Human soluble TRAIL/Apo2L induces apoptosis in a subpopulation of chemotherapy refractory nodal diffuse large B-cell lymphomas, determined by a highly sensitive in vitro apoptosis assay
Human soluble TRAIL/Apo2L induces apoptosis in a subpopulation of chemotherapy refractory nodal diffuse large B-cell lymphomas, determined by a highly sensitive \textit{in vitro} apoptosis assay

Saskia A.G.M. Cillessen,1 Chris J.L.M. Meijer,1 Gert J. Ossenkoppele,1 Kitty C.M. Castricum,1 August H. Westra,2 Petra Niesten,3 Jettie J.F. Muris,1 Hoite F. Nijdam,1 Klaas G. van der Hem,4 Marcel Flens,3 Erik Hooijberg3 and Joost J. Oudejans1

1Department of Clinical Pathology and Haematology, VU University Medical Centre, Amsterdam, 2Department of Otolaryngology, Westfries Gasthuis, Hoorn, 3Department of Internal Medicine and 4Clinical Pathology, Zaan Medical Centre de Heel, Zaandam, the Netherlands

Summary

Resistance to chemotherapy in therapy-refractory diffuse large B-cell lymphomas (DLBCL) is related to inhibition of the intrinsic apoptosis pathway. Human soluble tumour necrosis factor (TNF)-related apoptosis-inducing ligand (hsTRAIL/Apo2L) induces apoptosis via the alternative, death-receptor mediated apoptosis pathway and might be an effective alternative form of therapy for these lymphomas. This study investigated whether hsTRAIL/Apo2L could actually induce apoptosis in isolated lymphoma cells of DLBCL biopsies of patients with chemotherapy refractory DLBCL. Twelve out of a total of 22 DLBCL samples were sensitive to hsTRAIL/Apo2L. These sensitive lymphomas included seven clinically chemotherapy-refractory lymphomas. Furthermore, hsTRAIL/Apo2L induced apoptosis in DLBCL cells and in B-cell lines that showed high expression levels of inhibitors of the intrinsic apoptosis pathway: Bcl-2 and/or X-linked inhibitor of apoptosis (XIAP). hsTRAIL/Apo2L-sensitive lymphoma cells showed expression of the TRAIL receptors R1 and/or R2 and absence of R3 and R4. We conclude that hsTRAIL/Apo2L induced apoptosis in a subpopulation of chemotherapy-refractory nodal DLBCL and that disruption of the intrinsic apoptosis-mediated pathway and expression of Bcl-2 and XIAP did not confer resistance to hsTRAIL/Apo2L-induced apoptosis in DLBCL. Thus, based on our results, further exploration of hsTRAIL/Apo2L as an alternative treatment for patients with chemotherapy refractory DLBCL should be considered.

Keywords: lymphoma, apoptosis, TRAIL/Apo2L, chemotherapy.

Although high-grade lymphomas, including the diffuse large B-cell lymphomas (DLBCLs) are potentially curable with multi-agent chemotherapy, the disease appears to be fatal in 30–50% of patients (Coiffier, 2005). In the majority of these patients, chemotherapy fails to induce complete tumour remission, suggesting that tumour cells are intrinsically resistant to chemotherapy-induced cell death (Lowe et al, 2004).

Many \textit{in vitro} studies have demonstrated that disruption of the apoptosis signalling cascade is an important causative factor in chemotherapy resistance in haematological malignancies (Miyashita & Reed, 1993; Lowe et al, 1994; Friesen et al, 1996; Los et al, 1997; Schmitt et al, 2000; Schmitt & Lowe, 2001). Two major apoptosis pathways have been elucidated: a stress-induced, caspase 9-mediated pathway and a death receptor, caspase 8-mediated pathway (Fig 1). Almost all chemotherapeutic drugs used in the treatment of aggressive lymphomas induce apoptosis via the stress-induced pathway (Johnstone et al, 2002). The death receptor-mediated pathway can be triggered by tumour necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL/Apo2L) (Wiley et al, 1995; Petti et al, 1996; Bodmer et al, 2000). TRAIL/Apo2L induces apoptosis by interaction with two receptors TRAIL-R1 (or death receptor DR4) and TRAIL-R2 (or DR5). However,
The role of c-FlipS as an inhibitor of death receptor-inducing tumour cell death in many different human cancer cells has been shown previously that TRAIL/Apo2L is capable of inducing apoptosis in these cells. In this respect, TRAIL/Apo2L is the most promising candidate because it seems to exert both pro- and anti-apoptotic effects when expressed at physiological levels (Chang et al., 2002; Micheau et al., 2002; Boatright et al., 2004). Although the death receptor-mediated pathway has also been implicated in chemotherapy-induced apoptosis (Debatin & Krammer, 2004), it is possible that this pathway should be intact and can possibly be triggered by TRAIL/Apo2L (Muris et al., 2005).

