

Kupffer Cell-Expressed Membrane-Bound TNF Mediates Melphalan Hepatotoxicity via Activation of Both TNF Receptors¹

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Isolated hepatic perfusion of nonresectable liver cancer using the combination of TNF and melphalan can be associated with a treatment-related hepatotoxicity. We investigated whether, apart from TNF, also melphalan is cytotoxic in primary murine liver cells *in vitro* and investigated mediators, mode of cell death, and cell types involved. Melphalan induced a caspase-dependent apoptosis in hepatocytes, which was not seen in liver cell preparations depleted of Kupffer cells. Neutralization of TNF prevented melphalan-induced apoptosis and liver cells derived from mice genetically deficient in either TNFR 1 or 2, but not from *lpr* mice lacking a functional CD95 receptor, were completely resistant. Cell-cell contact between hepatocytes and Kupffer cells was required for apoptosis to occur. Melphalan increased membrane-bound but not secreted TNF in Kupffer cells and inhibited recombinant TNF- α converting enzyme *in vitro*. Melphalan induced also severe hepatotoxicity in the isolated recirculating perfused mouse liver from wild-type mice but not from TNFR 1 or 2 knockout mice. In conclusion, this study shows that melphalan elicits membrane TNF on Kupffer cells due to inhibition of TNF processing and thereby initiates apoptosis of hepatocytes via obligatory activation of both TNFRs. The identification of this novel mechanism allows a causal understanding of melphalan-induced hepatotoxicity. *The Journal of Immunology*, 2005, 175: 4076–4083.

The occurrence of primary or metastatic cancers confined to the liver, during hepatocellular or colorectal cancer and ocular melanoma, represents a major life-limiting factor for oncologic patients (1, 2). Despite aggressive treatment, the median survival after liver metastases is only between 12 and 24 mo for patients with colorectal cancer and between 2 and 7 mo for patients with ocular melanoma.

Melphalan is a bifunctional alkylating agent, which induces DNA cross-linking in various cancer cells (3, 4). Moreover, melphalan was shown to inhibit transcription and translation in a cell-free system (5). A novel treatment strategy was developed to apply melphalan together with the potent endogenous antitumor principle TNF, and phase I and II clinical trials were conducted with isolated limb perfusion for advanced refractory in-transit melanoma (6, 7) and soft tissue sarcoma (8, 9). For the treatment of patients with ocular melanoma metastatic to the liver as well as with unresectable colorectal cancer confined to the liver, a regional hepatic treatment regimen by isolating and perfusing the vascular supply to the liver, i.e., isolated hepatic perfusion was used (10–12).

The results of these trials have indicated that melphalan can induce, by means of a still unknown mechanism, a reversible grade III or grade IV (National Cancer Institute Common Toxicity Criteria) hepatic toxicity in a substantial portion of the patients (13), especially when combined with TNF. Although the interaction between TNF and its TNF-R1 is required for the priming of hepatocytes for responsiveness to their growth factors upon partial hepatectomy (reviewed in Ref. 14), this cytokine, during ischemia (15) or in combination with transcriptional inhibitors, can also induce a severe TNF-R1-dependent hepatotoxicity (16, 17). In this study, using an *in vitro* primary mouse liver cell model and an *ex vivo* isolated perfused mouse liver model, we investigated whether, apart from TNF, also melphalan is cytotoxic in primary hepatocytes, with special emphasis on the mode of cell death and its possible interaction with TNF-induced apoptosis.

Our results show that melphalan as such induces apoptosis in primary liver cells *in vitro* and hepatotoxicity in the isolated perfused liver *ex vivo*. Melphalan toxicity is mediated by the induction of membrane TNF in Kupffer cells caused by inhibition of TNF processing by the drug. Membrane-bound TNF then can interact by means of cell-cell contact with both TNF-R1 and TNF-R2 on hepatocytes, on top of which soluble TNF can further increase cell death via activation of TNF-R1. This explains the resistance of hepatocytes deficient in TNF-R1 or TNF-R2 toward melphalan toxicity. These findings reveal a novel biochemical mechanism for the hepatotoxic action of an agent used in cancer treatment that allows us to propose a potential strategy to rescue healthy tissue from the adverse effects of drug treatment.

Materials and Methods

Materials

Recombinant murine TNF was purchased from Innogenetics and had a specific activity of 2.10^8 IU/mg. Melphalan was obtained from Glaxo-SmithKline. The murine TNF ELISA (OptEIA) was purchased from BD

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Pharmingen. The caspase inhibitor benzoyloxycarbonyl-val-ala-aspartyl-fluoro-methylketone (zVAD-fmk)³ was purchased from Alexis Biochemicals. Collagen was obtained from Serva, and materials needed for cell culture were purchased from Greiner. Cell culture medium RPMI 1640 medium was purchased from BioWhittaker.

Animals

Specific pathogen-free male C57BL/6 wild-type mice, TNF-R1 knockout mice, TNF-R2 knockout mice, and TNF-R1R2 knockout mice (25 g), originally provided by Dr. H. Bluethmann (F. Hoffmann-LaRoche, Basel, Switzerland), were obtained from the in-house breeding stock at the University of Konstanz. *LPR* mice were obtained from Harlan Sprague Dawley. Animals were held at 22°C and 55% humidity and given a constant day-night cycle of 12 h. All steps of animal handling were conducted according to the Guidelines of the European Council (directive 86/609/EEC) and the national German authorities and followed the directives of the University of Konstanz Ethical Committee.

