Switch from Type II to I Fas/CD95 Death Signaling on *In Vitro* Culturing of Primary Hepatocytes

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Fas/CD95-induced apoptosis of hepatocytes *in vivo* proceeds through the so-called type II pathway, requiring the proapoptotic BH3-only Bcl-2 family member Bid for mitochondrial death signaling. Consequently, Bid-deficient mice are protected from anti-Fas antibody injection induced fatal hepatitis. We report the unexpected finding that freshly isolated mouse hepatocytes, cultured on collagen or Matrigel, become independent of Bid for Fas-induced apoptosis, thereby switching death signaling from type II to type I. In such *in vitro* cultures, Fas ligand (FasL) activates caspase-3 without Bid cleavage, Bax/Bak activation or cytochrome c release, and neither Bid ablation nor Bcl-2 overexpression is protective. The type II to type I switch depends on extracellular matrix adhesion, as primary hepatocytes in suspension die in a Bid-dependent manner. Moreover, the switch is specific for FasL-induced apoptosis as collagen-plated Bid-deficient hepatocytes are protected from tumor necrosis factor alpha/actinomycin D (TNFα/ActD)-induced apoptosis. *Conclusion:* Our data suggest a selective crosstalk between extracellular matrix and Fas-mediated signaling that favors mitochondria-independent type I apoptosis induction. (HEPATOLOGY 2008;48:1942-1953.)

The death ligand FasL/CD95L is a member of the tumor necrosis factor (TNF) cytokine family that plays a crucial role in regulating cell death for controlling T-cell as well as B-cell homeostasis, the cytolysis of virally infected cells and the resolution of immune responses.[1] The highest constitutive expression of Fas, the receptor for Fas ligand (FasL), is found in hepatocytes.[2,3] Mice injected with a lethal dose of the agonistic monoclonal anti-Fas antibody (Jo2) exhibit massive apoptosis of liver cells and die within a few hours from fulminant hepatitis.[3-5] In addition, liver cells are thought to die, at least in part, through Fas-mediated apoptosis during viral and autoimmune hepatitis, alcoholic liver disease as well as endotoxin- or ischemia/reperfusion-induced liver damage.[2,6,7] Perplexingly, Fas is also critical for liver regeneration after partial hepatectomy[8] when liver damage

Abbreviations: ActD, actinomycin D; DISC, death-inducing signaling complex; FADD, Fas-associated protein with death domain; FAK, focal adhesion kinase; FasL, Fas ligand; FLIP, c-FLICE-inhibitory protein; GFP, green fluorescent protein; HGF, hepatocyte growth factor; ILK, integrin-linked kinase; JNK, c-Jun N-terminal kinase; MEF, mouse embryo fibroblast; TNF, tumor necrosis factor; WME, Williams medium E; XIAP, X-linked inhibitor of apoptosis protein.

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activates anti-apoptotic signaling pathways that switch Fas-mediated signals from primarily apoptotic to hepatocyte growth inducing ones.[9] These findings indicate that, as for the related TNFα/TNF-R1 cytokine/receptor signaling pathway, the outcome of FasL/Fas-induced signaling is the result of a crosstalk between different signaling pathways dependent on cell type, cell state, and environmental factors.[9,10]

After binding, FasL causes Fas receptor multimerization and then recruitment of the adaptor Fas-associated protein with death domain (FADD)/MORT1 and the pro-form of caspase-8 to the cytoplasmic death domain of the receptor, leading to formation of the so-called death-inducing signaling complex (DISC).[11] The dimerization of caspase-8 at the DISC provokes its activation through conformational change, which is followed by autoproteolytic processing and release into the cytosol. The ensuing apoptotic program can kill cells by way of two different pathways, type I or type II.[12] In type I cells, caspase-8 directly processes and activates the effector caspase-3 and caspase-7, and this suffices to execute cell demolition. In type II cells, apoptosis induction also requires that caspase-8 cleaves the pro-apoptotic BH3-only Bcl-2 family member Bid, forming tBid, which translocates to mitochondria to activate the multi-BH domain pro-apoptotic Bcl-2 family members Bax and Bak by so far unknown mechanisms.[4,5,13] Bax/Bak-mediated mitochondrial membrane permeabilization results in the release of cytochrome c from the intermembrane space.[14] Once in the cytosol, cytochrome c binds to Apaf-1 and thereby recruits pro-caspase-9 into an apoptosomal complex, which processes and activates effector caspase-3 and caspase-7.[15] The reasons for the differences between type I and type II cells are still unclear,[12] although differences in the expression of inhibitors of the death receptor signaling cascade, such as c-FLICE-inhibitory protein (FLIP) or X-linked inhibitor of apoptosis protein (XIAP), have been proposed to be responsible. Whereas FLIP is an inhibitory caspase-8 analog, acting at the DISC,[16] XIAP blocks the enzymatic activity of caspase-9, caspase-3, and caspase-7.[15,17] The caspase inhibitory effect of XIAP can be overcome by apoptogenic proteins, such as Smac/DIABLO and Htr2A/Omi, which are released from mitochondria together with cytochrome c.[18-20] They bind to XIAP and neutralize its caspase binding activity or target it for proteasomal degradation. It has been postulated, therefore, that cells with high levels of XIAP would require tBid/mitochondrial-mediated amplification of the caspase cascade to overcome the caspase-inhibitory function of XIAP.[11]

In vivo and *in vitro*, many Fas-sensitive cells, including lymphocytes, die by way of the type I pathway, shown by the findings that neither loss of Bid nor Bcl-2 overexpression protects these cells from Fas-induced apoptosis.[5,21] In contrast, hepatocytes require the type II amplification loop for cell killing because Bid-deficient mice are resistant to agonistic anti-Fas antibody (Jo2)-induced hepatocyte apoptosis and fatal hepatitis.[4,5]

To understand the detailed mechanisms of hepatocyte killing, regeneration, and differentiation, it is necessary to develop *in vitro* culture models. The standard protocol has been to plate hepatocytes, after collagenase assisted isolation from the liver, on collagen I or certain other extracellular matrices, such as Matrigel, and to use the cells within 2 to 3 days for cellular or biochemical analysis.[22-24] By applying this protocol, we found that Fas-induced cell death signaling is switched from a type II to I pathway.

Materials and Methods

Collagen and Matrigel Coating. Six-well plates or 12-mm glass coverslips were incubated with 1 mL of collagen I (4.5 mg/mL) (diluted 1:10 in phosphate-buffered saline) per well for 30 minutes at 37°C. Subsequently, the collagen solution was removed before seeding the cells. The coating with Matrigel Matrix was carried out as described by the manufacturer (BD Biosciences). At room temperature, Matrigel matrix polymerizes to produce biologically active matrix material, resembling the mammalian cellular basement membrane.

Isolation and Cultivation of Primary Mouse Hepatocytes. Primary mouse hepatocytes were isolated from 6-week-old to 12-week-old wild-type (wt), *bid*−/−, *bak*−/− (Jackson Laboratories), *fas*−/− (from M. Simon, Freiburg), or *xiap*−/− (from J. Silke, Melbourne) mice (all kept on a C57BL/6 genetic background) based on the collagenase perfusion technique,[24] with the following modifications. The isolated hepatocytes were plated on collagen I-coated or Matrigel-coated plates containing Williams medium E (WME) supplemented with 10% fetal bovine serum, 100 nM dexamethasone, 2 mM L-glutamine, and 1% penicillin/streptomycin solution (complete WME, Biochrom). After 4 hours of adherence, the cells were washed twice in phosphate-buffered saline and incubated overnight in complete WME but without dexamethasone in a 5% CO_2 incubator at 37°C before treatment with FasL.

