Selective Apoptotic Killing of Malignant Hemopoietic Cells by Antibody-Targeted Delivery of an Amphipathic Peptide

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Abstract
The α-helical amphipathic peptide D-(KLAKLAK)₂ is toxic to eukaryotic cells if internalized by a suitable targeting mechanism. We have targeted this peptide to malignant hemopoietic cells via conjugation to monoclonal antibodies, which recognize lineage-specific cell surface molecules. An anti-CD19/peptide conjugate efficiently killed 3/3 B lymphoid lines. However, an anti-CD33/peptide conjugate was cytotoxic to only one of three CD33-positive myeloid leukemia lines. The IC₅₀ towards susceptible lines were in the low nanomolar range. Conjugates were highly selective and did not kill cells that did not express the appropriate cell surface cognate of the antibody moiety. Anti-CD19/peptide conjugates efficiently killed cells from patients with chronic lymphocytic leukemia but anti-CD33/peptide reagents were less effective against fresh acute myeloid leukemia cells. We therefore suggest that amphipathic peptides may be of value as targeted therapeutic agents for the treatment of a subset of hematologic malignancies.

Introduction
Targeting of toxic moieties to leukemic cells via attachment to antibodies, antibody fragments, or peptides that recognize selectively expressed cell surface structures is an important therapeutic goal (1, 2). Examples of targeted therapy include gentuzumab ozogamicin, a humanized anti-CD33 antibody linked to the potent toxin calicheamycin, which has yielded promising results in the treatment of relapsed acute myelogenous leukemia (3, 4). Anti-CD19 antibodies conjugated with blocked ricin (5) or tyrosine kinase inhibitors (6, 7) have been used in therapeutic trials for the treatment of B lymphoid malignancies. BL22, a recombinant immunotoxin consisting of the Fv domain of anti-CD22 fused to a fragment of Pseudomonas exotoxin has shown impressive activity against purine analogue–resistant hairy cell leukemia (8).

The cytotoxic actions of conventional antineoplastic agents often depend on the release of cytochrome c from mitochondria and the consequent assembly of an ~700-kDa complex, the Apaf-1 apoptosome, within which the apoptotic protease procaspase 9 is activated. Active caspase 9 cleaves and activates executioner caspsases, including caspases 3 and 7, resulting in the functional and morphologic changes of apoptotic cell death (9, 10).

Because the genetic changes resulting in malignant transformation often compromise the early stages of apoptotic signaling (10–12), there is considerable interest in developing reagents which induce apoptosis by interacting with relatively late components of the death signaling mechanism. The amphipathic peptide D-(KLAKLAK)₂ presents basic lysine residues on one face of an α helix (13, 14). This peptide does not disrupt the zwitterionic plasma membrane of eukaryotic cells. However, if internalized, D-(KLAKLAK)₂ can disrupt the negatively charged mitochondrial membrane, resulting in cell death. Ellerby et al. (14) used a targeting peptide to direct D-(KLAKLAK)₂ to the neovasculature of a human mammary carcinoma xenograft carried in nude mice. This strategy resulted in inhibition of angiogenesis and thereby caused regression of the tumor (14).

The susceptibility of malignant hemopoietic cells to D-(KLAKLAK)₂ has not been studied. Here we conjugated this moiety to anti-CD19 and anti-CD33 antibodies, with the consequent generation of reagents designated as anti-CD19:K and anti-CD33:K. These antibodies were chosen because they are known to internalize consequent to binding their cognate ligands, which are selectively expressed on B lymphoid (15) and myeloid (16) cells respectively. We show here that anti-CD19 conjugates potently and selectively killed malignant B lymphoid cells in vitro. In contrast, the anti-CD33 conjugates showed a more restricted spectrum of activity against myeloid cells. Some of the data here have been presented in a preliminary form (17).

Materials and Methods
Cell Lines. The following Mycoplasma-free leukemia/lymphoma cell lines were used in this study. B lymphoid lines: Raji, Daudi, 721.221 (CD2⁺ CD19⁺ CD33⁺); myeloid lines: THP1, HL60, U937 (CD2⁺ CD19⁻ CD33⁺), and K562 (CD2⁺ CD19⁻ CD33⁺); T lymphoid line Jurkat (CD2⁻ CD19⁻ CD33⁻). Cells were routinely cultured in RPMI 1640 supplemented with 10% FCS, 100 units/mL penicillin, and 100 μg/mL streptomycin (all from Invitrogen, Paisley, United Kingdom).

