Transforming growth factor α protects against Fas-mediated liver apoptosis in mice

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Abstract The Fas/Fas ligand interaction plays a crucial role in various liver diseases, and administration of agonistic anti-Fas antibody to mice causes massive hepatic apoptosis and fulminant hepatic failure. Several growth factors have recently been found to function in preventing apoptosis. In this study, we demonstrated that overexpression of transforming growth factor α (TGFα) has a dramatic protective effect on Fas-mediated hepatic apoptosis at the biochemical and histological levels. Moreover, 85.7% (six out of seven) of TGFα transgenic mice survived the lethal liver damage, whereas all wild-type mice died. Expression of Bcl-xL, an anti-apoptotic protein, was greatly increased in the transgenic mice. Taken together, our findings suggest that TGFα protects against Fas-mediated liver apoptosis in vivo and up-regulation of Bcl-xL may participate in protective effect of TGFα.

Key words: Fas/APO-1/CD95; Apoptosis; Transforming growth factor α; Bcl-xL

1. Introduction

Apoptosis, programmed cell death, is the most common form of eukaryocytic cell death [1] and plays an important role during embryonic development and in adult tissues. Disruption of apoptosis, however, can contribute to illegitimate cell survival and cause tumors to develop [2].

Fas/APO-1/CD95 is a 45 kDa glycoprotein and a member of the tumor necrosis factor (TNF)/nerve growth factor (NGF) receptor family [3]. Fas ligand, which is predominantly expressed in activated T cells, binds to Fas and can transmit the signals for cell death to target cells, resulting in the induction of apoptosis [3,4]. It is now clear that the Fas/Fas ligand interaction is related to a number of diseases, including viral hepatitis, alcoholic liver disease, and hepatocellular carcinoma [5–13]. Constitutive expression of Fas has been observed in normal mouse and human hepatocytes [14], and treatment with agonistic Fas antibody results in massive liver apoptosis and fulminant liver failure in mice [15–17].

Transforming growth factor alpha (TGFα) is structurally and functionally related to members of the epidermal growth factor (EGF) family that bind to EGF receptor [18], and it is a potent mitogen for epithelial cells in many organs. TGFα promotes wound healing [19] and plays a pivotal role in liver regeneration after partial hepatectomy [20,21]. Numerous human solid tumors, including hepatocellular carcinoma, express high levels of TGFα [22–25], and we previously reported that TGFα transgenic mice developed liver neoplasia [26,27].

Some growth factors, such as hepatocyte growth factor (HGF) and NGF, have been found to be capable of protecting cell against apoptosis [28–30]. Recent studies have shown that TGFα and EGF also relate to suppress apoptosis induced by various stimuli in vitro [31–35]. The mechanism of this effect in vivo, however, remains poorly understood. To clarify the relationship between TGFα and apoptosis in vivo, TGFα transgenic mice were used to analyze the protective effect of TGFα against liver apoptosis, particularly Fas-mediated apoptosis in this study.

2. Materials and methods

2.1. Mice

Transgenic mouse line MT100 bearing the metallothionein–TGFα fusion gene was created as described previously [36]. MT100 mice have expressed human TGFα transgene under the control of mouse metallothionein 1 promoter on a FVB mouse background. In this study we used 6- to 8-week-old male MT100 mice (TG) and non-transgenic littermates (WT). All animal studies were performed according to the guidelines for animal care and use established by Gunma University School of Medicine.

2.2. Experimental protocol and histological examination

Mice were injected intraperitoneally with 0.15 mg/kg of a mouse monoclonal agonistic Fas antibody (Jo2, PharMingen, CA, USA). At specified time points, mice were sacrificed and their serum and organs were collected. Serum alanine aminotransferase (ALT) levels were measured at each time point, and liver samples were fixed in 10% formalin, embedded in paraffin and stained with hematoxylin and eosin. For hepatic caspase 3-like activity assay, 0.3 mg/kg of agonistic Fas antibody was administered intraperitoneally and mice were sacrificed 5 h later. To assess survival times, mice were injected intraperitoneally with 0.3 mg/kg of Jo2 antibody and observed for survival up to 72 h.