Hepatocytes in suspension were used for apoptosis assays directly after isolation without plating on dishes. For this purpose, the cells were resuspended in complete WME without dexamethasone for cell counting, treated with FasL or left untreated and carefully shaken in a 5% CO_2 incubator at 37°C for different time points.

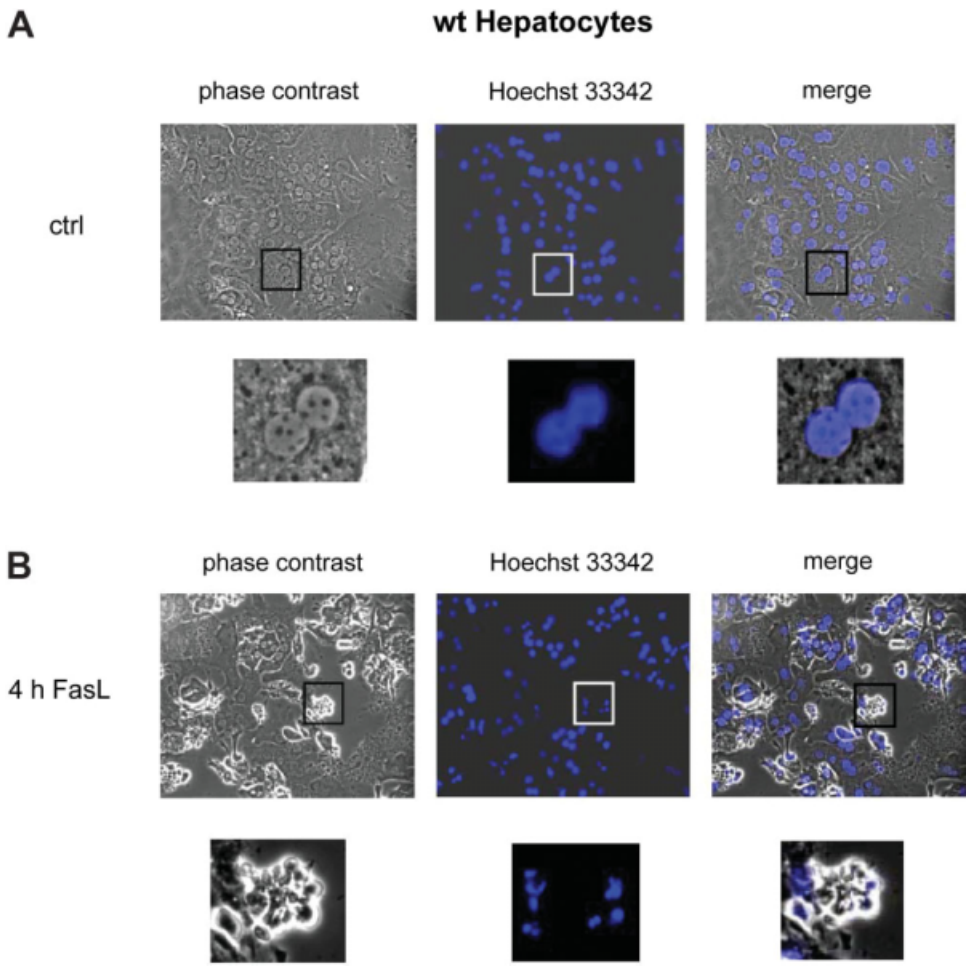


Fig. 1. Apoptotic morphology and annexin-V/PI staining of FasL-treated primary hepatocytes plated on collagen. (A–D) Phase contrast and DNA fluorescence (Hoechst 33342) microscopy of (A, C) untreated and (B, D) N2A FasL-treated (4 hours, 50 ng/ml) (A, B) wt and (C, D) *bid*−/− primary mouse hepatocytes cultured on collagen. Magnifications are 200× and 800×, respectively. (E) FACS analysis of GFP-annexin-V/PI stained wt, ***bid*−/−**, *bak*−/−, *xiap*−/− primary mouse hepatocytes and wt 3T9 MEFs treated with 50 ng/mL N2A FasL for 0 to 10 hours. The percentages of GFP-annexin-V/PI double negative cells are depicted. The values represent the means of three independent experiments ± SD.

For additional Materials and Methods, please see the online version of this article.

Results

In Contrast to* In Vivo *Conditions, FasL-Induced Caspase-3 Activation and Apoptosis of Collagen-Plated Hepatocytes Does Not Depend on Bid. We first confirmed in newly generated *bid*−/− mice[5] that anti-Fas-induced caspase-3 and caspase-7 activation and hepatocyte apoptosis in vivo required Bid and hence the mitochondrial type II pathway (Supporting Material; Supporting Fig. S1). We then isolated hepatocytes from C57BL/6 mice and plated them on collagen I. A total of 95% of these cells exhibited a binuclear morphology, typical of hepatocytes (Fig. 1A), and they survived for up to 3 days (data not shown). After 24 hours of culture the cells were treated with 50 ng/mL FasL from the supernatant of Neuro2A cells secreting a bioactive form of membrane-bound FasL[25] (quantitation and controls, Supporting Figs. S2, S3). Alternatively, the cells were exposed to recombinant Fc-FasL,[26] which produced identical results (Supporting Fig. S2). As shown in Figure 1B, FasL-treated primary hepatocytes showed typical signs of apoptosis, such as cell shrinkage, plasma membrane blebbing, and nuclear condensation and fragmentation. To quantify apoptosis, we carried out FACS analysis after staining cells with green fluorescent protein (GFP)-annexin-V plus propidium iodide (PI), counting the GFP-annexin-V/PI-double negative cells as "surviving cells." The number of surviving cells decreased gradually on FasL treatment, reaching 20% after 10 hours (Fig. 1E). Con-

