CD20 monoclonal antibody therapy for B-cell lymphoma
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Intracellular pathways of CD20-induced apoptosis

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Abstract

The apoptotic pathway activated by chimeric CD20 monoclonal antibodies (mAbs) was analyzed using the Burkitt-lymphoma cell line Ramos. Crosslinking of CD20 with goat anti-human mAbs (CD20XL), but not ligation of CD20 alone, induced apoptosis in Ramos cells, as demonstrated by annexin V binding and DNA fragmentation. Ramos subclones, selected for resistance to either Fas- or B-cell receptor (BCR)-induced apoptosis, were all sensitive to CD20XL-induced apoptosis, indicating that the pathway of CD20-induced apoptosis is different from those activated by Fas or the BCR. CD20XL-induced apoptosis involved mitochondrial membrane permeabilisation (MMP), as shown by loss of mitochondrial membrane potential ($\Delta \psi_m$), the release of cytochrome c, and activation of caspases-9 and -3. Preincubation with the broad-spectrum caspase inhibitor zVAD-fmk completely inhibited processing of caspase-3, -9 and PARP as well as DNA-fragmentation, but only partially inhibited CD20XL-induced apoptosis (as measured by annexin V staining). CD20XL-induced MMP was not prevented by zVAD-fmk. Furthermore, Bcl-2-overexpression did not affect sensitivity to CD20XL-induced apoptosis. We conclude that CD20XL most likely initiates a mitochondria-dependent apoptosis pathway that is different from both Fas- and BCR-mediated pathways. Furthermore, CD20XL-induced apoptosis is only partially dependent on active caspases. Because most chemotherapeutic drugs require the activation of caspases to exert their cytotoxicity, our data provide an important rationale for the use of CD20 mAbs in chemoresistant malignancies.
Introduction

The chimeric CD20 monoclonal antibody (mAb) IDEC-C2B8 (rituximab) has become an important treatment modality in low-grade non-Hodgkin’s lymphoma (NHL). Its application in other CD20-positive B-cell malignancies (e.g. aggressive lymphoma’s, post-transplant lymphoma, or chronic lymphocytic leukemia (CLL)), is rapidly expanding. Because it has been reported that CD20 mAbs can induce apoptosis in B-cell lines, this effector mechanism, in addition to complement-dependent cytotoxicity and antibody-dependent cellular cytotoxicity, may very well contribute to the clinical results.

Apoptosis, or programmed cell death, is characterized by typical morphological and biochemical changes like cell shrinkage, membrane blebbing, exposure of phosphatidyl serine (PS) to the outer leaflet of the cytoplasmic membrane, chromatin condensation and DNA fragmentation. The execution of apoptosis is mediated by a cascade of cell-death effector molecules in which caspases, a family of cysteine proteases that cleave their substrate after aspartate residues, play a crucial role. Caspases can be divided into initiator caspases (caspase-2, -8, -9, -10), which exert a regulatory role during apoptosis by activating downstream (effector) caspases, and effector caspases (caspase-3, -6, -7), which function as executioners of apoptosis by cleaving essential cellular and nuclear structures like lamins, histones, DNA-repair enzymes or inhibitors of endonucleases.

Two major caspase-dependent pathways leading to apoptosis have been identified (reviewed by Budihardjo et al. ref 19): the classical death-receptor mediated form of apoptosis, and a mitochondria-dependent pathway. The death-receptor mediated pathway is activated after ligation of death receptors, e.g. CD95 (Fas), the TNF receptor or other TNF-receptor family members, leading to the formation of a death-inducing signaling complex (DISC). The activation of the initiator caspase-8, after its recruitment to the DISC, is the critical event in this pathway. Caspase-8 activates downstream effector caspases, either directly (type I cells) or by cleaving Bid, which induces the release of cytochrome c (cyt-c) from the mitochondria (type II cells). The second form of caspase-dependent apoptosis is mainly controlled by mitochondria and is triggered by for example gamma-irradiation, cytotoxic drugs or p53. These stimuli induce mitochondrial release of cyt-c that, in the presence of ATP and apoptosis promoting factor 1 (Apafl) activates the initiator caspase-9. Caspase-9 then activates downstream effector caspases (e.g. caspase-3, -6, -7), resulting in the execution of cell death. Thus, caspase-9 plays a central role in the mitochondrial pathway.

Recently, several studies have been published describing caspase-independent forms of cell death, in which caspases are either not activated, or are activated but not essential for the induction of cell death. Although in these latter studies cell death was not
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prevented by caspase-inhibitors, the nuclear features of apoptosis (DNA fragmentation, chromatin condensation) disappeared, indicating that active caspases are required for the characteristic nuclear features of apoptosis.