In this study, we used isolated lymphoma cells of nodal DLBCL biopsies, including chemotherapy-refractory samples, and B-cell lymphoma/leukaemia-derived cell lines to investigate (1) whether human soluble (hs) TRAIL/Apo2L could induce apoptosis in chemotherapy-resistant DLBCL and B-cell lines; (2) whether inhibition of the caspase 9-mediated pathway and expression of Bcl-2 and/or XIAP interfere with sensitivity to hsTRAIL/Apo2L-induced apoptosis; and (3) whether sensitivity to hsTRAIL/Apo2L-induced apoptosis correlated with expression of c-Flip and the TRAIL receptors.

Materials and methods

Patient samples

Biopsies taken from 22 DLBCL patients that were diagnosed between 2000 and 2005 in the Comprehensive Cancer Centre Amsterdam, according to the World Health Organisation (WHO) criteria (Jaffe et al., 2001) were studied. Both primary lymphomas and lymph node samples of relapsed lymphomas were used (see Table I, clinical characteristics). Clinical follow-up data was available from 20 of 22 samples. De novo DLBCL was diagnosed in 16 biopsies and six other biopsies were from patients with relapsed DLBCL. Primary lymphoma samples were divided into chemotherapy-responsive and chemotherapy-refractory cases. DLBCL was considered refractory if no complete remission was achieved or if relapse occurred (according to standard clinical evaluation, including physical examination, chest X-ray, and computed tomography of chest, abdomen and pelvis). All other cases were considered responsive (follow-up period 9–43 months).

Lymphoma cell suspensions were isolated from tissue biopsies by a combination of mechanical and enzymatic dissociation and frozen until further testing. Cells were thawed 1 h before experimental testing and cultured in Iscove’s modified Dulbecco’s medium (IMDM; BioWhittaker, Cambrex, Belgium) supplemented with 10% fetal calf serum, 1% penicillin and streptomycin at 37°C with 5% CO2 in a humidified atmosphere. Non-neoplastic T-cells were removed by using super paramagnetic dynabeads coated with anti-CD3 antibodies (Dynal, Wirral, UK). The purity of the resulting tumour cell fractions was determined by morphological

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**Image:** Fig 1. Schematic representation of both major apoptosis signalling pathways. DNA damage induces p53 controlled cytochrome c release from mitochondria, which then binds to apoptosis-activating factor-1 (Apaf-1), resulting in activation of caspase 9, followed by activation of effector caspases including caspase 3 (Opperman & Korsmeyer, 2003). Bcl-2 inhibits apoptosis at the level of cytochrome c release, while XIAP exerts its anti-apoptotic effect via interaction with active caspase 9 and active caspase 3 (Schimmer et al., 2004). Following ligation, hsTRAIL/Apo2L induces a Death-Inducing Signalling Complex (DISC) composed of the cytoplasmic adapter protein FADD (Fas-associated death domain) and caspase 8 (Bodmer et al., 2000). Activated caspase 8 can activate caspase 3 directly and indirectly by truncation of Bid. The truncated form of Bid (t-Bid) translocates to mitochondria, leading to cytochrome c release and activation of the caspase 9-mediated pathway (Luo et al., 1998). C-Flip regulates caspase 8 activation via interfering with both recruitment and processing of pro-caspases within the DISC (Thome & Tschopp, 2001).
Assessment of caspase dependent cell death in isolated lymphoma cells