2.3. In situ analysis of liver apoptosis by terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate biotin nick end labeling (TUNEL) assay

TUNEL assay was performed to detect apoptotic cells. Briefly, paraffin-embedded sections were dewaxed in xylene and rehydrated

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Abbreviations: TGFα, transforming growth factor α; EGF, epidermal growth factor; TNF, tumor necrosis factor; NGF, nerve growth factor; HGF, hepatocyte growth factor; ALT, alanine aminotransferase; TUNEL, terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate biotin nick end labeling; PI3-k, phosphatidylinositol 3′-kinase

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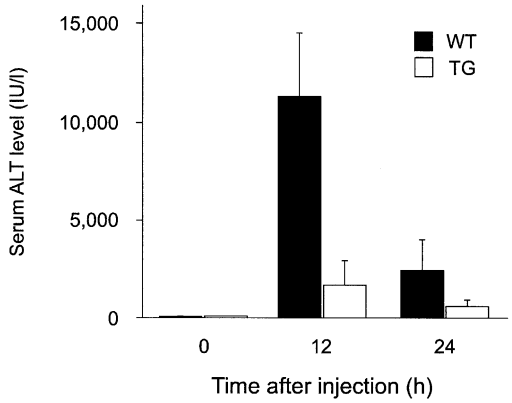

Fig. 1. Serum ALT levels following anti-Fas antibody injection. The TG mice had significantly lower serum ALT levels after Fas-mediated liver injury than the WT mice ($n = 5$ per group). $P = 0.0178$.

by passage through a graded series of ethanol solutions, ending with PBS. Sections were permeabilized with proteinase K. After washing, the 3′-OH ends of DNA fragments were stained by the method described by the manufacturer (In situ Apoptosis Detection kit; ApopTag Direct, Oncor, Gaithersburg, Germany). Sections were viewed and photographed by standard fluorescence microscopy techniques. TUNEL positive cells per ×200 high power field were counted.

2.4. Hepatic caspase 3-like protease activity assay

Liver tissues were homogenized and the lysate was centrifuged at 15 000 rpm for 15 min. Supernatant proteins were incubated at 37°C with caspase 3 substrates, Ac-DEVD-pNA (Medical and Biological Laboratories, Nagoya, Japan) and the liberated p-NA was measured at 405 nm. Non-specific reaction was corrected by subtracting the background readings from the combination of the lysates and buffers.

2.5. Western blotting analysis

Total liver protein from the mice was homogenized in RIPA buffer containing phenylmethylsulfonyl fluoride, pepstatin, leupeptin, and aprotinin (Roche Diagnostics GmbH, Mannheim, Germany). The supernatants were used for Western blotting analysis. Total protein (20 μg) was separated on a SDS–PAGE gel, transferred to polyvinylidene difluoride membranes, and blocked in 5% skim milk in Tris-buffered saline containing 0.1% Tween 20 before incubation with antibody (anti-mouse Bcl-xL antibody; a kind gift from Dr. Y. Tsujimoto, Osaka University, anti-mouse β-actin and Bax; Santa Cruz Biotechnology, CA, USA, anti-mouse Bcl-2 and Bad; BD Transduction Laboratories, NJ, USA). Enhanced chemiluminescense (Amersham Pharmacia, UK) was used to detect apoptosis.

2.6. Statistical analysis

Statistical analysis was performed by two-way analysis of variance for serum ALT levels and hepatic caspase 3-like protease activity, by unpaired *t*-test for the number of TUNEL positive cells. All values are expressed as means ± S.E.M. Survival rate was assessed by the Kaplan–Meier method, and differences in curves were tested by the log rank test. $P < 0.05$ was accepted as statistically significant.