The aim of this study was to establish apoptotic pathways activated by chimeric CD20 mAbs. To this end, the Burkitt-lymphoma cell line Ramos was used, as well as clones of Ramos with different sensitivities to Fas and/or B-cell receptor (BCR)-mediated apoptosis. Ligation of Fas results in activation of the death-receptor pathway, whereas cross-linking of the BCR is likely to activate a mitochondria-dependent pathway, since it induces the release of cyt-c and does not involve cleavage of caspase-8. However, both pathways eventually converge at the cleavage of caspase-3.

Materials and Methods

Antibodies and reagents

Chimeric CD20 mAbs (IDE-C2B8, rituximab) were supplied by Roche Nederland B.V. (Mijdrecht, The Netherlands). Fas10 mAbs and Fas2 mAbs (blocking antibodies to the Fas receptor) were a kind gift from Prof. Dr. L. Aarden (CLB Sanquin Blood Supply Foundation, Amsterdam, The Netherlands). Anti-human IgM mAbs (CLB/MH15) and CD40 mAbs (CLB-CD40/1, clone 14G7) were obtained from the CLB Sanquin Blood Supply Foundation (CLB, Amsterdam, The Netherlands). Goat anti-human IgG (Fcγ-fragment specific) mAbs were used as crosslinking antibodies (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA). The broad-spectrum caspase inhibitor zVAD-fmk was obtained from Alexis Biochemicals (Läuferlingen, Switzerland). FITC-labeled annexin V was from Nexins Research B.V. (Kattendijke, The Netherlands). Propidium iodide (PI) was from Sigma Chemical Co. (St. Louis, MO, USA).

The following antibodies were used in immunoblot analysis: polyclonal antibodies to caspase-3, monoclonal antibodies to cyt-c (7H8.2C12) (both from Pharmingen, San Diego, CA, USA), polyclonal antibodies to caspase-8 (a kind gift from Prof. Dr. J. Borst, The Netherlands Cancer Institute, Amsterdam, The Netherlands), monoclonal antibodies to poly (ADPribose) polymerase (PARP) (clone C-2-10, Biomol. Research Laboratories Inc. Plymouth Meeting, PA, USA), polyclonal antibodies to caspase-9 (New England Biolabs. Inc., Beverly, MA, USA), and monoclonal antibodies to Bcl-2 (DAKO A/S, Glostrup, Denmark). Horseradish peroxidase (HRP)-conjugated goat anti-mouse (GAM) immunoglobulins (Ig) were from the CLB (Amsterdam, The Netherlands), HRP-conjugated swine anti-rabbit (SwAR) Ig were from DAKO A/S (Glostrup, Denmark). Hoechst 33258 and MitoTracker Orange were from Molecular Probes (Leiden, The Netherlands).
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Cell lines

The Burkitt-lymphoma cell line Ramos (obtained from the ATCC; Ramos wild type, further referred to as Ramos) as well as Ramos-derived clones with different sensitivities for apoptosis induced via either Fas or the BCR\(^{35}\) (generated in our own laboratory) were used to study CD20-induced apoptosis. The clones used in this study are Ramos.FSA (highly sensitive to Fas), Ramos.FR3 (resistant to Fas) and Ramos.M3 (resistant to anti-IgM and Fas; S. Lens, unpublished data). All clones consistently displayed a comparable expression of CD95 (Fas), sigM and CD20\(^{35}\) (and own observations, data not shown). Cells were cultured in Iscove's modified Dulbecco's medium (IMDM; Gibco Life Technologies, Paisly, Scotland), supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS; ICN Biomedicals GmbH, Meckenheim, Germany), 100 U/mL penicillin, 100 mg/mL streptomycin and L-glutamin (Gibco). Bcl-2 cDNA was a gift from John Reed (Burnham Institute, La Jolla, CA) and cloned into the pCEP4 vector (Invitrogen, San Diego, CA). Sequences were confirmed by automated sequencing. Stable Ramos transfectants were generated by electroporation, and hygromycin B resistant clones were isolated by limiting dilution. Stable transfectants were screened by RNA blots using probes for Bcl-2 probe and by Western Blots using anti-Bcl-2 (Ancell). The Bcl-2 overexpressing Ramos cells were cultured in IMDM culture medium as described above, supplemented with hygromycin B (300 μg/mL).

Induction of apoptosis

Cells were stimulated in 48-wells plates or 25 mm\(^2\) culture flasks (0.25 - 1.0 x10\(^6\)/mL) with the indicated mAbs (5 μg/mL) for 4 hrs (Fas) or 24 hrs (chimeric CD20 mAbs and anti-IgM) unless indicated otherwise. Crosslinking mAbs (final concentration 50 μg/mL), CD40 mAbs (ascites dilution 1/500) and/or Fas2 mAbs (5 μg/mL) were added 5 min after the indicated mAbs. The broad-spectrum caspase inhibitor zVAD-fmk (final concentration as described in results) was added 60 min before the indicated mAbs.