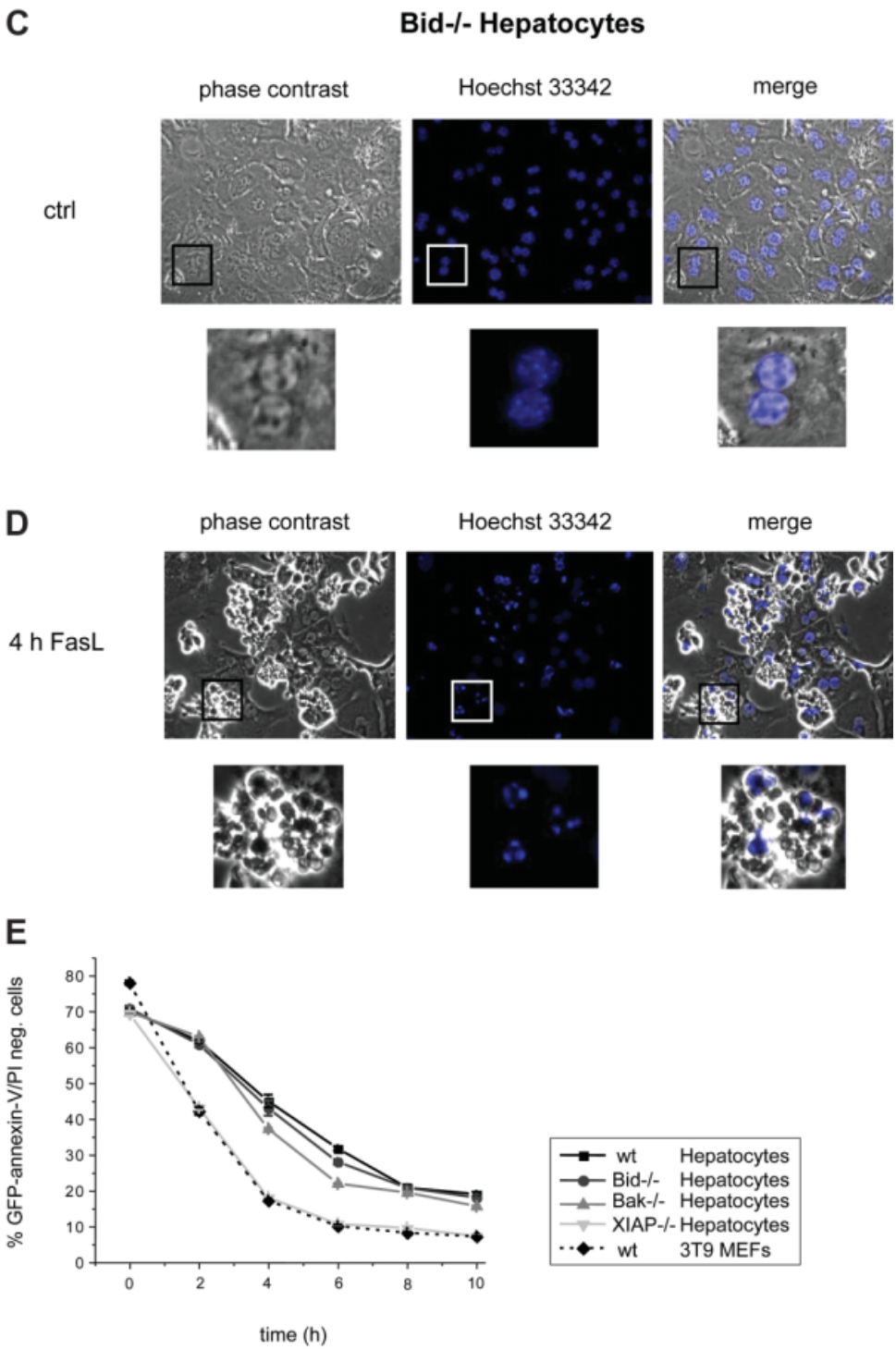


Fig. 1 (Cont'd.)

sistent with apoptosis induction, the activity of the effector caspase-3 and caspase-7 (Fig. 2A) and the processing of the p32 proform of caspase-3 to its active p20/p17 fragments (Fig. 2C) increased progressively over time. Immunofluorescence staining confirmed the intracellular activation of caspase-3 in FasL-treated hepatocytes (Fig. 3D). Unexpectedly, the kinetics of apoptosis, as assessed by morphologic examination (Fig. 1C,D), by GFP-annexin-V/PI staining (Fig. 1E) and the processing and activation of caspase-3, measured both *in vitro* (Fig. 2A,C) and inside the cells (Fig. 3E) were indistinguishable between *bid*−/− and wild-type primary hepatocytes at var-

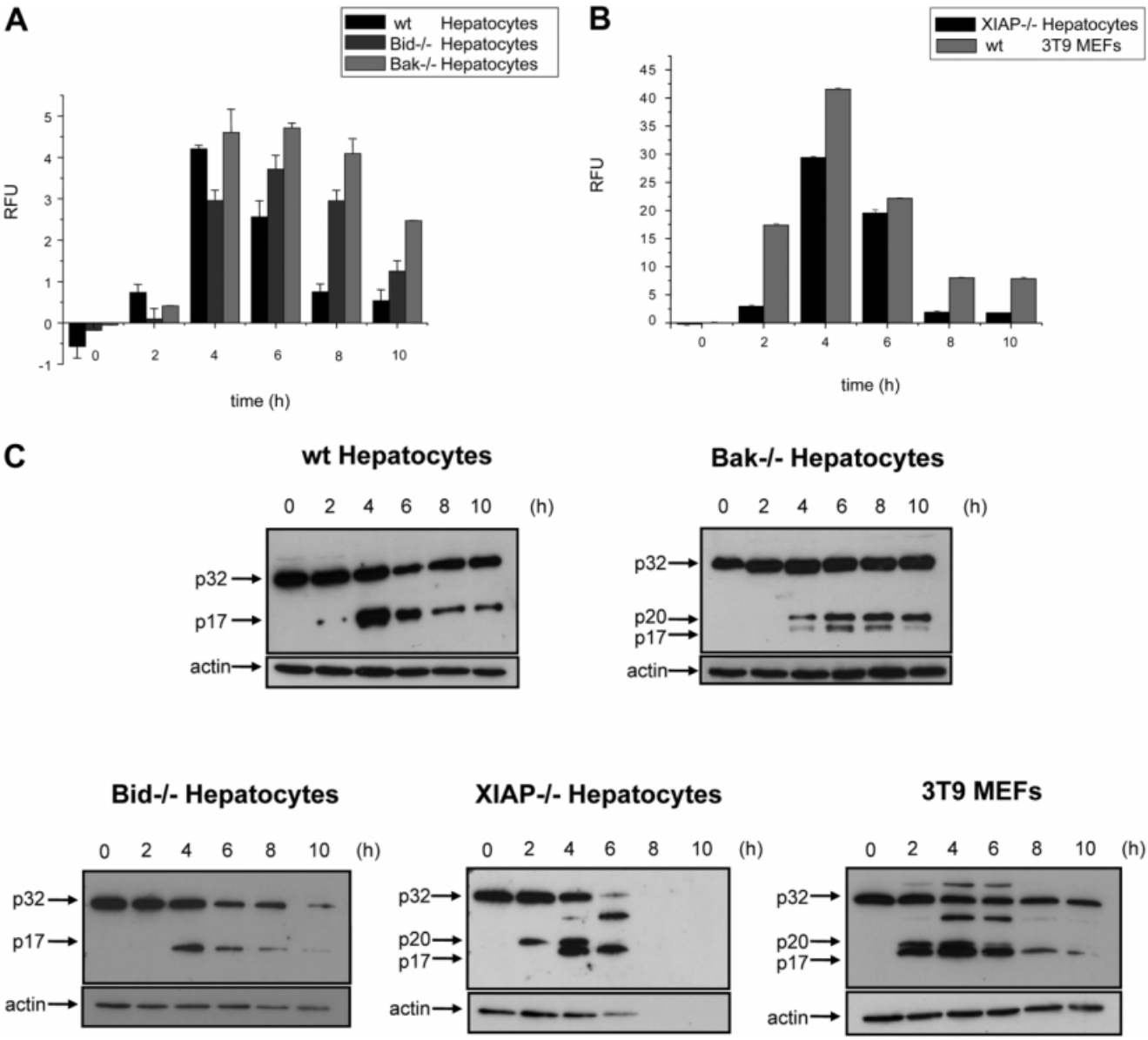


Fig. 2. Kinetics of caspase-3 processing and activation are similar between wt, *bid*−/−, and *bak*−/− hepatocytes but are accelerated in *xiap*−/− cultured hepatocytes and 3T9 MEFs. (A, B) Fluorogenic caspase-3/-7 activity assay (relative fluorescence units) of wt, Bid−/− and *bak*−/− hepatocytes (A) and *xiap*−/− hepatocytes and 3T9 MEFs (B). The graphs are shown separately because of the 10-fold difference in the caspase activities. (C) Western blot analysis for inactive p32 and active p20/p17 caspase-3 in the cytosols of the indicated cells. Probing for actin is shown as a loading control. The values in (A, B) represent the means of three independent experiments ± SD.

ious FasL concentrations (10–100 ng/mL) (Supporting Fig. S4A,B). This finding indicated that freshly isolated hepatocytes plated on collagen I switch Fas-induced death signaling from a Bid-dependent type II to a Bid-independent type I pathway.