Cell culture experiments

Hepatocytes were isolated from 8-wk-old mice using the method previously described by Seglen et al. (18) and cultured as described previously. For some experiments, cells were additionally purified by centrifugation using a Percoll gradient. In brief, hepatocytes were plated in 200 μ l of RPMI 1640 medium, including 10% FCS in collagen-coated 24-well plates at a density of 8×10^4 cells/well. Cells were allowed to adhere to the plate for at least 4 h before the medium was changed to RPMI 1640 medium without FCS. Incubation of murine hepatocytes with melphalan started 30 min after medium exchange alone or in the presence of other mediators mentioned in the text. Incubations were conducted at 37°C in an atmosphere of 40% O₂, 5% CO₂, and 100% humidity.

For some experiments, Kupffer cells were depleted *in vivo* by i.v. injection of 150 μ l of liposome-encapsulated clodronate 1 and 2 days before liver cell preparation. Clodronate liposomes were prepared and injected as described previously (19).

For Transwell experiments, Kupffer cells were left to adhere for 24 h on cell culture inserts with 1- μ m pore size (BD Biosciences) and were placed in wells containing freshly isolated and adhered purified hepatocytes.

Isolated liver perfusion

Upon a lethal i.v. injection with 150 mg/kg pentobarbital-natrium and 0.8 mg/kg heparin, the vena portae and the vena cava inferior of the mouse liver were cannulated. The organ was perfused blood-free with a Krebs-Henseleit buffer with a total volume of 25 ml of buffer in a closed circulation mode under constant pressure conditions. The temperature of the perfusate was kept constant at 37°C, and oxygenation with pure oxygen at a pressure of 500 mbar was performed. During perfusion, the perfusate flow through the liver, as well as the pressure, were constantly measured and recorded. Samples for metabolite and enzyme measurements were taken from the perfusate at different time points, as indicated in the text.

Caspase-3-like protease activity

Primary hepatocytes were washed three times with PBS and lysed with hypotonic extraction buffer. After centrifugation (15 min, 13,000 \times g, 4°C) of the lysates, supernatants were frozen immediately and stored at -80°C until measurement. Cytosolic extracts of liver tissue were prepared by Dounce homogenization of ~100 mg of frozen perfused liver tissue in hypotonic extraction buffer (25 mM HEPES (pH 7.5), 5 mM MgCl₂, 1 mM EGTA, 1 mM Pefabloc and pepstatin, leupeptin and aprotinin (1 μ g/ml each), and 0.1% Triton X-100) and subsequently centrifuged (15 min, 13,000 \times g, 4°C).

Generation of free 7-amino-4-trifluoromethylcoumarin (afc) was followed in assay buffer (50 mM HEPES (pH 7.4), 1% sucrose, 0.1% CHAPS, 10 mM DTT, and 50 μ M fluorogenic substrate *N*-acetyl-asp-glu-val-asp-afc) for 30 min at 37°C using a fluorometer plate reader VICTOR² (PerkinElmer Wallac) and set at an excitation wavelength of 385 nm and an emission wavelength of 505 nm. Protein concentrations of corresponding samples were quantified with the Pierce assay (Pierce), and the specific caspase-3-like activity was calculated in picomoles of free afc per minute (μ U) and milligram of protein, using serially diluted standards (0–5 μ M afc). Control experiments confirmed that the activity was linear with time and with protein concentration under the conditions described above.

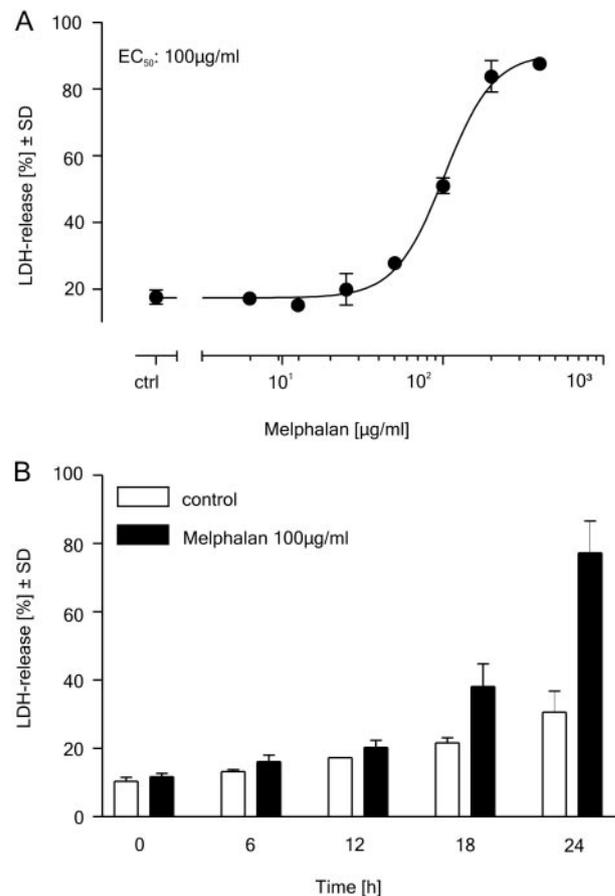


FIGURE 1. Melphalan induces cytotoxicity in primary liver cells. *A*, Isolated liver cells were incubated with various concentrations of melphalan. Cytotoxicity, as measured by LDH release, was assessed after 18 h. *B*, Time dependency of cytotoxicity of 200 μ g/ml melphalan, as compared with control conditions, was assessed by means of LDH release. All data represent means \pm SD of three independent experiments.