Detection of apoptotic cells

Phosphatidyl serine (PS) exposure on apoptotic cells was measured as described previously.\(^{39}\) In brief, cells were harvested and washed in ice-cold HEPES buffer (10 mM HEPES, 150 mM KCl, 1 mM MgCl\(_2\) and 1.3 mM CaCl\(_2\), pH 7.4) supplemented with 1 mg/mL glucose and 0.5% BSA. Cells were then incubated with FITC-labeled annexin V (diluted 1/500 in HEPES buffer) for 15 min and washed again in HEPES buffer. Just before analysis of the samples by flow cytometry (FACS-scan, Becton Dickinson, San Jose, CA, USA), propidium iodide (PI) was added (final concentration 5 μg/mL) to distinguish necrotic cells (annexin V+/PI+) from apoptotic cells (annexin V+/PI- (early apoptotic cells) and annexin V+/PI+ (late apoptotic cells).
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Immunoblot analysis

Lysates were prepared by suspending stimulated or unstimulated cells (2.0 x 10^6) in 50 µL of lysis buffer (1% NP40, 0.01 M triethanolamine-HCl pH 7.8, 0.15 M NaCl, 5 mM EDTA, 1 mM TLCK, 0.02 mg/mL ovomucoid trypsin inhibitor, 1 mM PMSF, 0.02 mg/mL leupeptin and 25 µM phenylarsine oxide). Lysates were cleared by centrifugation at 13,000g for 15 min. Protein contents in cell lysates were determined using BCA protein assay (Pierce, Rockford, Illinois). Thirty to 50 µg of protein was separated by electrophoresis on SDS-polyacrylamide gels (SDS-PAGE) (10% (caspase-9), 11% (caspase-8), 12.5% (caspase-3 and Bcl-2) or 13% (cyt-c)) under reduced conditions. Proteins were then transferred onto nitrocellulose or PVDF membranes (Hybond-C and Hybond-P respectively, Amersham, Buckinghamshire, U.K.) and blots were blocked with 5% non-fat dry milk in TBST (10 mM Tris, 150 mM NaCl and 0.05% Tween-20, pH 8.0). Blots were probed with the indicated antibodies diluted in TBST containing 2.5% non-fat dry milk. Immunoreactive proteins were visualized using HRP-conjugated Ig (GAM or SwAR) and enhanced chemiluminescence (ECL, Amersham, Buckinghamshire, U.K.).

Preparation of cytosolic cell extracts for analysis of cyt-c release

Cells (5.0 x 10^6 per sample) were washed twice in ice-cold PBS and resuspended in 100 µL of extraction buffer (50 mM PIPES-KOH pH 7.4, 220 mM mannitol, 68 mM sucrose, 50 mM KCl, 5 mM EGTA, 2 mM MgCl_2, 1 mM dithiothreitol and the protease inhibitors leupeptin, trypsin and PMSF, concentrations as described for NP40 lysis buffer) and allowed to swell on ice for 30 min. This was followed by incubation with digitonin (final concentration 200 µg/mL) for 15 min at 4°C. Lysates were centrifuged at 13,000g for 15 min and supernatants were harvested and stored at −20°C until use.

Analysis of DNA fragmentation by TUNEL assay

DNA strand breaks were detected using the APO-DIRECT™Kit (Phoenix Flow Systems Inc., San Diego, USA) according to the manufacturer’s recommendations. In brief, cells (1.0 x 10^6) were washed in PBS, taken up in 1% paraformaldehyde (cell fixation), and permeabilized in 70% ethanol. Fluorescin-tagged dUTP was coupled to the DNA strand breaks using terminal deoxynucleotidyl transferase (TdT).

Evaluation of mitochondrial membrane potential (Δψ_m)

To determine Δψ_m, MitoTracker Orange (CMTMRos) was used according to the manufacturer’s recommendations. A decrease in MitoTracker fluorescence as compared to non-stimulated cells is considered to be a measure for loss of Δψ_m. In brief, cells (0.25 x 10^6/mL) were either not stimulated or incubated with the indicated
mAbs for 4 hrs (Fas) or 24 hrs (anti-IgM and CD20). Cells were incubated with MitoTracker (final concentration 200 nM), for 30 min at 37°C. Fluorescence intensity of MitoTracker was visualized by FACScan-analysis. For flow cytometry, cells were washed twice in HEPES buffer, (double-)stained with FITC-labeled annexin V (as described above) and immediately analyzed on a FACS-scan.