Sensitivity to hsTRAIL/Apo2L-induced apoptosis was determined by incubating cells with hsTRAIL/Apo2L produced by Genentech (USA) as described by Ashkenazi et al (1999). Etoposide (VP16; Sigma, St. Louis, MO, USA) was used to assess the functional status of the stress-induced caspase 9 mediated pathway. In simultaneous experiments, isolated lymphoma cells were pre-incubated with 25 μmol/l of the caspase blocker Z-Val-Ala-Asp-fluoromethylketone (z-VAD-fmk, Alexis Biochemicals, Lausen, Switzerland) 1 h prior to exposure to Etoposide or hsTRAIL/Apo2L to investigate whether cell death was caspase dependent. Dose response curves of Etoposide and hsTRAIL/Apo2L in isolated lymphoma cells of eight tested DLBCL resulted in maximal apoptosis inducing concentrations of 300 nmol/l for Etoposide and 100 ng/ml for hsTRAIL/Apo2L consistent with data in literature (Ashkenazi et al, 1999). These concentrations were used for further analyses.

In isolated lymphoma cells, standard fluorescence-activated cell sorting (FACS) analysis with 7-amino-actinomycin D (7AAD) and Annexin V was of little value for the detection of Etoposide- and hsTRAIL/Apo2L-induced apoptosis, as these markers detect (pre-)apoptotic cells that are still intact, but fail to detect fully degraded or dissolved lymphoma cells. To overcome this problem, we added a standard number of fluorescent beads (Fluorospheres; Becton Dickinson, San Jose, CA, USA) to the cells after induction with hsTRAIL/Apo2L or Etoposide. Cell death was determined by FACS analysis to determine the number of viable (7AAD) and Annexin V negative cells in untreated samples with z-VAD; C the number of viable cells after Etoposide or hsTRAIL/Apo2L treatment without z-VAD; D the number of viable cells in untreated samples without z-VAD.

Induction and detection of apoptosis in B-cell lines

The following B-cell lines were used: Nalm6 and SMS-SB (both originating from B-lymphoblastic leukaemia), Ramos, Raji, JVM-3, DoHH2 and JY (all originating from germinal centre B-cell derived lymphomas) and 8226 (a multiple myeloma cell line). Nalm6, Raji, JVM-3 and 8226 were cultured in RPMI 1640 medium (BioWhittaker) containing 10% fetal calf serum (Hyclone, Perbio, Sweden) and 100 IU penicillin/100 μg/ml.

Table I. Characterisation of DLBCL patients.

<table>
<thead>
<tr>
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<th>hsTRAIL/Apo2L</th>
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<tr>
<td></td>
<td>Sensitive</td>
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<tr>
<td>(n = 12)</td>
<td>(n = 10)</td>
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<td>P-value</td>
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Clinical characteristic

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<th>Response to chemotherapy of primary lymphoma biopsies†</th>
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<tr>
<td>Responsive</td>
</tr>
<tr>
<td>Refractory</td>
</tr>
<tr>
<td>No follow-up</td>
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<tr>
<td>Relapses</td>
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Tumour cell characteristic

| XIAP | Negative | 5          | 8          |
|      | Positive  | 5          | 1          |
|      | NS        | 2          | 1          |
| c-Flip | Negative | 9          | 7          |
|       | Positive  | 2          | 2          |
|       | NS        | 1          | 1          |
| GCB or ABC phenotype§ |              |
| GCB | 5          | 4          |
| ABC | 6          | 5          |
| NS  | 1          | 1          |

NS, not significant; NI, not interpretable; GCB, germinal centre B-cell-like; ABC, activated B cell-like.

†Clinical data could be retrieved from 20 of 22 patients.

‡Primary lymphoma samples were divided into chemotherapy responsive and chemotherapy refractory cases. DLBCL were considered responsive if therapy induced complete remission (according to standard clinical evaluation) and no relapse occurred during the follow-up time.

§Including two DLBCL samples which were transformed follicular lymphomas.