Cytotoxicity assay

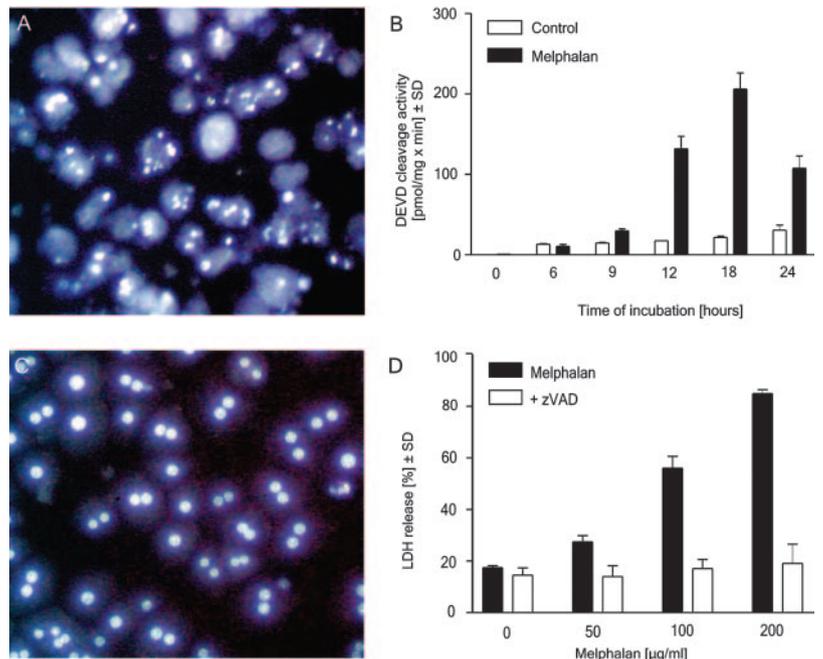
Cytotoxicity was quantified by measurement of lactate dehydrogenase (LDH) activity in culture supernatants (S) and in the remaining cell monolayer (C). After lysis with 0.1% Triton X-100, and calculation of the percentage of the LDH release from the ratio of $S/(S+C)$. Cytotoxicity after liver perfusion was quantified by LDH and the liver specific enzyme alanine aminotransferase (ALT)-measurement out of perfusate samples taken at different time points during the experiment and stored at 4°C at the end of the experiment until measurement.

Cell-based ELISA

This assay was performed as described previously (20). To assess the expression of membrane-bound TNF, Kupffer cells (10^5 cells/well) were left to adhere in 96-well plates for 24 h and were then either left untreated or were treated with LPS (10 ng/ml) or melphalan (200 μ g/ml) for various times of incubation. Subsequently, cells were washed with HBSS (BioWhittaker), fixed for 45 min at room temperature with HBSS + 1% paraformaldehyde, and washed again, after which unspecific binding was blocked by means of incubating the wells with HBSS + 5% BSA (Serva). Subsequently, cells were incubated for 60 min at room temperature under mild shaking with either 30 μ l/well HBSS + 5% BSA, with 10 μ g/ml of the 1F3F3-neutralizing rat-anti-mouse TNF mAb (21) or with 10 μ g/ml of an isotype-matched control rat IgM (Innogenetics) or with 10 mg/ml of a polyclonal sheep-anti-mouse TNF Ab (16, 17), all diluted in HBSS + 5% BSA. After two washing steps, cells were incubated for 45 min under mild shaking with 3 mg/ml of either a goat-anti-rat IgG-alkaline phosphatase conjugate or a rabbit-anti-sheep IgG-alkaline phosphatase conjugate and washed twice with HBSS + 5% BSA and once with 2.5 M diethanolamine (pH 9.5). Finally, the substrate solution consisting of 0.58 mg/ml of the

³ Abbreviations used in this paper: zVADfmk, benzoyloxycarbonyl-val-ala-aspartyl-fluoromethylketone; afc, 7-amino-4-trifluoromethylcoumarin; LDH, lactate dehydrogenase; TACE, TNF- α converting enzyme; ALT, alanine aminotransferase.

FIGURE 2. Melphalan induces caspase-dependent apoptosis in primary liver cells. *A*, Primary liver cells treated with 200 $\mu\text{g/ml}$ melphalan became apoptotic, as characterized by chromatin condensation visualized by Sytox/Hoechst staining. *B*, The melphalan-induced apoptosis was preceded by caspase-3-like protease activation, arising from 9 h posttreatment on. In contrast, melphalan-treated (200 $\mu\text{g/ml}$) liver cells were protected from apoptosis when pretreated with the general caspase inhibitor z-VAD-fmk (10 μM), as shown by morphological analysis of Hoechst-dyed cells (*C*) or measurement of LDH release (*D*).



fluorescent substrate Attophos (Promega) and of 2.4 mg/ml of the endogenous phosphatase activity blocking agent levamisole (Sigma-Aldrich), diluted in diethanolamine buffer, was added. After 5 min, fluorescence was measured at an excitation wavelength 485 nm and an emission wavelength 530 nm in the Victor fluorometer plate reader (PerkinElmer Life Sciences). Background values due to the unspecific control IgM Ab were subtracted from the antimembrane TNF IgM signal.

FACS analysis

RAW cells were seeded at a number of 2×10^5 cells/well in a 24-well plate and allowed to settle for 2 h at 37°C in humid atmosphere with 5% CO_2 before addition of melphalan to adjust the indicated concentrations. After 16 h of incubation in the presence of melphalan, cells were harvested, and TNF- α expression was analyzed by FACS. Cells were washed twice with FACS staining buffer (PBS with 1% BSA and 0.05% NaN_3) before staining of membrane-bound TNF- α with biotinylated anti-TNF- α -Ab clone MP6-XT3 (BD Pharmingen). After 1 h of incubation at 4°C, cells were washed twice, and R-PE (PR)-labeled streptavidin was added for another incubation period of 30 min at 4°C before final washing. Flow cytometric analysis

was performed using a FACScan flow cytometer (BD Biosciences). Data were recorded and analyzed using the BD CellQuest software provided with the flow cytometer and WinMDI 2.8 software (J. Trotter, The Scripps Research Institute, La Jolla, CA).