Morphology and immunofluorescence
Cells (0.25 x 10^6/mL) were either not stimulated or stimulated with the indicated mAbs. After 4 hrs (Fas) or 24 hrs (anti-IgM and CD20), cytospin slides were prepared. To evaluate morphology of the cells, slides were stained with Jenner/Giemsa and evaluated by light microscopy. To detect apoptotic nuclei, slides were stained with Hoechst 33258 according to the manufacturer’s recommendations. In brief, slides were fixed (with paraformaldehyde 4%) and stained with Hoechst 33258 (final concentration 0.5 μg/mL) for 30 min. Cells were analyzed by fluorescence microscopy.

Results

Apoptotic features induced by crosslinking of CD20 mAbs
Ramos cells were incubated with chimeric CD20 mAbs (5 μg/mL) in the presence or absence of goat anti-human IgG (50 μg/mL) and apoptotic cells were detected by annexin V staining (fig.1). Incubation with either CD20 or crosslinking antibodies alone did not induce apoptosis in Ramos cells. However, crosslinking of CD20 mAbs with goat anti-human IgG (further referred to as CD20XL) did induce apoptosis. Time course experiments showed that apoptosis

Fig. 1 CD20-induced apoptosis requires crosslinking
Ramos cells were untreated (A) or incubated for 24 hrs with chimeric CD20 mAbs (5 μg/mL) (B) or CD20 + goat anti-human IgG (5 μg/mL + 50 μg/mL) (C). Apoptosis was detected by annexin V staining as described in Materials and Methods. Quadrant markers were set using the medium control sample. Percentages for each quadrant are given.
started 8 hrs after incubation, reaching a maximum (~ 50% apoptotic cells) after 24 hrs (data not shown).

Characteristic nuclear features of apoptosis, like DNA fragmentation and chromatin condensation, were observed during CD20XL-induced apoptosis (fig. 2). DNA fragmentation was evidenced by TUNEL assay (fig. 2A) and chromatin condensation was observed after staining of the CD20XL-treated cells with Hoechst 33 258 (fig. 2B). Furthermore, cytospin slides stained with Jenner/Giemsa also demonstrated pyknotic nuclei (data not shown).

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**Fig. 2 Nuclear features of CD20XL-induced apoptosis**

(A) Ramos.FSA cells were incubated with Fas (4 hrs) or CD20XL (24 hrs) in the presence or absence of zVAD-fmk (concentrations of zVAD-fmk as described in legends of fig. 4). Cells were stained with fluorescin-tagged dUTP and PI to detect DNA strand breaks as described in ‘Materials and Methods’. Quadrant markers were set using the medium control sample. Percentages of dUTP positive cells are depicted. The percentage of annexin V positive cells for each sample is given in parenthesis. (B) Ramos.FSA cells were incubated with CD20XL for 24 hrs. Cytospin slides were prepared and stained with Hoechst 33258. Apoptotic nuclei are indicated with an arrow.
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The apoptosis-inducing pathway activated by CD20XL is different from the pathways activated by Fas or the BCR.

To investigate a possible role for the Fas/FasL system in CD20XL-induced cell death, the influence of antagonistic antibodies to the Fas receptor on CD20XL-induced apoptosis was studied. Fas2 mAbs completely inhibited Fas-induced apoptosis in Ramos, whereas they did not affect CD20XL-induced apoptosis (fig. 3A) showing that CD20XL-induced cell death is not dependent on the Fas/FasL interaction.

In order to investigate whether CD20XL-induced apoptosis is executed via the intracellular part of the Fas pathway, we used Ramos cells and Ramos clones with a different sensitivity to Fas-induced cell death. CD20XL-induced apoptosis was comparable in Ramos.FSA, which is highly sensitive to Fas-induced apoptosis, Ramos.FR3, which is resistant to Fas-induced apoptosis and Ramos (wild type) (fig. 3A and 3B), suggesting that CD20XL-induced apoptosis is not executed through the Fas pathway.

Next, CD20XL-induced apoptosis was compared with BCR-induced apoptosis. CD40 mAbs, which are known to prevent BCR-induced apoptosis, did not inhibit CD20XL-induced apoptosis (fig. 3C). Rather, CD40 mAbs slightly stimulated CD20XL-induced apoptosis. Furthermore, Ramos.M3, which is resistant to BCR-induced apoptosis, was sensitive to CD20XL-induced apoptosis (fig. 3D).

Fig. 3 The apoptosis-inducing pathway used by CD20XL is different from the pathways activated after ligation of Fas or the BCR

Cells were incubated with Fas, anti-IgM or CD20XL, for 4 hrs (Fas) or 24 hrs (anti-IgM and CD20XL). Fas2 mAbs and CD40mAbs were added 5 min after the indicated antibodies. Apoptosis was detected by annexin V staining as described in Materials and Methods. Data are mean ± SEM of at least 3 experiments. (A) Fas-induced apoptosis and not CD20XL-induced apoptosis of Ramos (wild type) can be inhibited by Fas2 mAbs. (B) Ramos.FSA (highly sensitive to Fas-induced apoptosis) and Ramos.FR3 (resistant to Fas-induced apoptosis) are both sensitive to CD20XL-induced apoptosis. (C) BCR-induced apoptosis and not CD20XL-induced apoptosis in Ramos (wild type) is inhibited by CD40 mAbs. (D) Ramos.M3 (resistant to BCR-induced apoptosis) is sensitive to CD20XL-induced apoptosis.
These results together suggest that the pathway activated by CD20XL is different from both the Fas and the BCR-induced apoptosis pathways.