Lack of Bid Cleavage and Activation of Mitochondrial Effectors During FasL-Induced Apoptosis of Collagen-Plated Hepatocytes. To further substantiate our findings, we carried out a detailed biochemical analysis of the components of type I and type II signaling in FasL-treated primary hepatocytes in culture. As a positive control, we used primary as well as transformed (3T9) mouse embryo fibroblasts (MEFs), which showed a typical type II signaling behavior. In these fibroblasts, FasL caused the formation and mitochondrial translocation of p15 tBid (Fig. 3A), the release of cytochrome c (Fig. 3B), and Smac (Fig. 3C) and caspase-3 activation (Fig. 2B,C). Moreover, apoptosis occurred even faster than in hepatocytes and was largely inhibited by loss of Bid (Supporting Fig. S5). In contrast, in cultured wt as well as *bid*−/− hepatocytes, neither p15 tBid formation/mitochondrial translocation (Fig. 3A)

nor the release of cytochrome c (Fig. 3B) could be detected by western blotting. Remarkably, Smac was abundant in the cytosol of hepatocytes for unknown reasons, but its level was not affected by the presence or absence of Bid or by FasL treatment (Fig. 3C). Immunofluorescence staining confirmed that, on FasL treatment, both wt and *bid*−/− hepatocytes retained cytochrome c in punctuate structures, although the overall fluorescence intensity increased due to cell shrinkage (Fig. 3D,E). Consistent with this notion, we did not observe activation of the downstream target of cytochrome c, caspase-9, in FasL-treated hepatocytes in culture (Fig. 3F). Although low amounts of the partially processed forms of caspase-9 were detected between 2 to 6 hours of treatment, this was also seen in *bid*−/− hepatocytes. By contrast, FasL-treated MEFs displayed a clear processing of caspase-9 to the p37/p35 fragments (Fig. 3F).

Mitochondrial outer membrane permeabilization depends on the multi-BH domain pro-apoptotic Bcl-2 family members Bax and Bak.[27] Although Bak is constitutively localized to mitochondria, Bax is mostly found in the cytosol of healthy cells.[28] Indeed, we found that wt hepatocytes contained all Bax in the cytosol (Supporting Fig. S6A). In response to treatment with FasL, we did not observe translocation of Bax to mitochondria (Supporting Fig. S6A), suggesting that Bax was not activated in FasL-treated hepatocytes. This was not due to a technical problem to detect Bax translocation, as Bax migration to mitochondria was readily detectable in FasL-treated MEFs (Supporting Fig. S6A). To examine the role of Bak in FasL-induced apoptosis, we isolated hepatocytes from Bak-deficient mice. On treatment with FasL, these cells showed similar kinetics of apoptosis (Fig. 1E) and caspase-3 activation (Fig. 2A) and processing (Fig. 2C) and a failure to translocate Bax (Supporting Fig. S6A) as wt cells. Moreover, overexpression of Bcl-2 could not protect primary hepatocytes from FasL-induced apoptosis (Supporting Fig. S6B), consistent with the notion that cell killing occurred through the type I pathway[12,20].

XIAP Is Not Degraded in FasL-Treated Hepatocytes in Culture and Its Deletion Sensitizes the Cells to Caspase-3 Activation and Apoptosis. XIAP is an endogenous inhibitor of caspase-3, caspase-7, and caspase-9.[17] In cells undergoing mitochondria dependent apoptosis, XIAP is neutralized or degraded by the proteasome.[18-20] We examined the expression and fate of XIAP in FasL-treated 3T9 MEFs and primary hepatocytes. Healthy MEFs expressed high levels of XIAP, but this was rapidly degraded in response to FasL due to activation of the type II pathway (Fig. 4). By contrast, in healthy, primary hepatocytes plated on collagen (from both wt and *bid*−/− mice), XIAP levels were much lower than in MEFs and they remained low for up to 8 hours of FasL treatment (Fig. 4). Thereafter, XIAP levels started to diminish, but at that time 70% to 80% of the hepatocytes had already undergone apoptosis (Fig. 1E), indicating that XIAP degradation was a consequence and not a cause of cell killing. Nevertheless, some caspase-3 in hepatocytes was held in check by XIAP, because *xiap*−/− cells displayed a faster and ~10 times higher caspase-3 activation (Fig. 2B,C) and accelerated apoptosis (Fig. 1E) in response to FasL treatment than wt hepatocytes.

The Type II to Type I Switch Is Specific for FasL and Does Not Occur with TNF/Actinomycin-D-Induced Apoptosis. Type I and II apoptosis pathways were also reported for TNFα/TNF-R1 signaling.[29] To investigate whether collagen plating affected components, which are common to TNFα/TNF-R1 and FasL/Fas signaling, we treated wt and *bid*−/− primary hepatocytes with TNFα plus actinomycin-D (ActD). The presence of actinomycin D was necessary to block NFκB-transduced survival signaling, which is normally triggered by TNFα-TNF-R1 stimulation.[29] Importantly, TNFα/ActD (and also FasL/ActD) induced classical apoptosis of primary hepatocytes because cell death was effectively inhibited by the broad spectrum caspase inhibitor Q-VD-OPH (Supporting Fig. S7A,B). Strikingly, whereas wt hepatocytes showed high caspase-3 activity between 6 to 8 hours of TNFα/ActD treatment, only little caspase-3 activation was detected in the Bid−/− cells (Fig. 5A). This shows that collagen-plated hepatocytes retained mitochondria-mediated type II signaling in response TNFα/ActD. This finding was independent of the dose of TNFα used (Supporting Fig. S4C). Consistent with type II signaling, TNFα/ActD caused rapid degradation of XIAP (data not shown), and apoptosis sensitivity was similar between wt and XIAP−/− hepatocytes (Fig. 5B).

The Type II to Type I Switch Occurs on Plating Hepatocytes on Matrigel But Not if the Cells Are Kept in Suspension. As the FasL type II to type I apoptosis signaling switch was seen with cell culturing on collagen, we wondered whether it also occurred when freshly isolated hepatocytes were plated on other extracellular matrices, such as Matrigel. This matrix is a solubilized basement membrane preparation extracted from EHS mouse sarcoma, a tumor rich in ECM proteins. Its major component is laminin, followed by collagen IV, heparan sulfate proteoglycans, and entactin. When seeded on Ma-

trigel, primary hepatocytes showed typical features of type I apoptotic signaling in response to FasL treatment (Supporting Fig. S8), indicating that other extracellular matrix components were as potent as collagen to induce the type II to I switch of apoptosis signaling in freshly isolated hepatocytes.

We then examined the possibility that the differences in FasL signaling in hepatocytes between *in vivo* and *in vitro* might be due to the fact that the cells were forced to re-attach to an extracellular matrix after detachment from their natural environment. For this purpose, we kept primary hepatocytes in suspension cultures and treated them with FasL directly after isolation. (This protocol had to be used because it was reported that hepatocytes in suspension survive only for 1 to 2 days.[22]) As shown in Fig. 6A, hepatocytes in suspension cultures were rapidly killed by FasL (within 4 hours) and showed fast and efficient caspase-3 processing (Fig. 6C) and activation (Fig. 6B). Interestingly, we clearly detected FasL-induced release of cytochrome c into the cytosol (Fig. 6D) and the processing of caspase-9 into its p37 form (Fig. 6C). Remarkably, cytochrome c release (Fig. 6D), caspase-9 processing and caspase-3 activation/processing (Fig. 6B,C) as well as apoptosis induction (Fig. 6A) were all inhibited significantly by loss of Bid. These results indicate that hepatocytes retain the mitochondria-mediated type II FasL signaling pathway for apoptosis when they are maintained in single-cell suspension.

Discussion

We report that FasL/Fas signaling is switched from the Bid/mitochondria-dependent type II to the Bid/mitochondria-independent type I pathway when freshly isolated mouse hepatocytes are plated on collagen or Matrigel. Consistently, the switch does not occur when hepatocytes are kept in single-cell suspension after isolation. These results indicate that extracellular matrix components trigger signaling cascades that crosstalk with FasL/Fas-signaling to abrogate its dependence on the Bid-mediated mitochondrial amplification loop for apoptosis induction.