Examination of the tumour cells in cytospin preparations stained with CD3 (clone C8B.37, DakoCytomation, Glostrup, Denmark) and CD20 (clone L26, DakoCytomation). This procedure resulted in <5% contaminating, non-neoplastic CD20 negative cells in all cases tested. Approval of the study was obtained from the ethics review board of the VU University Medical Centre. Informed consent was provided according to the Declaration of Helsinki.
streptomycin (Gibco, Invitrogen, Grand Island, NY, USA). SMS-SB, Ramos, DoHH2 and JY were cultured in IMDM medium (BioWhittaker) supplemented with 10% fetal calf serum and penicillin (100 IU)/streptomycin (100 μg/ml).

Dose response curves of cell lines demonstrated that, similar to that found for DLBCL cells, 100 ng/ml hsTRAIL/Apo2L was the optimal concentration for inducing apoptosis. For Etoposide-induced apoptosis, dose response curves showed that 25 μmol/l Etoposide was the optimal concentration for inducing apoptosis (data not shown). When lower concentrations of Etoposide were used (0.5 and 5 μmol/l) no induction of apoptosis was observed, whereas higher concentrations (100 and 200 μmol/l Etoposide) resulted in caspase-independent cell death. Cells lines were incubated for 4, 8, 16 and 24 h with 25 μmol/l Etoposide or 100 ng/ml hsTRAIL/Apo2L.

In cell lines, apoptosis was measured by phosphatidylserine (PS) externalisation with Annexin V-fluorescein isothiocyanate (FITC) (250 μg/ml; VPS Diagnostics, Moeven, the Netherlands) and cell viability with 7AAD (Via-probeTM, 2 μg/ml; Pharmingen, San Diego, CA, USA), according to the manufacturer’s recommendation. Fluorescence was detected by the FACS Calibur flowcytometer and analysed using cell-quest software.

Fig 2. Detection of sensitivity to hsTRAIL/Apo2L in isolated lymphoma cells. (A) FACS analysis of isolated lymphoma cells treated with and without 100 ng/ml hsTRAIL/Apo2L for 24 h (DLBCL2). By standard FACS analysis no decrease in Annexin V-negative, 7AAD-negative cells was observed after induction with hsTRAIL/Apo2L (compare R1 with R2). Also no increase in Annexin V positive, 7AAD positive cells was observed. However, when the number of 7AAD-negative cells was determined per constant number of fluorescent beads a clear decrease in 7AAD-negative cells was observed (compare R3 with R4). (B) Time response curves of isolated lymphoma cells. Shown are 2 examples of DLBCL with high levels of spontaneous apoptosis (top, DLBCL6 ▲ and DLBCL17 ▼) and two examples of DLBCL with low levels of spontaneous apoptosis (bottom, DLBCL2 ▲ and DLBCL18 ▼) induced with 100 ng/ml hsTRAIL/Apo2L. Using the fluorescent beads method it was still possible to detect hsTRAIL/Apo2L sensitivity at t = 4 h in DLBCL6 with high levels of spontaneous apoptosis, hsTRAIL/Apo2L-sensitive DLBCL2 with a low level of spontaneous apoptosis showed maximal apoptosis induction at 24 h, whereas hsTRAIL/Apo2L-resistant DLBCL18 remained resistant at 24 h. Induction in combination with zVAD-FMK showed that hsTRAIL/Apo2L-induced cell death was always caspase-dependent. (C) Spontaneous cell death in relation to hsTRAIL/Apo2L-induced cell death at t = 4 h. DLBCL cases sensitive to hsTRAIL/Apo2L-induced apoptosis usually showed low levels of spontaneous apoptosis.