Caspase activation in CD20XL-induced apoptosis

To further explore the pathway of CD20XL-induced cell death, the involvement of caspases was investigated. First, the processing of caspase-3 upon CD20XL was studied. During CD20XL-induced cell death caspase-3 was processed into two fragments (17 kDa and 19 kDa) (fig. 4A). These products are formed after initial cleavage of the caspase-3 proform at Asp-175 (resulting in a 20 kDa product), followed immediately by autocatalytic cleavage at Asp-9 and Asp-28, resulting in the observed 17 kDa and 19 kDa products. PARP, a DNA-repair enzyme that is cleaved by effector caspases and is considered to be a hallmark of apoptosis, was cleaved during CD20XL-induced apoptosis (fig. 4A), as well as during Fas- and BCR-induced apoptosis, confirming the involvement of active caspases in CD20XL-induced cell death.

To investigate the apoptosis pathway upstream of caspase-3, the activation of several other (initiator) caspases, as well as the involvement of the mitochondria were investigated. As is shown in fig. 4B, CD20XL induced the release of cyt-c and the activation of caspase-9. Furthermore, in contrast to Fas-induced apoptosis, CD20XL-induced apoptosis was accompanied by a decrease in MitoTracker fluorescence as compared to untreated cells (fig. 4C), indicating loss of ΔΨm. Time course experiments showed that in Fas-induced apoptosis a decrease in MitoTracker staining occurred after 8 hrs, i.e. after PS-exposure (not shown).

CD20XL also induced the processing of caspase-8. However, since time course experiments demonstrated that cleavage of caspase-8 occurred subsequent to caspase-3 processing (data not shown), we conclude that caspase-8 is not instrumental in the initiation phase of CD20XL-induced apoptosis.

Taken together, these findings demonstrate that CD20XL-induced apoptosis involves the induction of mitochondrial membrane permeabilisation (MMP) and the activation of caspases-9 and -3.

ZVAD-fmk only partially inhibits CD20XL-induced apoptosis

Several studies have reported a form of cell death that is independent of active caspases. Although during this form of caspase-independent cell death caspases might be activated, caspase-inhibitors do not prevent cell death. To study whether caspase activation is required for CD20XL-induced apoptosis, we used the broad-spectrum caspase inhibitor ZVAD-fmk. Interestingly, pre-incubation with 200 μM ZVAD-fmk only minimally prevented CD20XL-induced apoptosis, as measured by annexin
Intracellular pathways of CD20-induced apoptosis

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- Caspase-3
  - p20
  - p19
  - p17

- PARP
  - p116
  - p89

**B**

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- Cyt-c
  - p15

- Caspase-9
  - p37

**C**

![propidium iodide](image)

- Fas
- Fas + zVAD-fmk
- CD20XL
- CD20XL + zVAD-fmk

![Mito Tracker](image)

- annexin V

Fig. 4 CD20XL-induced apoptosis involves the cleavage of caspases-3 and -9, preceded by mitochondrial membrane permeabilisation

(A) Ramos.FSA cells were incubated with Fas, anti-IgM or CD20XL, for 4 hrs (Fas) or 24 hrs (anti-IgM and CD20XL) in the presence or absence of zVAD-fmk. Different concentrations of zVAD-fmk were used: Fas: 50μM of zVAD-fmk, anti-IgM: 100μM, CD20XL: 200μM. Western blots were performed using polyclonal antibodies to caspase-3 and monoclonal anti-PARP antibodies. The cleaved forms of caspase-3 (20, 19 and 17 kDa) and the pro- and cleaved forms of PARP (116 kDa/89 kDa) are indicated.

(B+C) Ramos.FSA cells were incubated with Fas (4 hrs) or CD20XL (24 hrs) in the presence or absence of zVAD-fmk (concentrations as described above). (B) For detection of cyt-c (15 kDa), cytosolic lysates were prepared as described and blots were stained with monoclonal antibodies to cyt-c. Cleavage of caspase-9 was demonstrated using polyclonal antibodies detecting only the cleaved form of caspase-9 (37 kD).

