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

Matthias Kresse,* Markus Latta,* Gerald Künstle,* Hans-Martin Riehle,† Nico van Rooijen,‡ Hannes Hentze,* Gisa Tiegs,§ Markus Biburger,§ Rudolf Lucas,2* and Albrecht Wendel2*

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 recombiant TFN-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 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 GlaxoSmithKline. The murine TNF ELISA (OptEIA) was purchased from BD
Pharmacology. The caspase inhibitor benzoylloxycarbonyl-val-alal-asp-fluoro-methylketone (zVAD-fmk) was purchased from Alexis Biochemica.
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 C57BL6 wild-type mice, TNF-R1 knockout mice, TNF-R2 knockout mice, and TNF-R1R2 knockout mice (25 g), originally provided by Dr. H. Bluthmann (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 µl 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 µl 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-µm 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 × g, 4°C) of the lysates, supernatants were frozen immediately and stored at −80°C before measurement. Cytosolic extracts of liver tissue were prepared by Direct 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 aprotime (1 µg/ml each), and 0.1% Triton X-100) and subsequently centrifuged (15 min, 13,000 × 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 µM fluorogenic substrate N-acetyl-asp-glu-val-asp-afc) for 30 min at 37°C using a fluorometer plate reader VICTOR (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 (µM) and milligram of protein, using serially diluted standards (0–5 µM afc). Control experiments confirmed that the activity was linear with time and with protein concentration under the conditions described above.

**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 µg/ml) for various times of incubation. Subsequently, cells were incubated for 60 min at room temperature under mild shaking with either 30 µl/well HBSS (BioWhittaker), fixed for 45 min at room temperature with HBBS + 1% paraformaldehyde, and washed again, after which unspecific binding was blocked by means of incubating the wells with HBBS + 5% BSA (Serva). Subsequently, cells were treated with 10 µg/ml of a polyclonal sheep-anti-mouse TNF Ab (16, 17), all diluted in HBBS + 5% BSA. After two washing steps, cells were incubated for 45 min under mild shaking with 3 ng/ml of either a goat-anti-rat IgG-alkaline phosphatase conjugate or a rabbit-anti-sheep IgG-alkaline phosphatase conjugate and washed twice with HBBS + 5% BSA and once with 2.5 M diethanolamine (pH 9.5). Finally, the substrate solution consisting of 0.58 mg/ml of the

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**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 µg/ml melphalan, as compared with control conditions, was assessed by means of LDH release. All data represent means ± SD of three independent experiments.

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3 Abbreviations used in this paper: zVAD-fmk, benzoylloxycarbonyl-val-alal-asp-fluoro-methylketone; afc, 7-amino-4-trifluoromethylcoumarin; LDH, lactate dehydrogenase; TACE, TNF-α converting enzyme; ALT, alanine aminotransferase.
fluorescent substrate Attophas (Promega) and of 2.4 mg/ml of the endog-

enzous phosphatase activity blocking agent levamisole (Sigma-Aldrich), di-

luted 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 $2 \times 10^5$ 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-$\alpha$ 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-$\alpha$ with biotinylated anti-TNF-$\alpha$-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 incuba-

tion period of 30 min at 4°C before final washing. Flow cytometric analysis

was performed using a FACSScan 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-$\alpha$ 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 moni-
toring TACE activity, an internally quenched fluorogenic substrate (Sub-
strate IV; Merck Biosciences) was used with an excitation maximum of

320 nm and an emission maximum of 420 nm. Experiments were con-
ducted at a temperature of 37°C in a total volume of 1.0 ml containing 200
ng/ml of the recombinant protein, 5 M TACE substrate IV, and various
concentrations of a known inhibitor for TACE (TAPI-1; Merck Bio-
sciences) as positive control or melphalan in concentrations as indicated in
the text. Spectra were recorded over time using the Luminescence Pho-
tometer LS 50B (PerkinElmer) with a slot of 5 nm.

FIGURE 2. Melphalan induces caspase-dependent apoptosis in primary liver cells. A, Primary liver cells treated with 200 $\mu$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 activa-
tion, arising from 9 h posttreatment on. In contrast, mel-

phalan-treated (200 $\mu$g/ml) liver cells were protected

from apoptosis when pretreated with the general caspase

inhibitor z-VAD-fmk (10 $\mu$M), as shown by morpho-

logical analysis of Hoechst-dyed cells (C) or measure-

ment of LDH release (D).

FIGURE 3. Melphalan-induced apoptosis in primary

liver cells is due to TNF. A, Neutralizing rat-anti-mouse

TNF mAb (1F3F3 (10 $\mu$g/ml); Ref. (21) completely ab-

rogated 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 ± SD in percentage of control of

three independent experiments. Experiments with basal

toxicities > 15% were excluded.
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 subjection 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. 1A), leading to a steady increase of LDH release over control incubations from 9 h on after exposure (Fig. 1B). As shown in Fig. 2A, 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 associated by an increase of caspase-3-like protease activity, measured in liver cell lysates (Fig. 2B), 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 IF3F3, 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. 3A). The causal role of TNF in melphalan toxicity

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 × 105 cells/ml) were incubated with 200 μg/ml melphalan for 18 h before measuring LDH release.

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.
adherent Kupffer cells. Cells were incubated with 200 hepatocytes. For control experiments, these hepatocytes were directly plated on the
these inserts were placed on wells containing freshly isolated and additionally purified
dependent measurements, and IC50 values were calculated for TAPI-1 and
B
IC50 transition of melphalan (Fig. 5A) or
A
presence and in the absence of various concentrations of melphalan (Fig. 5B). 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.

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

<table>
<thead>
<tr>
<th>Incubation Conditions</th>
<th>LDH Release (% ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insert with hepatocytes alone</td>
<td>11.7 ± 3.2</td>
</tr>
<tr>
<td>Hepatocytes + melphalan</td>
<td>15.9 ± 5.8</td>
</tr>
<tr>
<td>Hepatocytes + MP contact</td>
<td>14.0 ± 2.4</td>
</tr>
<tr>
<td>Hepatocytes + MP separated + melphalan</td>
<td>24.3 ± 7.4</td>
</tr>
<tr>
<td>Hepatocytes + MP contact + melphalan</td>
<td>75.4 ± 5.5</td>
</tr>
</tbody>
</table>

* Isolated Kupffer cells were plated in a concentration of 10^5 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.

**MP, 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 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 transla-

![FIGURE 6. Inhibition of recombinant TACE by melphalan. Recombi-
nant 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.](http://www.jimmunol.org/)

![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.](http://www.jimmunol.org/)
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. 5B, 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. 5C).

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, 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. 7A). Moreover, the causal role of TNF action in the hepatotoxicity was confirmed by the resistance of livers from TNF-R1−/− or TNF-R2−/− mice against melphalan, as assessed by the release of ALT (Fig. 7B). 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−/−/− or TNF-R2−/−/− 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 recombiant 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), 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 tumoristatic 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
The authors have no financial conflict of interest.

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