homogenates (Fig. 4), which indicates inhibition of caspase-3 and caspase-7 activation. In Fig. 5, we confirmed that protection conferred by both acute-phase proteins in the TNF/GalN model is also reflected in liver DNA ladder patterns and that the activation of procaspase-3 and procaspase-7 is inhibited. In the TNF/ActD model, only α_1-AGP can inhibit the cleavage of procaspase-3 and procaspase-7. In the anti-Fas model, none of the acute-phase proteins influence the activation of executioner caspases (data not shown).

Endogenous protection by Bcl-2 overexpression

It has been described that transgenic overexpression of Bcl-2 in hepatocytes of mice prevents anti-Fas-induced apoptosis of hepatocytes (27, 28). We were interested to study the effect of Bcl-2 overexpression on induction of apoptosis and lethality by the three models. In Tables II and III, we demonstrate that Bcl-2 transgenic mice are protected very well against a lethal injection of anti-Fas: hypothermia, apoptosis, transaminase release, and lethality are completely prevented. Against TNF/GalN, transgenic Bcl-2 was also able to protect, but clearly over a less broad dose range, while no protection was observed in the TNF/ActD model.

Discussion

TNF and related members of the TNF family are very pleotropic cytokines. Many of them show an interesting antitumor activity, in

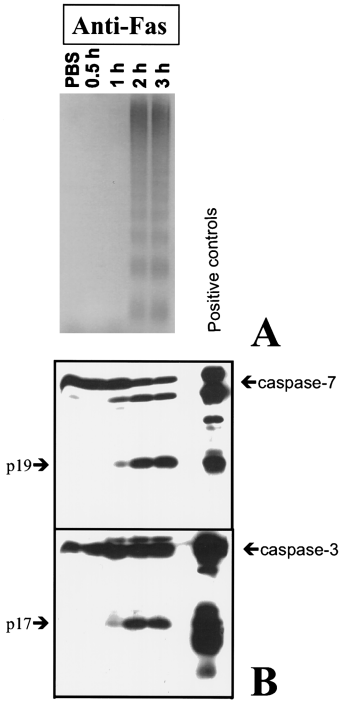

FIGURE 3. DNA laddering and activation of caspases occurs from 2 h after anti-Fas challenge. Mice were injected i.v. with 15 μg anti-Fas. Then, 0.5, 1, 2, or 3 h after challenge, livers were removed and homogenized. *A*, Agarose gel (1.8%) from DNA extracted from liver homogenates after ethidium bromide staining. *B*, Western blot of liver homogenates for caspase-3 and -7. Positive controls are caspase-3- and caspase-7-transfected HEK cells. Samples represent pooled homogenates of two mice. Shown is a representative example of at least three independent experiments.

vivo as well as in vitro. However, application of e.g. TNF in therapy is currently limited due to the toxicity, which is basically the result of the proinflammatory nature of TNF (29). It has been demonstrated that TNF, but also Abs triggering CD95/Fas, cause extreme toxicity in the liver (2–6). TNF alone is not hepatotoxic (our unpublished observations), but requires the presence of a liver-specific or general transcription-blocking agent (GalN and ActD, respectively). GalN is a liver-specific transcription-blocking agent, which depletes hepatocytes from UTP, UDP, and UMP by interfering in the galactose pathways (15). Moreover, it was suggested that toxicity in the TNF/GalN, TNF/ActD, and anti-Fas model resemble viral forms of acute hepatic failure (15–18). We are interested to identify on the one hand the factors that mediate the toxicity induced in these models and on the other hand potentially inhibitory factors. We demonstrated earlier that two acute-phase proteins, α_1-AGP and α_1-AT, are able to protect in a differential fashion, in the sense that α_1-AGP protects against the lethality induced by TNF/GalN and TNF/ActD, while α_1-AT confers protection only in the former model (2, 5, 24). We had hoped to shed more light on the mechanism of induction of hepatitis and on the basis of the differential protection by 1) identifying the induction of active caspases in the hepatitis models, 2) studying the effect of the acute-phase reactants on the activation of caspases, and 3) looking at the effect of overproduction of Bcl-2 on the induction of acute hepatitis.