(C) Cells were either stained with annexin V and PI (upper panel) or with annexin V and Mito Tracker Orange (lower panel), and analyzed by flow cytometry. In contrast to Fas-induced apoptosis, CD20XL-induced apoptosis was accompanied by a decrease in Mito Tracker fluorescence, indicating the loss of Δψm. In the presence of zVAD-fmk, the percentage of annexin V positive cells decreased, whereas CD20XL-induced loss of Δψm was not inhibited. During Fas-induced apoptosis, loss of Δψm was found to occur after 8 hrs, i.e. after PS exposure (not shown). Both Fas-induced PS exposure and loss of Δψm were completely prevented by zVAD-fmk.
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**Fig. 5** CD20XL-induced apoptosis can only partially be inhibited by the broad-spectrum caspase inhibitor zVAD-fmk

Ramos, FSAA cells were incubated with Fas, anti-IgM or CD20XL for 4hrs (Fas) or 24 hrs (anti-IgM and CD20XL) in the presence or absence of zVAD-fmk (concentrations as described in legend of fig. 4). Cells were stained with annexin V and PI and the percentage of apoptotic cells was measured.

V staining (fig. 5). Even the highest concentration of zVAD-fmk tested (500 μM) only partially prevented CD20XL-induced apoptosis, similar to inhibition with 200 μM zVAD-fmk. Therefore, the concentration of 200 μM was used in further experiments. The percentage of apoptosis was 49 ± 3% in the absence of zVAD-fmk, versus 28 ± 2% in the presence of zVAD-fmk (n=11; the percentages are corrected for the amount of spontaneous apoptosis in untreated samples). This was in sharp contrast to Fas- and BCR-induced apoptosis, which were both completely prevented by lower concentrations of zVAD-fmk (50 μM and 100 μM of zVAD-fmk respectively for Fas and BCR-induced apoptosis, fig. 5).

To confirm these observations, we verified whether zVAD-fmk indeed prevented the activation of caspases in these experiments. Although BCR-triggering and CD20XL in the presence of zVAD-fmk resulted in a 20 kDa cleavage product of caspase-3, this was not accompanied by processing of PARP (fig. 4A), indicating that this cleavage product of caspase-3 was not active. Furthermore, caspase-9 remained in precursor state when zVAD-fmk was present (fig. 4B). Therefore we concluded that caspases were indeed not activated in the presence of zVAD-fmk.

The nuclear features of apoptosis that were observed during CD20XL-induced cell death were prevented by zVAD-fmk (fig. 2 and data not shown). These results are in agreement with other studies demonstrating that the nuclear features of apoptosis are mediated by caspases. Cyt-c release and loss of Δψₘ were not inhibited by the caspase-inhibitor zVAD-fmk (fig. 4B+C). However, a considerable amount of the cells that showed loss of Δψₘ in the presence of zVAD-fmk, were annexin V negative (fig. 4C). In summary, zVAD-fmk completely inhibited CD20XL-induced caspase-activation and nuclear features of apoptosis, partially inhibited CD20XL-induced exposure of phosphatidyl serine, but did not inhibit cyt-c release and loss of Δψₘ.

CD20XL-induced apoptosis is not inhibited by overexpression of Bcl-2

Bcl-2 is known to prevent apoptosis by preventing cyt-c release. Since CD20XL-induced apoptosis involved cyt-c release, we investigated the influence of Bcl-2 expression on CD20XL-
induced apoptosis. To this end, Ramos cells transfected with Bcl-2 (Ramos.Bcl-2) were used. The overexpression of Bcl-2 in this clone was confirmed by western blotting (fig. 6A). As is shown in fig. 6B, Ramos.Bcl-2 cells were sensitive to CD20XL-induced apoptosis and resistant to Fas-induced apoptosis. Furthermore, CD20XL induced the release of cyt-c (fig. 6C) and loss of ΔΨm (not shown) in Ramos.Bcl-2.

**Fig. 6 CD20XL-induced apoptosis is not inhibited by Bcl-2**
(A) Lysates were prepared from unstimulated Ramos (wt) and Ramos.Bcl-2 cells. Western blots were prepared using monoclonal anti-Bd-2 antibodies. (B) Ramos.Bcl-2 cells were incubated with Fas or CD20XL (both for 24 hrs) and the percentage of apoptosis was measured by annexin V staining. Data are mean ± SEM of 4 experiments. (C) For detection of cyt-c, cytosolic lysates were prepared as described and blots were stained with monoclonal antibodies to cyt-c.

**Discussion**

In the present study, we investigated the apoptosis pathway activated by chimeric CD20 mAbs. The main observations made in this study are: chimeric CD20 mAbs only induce apoptosis after crosslinking (CD20XL); CD20XL-induced apoptosis is initiated via a pathway that is different from the pathways activated by Fas or the BCR; CD20XL induces mitochondrial membrane permeabilisation (MMP) and activation of caspases-9 and -3; CD20XL-induced apoptosis is only partially prevented by zVAD-fmk and CD20XL-induced apoptosis not inhibited by high Bcl-2 expression.