3. Results

3.1. Changes in serum ALT levels following anti-Fas antibody injection

As shown in Fig. 1, the mean serum ALT levels of the WT mice at 0, 12 and 24 h were 50 ± 3, 11 292 ± 3286, 2446 ± 1554 IU/l, respectively, and those of the TG mice were much lower: 48 ± 9, 1647 ± 1308 and 550 ± 342 IU/l, respectively ($P = 0.0178$). It was particularly striking that the serum ALT levels of TG mice at 12 h were suppressed to approximately one-seventh their level of the WT mice.

3.2. Histopathological examination after anti-Fas antibody injection

Histological examination revealed that injection with anti-Fas antibody induced more massive hepatocyte apoptosis

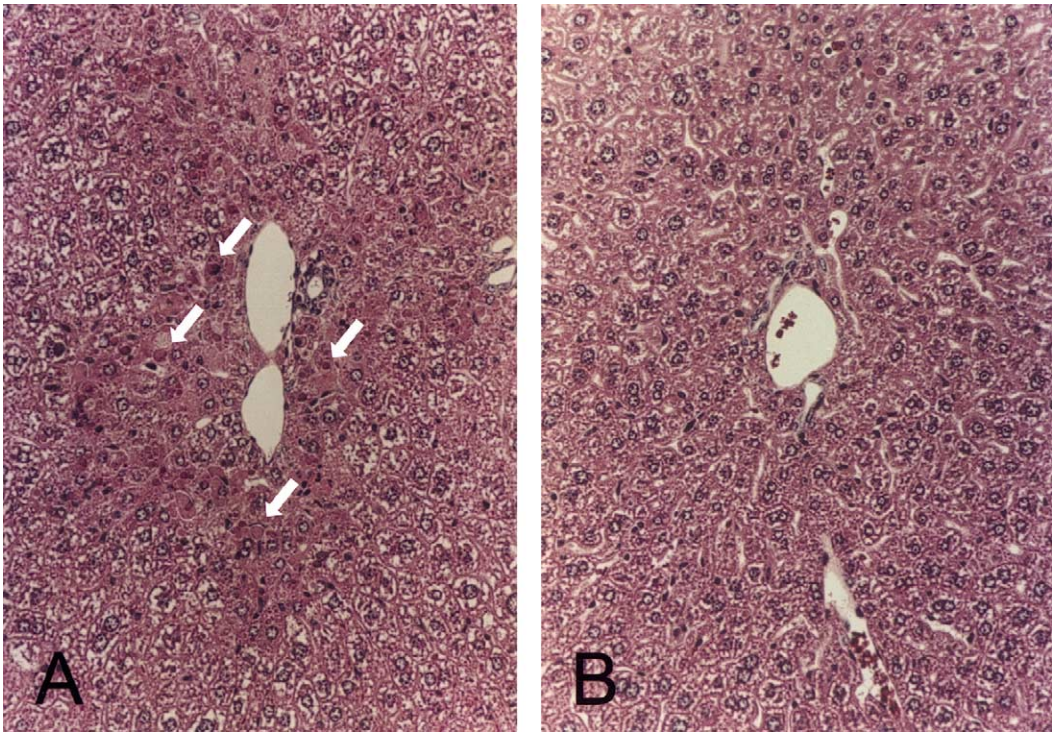

Fig. 2. Histological findings in the liver 12 h after anti-Fas antibody injection. A: Liver from WT mouse. B: Liver from TG mouse. Arrows point to typical apoptosis of hepatocytes: acidophilic bodies. The liver apoptosis was much less severe in the TG mouse than in the WT mouse. (HE staining; original magnification ×200)