In the three hepatitis models, we first studied the kinetics of apoptotic events by determining the appearance of fragmented DNA in an ELISA system as well as on agarose gels and found that, as compared with the release of transaminases, apoptosis is an early event, followed by necrosis (which is also macroscopically

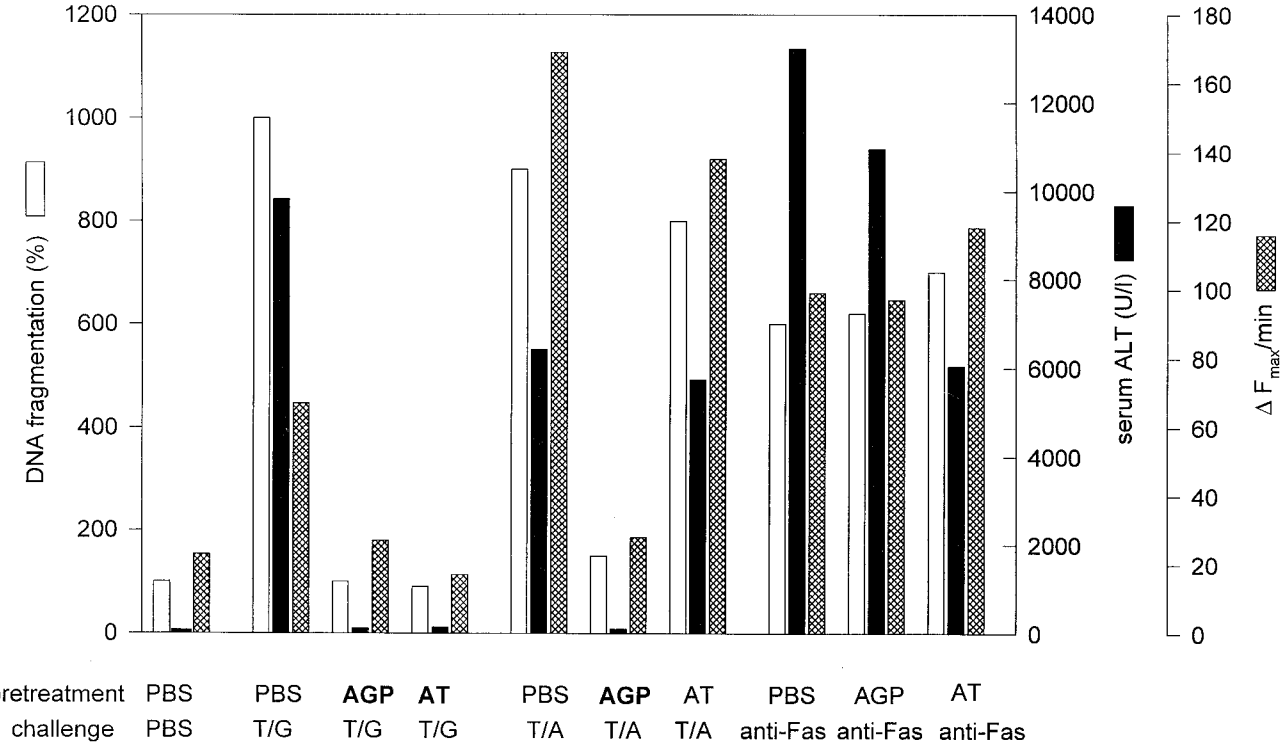

FIGURE 4. Effect of α_1-AGP and α_1-AT on TNF and anti-Fas-induced apoptosis, ALT release, and caspase activation. Mice were pretreated i.p. with PBS, 5 mg α_1-AGP, or 1 mg α_1-AT 2 h before a challenge with 0.5 μg TNF/20 mg GalN (i.p.), 0.3 μg TNF/20 μg ActD (i.p.), or 10 μg anti-Fas (i.v.). Then, 3 h (anti-Fas) or 6 h (TNF/GalN and TNF/ActD) after the challenge, blood was withdrawn and livers were excised. Open bars represent DNA fragmentation, closed bars represent ALT levels, and hatched bars represent Ac-DEVD-amc cleavage activity. Samples represent pooled homogenates of two mice. Shown is a representative example of at least three independent experiments.