In agreement with previous observations\(^45\), we found that chimeric CD20 mAbs only induce apoptosis after crosslinking (CD20XL). Apoptosis started after 8 hours and reached a maximum of ~50% of apoptotic cells after 24 hours.

Several observations lead to the conclusion that the apoptosis pathway activated by CD20XL is different from the well-known Fas death-receptor pathway. First, CD20XL-induced apoptosis could not be inhibited by antagonistic antibodies interfering with the Fas/FasL interaction. Second, it was demonstrated that crosslinking of CD20 induced apoptosis in a
Fas-resistant clone of Ramos. Finally, in CD20XL-induced apoptosis the cleavage and activation of caspase-8, the central caspase of the death receptor pathway, only occurred several hours after activation of caspase-3, indicating that caspase-8 is not involved in the initiation phase of CD20XL-induced apoptosis. Caspase-8 is likely to be cleaved by previously activated caspases, like caspase-3 or caspase-7.34-46

CD20XL-induced apoptosis was shown to induce the mitochondrial release of cyt-c and loss of ΔΨ$_m$ and activation of caspase-9 and the effector caspase-3. These characteristics have also been described for BCR-induced apoptosis.37,38 However, our data also clearly demonstrate differences between CD20XL- and BCR-induced apoptosis. First, CD40 mAbs, which completely inhibited BCR-induced apoptosis of Ramos cells, did not prevent CD20XL-induced apoptosis. Second, Ramos.M3 cells, which are resistant to BCR-induced apoptosis, were sensitive to CD20XL-induced apoptosis. Finally, the broad-spectrum caspase inhibitor zVAD-fmk completely prevented BCR-induced apoptosis, whereas CD20XL-induced apoptosis was only partially inhibited by zVAD-fmk. Thus, although similarities between the CD20XL- and BCR-induced apoptosis pathways do exist, it is evident that these pathways are at least partially different.

CD20XL induced the release of cyt-c and loss of ΔΨ$_m$ in Ramos cells. These phenomena are both signs of mitochondrial membrane permeabilisation (MMP), which is considered to constitute a point of commitment to cell death.47 In different apoptosis pathways, caspases can either be activated prior to MMP, or as a result of MMP. In death-receptor mediated apoptosis (via the so-called 'extrinsic pathway'), caspases are upstream inducers of MMP.

In contrast, during the mitochondrial-dependent (or 'intrinsic') pathway of apoptosis, MMP is induced independent of caspases, and caspase activation (only) is a downstream consequence of MMP. In this setting, caspases are responsible for the accomplishment of the apoptotic phenotype but not strictly required for cell death. Thus, when caspases are inhibited in this latter system (for example by caspase inhibitors like zVAD-fmk), characteristic nuclear features of apoptosis do not occur. However, since MMP does occur, cells are committed to cell death.48

We found that CD20XL-induced loss of ΔΨ$_m$ and release of cyt-c were not inhibited by the caspase inhibitor zVAD-fmk. This strongly suggests that CD20XL can induce MMP independent of active caspases, and thus activates a mitochondrial-dependent, or 'intrinsic', pathway of apoptosis.

Although CD20XL-induced apoptosis involved the activation of caspases-9 and -3, CD20XL-induced apoptosis was only partially inhibited by the broad-spectrum caspase inhibitor zVAD-fmk. In the presence of zVAD-fmk, the percentage of apoptotic cells decreased (from ~50%) to ~30%, and these cells still displayed characteristic cellular features of apoptosis, like cell shrinkage, PS exposure and loss of membrane integrity. However, chromatin condensation
Intracellular pathways of CD20-induced apoptosis

and DNA fragmentation were both prevented by zVAD-fmk. This indicates, in agreement with previous studies\(^{32,34}\), that active caspases were required for the nuclear features of CD20XL-induced apoptosis.

Importantly, although CD20XL-induced apoptosis involved the release of cyt-c, CD20XL-induced apoptosis was not prevented by overexpression of Bcl-2. CD20XL induced \(-50\%\) of apoptosis in a Bcl-2 overexpressing clone of Ramos, and this was accompanied by the release of cyt-c and loss of \(\Delta \psi_m\). This is in contrast to the majority of previous studies demonstrating that Bcl-2 confers protection against apoptosis induced by a variety of stimuli, including many chemotherapeutic drugs.\(^{43,44,47,49-52}\) It is assumed that the ratio of pro- and anti-apoptotic members of the Bcl-2 family determines the susceptibility of a cell to undergo apoptosis in response to a certain death stimulus.\(^{52}\) Thus, the anti-apoptotic effect of Bcl-2 may be inhibited by pro-apoptotic members of the Bcl-2 family, like Bax, Bak or Bid. In addition, certain agents were reported to induce apoptosis irrespective of the level of Bcl-2 expression, by acting directly on components of the mitochondrial membrane.\(^{53-55}\)

Whether the signal induced by CD20XL supercedes the inhibitory capacity of Bcl-2, for example by activating a pro-apoptotic member of the Bcl-2 family, or whether CD20XL damages the mitochondrial membrane beyond control of the Bcl-2 family, remains to be investigated.