Given that death receptors use common downstream signaling components, it was surprising that we did not observe a type II to type I apoptosis signaling switch when hepatocytes were treated with TNFα/ActD. One might argue that the presence of ActD may have prevented this switch. However, this seems unlikely, as FasL/ActD-induced caspase activation was also Bid-independent (type I) in these cells (Fig. 5 and

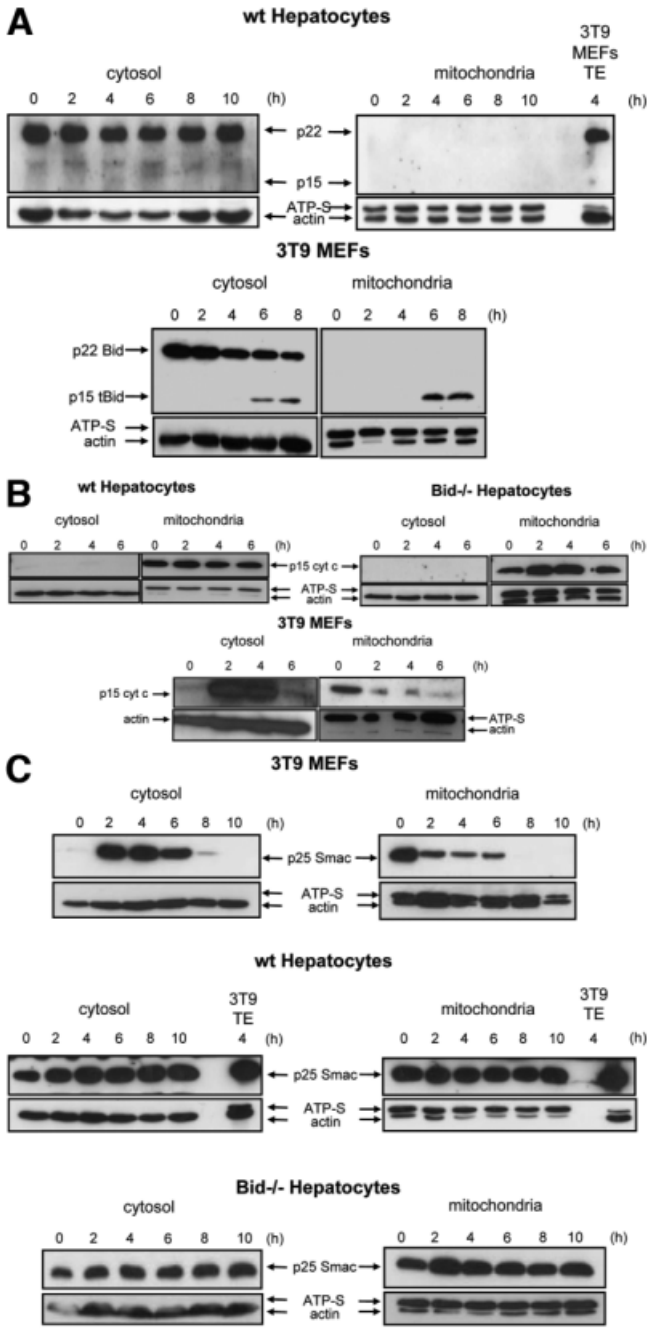
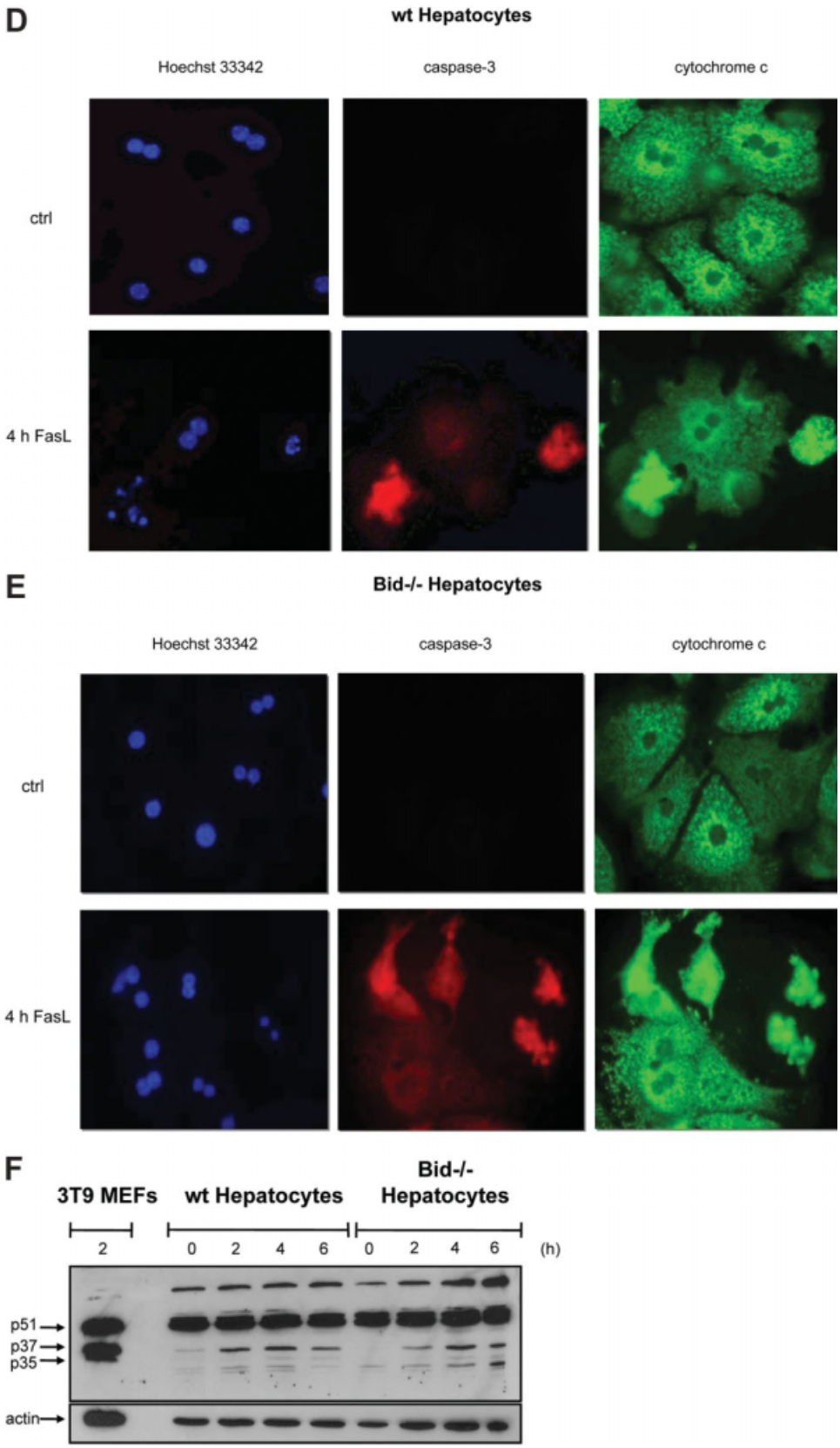