Isolation of Malignant Cells from Leukemia Patients. Cells were isolated from the peripheral blood of patients with chronic lymphocytic leukemia (CLL) or acute myeloid leukemia (AML) by centrifugation on lymphoprep gradients (Axis-Shield, Oslo, Norway). Their purity was determined as described (18, 19). Cells were cultured in supplemented RPMI 1640 as described above.

Antibodies. Mouse monoclonal anti human CD19 antigen (clone B-C3) was from Immunodiagnostic Systems (Bolton, United Kingdom). The mouse monoclonal anti-human CD33 antigen (clone NM 30.1.3.1) was generously provided by the Therapeutic Antibody Centre (Oxford, United Kingdom).

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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Anti-CD33 conjugated to calicheamycin (gentzumab ozogamycin) was from Wyeth (Maidenhead, United Kingdom).

Conjugation Protocol. A modified amphipathic peptide containing additional NH2-terminal biotin, cysteine, and linker residues was conjugated to antibodies using the bifunctional cross-linker N-succinimidyl-3-(2-pyridylthio) propionate (20). Conjugation ratios of between 6 and 10 mol of peptide per mole antibody were achieved. Complete details of the conjugation protocol and characterization of the conjugates by HPLC and Western blot analysis are provided in the supplementary section (Supplementary Fig. 1 and B).

Dye Reduction Assay. Cells (0.25 × 10^6 mL^-1) were cultured with increasing concentrations of antibody-peptide conjugates. At 24 and 48 hours of culture, 100 μL aliquots were transferred into 96-well round-bottomed microtiter plates and 10 μL of 5 mg mL^-1 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, Sigma, Poole, United Kingdom) were added and incubation continued for a further 4 hours at 37°C. Plates were centrifuged at 800 × g for 5 minutes, the supernatants were withdrawn, and 100 μL of DMSO were added per well. The absorbance of the purple formazan generated by reduction of MTT by viable cells was quantified by recording absorbance at 540 nm.

Clonogenic Assay. One-milliliter aliquots of cells (2.5 × 10^5 mL^-1) were incubated at 37°C in the presence or absence of antibody-peptide conjugate for 48 hours. Cultures were then diluted 10-fold. Ten microliters of the diluted suspensions were mixed with 290 μL of Iscove's modification of Dulbecco's medium (Irvine Scientific, Garden Grove, CA) containing 10% fetal calf serum, 100 U/mL penicillin, 100 μg/mL streptomycin, and 2 mM L-glutamine. Triplicate aliquots (1 mL) were plated in 35-mm Petri plates and incubated further at 37°C. Colonies were counted at 7 days.

Immunofluorescence Microscopy. Cytospin preparations of Raji cells (2.5 × 10^5 mL^-1) incubated with 20 nmol/L anti-CD19:K were made. Slides were fixed in ice-cold methanol overnight and rehydrated in PBS containing 1% bovine albumin. Each cell spot was incubated with 100 μL of a 1/10 dilution of FITC-labeled anti-mouse immunoglobulin (DAKO, Glostrup, Denmark) for 30 minutes. Following the addition of 10 μL Hoechst 33342, slides were rinsed thrice in PBS, mounted, and viewed in an Olympus IX70 confocal microscope.

Flow Cytometry. Raji cells incubated with anti-CD19 conjugate were stained with FITC-labeled anti-mouse immunoglobulin, washed with PBS and analyzed in a FACScan flow cytometer (Becton Dickinson, Cowley, United Kingdom). Each sample was analyzed without permeabilization or following permeabilization and fixation with IntraStain (DAKO).

Western Blotting. Cells were extracted and the lysates analyzed by Western blotting as described (21). The following antibodies were used: poly(ADP-ribose) polymerase (Becton Dickinson), p85 fragment of poly(ADP-ribose) polymerase (Promega, Southampton, United Kingdom), and β actin (Sigma). Horseradish peroxidase–labeled anti-mouse immunoglobulin was from DAKO and horseradish peroxidase-streptavidin from Becton Dickinson.

Statistical Methods. The significance of differences between means was evaluated using Student's t test for unpaired samples.

Results

Actions of Anti-CD19:K on B Lymphoid Cell Lines. Anti-CD19 conjugated to D-(KLAKLAK)2 efficiently killed the CD19-positive malignant B lymphoid lines 721.221, Daudi, and Raji with IC50 values of 5.1, 3.9, and 2.0 nmol/L, respectively. The CD19-negative Jurkat cell line was completely resistant to this reagent (Fig. 1A). Raji cells were unaffected by unconjugated anti-CD19 antibody (Fig. 1B) or by a mixture of anti-CD19 and peptide presented at the same ratio as in the conjugate (Fig. 1C).