Detection of caspase 3/7 activity

Caspase 3/7 activity was determined using a fluorimetric homogeneous caspase assay (Roche, Mannheim, Germany), according to the manufacturer’s instructions. Briefly, cell lines were lysed and incubated with DEVD-rhodamine 110 substrate for 1 h at 37°C. Lymphoma cells were lysed and incubated with this substrate for 18 h at 37°C. Subsequently, the amount of free rhodamine was determined with excitation filter 492 nm and emission filter 535 nm at a micro-plate fluorescence reader (TECAN spectrafluor, Männedorf, Switzerland). The developed fluorochrome was proportional to the concentration of activated caspase 3/7 and could be quantified by comparing the detected fluorescence with a calibration curve of diluted free rhodamine. For cell lines caspase 3/7 activation levels were indicated as caspase activation levels of treated samples minus caspase activation levels of untreated samples. For isolated lymphoma cells caspase 3/7 activation levels are reported as ratio of treated cells to untreated cells to correct for the high levels of spontaneous apoptosis. For cell lines and lymphomas samples determination of caspase activation levels was performed in triplicate.
Immunocyto-histochemistry

Immunohistochemical analysis of Bcl-2, XIAP and c-Flip in DLBCL samples was performed as described previously (Muris et al, 2005). Three μm thick sections of paraffin-embedded biopsies were stained with the following antibodies; monoclonal mouse anti c-Flip (clone NF6; Alexis, Lausen, Switzerland), mouse anti-Bcl-2 (clone 124; DakoCytomation) and mouse anti-XIAP (clone 2F1; MBL Nagoya, Japan). For detection of Bcl-2 and XIAP, antigen retrieval in citrate buffer (10 mmol/l, pH 6.0) was performed. Antigen retrieval in TRIS EDTA buffer (10 mmol/l/1 mmol/l, pH 9.0) was required for detection of c-Flip. The catalysed reported deposition (CARD) method (DakoCytomation) was used to detect Bcl-2. For c-Flip and XIAP, a standard, highly sensitive EnVision™ horseradish peroxidase system (DakoCytomation) was used. Visualisations were performed using diaminobenzidine (DAB) as chromogen. C-Flip, XIAP and Bcl-2 were evaluated semi-quantitatively as percentages of all tumour cells as described (Muris et al, 2005). Non-neoplastic lymphoid tissues served as positive control for all tested antibodies. In addition, cytoospin preparations of cell lines were incubated with a specific polyclonal rabbit anti-active caspase 3 antibody (Cell signalling Technology, Beverly Hills, CA, USA) overnight, as described previously (Dukers et al, 2005). The primary antibody was visualised using the EnVision™ horseradish peroxidase system (DakoCytomation) and DAB. Active caspase 3 positive staining was analysed microscopically.

Western blot analysis

Protein extracts of untreated B-cell lines were prepared for Western blot analysis. Cells were washed with phosphate-buffered saline and resuspended in lysis buffer (50 mmol/l Tris/HCl, pH 8.0, 0.5% NP-40, 5 mmol/l EDTA) containing protease inhibitors. Antibodies against the following proteins were used: Bcl-2 (clone 124, 0.225 μg/ml; DAKO, Glostrup, Denmark), FADD (clone 1, 4 μg/ml; BD Biosciences, San Jose, CA, USA), c-Flip (clone DAVE III, 1 μg/ml; Kamiya Biomedical Co., Seattle, WA, USA), pro-caspase 8 (clone 12F5, 1 μg/ml; Imgen, Alexandria, VA, USA) and XIAP (clone 2F1, 1 μg/ml; MBL, Woburn, MA, USA). The proteins were visualised with the enhanced chemoluminescence technique (Amersham Pharmacia Biotech, Piscataway, NJ, USA). For semi-quantitative analysis of the Western blots, sub-saturated auto radiograms were scanned and the obtained signals were analysed using TINA 2.09 software (Raytest, Isotopenmessgeräte, Straubenhardt, Germany). The relative expression levels were correlated to cellular β-actin (AB-1 kit, 6.25 ng/ml; Oncogene Research Products, Darmstadt, Germany) levels, measured as a reference in each sample on the blots.

Detection of TRAIL receptors

For analysis of TRAIL receptor expression, cells were incubated with antibodies against TRAIL receptors R1, R2 and the decoy receptors R3 and R4 (clone HS101, HS201, HS301 and HS402, 10 μg/ml; Alexis) for 15 min at room temperature. An isotype matched antibody was used as the negative control. After washing, cells were incubated with rabbit anti-mouse PE labelled (DAKO) for 15 min at room temperature. Cells were washed and fluorescence was measured on a FACSCalibur flowcytometer. The Relative Fluorescence Intensity (RFI) was calculated as [mean fluorescence intensity (MFI) of the TRAIL/Apo2L receptor] / (MFI IgG1 isotype control).