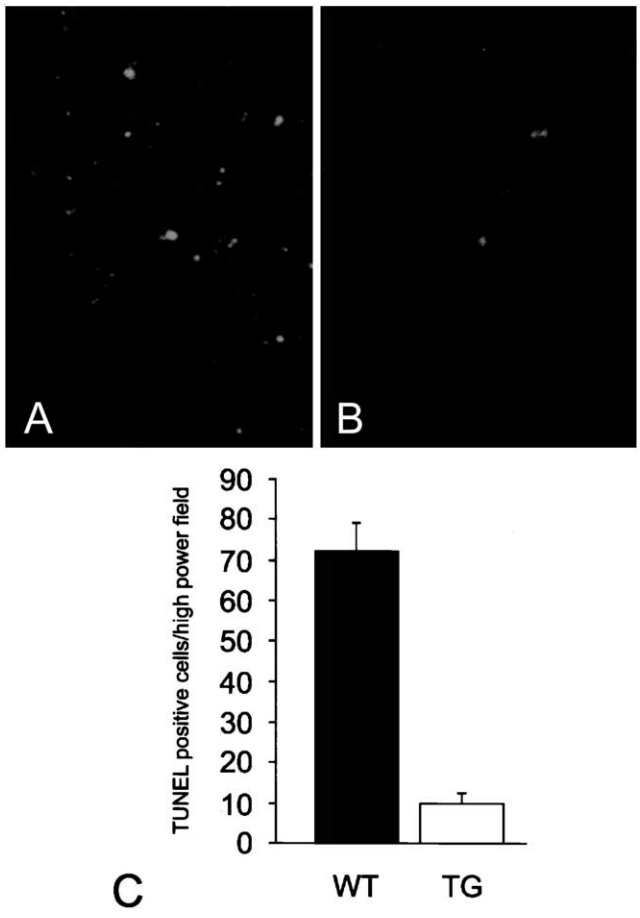

Fig. 3. TUNEL staining of a WT and TG mouse after injection of anti-Fas antibody. Section of WT (A) and TG (B) mouse liver taken 12 h after injection. Many TUNEL positive cells were detected in the WT mouse, but a few TUNEL positive cells were seen in the TG mouse (original magnification ×200). TUNEL positive cells per ×200 high power field were counted (C). TUNEL positive cells were significantly decreased in TG mice. $P < 0.001$.

around the portal area in the WT mice (Fig. 2A) than in the TG mice (Fig. 2B). Numerous acidophilic bodies resulting from Fas-mediated hepatocyte apoptosis were observed in the WT mice. The livers of the WT mice (Fig. 3A) and the TG mice (Fig. 3B) mice were analyzed by TUNEL staining to further assess the induction of apoptosis in the liver following anti-Fas injection. More than 70 TUNEL positive cells/high power field on average were found in WT mice. In contrast, only 10 TUNEL positive cells/high power field were found in TG mice. TG mice suppressed Fas-mediated liver apoptosis in compared with WT mice (Fig. 3C; $P < 0.001$).

3.3. Hepatic caspase 3-like protease activity after anti-Fas antibody injection

Caspase 3-like protease activity was assessed by cleavage of the peptide substrate Ac-DEVD-pNA in the liver homogenates. As shown in Fig. 4, this activity was almost equal between WT and TG mice before injection and there was a four-fold increase in WT mice 5 h after injection. However, this activity in TG mice 5 h after injection was significantly decreased relative to WT mice at same time ($P = 0.0043$).

3.4. Cumulative survival after Fas-induced lethal liver injury

Severe liver failure occurred rapidly after injection of a

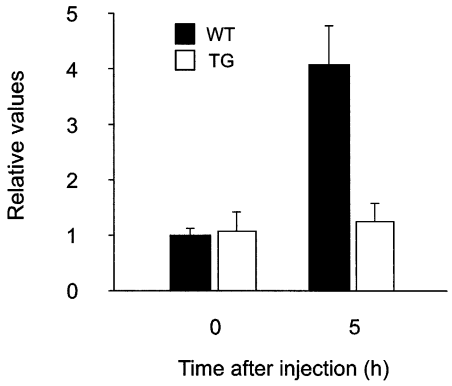

Fig. 4. Hepatic caspase 3-like protease activity after injection of anti-Fas antibody. Caspase 3-like protease activities were measured 0 and 5 h after injection of 0.3 mg/kg anti-Fas antibody ($n = 4$ per group). All values were normalized to WT mice at 0 h (set at 1.0). There was a four-fold increase of caspase 3-like protease activity in the liver of WT mice 5 h after injection. In contrast, this activity in the TG mice 5 h after injection was significantly suppressed. $P = 0.0043$

lethal dose (0.3 mg/kg) of anti-Fas antibody injection, and all of the WT mice were dead within 28 h. By contrast, six out of seven TG mice (85.7%) survived the lethal challenge (Fig. 5), showing that the lethal liver failure following anti-Fas antibody injection was significantly inhibited in the TG mice compared to the WT mice ($P = 0.0029$).