TNF- α converting enzyme (TACE) activity

Recombinant TACE enzyme (Merck Biosciences) was dissolved in a buffer containing 50 mM Tris-HCl, 50 mM NaCl, and 4% glycerol. For monitoring TACE activity, an internally quenched fluorogenic substrate (Substrate IV; Merck Biosciences) was used with an excitation maximum of 320 nm and an emission maximum of 420 nm. Experiments were conducted at a temperature of 37°C in a total volume of 1.0 ml containing 200 ng/ml of the recombinant protein, 5 μM TACE substrate IV, and various concentrations of a known inhibitor for TACE (TAPI-1; Merck Biosciences) as positive control or melphalan in concentrations as indicated in the text. Spectra were recorded over time using the Luminescence Photometer LS 50B (PerkinElmer) with a slot of 5 nm.

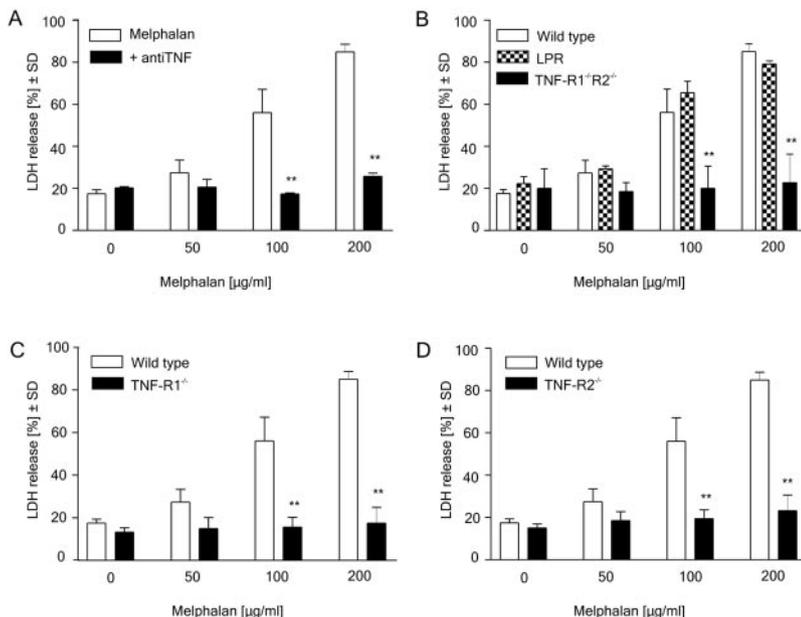


FIGURE 3. Melphalan-induced apoptosis in primary liver cells is due to TNF. *A*, Neutralizing rat-anti-mouse TNF mAb (1F3F3 (10 $\mu\text{g/ml}$); Ref. (21)) completely abrogated melphalan-induced LDH release in primary liver cells, after 18 h of incubation. Primary liver cells isolated from TNF-R1^{-/-}R2^{-/-} (*B*), TNF-R1^{-/-} (*C*), or TNF-R2^{-/-} (*D*) mice treated with melphalan were completely resistant, as measured by LDH release. All data represent means \pm SD in percentage of control of three independent experiments. Experiments with basal toxicities > 15% were excluded.

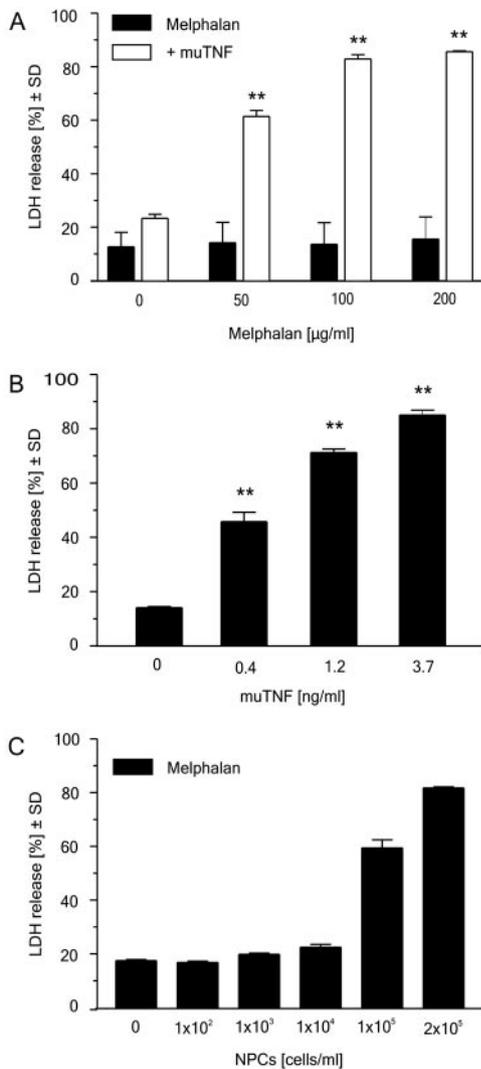


FIGURE 4. Melphalan-induced toxicity requires the presence of Kupffer cells. *A*, Hepatocytes from macrophage-depleted livers were stimulated with melphalan alone or together with soluble murine TNF (50 ng/ml). Apoptosis was determined 18 h after addition of melphalan. Data represent means ± SD derived from 10 independent experiments. *B*, Isolated purified hepatocytes were incubated with 200 µg/ml melphalan, and different concentrations of soluble murine TNF are indicated in the graph. Data represent means ± SD derived from five independent experiments. *C*, Cocultures of defined numbers of nonparenchymal cells and purified hepatocytes (4×10^5 cells/ml) were incubated with 200 µg/ml melphalan for 18 h before measuring LDH release.

Statistical analysis

Statistical differences were determined using an unpaired *t* test, if applicable, or were analyzed by one-way ANOVA followed by Dunnett’s multiple comparison test. In the case of nonhomogeneous variances, data were transformed before subsection to further analysis.