Statistical analysis

Correlations were calculated using the Mann–Whitney U-test. P-values below 0.05 were considered significant. All analyses were performed using the Statistical Package for the Social Sciences (SPSS) statistical software package (version 10; SPSS Inc., Chicago, IL, USA).

Results

Assessment of caspase-dependent cell death in isolated lymphoma cells

Thirteen samples of isolated lymphoma cells were incubated with Etoposide and hSTRAIL/Apo2L for various periods of time to optimise the conditions. The levels of spontaneous apoptosis were highly variable, ranging from <5% to 20% after 4 h of incubation and from <5% to >80% after 24 h of incubation. Cases with high levels of spontaneous apoptosis in vitro also usually showed high levels of spontaneous apoptosis on the paraffin slides of the corresponding tumours, as demonstrated by staining for active caspase 3 and p89 (data not shown). Time response curves of isolated lymphoma cells showed that, even in five of six tested cases with high levels of spontaneous apoptosis (DLBCL2, 4, 18, 25 and 31), but always already detectable after 4 h of incubation. Detection of apoptosis in these cases at later time points resulted in smaller differences, due to the increasing levels of spontaneous apoptosis observed between 7 and 24 h of incubation (Fig 2B, top).

In sensitive cases with low levels of spontaneous apoptosis, induction of apoptosis was higher at 24 h for all tested cases (DLBCL2, 4, 18, 25 and 31), but always already detectable after 4 h (Fig 2B, bottom). For that reason and for optimal comparison between the different biopsy samples 4 h of incubation was used for further analysis. Levels of spontaneous cell death were not related to differences in sensitivity to Etoposide- or hSTRAIL/Apo2L-induced apoptosis, although relatively low levels of hSTRAIL/Apo2L-induced apoptosis were detected in cases with high levels of spontaneous apoptosis (see Fig 2C).
hsTRAIL/Apo2L induces apoptosis in chemotherapy-refractory DLBCL

In order to determine whether DLBCL tumour cells were sensitive for hsTRAIL/Apo2L- and Etoposide-induced apoptosis, isolated lymphoma cells of 22 DLBCL samples were incubated for 4 h with Etoposide and hsTRAIL/Apo2L in the absence or presence of z-VAD-FMK. Taking 5% apoptosis after 4 h of incubation as a cut-off value, 12 of 22 lymphoma samples appeared to be sensitive to hsTRAIL/Apo2L, including seven chemotherapy refractory lymphomas (Fig 3A and Table I). A cut-off value of 5% apoptosis was used because in hsTRAIL/Apo2L sensitive cases tested (DLBCL2, 4, 25 and 31), 5% or more apoptosis after 4 h of incubation correlated to 20–40% apoptosis after 24 h, which indicated that the cases showing 5% apoptosis after 4 h were sensitive to hsTRAIL/Apo2L.

A fluorimetric assay was used to determine hsTRAIL/Apo2L-induced caspase 3/7 activity in eight DLBCL samples. As expected, hsTRAIL/Apo2L-sensitive lymphoma cells showed increased levels of caspase 3/7 activity after induction with hsTRAIL/Apo2L whereas hsTRAIL/Apo2L-resistant cells showed little increase in caspase 3/7 activity (Fig 3B).

Expression of Bcl-2 and/or XIAP in DLBCL and B-cell lines does not confer resistance to hsTRAIL/Apo2L-induced apoptosis

To investigate the involvement of Bcl-2 and XIAP expression in resistance to hsTRAIL/Apo2L-induced apoptosis, expression of Bcl-2 and XIAP in DLBCL was determined by immunohistochemical analysis and was interpreted as published...
previously (Muris et al., 2005). Sensitivity to hsTRAIL/Apo2L-induced apoptosis was found in both Bcl-2 and/or XIAP positive and negative cases (Table I). Furthermore, no difference in sensitivity to hsTRAIL/Apo2L-induced apoptosis was observed between Germinal Centre B-cell-like (GCB) DLBCL and Activated B-cell-like (ABC) DLBCL (Table I) as determined by the algorithm described by Hans et al. (2004).