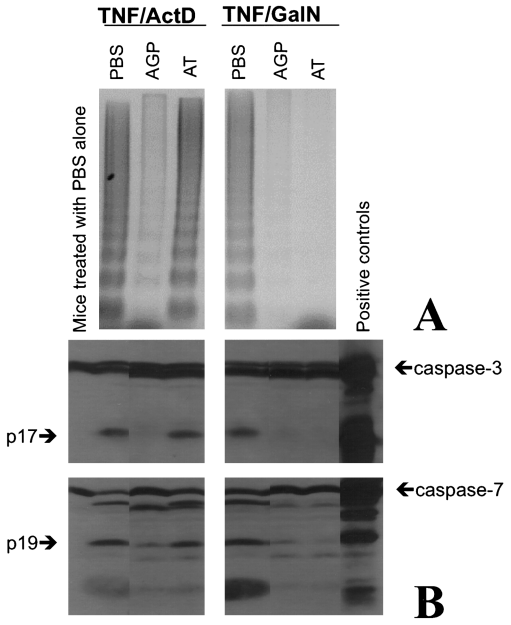

FIGURE 5. Pretreatment with α_1-AGP or α_1-AT prevents TNF-induced DNA laddering and caspase activation (samples as in Fig. 4). *A*, Agarose gel (1.8%) from DNA extracted from liver homogenates after ethidium bromide staining. *B*, Western blot of liver homogenates for caspase-3 and -7. Positive controls are caspase-3- and caspase-7-transfected HEK cells. Samples represent pooled homogenates of two mice. Shown is a representative example of at least three independent experiments.

visible as the livers look entirely black). The anti-Fas-induced hepatitis develops more rapidly than the TNF-induced ones. We also found that active executioner caspases-3 and -7 appear in close time relation with the appearance of ladder patterns and fragmented DNA. Clearly the strongest signals were found after anti-Fas compared with TNF/ActD and TNF/GalN, which, together with the kinetics of events, illustrates the aggressive nature of the anti-Fas model. In this latter model, similar observations were reported recently (30). We could find no active forms of other important caspases, like caspase-1, -6, or -8, perhaps because their concentrations are below detection limits. From these studies, we conclude that no fundamental differences could be found between the three models of cytokine-induced hepatitis, as far as caspases-3 and -7 are concerned, except on the level of kinetics and quantity.

We then looked at the effects of the protective acute-phase proteins, α_1-AGP and α_1-AT, in the different models. We describe here that all apoptotic events (DNA laddering, DNA fragmentation in ELISA, AST release, and activation of caspase-3 and -7) are prevented by the acute-phase reactants in those cases where protection against lethality is observed. These data are remarkable not only because they demonstrate a nice correlation between apoptosis and subsequent lethality, but also because here we see that acute-phase protein are able to prevent the activation of caspases by certain apoptotic triggers. Acute-phase proteins are essential components of the acute-phase response, a fundamental reaction during stress, trauma, and disease (reviewed in Ref. 31). In other words, during the acute-phase response, factors are produced that have an inherent capacity of blocking apoptosis, a process that may be active at certain sites of inflammation, e.g., during hepatitis, inflammatory bowel disease, etc.

Although the protection by acute-phase proteins against apoptosis per se is very interesting, we are still left with the question why this prevention is seen only in certain models and not in others. In the anti-Fas-triggered model, for instance, we were unable to see any effect of these protective proteins. We believe that the differential effect of the acute-phase proteins reflects a different mechanism of induction of apoptosis (although the executioner last-stage caspase-3 and -7 are present in all three models). We believe that the results obtained with the Bcl-2 transgenic mice illustrate this hypothesis: we described the interesting observation that liver-specific overexpression of Bcl-2 confers complete protection against anti-Fas-induced lethality, as already seen by one of us (27) and another group (28). Others were not able to find protective effects of Bcl-2 against anti-Fas, but this was in a cell-free system

Table II. *Bcl-2 transgenic mice are partially protected from a lethal challenge with TNF/GalN and fully protected from a lethal challenge with anti-Fas*

Challenge[a]	Lethality (24 h)	
	Control mice	Transgenic mice
0.1 μg TNF/20 mg GalN	0/5	0/5
0.3 μg TNF/20 mg GalN	4/5	0/5*
0.5 μg TNF/20 mg GalN	5/5	5/5
0.1 μg TNF/20 μg ActD	4/5	3/5
0.3 μg TNF/20 μg ActD	5/5	5/5
0.5 μg TNF/20 μg ActD	5/5	5/5
10 μg anti-Fas	2/5	0/5
15 μg anti-Fas	5/5	0/5†
20 μg anti-Fas	ND	0/5

[a] TNF/GalN and TNF/ActD were administered i.p., anti-Fas i.v. All doses were adjusted to 20 g of body weight.
*, $p = 0.0476$ for transgenic vs wild-type mice.
†, $p = 0.0079$ for transgenic vs wild-type mice.