Results

Melphalan induces apoptosis in primary murine hepatocytes

As shown in Fig. 1, melphalan induced a concentration-dependent cytotoxicity in primary murine liver cells with an EC₅₀ of ~100 µg/ml (Fig. 1*A*), leading to a steady increase of LDH release over control incubations from 9 h on after exposure (Fig. 1*B*). As shown in Fig. 2*A*, the mode of cell death induced by melphalan was apoptotic, as characterized by the typical nuclear alterations due to chromatin condensation. Melphalan-induced apoptosis was asso-

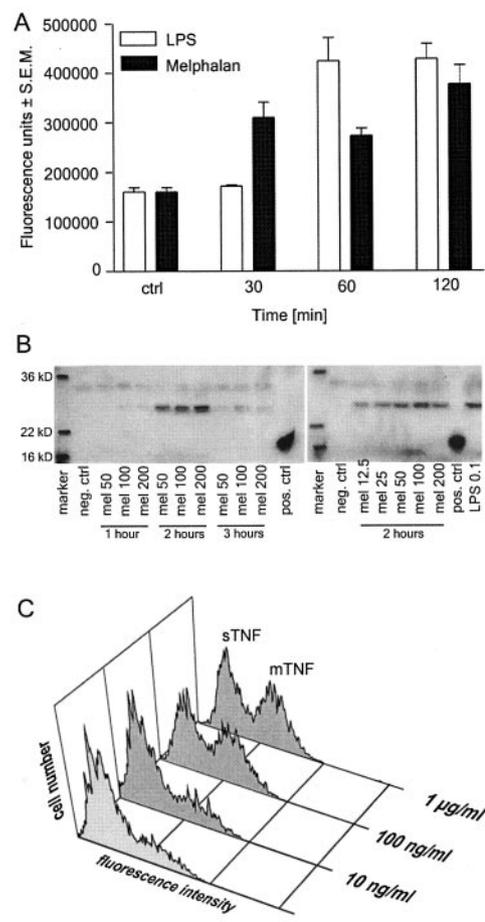


FIGURE 5. Expression of membrane-bound TNF (memTNF) on isolated Kupffer cells and on RAW cells. Melphalan-induced expression of memTNF was assessed by cell-based ELISA in isolated Kupffer cells (*A*) or by Western blot (*B*) and FACS analysis (*C*) in RAW cells. *A*, Ten nanograms per milliliter LPS (*Salmonella abortus equi*) or 200 µg/ml melphalan were added to the Kupffer cells, and memTNF was measured at different time points, as indicated in the graph. Data represent means ± SEM of three independent experiments. *B*, Time- and concentration-dependent melphalan-induced memTNF expression in RAW cells. Cells were treated with three doses of melphalan (50, 100, and 200 µg/ml) for 1, 2, or 3 h, upon which memTNF expression was assessed in Western blot analysis (*left lower panel*). Because the highest expression was observed upon 2 h of incubation, this time point was selected for a more detailed study of the concentration-dependent activity of melphalan, using five different concentrations in comparison to 100 ng/ml LPS (*right lower panel*). Equal amounts of protein loaded onto the gels were confirmed by detection of β-actin (*upper panels left and right*). *C*, Concentration-dependent melphalan-induced memTNF expression in RAW cells, as assessed in FACS.

ciated by an increase of caspase-3-like protease activity, measured in liver cell lysates (Fig. 2*B*), and was blocked in liver cell incubations in the presence of the pan-caspase inhibitor zVAD-fmk (Fig. 2, *C* and *D*).

TNF mediates melphalan toxicity in hepatocytes

Because TNF, together with CD95 ligand, represents one of the most important known apoptosis-inducing cytokines in hepatocytes, we subsequently investigated the effect of a neutralizing monoclonal rat anti-mouse TNF Ab 1F3F3, which interacts with both soluble and membrane-bound TNF (21), on the melphalan-induced apoptosis. Neutralization of TNF completely abrogated the melphalan-increased LDH release to the level of control incubations (Fig. 3*A*). The causal role of TNF in melphalan toxicity

Table I. Cytotoxicity of melphalan during coculture and Transwell incubations of isolated hepatocytes and Kupffer cells^a

| Incubation Conditions | LDH Release (%) ± SD |
|--|----------------------|
| Insert with hepatocytes alone | 11.7 ± 3.2 |
| Hepatocytes + melphalan | 15.9 ± 5.8 |
| Hepatocytes + MΦ ^b contact | 14.0 ± 2.4 |
| Hepatocytes + MΦ separated + melphalan | 24.3 ± 7.4 |
| Hepatocytes + MΦ contact + melphalan | 75.4 ± 5.5 |

^a Isolated Kupffer cells were plated in a concentration of 10⁵ cells/ml in cell culture inserts with a pore size of 1.0 μm or directly in 24-well plates. After 24 h, these inserts were placed on wells containing freshly isolated and additionally purified hepatocytes. For control experiments, these hepatocytes were directly plated on the adherent Kupffer cells. Cells were incubated with 200 μg/ml melphalan, and cytotoxicity, as measured by LDH release, was assessed after 18 h.