Fig. 3. Lack of Bid cleavage, cytochrome c, and Smac release and caspase-9 processing in FasL-treated primary hepatocytes. Western blot analysis of (A) Bid, (B) cytochrome c, (C) Smac/DIABLO, and (F) caspase-9 in the cytosolic and mitochondrial fractions of wt and *bid*−/− primary hepatocytes and 3T9 MEFs treated with 50 ng/mL N2A FasL for up to 10 hours. 3T9 TE, total extract of 3T9 MEFs as control. Probing for actin (cytosol) or the F1 subunit of the mitochondrial ATP synthase (ATP-S) is shown as a loading control. (D, E) Hoechst 33342 staining (blue) and activated caspase-3 (red) and cytochrome c (green) immunofluorescence analysis of untreated and N2A FasL-treated wt and *bid*−/− hepatocytes. Magnification is 630×.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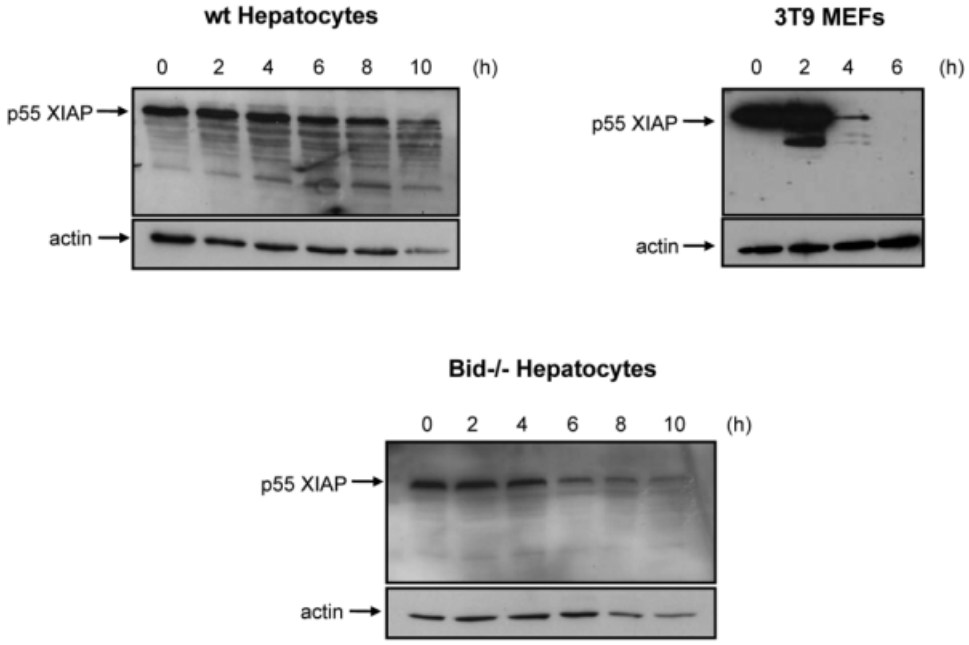

Fig. 4. Treatment with FasL triggers degradation of XIAP in MEFs but not in hepatocytes. Western blot analysis of XIAP in cytosolic fractions of wt and Bid−/− hepatocytes and wt 3T9 MEFs treated with 50 ng/mL N2A FasL for up to 10 hours. Probing for actin is shown as a loading control.

Supporting Fig. S4B). How might then extracellular matrix components specifically affect FasL/Fas signaling? Possibly, these components could activate intracellular signaling pathways through integrins that intercept the FasL/Fas pathway at discrete signaling modules. Integrins are known to associate on their cytoplasmic side with integrin-linked kinase (ILK) and focal adhesion kinase (FAK), which activate downstream signaling through the phosphoinositide 3-kinase/AKT/glycogen synthase kinase-3, MEK/mitogen-activated protein kinase, and c-Jun N-terminal kinase (JNK) pathways.[30,31] Any of these kinases or kinases activated further downstream might phosphorylate and thereby change the activity or stability of Fas signaling components, such as FADD, caspase-8, Bid, the caspase-8 inhibitor FLIP, or the caspase-3/-9 inhibitor XIAP. For example, phosphorylation of Bid by casein kinases I and II has been shown to prevent its cleavage by caspase-8.[32,33] Moreover, FLIP phosphorylation by JNK marks the protein for proteasomal degradation by way of the E3 ubiquitin ligase Itch.[34] Preliminary results from our lab showed, however, that pharmacologic inhibition of phosphoinositide 3-kinase, mitogen-activated protein kinase, casein kinase I and II, JNK, or glycogen synthase kinase-3 did not affect the collagen plating-induced signaling switch of hepatocytes from type II to type I (Supporting Fig. S9). Moreover, cytosolic and membrane-associated levels of caspase-8 were unchanged (Supporting Fig. S10) and FLIP protein was undetectable (data not shown) in extracts from either collagen-plated or suspension hepatocytes. This indicates that the type II to I signaling switch was not caused by alterations in the levels of FLIP or caspase-8, in contrast to a previous report.[12]

An attractive possibility for integrin signals to interfere with the FasL/Fas signaling pathway, without the need for modifying downstream signaling components, would be by way of a direct interaction between integrins and Fas. Such a mechanism has been reported for the hepatocyte growth factor (HGF) receptor c-Met. In healthy hepatocytes c-Met was found to physically interact with Fas, preventing its activation.[35,36] In response to high levels of HGF or FasL, c-Met is released from Fas, allowing FADD and caspase-8 recruitment and apoptosis induction.[35,36] Interestingly, a recent report showed that high doses of HGF could bypass the mitochondrial requirement for Fas-induced apoptosis in Jurkat cells and hepatocytes, leading to a type I signaling phenotype.[37] Similarly, integrins could modulate the Fas, but not the TNF-R1 DISC to engage type I instead of type II signaling. It is also conceivable that integrin activation may modify lipid rafts on the plasma membrane. These specialized cholesterol-rich microdomains have been reported to recruit or

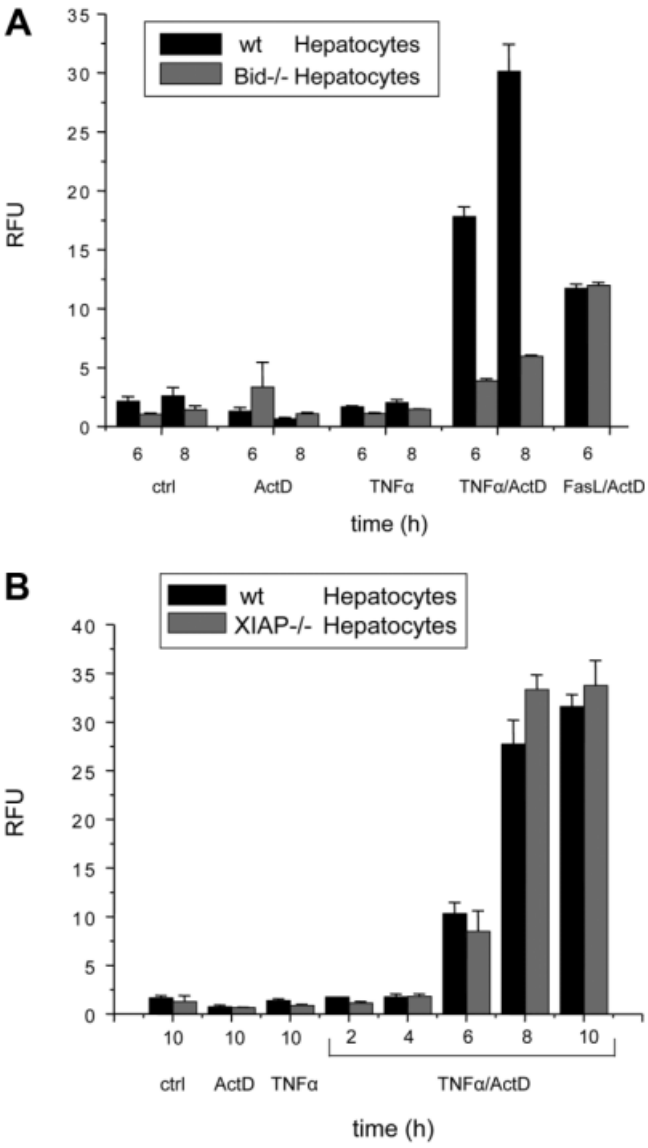

Fig. 5. Caspase-3 activation requires Bid when primary hepatocytes are treated with TNFα/ActD instead of FasL ($\pm$ActD). (A, B) Fluorogenic caspase-3/-7 activity assay on cytosolic extracts of (A) wt and Bid$-/-$ or (B) wt and XIAP$-/-$ primary mouse hepatocytes treated for the indicated times with 25 ng/mL TNFα plus 0.4 μg/mL ActD, 50 ng/mL N2A FasL plus 0.4 μg/mL ActD or TNFα and ActD alone. The values represent the means of three independent experiments $\pm$ SD.