Bcl-2 and XIAP expression in B-cell lines was detected by Western blot analysis. Similar to the DLBCL samples, hsTRAIL/Apo2L-induced apoptosis was also observed in Bcl-2 and/or XIAP positive B-cell lines (Fig 5).

hsTRAIL/Apo2L-sensitive DLBCL samples and B-cell lines express TRAIL/Apo2L receptors R1 and/or R2
We investigated whether differences in sensitivity to hsTRAIL/Apo2L correlated with variations in expression of TRAIL receptors by using FACS analysis. Most DLBCL samples showed weak expression of TRAIL receptors R1 and R2 (Fig 3C). If a cut-off value of 5% of hsTRAIL/Apo2L sensitivity was used, hsTRAIL/Apo2L-sensitive DLBCL showed higher levels of TRAIL receptor R1 (P = 0.03) and R2 (P = 0.02) when compared with hsTRAIL/Apo2L resistant-DLBCL. Expression of R3 was not detected in any of the samples, whereas expression of R4 was detected in a very small percentage of cells in two samples (2–5%).

Next, we examined if expression of c-Flip in DLBCL was responsible for resistance to hsTRAIL/Apo2L by immunohistochemistry and interpreted as published previously (Muris et al., 2005). In all, except two, hsTRAIL/Apo2L-sensitive DLBCL cases tested, no expression of c-Flip was detected. However, the most hsTRAIL/Apo2L resistant-DLBCL also showed no expression of c-Flip (Table I).

B-cell lines were also investigated to determine whether resistance to hsTRAIL/Apo2L-induced apoptosis could be due to defects in the caspase 8-mediated pathway or loss of TRAIL receptors. High levels of FADD and pro-caspase 8 were consistently expressed in all B-cell lines tested (Fig 6A). c-FlipS expression was detected in one hsTRAIL/Apo2L-resistant cell line (JVM-3). c-FlipL expression was detected in both sensitive and resistant cell lines (Fig 6B). Most hsTRAIL/Apo2L-sensitive cell lines demonstrated expression of R1 (P = 0.08) and R2 (P = 0.05) in contrast to hsTRAIL/Apo2L resistant cell lines (Fig 6C and D). Expression levels of decoy receptor R3 and R4 were very low in all cell lines tested, showing no differences between hsTRAIL/Apo2L sensitive and resistant cells (data not shown).

Discussion
Approximately 30–50% of DLBCL patients do not respond to chemotherapy or will relapse rapidly. Many studies have
indicated that inhibition of the apoptosis pathways play an important role in chemotherapy resistance (Miyashita & Reed, 1993; Lowe et al., 1994; Friesen et al., 1996; Los et al., 1997; Schmitt & Lowe, 2001). As chemotherapy induces apoptosis mainly via the stress-induced caspase 9-mediated pathway we investigated if apoptosis could be induced via the death receptor caspase 8-mediated pathway with hsTRAIL/Apo2L. Many groups have tested the functionality of the apoptosis cascade in cell lines, but in our study we examined the functional sensitivity to apoptosis in isolated lymphoma cells of DLBCL.

Testing of apoptosis sensitivity in DLBCL in vitro is hampered by the frequently high levels of spontaneous apoptosis, resulting in rapid cell death, sometimes within 8 h after thawing the isolated lymphoma cells. In cases with high levels of spontaneous apoptosis hsTRAIL/Apo2L- and Etoposide-induced apoptosis could be detected after 4 h of incubation, whereas detection of apoptosis at later time points resulted in smaller differences, due to high levels of spontaneous apoptosis. In all cases with low levels of spontaneous apoptosis, induction of apoptosis was higher at later time points, but already detectable within 4 h. In sensitive cell lines and in sensitive lymphomas with low levels of spontaneous apoptosis, increased levels of hsTRAIL/Apo2L- and Etoposide-induced apoptosis were observed after longer incubation times, indicating that the relatively low levels of Etoposide- and hsTRAIL/Apo2L-induced apoptosis after 4 h of incubation do indicate high sensitivity to hsTRAIL/Apo2L- and Etoposide-induced apoptosis. Moreover, detection of hsTRAIL/Apo2L-induced apoptosis by FACS analysis correlated well with induction of caspase 3/7 activity, which indicates strongly that hsTRAIL/Apo2L induced the apoptosis cascade in sensitive cases. Thus, our assay proved to be sensitive enough to detect Etoposide- and hsTRAIL/Apo2L-induced apoptosis also in cases showing very high levels of spontaneous apoptosis.