^b MΦ, macrophage.

was confirmed using hepatocytes isolated from mice deficient in both TNFR types (Fig. 3B). Surprisingly, although only TNF-R1 has been shown to mediate apoptosis by TNF in hepatocytes in the presence of transcriptional inhibitors (16, 17), not only hepatocytes lacking this receptor type (Fig. 3C), but also cells lacking only a functional TNF-R2 were completely protected from melphalan cytotoxicity (Fig. 3D). In contrast, hepatocytes isolated from *lpr* mice, which lack a functional CD95 receptor, were as sensitive to

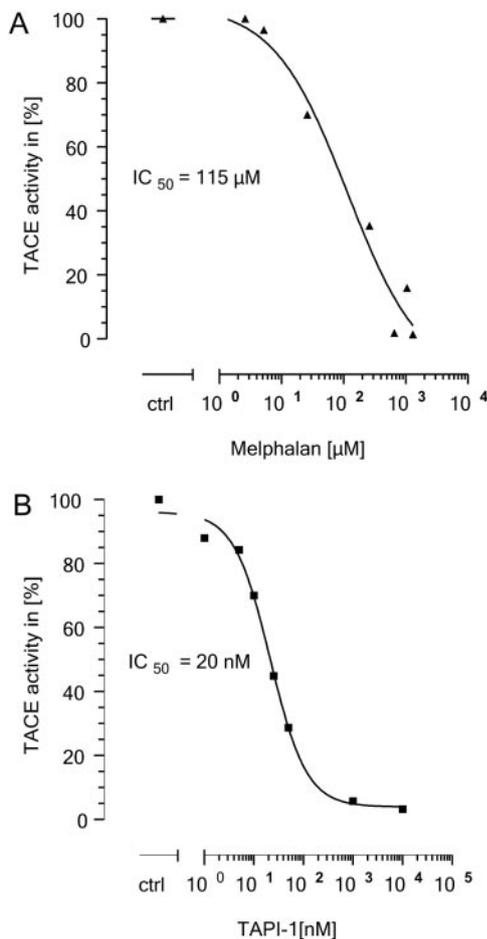


FIGURE 6. Inhibition of recombinant TACE by melphalan. Recombinant TACE was incubated with Substrate IV for 10 min at 37°C in the presence and in the absence of various concentrations of melphalan (A) or TAPI-1 (B). Specific enzyme activity was calculated for each of five independent measurements, and IC₅₀ values were calculated for TAPI-1 and melphalan as indicated in the graph.

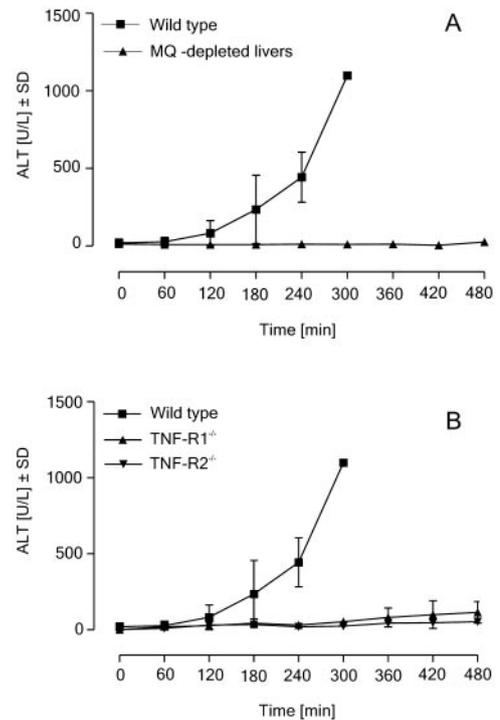


FIGURE 7. Melphalan-induced toxicity in isolated recirculating perfused mouse livers. Isolated mouse livers from wild-type (+) and macrophage-depleted mice (▲) (A) and from wild type (+), TNF-R1^{-/-} (▲) and TNF-R2^{-/-} (▼) mice (B) were perfused in situ with 150 mg/kg melphalan as described in *Materials and Methods*. Hepatotoxicity was determined by release of liver-specific enzyme ALT as indicated in the graph. Data represent means ± SD of six independent experiments.

melphalan-induced apoptosis as wild-type mice, thus stressing the selective dependence of melphalan toxicity on TNF (Fig. 3B).

Kupffer cells are the source of membrane-bound TNF induced by melphalan

We subsequently identified the source of TNF. As shown in Fig. 4A, upon depletion of Kupffer cells, achieved by i.v. injection of clodronate liposomes, melphalan-induced apoptosis was completely inhibited but was restored in the presence of exogenous TNF (Fig. 4, A and B). Notably, TNF as such fails to directly induce apoptosis in hepatocytes, unless transcriptional or translational inhibitors are also present (17). Thus, these findings indicate that in the absence of Kupffer cells the simultaneous presence of melphalan and TNF is a necessary condition to initiate hepatocyte apoptosis. Moreover, after addition of increasing concentrations of previously purified Kupffer cells to the hepatocytes, apoptosis induced by 200 μg/ml melphalan was restored (Fig. 4C), strongly indicating that this cell type is the source of TNF. In line with this observation, treatment of purified Kupffer cells with 200 μg/ml melphalan led to a significantly increased expression of membrane-bound TNF as detected by a cell-based ELISA for membrane TNF (Fig. 5A) but not of secreted TNF (< 20 pg/ml for all investigated time points; data not shown). Such increased expression of membrane-bound TNF was measurable after 30 min and was blunted after 120 min. In contrast, LPS (10 ng/ml) increased both the production of membrane-bound (Fig. 5A) and secreted TNF (30 min, <20 pg/ml; 120 min, 550 ± 70 pg/ml; 360 min, 750 ± 85 pg/ml; data not shown), with clearly different kinetics from melphalan. It should be noted here that the simultaneous addition of melphalan and LPS to isolated Kupffer cells did not induce measurable amounts of secreted nor membrane-bound TNF