So far, the factors involved in the initiation phase of CD20XL-induced apoptosis, upstream of the mitochondria, are unknown. Therefore, we investigated the involvement of several signaling molecules that have previously been shown to play a role in different (caspase-independent) apoptosis pathways, and are known to be able to induce mitochondrial membrane permeability. Ceramide, a sphingosine-based lipid signaling-molecule, has been reported to play a role in apoptosis induced by certain stimuli, including drug-induced apoptosis.\(^{56,57}\) Several inhibitors of the ceramide synthesis (fumonisin B1, deoxynojirimycin and conduritol B epoxide) did not inhibit CD20XL-induced apoptosis (data not shown), indicating that CD20XL probably does not interfere with the ceramide metabolism to induce apoptosis. Furthermore, Cyclosporin A (CsA), which was found to inhibit apoptosis by preventing MMP\(^{58,62}\), did not inhibit CD20XL-induced apoptosis (data not shown). This suggests that CD20XL-induced apoptosis is not dependent on calcineurin and does not act on cyclophylalin D to induce MMP. Furthermore, since CsA was also found to inhibit BCR-induced apoptosis\(^{36,61}\), it confirms our observation that BCR and CD20XL activate (at least partially) different apoptosis pathways.

The existence of apoptosis independent of active caspases has been observed before in in vitro studies.\(^{29,34}\) However, the clinical relevance of this form of cell death is unclear. Recently, a role for caspase-independent apoptosis in the treatment of chemoresistant tumors was suggested.\(^{29,32}\)

Chemotherapeutic drugs, although acting on a variety of intracellular targets, eventually kill
their targets via the induction of apoptosis. Drug-induced apoptosis may be dependent\textsuperscript{41,63,64} or independent of the Fas/FasL system.\textsuperscript{65,67} In the majority of studies, drug-induced apoptosis was inhibited by caspase-inhibitors, indicating that most chemotherapeutic agents, although functioning independent of the Fas death-receptor pathway, do require the activation of caspases to induce apoptosis in their tumor targets. Chemoresistance may be caused by a defect in apoptosis-inducing pathways, leading to the inability of drugs to activate caspases. For example, P-glycoprotein (P-gp), which is one of the main causes of multidrug resistance and also plays an important role in the chemoresistance of hematologic malignancies\textsuperscript{68,69}, has recently been shown to function not only as a drug efflux pump, but also to interfere with the Fas apoptosis pathway.\textsuperscript{32} Another cause of chemoresistance in hematologic malignancies may be the overexpression of Bcl-2.\textsuperscript{52} Cell lines overexpressing the Bcl-2 protein show decreased sensitivity to drug-induced apoptosis\textsuperscript{49,51}, and high Bcl-2 protein expression has been correlated with poor response to chemotherapy and poor prognosis in hematologic malignancies.\textsuperscript{70,73} Thus, from these data on drug-induced apoptosis it can be deduced that anti-neoplastic agents that act independent of Bcl-2 expression, independent of the Fas death-receptor pathway or independent of the activation of caspases in general, may circumvent chemoresistance of their tumor targets. These aspects are all combined in CD20XL-induced apoptosis.

In a study in 31 lymphoma patients with bulky disease treated with chimeric CD20 mAbs\textsuperscript{2}, it was demonstrated that the number of prior chemotherapy courses, the number of relapses, resistance to any or all courses of chemotherapy, prior aggressive therapy or prior anthracycin therapy, were not correlated to the response to CD20-therapy. Furthermore, in low-grade lymphoma patients treated with chimeric CD20 mAbs\textsuperscript{1}, baseline peripheral blood Bcl-2 negativity was correlated with a lower response to CD20-therapy, indicating that Bcl-2 positive tumors were sensitive to CD20-therapy. Thus, these clinical data are in line with our \textit{in vitro} results suggesting that CD20XL may induce apoptosis in tumor cells that acquired resistance to chemotherapeutic agents.

In conclusion, CD20XL-induced apoptosis is independent of the Fas/FasL apoptosis pathway, is not inhibited by overexpression of Bcl-2 and only partially depends on active caspases. Therefore, CD20XL-induced apoptosis is likely to circumvent important causes of chemoresistance. This may explain the favorable clinical results of chimeric CD20 mAbs in chemoresistant lymphomas.
Reference List