The most important observation in our study was that hsTRAIL/Apo2L induced apoptosis in isolated lymphoma cells of DLBCL, including lymphomas refractory to standard CHOP (cyclophosphamide/doxorubicin/vincristine/prednisone)-based chemotherapy, also expression of the apoptosis inhibitory proteins Bcl-2 and/or XIAP and functional inhibition of the Etoposide-induced apoptosis pathway did not necessarily interfere with hsTRAIL/Apo2L sensitivity. Similar data have been reported previously for chemotherapy-resistant multiple myeloma cells (Walczak et al., 1999; Mitsiades et al., 2001; LeBlanc & Ashkenazi, 2003), indicating that the caspase 8-mediated pathway could still be triggered with a functional blockade in the caspase 9-mediated pathway and vice versa. Furthermore, these data suggest that hsTRAIL/Apo2L-induced apoptosis does not necessarily involve activation of the damage-induced caspase 9-mediated pathway and this was consistent with a recent paper by Rudner et al. (2005).

As expected, hsTRAIL/Apo2L-sensitive cell lines and DLBCL cells demonstrated relatively high levels of the TRAIL/Apo2L receptors R1 and R2 and absence of the decoy receptors R3 and R4. However, differences in expression of TRAIL receptors R1 and R2 could not be an explanation of all the observed differences in sensitivity to hsTRAIL/Apo2L induced apoptosis, which was consistent with previous reports (Luciano et al., 2002; Kolb et al., 2003; Tafuku et al., 2006). This indicates that other factors are involved that determine the sensitivity to apoptosis. A probable factor that influences sensitivity is c-Flip. c-Flip expression was observed in one of the TRAIL/Apo2L resistant cell lines (JVM-3), indicating possible contribution to hsTRAIL/Apo2L resistance. C-Flip was also detected in both hsTRAIL/Apo2L sensitive cell lines, indicating that c-Flip alone is not a major inhibitor of hsTRAIL/Apo2L-induced apoptosis in B-cell lines, consistent with a previous study (Kang et al., 2003). In DLBCL, c-Flip expression was detected in both hsTRAIL/Apo2L-sensitive and -resistant cases, with immunohistochemistry cannot differentiate between c-FlipS and c-FlipL. Therefore, the contribution of c-Flip to resistance to hsTRAIL/Apo2L-induced apoptosis in DLBCL remains uncertain.
Based on expression levels of Bcl-2, XIAP, c-Flip and caspase 3 activity we have previously shown that primary nodal DLBCL can be separated into three groups: (1) one with a caspase 8 inhibition profile; (2) one with a caspase 9 inhibition profile; (3) one with a caspase 8 and caspase 9 inhibition profile (Muris et al., 2005). Clinical outcome in the group of lymphomas with a caspase 9 inhibition profile proved to be fatal in most cases. As it is likely that the caspase 8-mediated pathway in these lymphomas is fundamentally intact, hsTRAIL/Apo2L could be a valuable treatment in this specific group of DLBCL.

We conclude that hsTRAIL/Apo2L induces apoptosis in some chemotherapy refractory nodal DLBCL and that disruption of the caspase 9-mediated pathway and expression of Bcl-2 and XIAP do confer resistance to hsTRAIL/Apo2L-induced apoptosis in DLBCL and B-cell lines. Thus, based on our results, further exploration of hsTRAIL/Apo2L as an alternative treatment for patients with chemotherapy refractory DLBCL should be considered.

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