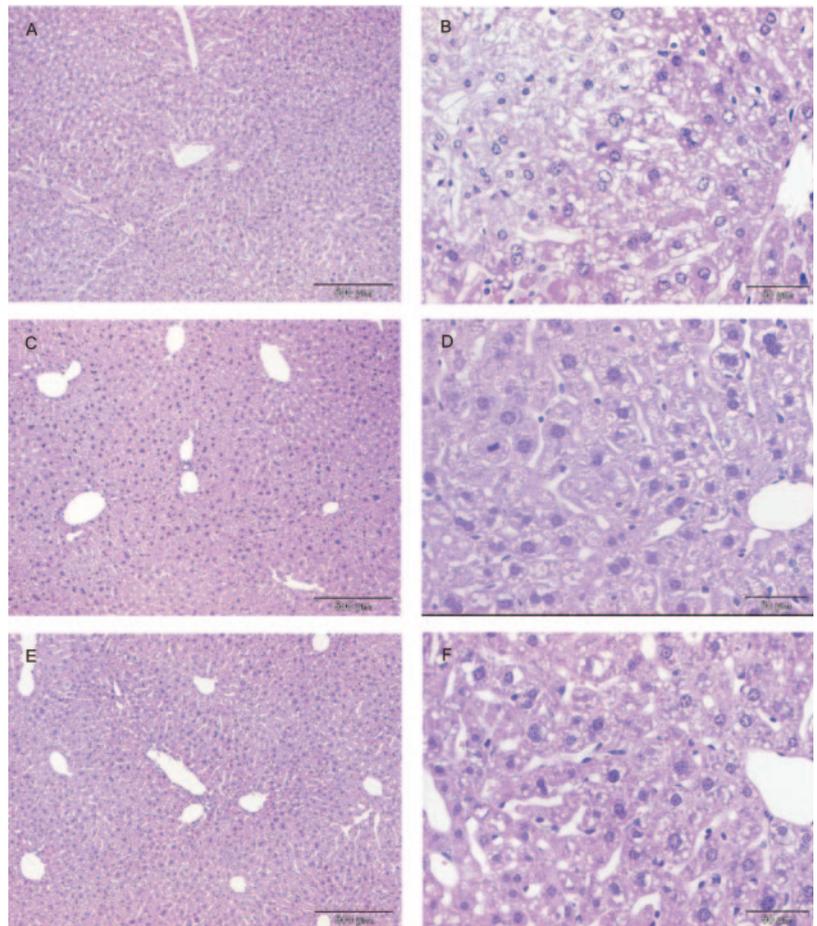


FIGURE 8. Histomorphology of murine livers perfused with melphalan. Isolated mouse livers from wild-type (*A* and *B*), TNF-R1^{-/-} (*C* and *D*), and TNF-R2^{-/-} (*E* and *F*) mice were perfused for 360 min with 150 mg/kg melphalan and subsequently preserved in phosphate-buffered neutral 4% formalin and embedded in paraplast. The samples were prepared for histological examination by routine methods (H&E staining, $5 \pm 2 \mu\text{m}$ thick). *A*, *C*, and *E* show a magnification of 60-fold, and *B*, *D*, and *F* show a magnification of 125-fold.

(data not shown). In a second, independent approach, we used RAW macrophages, i.e., an adherent murine macrophage cell line for protein expression and FACS analysis. Melphalan induced time and dose dependently the expression of membrane-bound TNF in this RAW macrophage cell line, as detected in Western blot analysis (Fig. 5*B*, lower panels). In an additional experiment, after exposure to increasing amounts of melphalan and staining of membrane-bound TNF- α with a biotinylated anti-TNF- α -Ab, these cells showed a concentration-dependent increase of membrane-bound TNF, as detected by FACS analysis (Fig. 5*C*).

The previous experiments indicated that cell-cell contact between membrane-bound TNF-expressing Kupffer cells and hepatocytes was necessary for the melphalan-induced apoptosis to occur. To confirm this hypothesis, we performed Transwell experiments, in which the Kupffer cells were cultivated in the inserts, that were spatially separated from the hepatocytes cultivated in the bottom wells. As shown in Table I, in this setting, in the presence of melphalan in the inserts containing the Kupffer cells, no cytotoxicity was induced in hepatocytes, indicating the necessity of cell-cell contact. Because the TNF converting enzyme TACE is responsible for the cleavage of TNF from its membrane-bound precursor form and because melphalan itself increased the expression of membrane-bound, but not secreted TNF, it was straightforward to check the hypothesis whether the melphalan directly inhibited TACE activity. We used the established TACE inhibitor TAPI-1 as a positive control in a published assay using substrate IV and determined an IC₅₀ of 20 nM for this compound, which is in agreement with published data (Fig. 6*B*). With this assay, we found a concentration-dependent inhibition of TACE activity by melphalan and determined an IC₅₀ of ~100 nM for melphalan (Fig. 6*A*). These data explain the accumulation of mem-

brane-bound TNF on TNF-producing cells in the presence of melphalan.

Melphalan induces TNF-mediated hepatotoxicity in situ

To confirm the toxicity of melphalan in the intact organ, we exposed the isolated perfused mouse liver to the drug. In this model, control organs did not undergo a significant hepatotoxicity for up to 480 min. In contrast, the perfusion with 150 mg/kg melphalan induced a significant hepatotoxicity, as evidenced by the release of ALT in naive but not in Kupffer cell-depleted livers (Fig. 7*A*). Moreover, the causal role of TNF action in the hepatotoxicity was confirmed by the resistance of livers from TNF-R1- or TNF-R2-deficient mice against melphalan, as assessed by the release of ALT (Fig. 7*B*). Analogous to these findings, organs isolated from wild-type mice displayed a significant disturbance of liver architecture (Fig. 8, *A* and *B*) 360 min after perfusion with melphalan, which was characterized by endothelial damage, edema formation, and apoptotic chromatin condensation in a subset of hepatocytes. In contrast, livers isolated from TNF-R1^{-/-} mice (Fig. 8, *C* and *D*) or TNF-R2^{-/-} mice (Fig. 8, *E* and *F*) displayed an intact liver architecture, with some light necrotic areas occasionally seen in the periportal fields but not in the central vein areas.