exclude Fas and their associated signaling molecules and thereby change intracellular signaling behavior.[38,39]

As XIAP needs to be inactivated or removed by mitochondrial type II signaling for effective apoptosis to occur, it was suggested that it determines the type of apoptotic signaling in response to FasL.[11,18-20] Interestingly, although MEFs express high levels of XIAP, which is rapidly degraded in response to FasL treatment, hepatocytes express only little XIAP that allows most caspase-3 to be directly activated by caspase-8. It is, however, unclear why this direct pathway is not used on treatment with TNFα/ActD in the very same hepatocytes. XIAP$-/-$ mice were found to be more susceptible to anti-Fas antibody induced hepatitis than wild-type mice (T. Kaufmann, J. Silke, C. Borner, A. Strasser, in preparation) although we expected that removal of XIAP would not further sensitize type II cells *in vivo*. Thus, although XIAP may determine the sensitivity of cells toward death ligand induced apoptosis, additional mechanisms must exist to regulate the switch from type I to II or vice versa. We envisaged the possibility that other IAPs, such as cIAP1 and cIAP2, could be involved in the conversion. As shown in Supporting Fig. S11A, whereas MEFs express cIAP1 in a stable form, no cIAP1 protein could be detected in healthy or FasL-treated hepatocytes, irrespective of whether death signaling used the type I (suspension) or type II (on collagen) pathway. By contrast, cIAP2 seems to be transcriptionally up-regulated in response to FasL treatment in collagen plated (type I) hepatocytes, but not in type II MEFs, indicating that this IAP may play a role in the apoptosis pathway signaling switch.

The question remains why hepatocytes in suspension (at least in part) resemble the *in vivo* situation despite the fact that they are not in their natural environment with respect to extracellular matrix, neighboring cells (stellate cells, Kupffer cells, etc.) and cytokines/growth factors. We speculate that collagen I and Matrigel may not provide the optimal (correct) extracellular matrix components that maintain the *in vivo* function of hepatocytes with respect to apoptosis sensitivity. We are in the process of screening arrays of different extracellular mixtures to identify the cocktail of components that retain type II FasL-induced apoptosis signaling in hepatocytes *in vitro*. This will be important for future studies with primary hepatocytes *in vitro* as the findings obtained from these cultures should reflect *in vivo* situations as close as possible.

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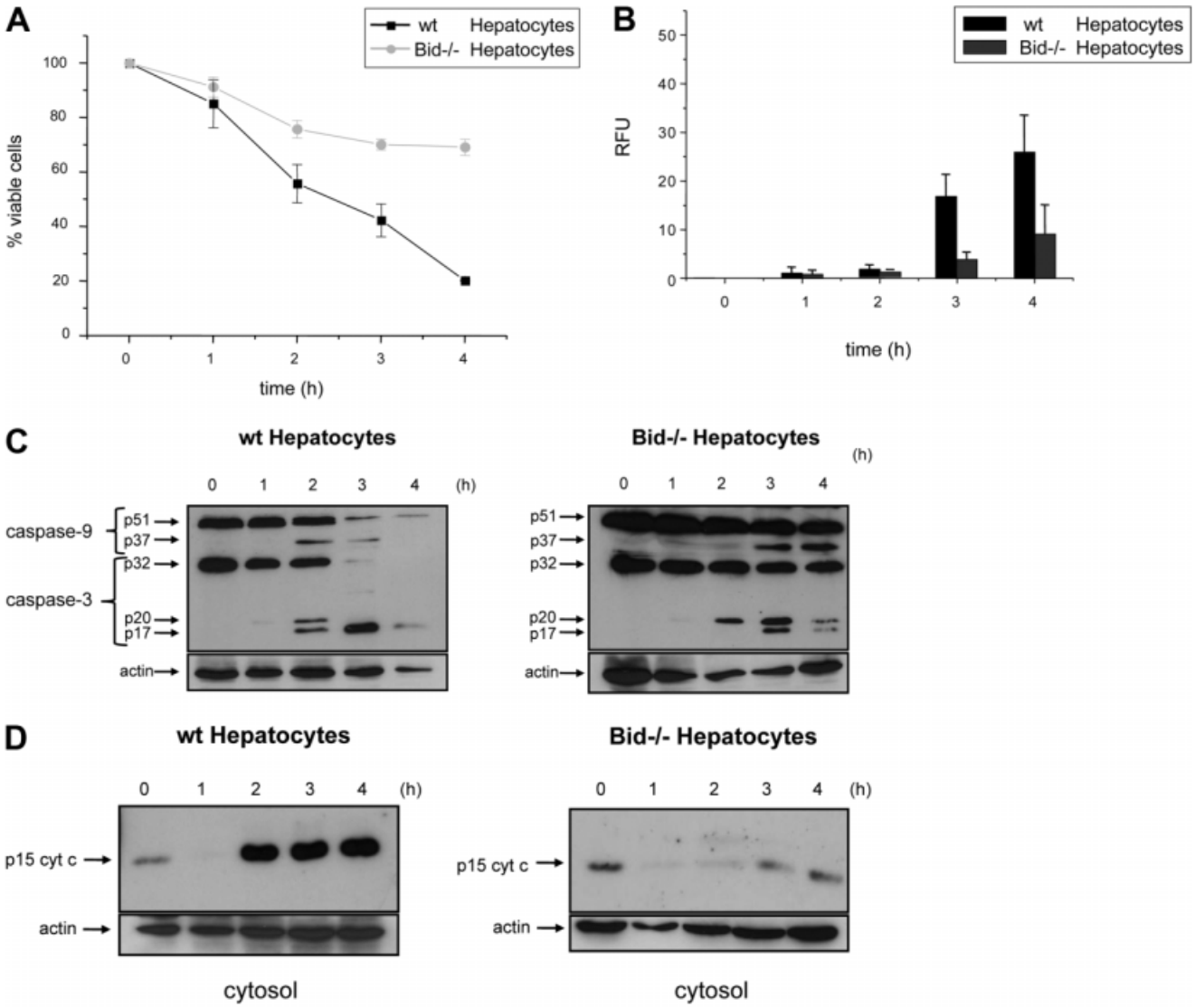

Fig. 6. Primary hepatocytes kept in suspension require Bid for FasL-induced cytochrome c release, caspase-9/-3 activation and apoptosis. (A) MTT assay. (B) Fluorogenic caspase-3/-7 activity assay (on cytosolic extracts, relative fluorescence units/RFU). (C) Caspase-3 and caspase-9 western blot analysis (on the same blot). (D) Cytochrome c subcellular localization by western blot analysis of wt and Bid−/− hepatocytes cultured in suspension and treated with 50 ng/mL N2A FasL for up to 4 hours. The values in (A, B) represent the means of three independent experiments ± SD. In (C, D) probing for actin is shown as a loading control.