Table III. *DNA fragmentation, release of ALT, and drop in body temperature induced by TNF/GalN and anti-Fas are prevented in Bcl-2 transgenic mice*

Challenge[a]	Mice	DNA Fragmentation (%)[b]	Serum ALT (U/L)[b]	Body Temperature (°C)[b]
PBS	Bcl-2 controls	100 ± 32	60 ± 20	36.9 ± 0.5
	Bcl-2 transgenics	100 ± 8	57 ± 21	36.9 ± 0.5
TNF/GalN	Bcl-2 controls	938 ± 225	4004 ± 1255	28.9 ± 2.1
	Bcl-2 transgenics	106 ± 14*	169 ± 24*	36.9 ± 0.5*
TNF/ActD	Bcl-2 controls	533 ± 46	6534 ± 1495	24.7 ± 0.8
	Bcl-2 transgenics	495 ± 83	7953 ± 5661	24.3 ± 2.2
Anti-Fas	Bcl-2 controls	988 ± 111	3432 ± 1186	25.1 ± 4.0
	Bcl-2 transgenics	154 ± 22*	182 ± 22*	36.8 ± 0.2*

[a] TNF/GalN and TNF/ActD were administered i.p., and anti-Fas was administered i.v. ($n = 5$ in all groups). All doses were adjusted to 20 g of body weight.
[b] Data are 4 h (anti-Fas), 5 h (TNF/ActD), or 6 h (TNF/GalN) after the challenge.
*, $p < 0.05$ for transgenic mice vs controls.

(32) or in an in vitro test using T cell hybridoma cells (33). We found also partial protection against TNF/GalN and no protection at all against TNF/ActD. These data may be an in vivo situation where two or perhaps three different pathways for induction of apoptosis are followed, all culminating in active caspase-3 and -7 and in lethality, but not all inhibitable by Bcl-2. The fact that different types of induction of apoptosis exist was recently shown by Scaffidi et al. (10). In analogy with their work, we could interpret our results as follows: 1) anti-Fas induces lethal apoptosis by a type 2 apoptosis, completely inhibitable by Bcl-2; 2) TNF/ActD works by a type1 apoptosis, not at all inhibitable by Bcl-2; 3) TNF/GalN uses a third pathway, perhaps consisting of elements of the former two types. Unfortunately, no further experimental data can be collected to underbuild this hypothesis. However, one should be cautious in interpreting data on Bcl-2-mediated inhibition of anti-Fas-induced apoptosis. There are indeed reports, using Bcl-2-transfected cell lines and hepatocytes in Bcl-2-transgenic mice, that describe protection by Bcl-2 against anti-Fas-induced apoptosis (27, 34–36); in contrast, data have been published showing lack of protection by Bcl-2 against anti-Fas-induced apoptosis (33, 37–39).

Additional differences between the three models of hepatitis come from unpublished work (C. Libert) showing that in the TNF/GalN model inflammation is present in all 18 different tissues sampled, while in the TNF/ActD model inflammation was observed in the lung and in the anti-Fas model only apoptosis was observed (in the liver, but also slightly in the lung). Because acute-phase proteins such as α_1-AGP are suspected to have antiinflammatory properties, we believe that in the TNF/GalN model apoptosis is prevented because inflammatory molecules are inhibited, some of which contribute to the induction of apoptosis.

The mechanisms by which α_1-AGP and α_1-AT prevent caspase activation are unclear. We have reported earlier that α_1-AGP and α_1-AT do not block TNF-induced gene expression in hepatocytes (5). Direct inhibition of caspases upstream of procaspase-3 and procaspase-7 is also excluded because α_1-AGP and α_1-AT do not contain any caspase-inhibitory activity on the proteolytic activity of recombinant caspases and in TNF cytotoxicity assays (our unpublished observations). Up-regulation of Bcl-2 or Bcl-x_L by α_1-AGP and/or α_1-AT, is very unlikely, because α_1-AGP nor α_1-AT protect in the anti-Fas model, while Bcl-2 does (our unpublished observations). In the TNF/GalN model, we have demonstrated the involvement of several mediators, such as PAF and Tx-A2 (7). The induced apoptosis of hepatocytes could be the result of a combined action of TNF, GalN, and TNF-induced mediators. The mechanism of protection conferred by α_1-AGP and α_1-AT might be the prevention of activity or release of these mediators. Anti-Fas-induced apoptosis results from direct binding on the receptor, without involvement of sensitizing mediators and therefore no targets for α_1-AGP or α_1-AT. The TNF/ActD model appears to behave intermediately and to involve a mediator that can be inhibited by α_1-AGP. The inhibition of apoptosis by α_1-AGP and α_1-AT appears to be an early inhibition of apoptotic mediators, and in that respect is distinct from late interventions, such as the inhibition of apoptotic events by Hsp70, which occurs even in the presence of active caspase-3 (40). However, the fact that acute-phase proteins are able to prevent apoptosis in vivo, whatever the mechanism, is interesting in view of their potential use as therapeutic agents in disorders involving unwanted apoptosis. The acute-phase proteins might also constitute an inducible anti-apoptotic, protective system that in pathology or disturbed homeostasis prevents excessive apoptosis.

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