These experiments demonstrate that the *in vitro* results are reproduced in the whole organ as a model, which is close to the clinical situation of the local therapeutic liver perfusion with melphalan.

Discussion

Hepatotoxicity, as well as hypotension, represent major side effects preventing the systemic use of TNF in cancer therapy (16, 22). One way to circumvent the systemic toxicity of TNF is its local

application, preferentially in combination with the DNA cross-linking alkylating agent melphalan during isolated limb or hepatic perfusion (6, 13, 23). This elegant procedure prevents random distribution of TNF in the circulation and therefore prevents systemic toxicity to a large extent. However, the hepatic perfusion of melphalan, especially in combination with TNF was shown to lead to a reversible hepatotoxicity in the majority of patients by a mechanism that remains elusive.

Although TNF and CD95 ligand represent the key cytokines implicated in hepatotoxicity and hepatocyte apoptosis, the former cytokine, the expression of which is up-regulated in many acute and chronic liver diseases, does not cause apoptosis in hepatocytes in vitro or in vivo, unless during conditions of ischemia (15) or transcriptional inhibition (16). A very recent example includes the cytostatic drug and topoisomerase inhibitor camptothecin, which rendered primary cultured murine hepatocytes sensitive toward apoptosis induction by TNF (24). In contrast, the interaction between TNF and its TNF-R1 has been shown to be crucial in the priming of hepatocytes during liver regeneration (25, 26) and in the proliferation of oval cells during the neoplastic phase of liver carcinogenesis (27). Recent results have also indicated a crucial role of TNF in classical toxicological processes of chemical exposure (28). TNF was shown to be implicated in the regulation of products inducing inflammation and fibrosis but not in direct hepatocyte damage in carbon tetrachloride hepatotoxicity (29). Also in alcohol-mediated liver toxicity, an important role for TNF was suggested recently (30). Moreover, the interaction between TNF and TNF-R2 was suggested to be implicated in fumonisin hepatotoxicity in mice (31).

In this study, we present evidence for a novel mechanism by which an antineoplastic drug induces a significant hepatotoxicity in mice, which is mediated by the induction of membrane-bound TNF expression in Kupffer cells. Notably, the increase of membrane-bound but not secreted TNF on Kupffer cells was shown to be due to direct inhibition by the drug of the enzyme, which cleaves soluble TNF from membrane-bound TNF, i.e., the TNF converting enzyme TACE (32–34). The importance of membrane-bound TNF in the observed melphalan cytotoxicity is not only demonstrated by the core data shown in the results section but also by our following additional observations (data not shown): 1) hepatocytes lacking TNF-R2 were protected from melphalan cytotoxicity, mediated by membrane-bound TNF expressed on Kupffer cells but not from the combined apoptotic effect of melphalan and exogenously added soluble TNF, in the absence of Kupffer cells; 2) melphalan, although increasing the expression of membrane-bound TNF, did not increase but rather inhibits the production of secreted TNF in Kupffer cells; and 3) soluble TNF induced apoptosis in hepatocytes in the presence but not in the absence of melphalan. This latter finding might be explained by the fact that melphalan is known to block also transcription in liver cells (3, 4), thus preventing the synthesis of antiapoptotic factors and allowing soluble TNF to activate apoptosis via TNF-R1 (16, 17). The demonstration of an in vitro inhibition of recombinant TACE by melphalan is to the best of our knowledge the first example of direct action of a low-m.w. drug on this important step of TNF processing, which completes here the interpretation of the toxic mechanism.

Although TNF-R1, which contains a death domain (35, 36), has been shown to mediate apoptosis induced by soluble TNF in hepatocytes (16, 17), not only this receptor type but also TNF-R2, which is preferentially activated by membrane TNF, is causally implicated in the melphalan-induced hepatotoxicity because livers from either TNF-R1- or TNF-R2-deficient mice were resistant. This means that TNF-R2, although devoid of a death domain, is

nevertheless implicated in melphalan-induced apoptosis. Others have obtained similar evidence for such a role of this TNFR type by showing that membrane-bound TNF is implicated in inflammation and degeneration in the CNS of transgenic mice (37–39), as well as in experimental cerebral malaria (20). Moreover, transmembrane TNF was found to be sufficient to mediate localized tissue toxicity and chronic inflammatory arthritis in transgenic mice (40), as well as in Con A hepatotoxicity (41). Because membrane-bound TNF, induced by melphalan, was suggested to preferentially trigger TNF-R2 and soluble TNF preferentially activates TNF-R1, this could explain why melphalan and soluble TNF have an additive effect in hepatotoxicity because the activation of TNF-R2 was reported to enhance the TNF-R1-mediated apoptosis (reviewed in Ref. 42). It is exactly this profile of action that might underlie the potent antitumor action of the drug.

The high extent of hepatotoxicity we observed in our isolated perfused mouse liver model does not correspond to the relatively mild hepatotoxicity reported in patients treated with melphalan and TNF via hepatic perfusion. Besides the reasons of species and dose, this could be because the clinical treatment frequently uses hyperthermia, i.e., a condition that could give rise to increased levels of heat shock proteins. Recent results suggest that induction of heat shock protein 70 in mice kept at 42°C can protect them from the systemic toxicity of TNF, without interfering with the tumoristic effect in a murine melanoma tumor model (43). Because we show in our preclinical setting that melphalan hepatotoxicity is mediated by TNF, hyperthermia could thus potentially confer protection under clinical conditions. The understanding of the mechanism and the time course of melphalan hepatotoxicity can thus provide a basis for the design of clinical treatment regimens that minimize or even abrogate the hitherto inevitable adverse effects of this therapy.

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Disclosures

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