References

1. Krammer PH. CD95's deadly mission in the immune system. Nature 2000;407:789-795.
2. Kanzler S, Galle PR. Apoptosis and the liver. Semin Cancer Biol 2000;10: 173-184.
3. Ogasawara J, Watanabe-Fukunaga R, Adachi M, Matsuzawa A, Kasugai T, Kitamura Y, et al. Lethal effect of the anti-Fas antibody in mice. Nature 2003;364:806-809.
4. Yin XM, Wang K, Gross A, Zhao Y, Zinkel S, Klocke B, et al. Bid-deficient mice are resistant to Fas-induced hepatocellular apoptosis. Nature 1999; 400:886-891.
5. Kaufmann T, Tai L, Ekert PG, Huang DC, Norris F, Lindemann RK, et al. The BH3-only protein bid is dispensable for DNA damage- and replicative stress-induced apoptosis or cell-cycle arrest. Cell 2007;129: 423-433.
6. Galle PR, Krammer PH. CD95-induced apoptosis in human liver disease. Semin Liver Dis 1998;18:141-151.
7. Canbay A, Friedman S, Gores GJ. Apoptosis: the nexus of liver injury and fibrosis. HEPATOLOGY 2004;39:273-278.
8. Desbarats J, Newell MK. Fas engagement accelerates liver regeneration after partial hepatectomy. Nat Med 2000;6:920-923.
9. Peter ME, Budd RC, Desbarats J, Hedrick SM, Hueber AO, Newell MK, et al. The CD95 receptor: apoptosis revisited. Cell 2007;129:447-450.
10. Diehl AM. Cytokine regulation of liver injury and repair. Immunol Rev 2000;174:160-171.
11. Peter ME, Krammer PH. The CD95(APO-1/Fas) DISC and beyond. Cell Death Differ 2003;10:26-35.
12. Scaffidi C, Fulda S, Srinivasan A, Friesen C, Li F, Tomaselli KJ, et al. Two CD95 (APO-1/Fas) signaling pathways. EMBO J 1998;17;1675-1687.
13. Gross A, Yin XM, Wang K, Wei MC, Jockel J, Milliman C, et al. Caspase cleaved BID targets mitochondria and is required for cytochrome c release, while BCL-XL prevents this release but not tumor necrosis factor-R1/Fas death. J Biol Chem 1999;274:1156-1163.

14. Youle RJ, Strasser A. The Bcl-2 protein family: opposing activities that mediate cell death. Nat Rev Mol Cell Biol 2008;9:47-59.
15. Green DR, Kroemer G. The pathophysiology of mitochondrial cell death. Science 2004;305:626-629.
16. Hyer ML, Samuel T, Reed JC. The FLIP-side of Fas signaling. Clin Cancer Res 2006;12:5929-5931.
17. Takahashi R, Deveraux Q, Tamm I, Welsh K, Assa-Munt N, Salvesen GS, et al. A single BIR domain of XIAP sufficient for inhibiting caspases. J Biol Chem 1998;273:7787-7790.
18. Du C, Fang M, Li Y, Li L, Wang X. Smac, a mitochondrial protein that promotes cytochrome c-dependent caspase activation by eliminating IAP inhibition. Cell 2000;102:33-42.
19. Li S, Zhao Y, He X, Kim TH, Kuharsky DK, Rabinowich H, et al. Relief of extrinsic pathway inhibition by Bid-dependent mitochondrial release of Smac in Fas-mediated hepatocyte apoptosis. J Biol Chem 2002;277: 26912-26920.
20. Sun XM, Bratton SB, Butterworth M, MacFarlane M, Cohen GM. Bcl-2 and Bcl-xL inhibit CD95-mediated apoptosis by preventing mitochondrial release of Smac/DIABLO and subsequent inactivation of X-linked inhibitor-of-apoptosis-protein. J Biol Chem 2002;277: 11345-11351.
21. Strasser A, Harris AW, Huang DC, Krammer PH, Cory S. Bcl-2 and Fas/APO-1 regulate distinct pathways to lymphocyte apoptosis. EMBO J 1995;14:6136-6147.
22. Block GD, Locker J, Bowen WC, Petersen BE, Katyal S, Strom SC, et al. Population expansion, clonal growth, and specific differentiation patterns in primary cultures of hepatocytes induced by HGF/SF, EGF and TGF alpha in a chemically defined (HGM) medium. J Cell Biol 1996;132: 1133-1149.
23. Hewitt NJ, Lechón MJ, Houston JB, Hallifax D, Brown HS, Maurel P, et al. Primary hepatocytes: current understanding of the regulation of metabolic enzymes and transporter proteins, and pharmaceutical practice for the use of hepatocytes in metabolism, enzyme induction, transporter, clearance, and hepatotoxicity studies. Drug Metab Rev 2007; 39:159-234.
24. Klingmüller U, Bauer A, Bohl S, Nickel PJ, Breitkopf K, Dooley S, et al. Primary mouse hepatocytes for systems biology approaches: a standardized in vitro system for modelling of signal transduction pathways. Syst Biol (Stevenage) 2006;152:193-200.
25. Shimizu M, Fontana A, Takeda Y, Yagita, H, Yoshimoto T, Matsuzawa A. Induction of antitumor immunity with Fas/APO-1 ligand (CD95L)-transfected neuroblastoma Neuro-2a cells. J Immunol 1999;162:7350-7357.
26. Holler N, Tardivel A, Kovacsovics-Bankowski M, Hertig S, Gaide O, Martinon F, et al. Two adjacent trimeric Fas ligands are required for Fas signaling and formation of a death-inducing signaling complex. Mol Cell Biol 2003;23:1428-1440.
27. Lindsten T, Ross AJ, King A, Zong WX, Rathmell JC, Shiels HA, et al. The combined functions of proapoptotic Bcl-2 family members bak and bax are essential for normal development of multiple tissues. Mol Cell 2000;6: 1389-1399.
28. Desagher S, Martinou JC. Mitochondria as the central control point of apoptosis. Trends Cell Biol 2000;10:369-277.
29. Wajant H, Pfizenmaier K, Scheurich P. Tumor necrosis factor signaling. Cell Death Differ 2003;10:45-65.
30. Hynes RO. Integrins: bidirectional, allosteric signaling machines. Cell 2002;110:673-687.
31. Hehlgans S, Haase M, Cordes N. Signaling via integrins: implications for cells survival and anticancer strategies. Biochem Biophys Acta 2007;1775:163-180.
32. Degli Esposti M, Ferry G, Masdehors P, Boutin JA, Hickman JA, Dive C. Post-translational modification of Bid has differential effects on its susceptibility to cleavage by caspase 8 or 3. J Biol Chem 2003;278: 15749-15757.
33. Desagher S, Osen-Sand A, Montessuit S, Magnenat E, Vilbois F, Hochmann A, et al. Phosphorylation of bid by casein kinases I and II regulates its cleavage by caspase 8. Mol Cell 2001;8:601-611.
34. Chang L, Kamata H, Solinas G, Luo JL, Maeda S, Venuprasad K, et al. The E3 ubiquitin ligase itch couples JNK activation to TNFα-induced cell death by inducing c-FLIP(L) turnover. Cell 2006;124:601-613.
35. Wang X, DeFrances MC, Dai Y, Pediaditakis P, Johnson C, Bell A, et al. A mechanism of cell survival: sequestration of Fas by the HGF receptor Met. Mol Cell 2002;9:411-421.
36. Smyth LA, Brady HJ. cMet and Fas receptor interaction inhibits death-inducing signaling complex formation in endothelial cells. Hypertension 2005;46:100-106.
37. Zhao Y, Difrancesca D, Wang X, Zarnegar R, Michalopoulos GK, Yin X-M. Promotion of Fas-mediated apoptosis in type II cells by high doses of hepatocyte growth factor bypasses the mitochondrial requirement. J Cell Physiol 2007;213:556-463.
38. Hueber AO, Bernard AM, Herincs Z, Couzinet A, He HT. An essential role for membrane rafts in the initiation of Fas/CD95-triggered cell death in mouse thymocytes. EMBO Rep 2002;3:190-196.
39. O'Reilly LA, Divisekera U, Newton K, Scalzo K, Kataoka T, Puthalakath H, et al. Modifications and intracellular trafficking of FADD/MORT1 and caspase-8 after stimulation of T lymphocytes. Cell Death Differ 2004; 11:724-736.