3.5. Changes in anti-apoptotic and pro-apoptotic proteins in mouse liver

To assess the expression of the Bcl-2 family apoptosis-related protein, Western blotting analysis was performed for Bcl-2, Bcl-xL, Bax, and Bad (Fig. 6). Two of the members of the Bcl-2 family, Bcl-2 and Bcl-xL proteins, act as anti-apoptotic agents, while Bax and Bad act as pro-apoptotic agents. The expression of the pro-apoptotic proteins, Bax and Bad, remained unchanged in the TG mice, and Bcl-2 protein expression was too weak to detect in either the WT or TG mice. Expression of the anti-apoptotic protein Bcl-xL, however, was markedly higher in the TG mice both before injection (TG 0 h) and 12 h after injection (TG 12 h).

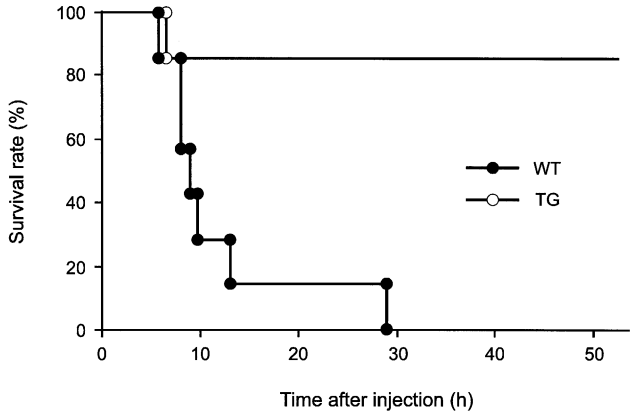

Fig. 5. Survival rate of TG and WT mice after administration of anti-Fas antibody, 0.3 mg/kg body weight. The TG mice show a drastically improved survival rate ($n = 7$ per group). $P = 0.0029$.

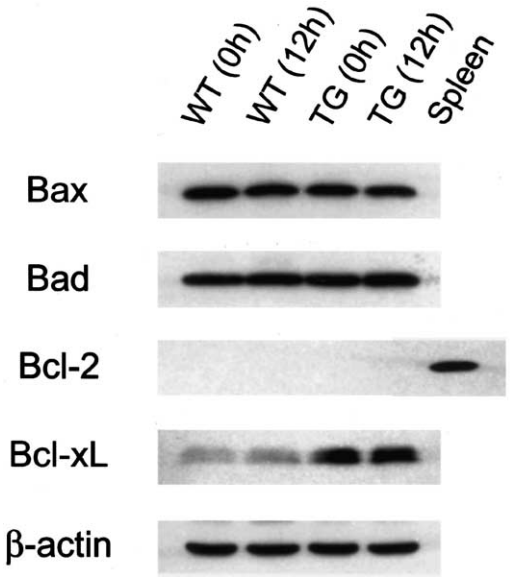

Fig. 6. Western blotting analysis of Bcl-2 family protein expression. Expression of Bcl-xL, a member of the anti-apoptotic Bcl-2 family proteins, was greatly increased in the TG mice each time measured after anti-Fas antibody injection. Expression of Bcl-2 was not prominent, and the spleen of untreated WT mice was used as positive control for Bcl-2. By contrast, expression of the pro-apoptotic Bcl-2 family proteins, Bax and Bad, was unaltered in either genotype.

4. Discussion

We demonstrated a protective effect of TGFα on liver apoptosis induced by the Fas pathway in vivo. Consistent with the results of vitro studies [31–34], TGFα protects against c-myc and TNFα-induced apoptosis in some cell types, although it was first identified as a potent mitogen resembling EGF [24]. EGF protects epithelial cells [35] and primary cultured hepatocytes [37] from Fas-induced apoptosis in vitro. Its protective effects were concentration-dependent [32] and greater overexpression of TGFα might completely suppress apoptosis in vivo.

It has recently been concluded that a variety of key events in apoptosis converge on mitochondria and that the release of cytochrome *c* from mitochondria triggers cell apoptosis [38]. The anti-apoptotic Bcl-2 family protein Bcl-xL is located on the outer mitochondrial membrane and inhibits the release of cytochrome *c*, resulting in maintenance of cell survival [39–41]. Boise et al. reported observing that Bcl-xL inhibits apoptosis in Jurkat T cells treated with Fas antibodies and maintains cell viability by preventing the loss of mitochondrial membrane potential [42]. We found that TGFα transgenic mice constitutively express more Bcl-xL protein than wild-type mice. Analysis of the Bcl-xL transgenic mice revealed that Bcl-xL could inhibit both Fas- and TNFα-induced hepatocyte apoptosis [43]. Accordingly, up-regulation of Bcl-xL may partially take part in suppressing liver apoptosis driven by Fas antibody injection.

Bcl-2 can also prevent liver apoptosis [44,45], but expression of Bcl-2 was so much lower than that of other Bcl-2 family proteins that it could not be detected on Western blots. The pattern of Bcl-2 expression in the transgenic mice was similar to that in WT mice. Bax and Bad, on the other hand, can promote cell death to regulate the release of cytochrome *c* [41,46], and Rb protein overexpression has been documented to down-regulate Bax protein and protect against Fas-mediated liver apoptosis in mice [47]. In our study, Bcl-2, Bax, and Bad expression were relatively constant, compared with Bcl-xL, in both the wild-type mice and the transgenic mice. Since the balance between anti-apoptotic and pro-apoptotic Bcl-2 family proteins is important for the regulation of apoptosis, the increased expression of Bcl-xL but similar expression of other Bcl-2 family proteins can cooperate to protection against liver apoptosis.

EGF receptor signaling controls the expression of Bcl-xL in keratinocytes [48,49], mammary epithelial cells [31], squamous cell carcinoma cells in the head and neck [50], and hepatocytes [37] in vitro. Activation of EGF receptor triggers transmission of signals involved in mitogen-activated protein kinases (MAPK), phosphatidylinositol 3′-kinase (PI3-k)/Akt, and the signal transducers and activators of transcription-3 pathway [35,49,51]. In hepatocytes, EGF signaling can up-regulate Bcl-xL expression via the MAPK and PI3-k/Akt pathways [37]. Thus, TGFα may control the expression of Bcl-xL protein through these pathways in this mouse model. HGF has been reported to prevent Fas-mediated liver apoptosis [29], and, the same as TGFα, HGF strongly induces expression of Bcl-xL but not of Bcl-2, thereby protecting against hepatocyte apoptosis in vivo. However, there is a difference in intracellular signaling pathways, with the PI3-k/Akt pathway having been reported to mainly contribute to Bcl-xL expression in hepatocytes stimulated by HGF [52].

In addition, accumulating evidence has shown that the mouse model of Fas-mediated liver injury may involve a mechanism similar to that in the fulminant hepatic failure caused by hepatitis virus infection [5,15,53]. Tomiya et al. reported that serum TGFα levels are correlated with the outcome of fulminant hepatic failure [54]. We have documented that TGFα transgenic mice dramatically survived Fas-mediated lethal hepatic failure. These findings seem to support the ability of TGFα to protect against fulminant failure. Administration of TGFα may be beneficial as a new option for treatment of fulminant liver failure. The clinical use of TGFα, however, needs further investigation because of its initiation and promotion of hepatocarcinogenesis.

In conclusion, the results of our study confirm that TGFα efficiently protects against Fas-mediated liver injury in vivo and suggest that elevated expression of Bcl-xL protein in response to TGFα may participate in suppressing liver apoptosis.

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