The selectivity of anti-CD19:K towards cells expressing the cognate cell surface antigen is further confirmed in Fig. 2. THP1 and Jurkat cells were completely resistant to this reagent (Fig. 2A). The killing of Raji cells by anti-CD19:K shows a closely similar dose dependence when measured by MTT assay (Fig. 2A) or by a clonogenic assay (Fig. 2C). The resistance of THP1 and Jurkat cells to anti-CD19:K was also confirmed by clonogenic assay (data not shown).

The D-(KLAKLAK)2 peptide alone was not toxic to either Raji, Jurkat, or THP1 cells at concentrations below 5 μmol/L, but induced nonselective killing of each of these lines with IC50 values in the range of 10 to 15 μmol/L (Fig. 2B). Therefore, targeting of the peptide via linkage to anti-CD19 augmented its toxicity towards B lymphoid lines and simultaneously conferred a high degree of selectivity.

Additional confirmation of the toxicity of anti-CD19:K towards the three lymphoid cell lines was obtained by parallel evaluation of cell killing by manual counting of viable cells able to exclude 0.1% trypan blue (Fig. 3A) and by MTT assay (Fig. 3B).

Internalization of the conjugate by Raji cells was studied by immunofluorescence microscopy using FITC-labeled anti-mouse immunoglobulin to detect anti-CD19:K. Whereas control cells showed no green fluorescence (Fig. 4A), cells incubated with conjugate for 30 minutes showed intense labeling of vesicle-like structures (Fig. 4B). When these cells were washed free of conjugate and incubated for a further 6 hours, cellular labeling was substantially reduced, compatible with degradation of the immunoglobulin moiety of anti-CD19:K (Fig. 4C).

Flow cytometric and Western blotting studies carried out using the same cell preparations as in Fig. 4 confirmed the binding and internalization of anti-CD19:K. Analysis of intact Raji cells incubated with the conjugate for 30 minutes showed cell surface labeling by FITC anti-mouse immunoglobulin which persisted 1 hour after the initiation of a "chase" period in medium alone (Fig. 5A). However, cell...
surface labeling was not detectable at 3 or 6 hours. Parallel analysis of permeabilized and fixed cells also provided evidence of cell-associated anti-CD19:K, which declined slowly over a 6-hour chase period (Fig. 5B). The presence of anti-CD19 reactivity in permeabilized cells at 3 and 6 hours of incubation, at which time cell surface labeling was undetectable (Fig. 5A) provides strong evidence for the internalization of the reagent.

Due to the high background reactivity of cellular biotinylated proteins (Fig. 5C), it was not possible to detect the biotinylated peptide moiety of anti-CD19:K by either immunofluorescence or by flow cytometry. However, a biotinylated band, detected using streptavidin-horseradish peroxidase and which comigrated with a band reactive with anti-mouse immunoglobulin was detectable in cells incubated with anti-CD19:K (Fig. 5C). Neither the streptavidin-horseradish peroxidase- nor anti-mouse immunoglobulin horseradish peroxidase–reactive bands were detected in control cells. The rapid disappearance of the biotinylated band during further incubation, despite the partial persistence of the immunoglobulin band is suggestive of the dissociation of the peptide from anti-CD19. However, we cannot exclude the possibility that the peptide moiety, which contains of D-amino acids and would be expected to resist proteolysis, is released consequent to degradation of the targeting immunoglobulin moiety. It was not possible to directly show the generation of free D-(KLAKLAK)₂ due to the small amounts of cell-associated label and the inefficient retention of the 2-kDa free peptide on Western blot membranes.

Immunoblot analysis of Raji cells incubated with anti-CD19:K provided clear evidence of apoptotic cell death. An antibody highly

Figure 2. Actions of anti-CD19:K and D-(KLAKLAK)₂ on B and T lymphoid and myeloid cell lines. Actions of anti-CD19:K (A) and D-(KLAKLAK)₂ (B) on Raji, Jurkat, and THP1 cells. SE of triplicate MTT assays at 48 hours were <10% of the means. C. action of anti-CD19:K on Raji cells determined by clonogenic assay. Representative of two independent experiments. Bars, SE of triplicate determinations.

Figure 3. Toxicity of anti-CD19:K towards B lymphoid cell lines. Incubations were for 48 hours. Cell killing was assessed by counting of trypan blue–negative cells in a hemocytometer (A) or by MTT assay (B). For each cell line, cell survival in the presence of 5 or 20 nmol/L conjugate was significantly different from control values (P < 0.01).

Figure 4. Internalization of anti-CD19:K by Raji cells. Cytospin preparations were stained with FITC-labelled anti-mouse immunoglobulin (green). Nuclei were stained with Hoechst 33342 (purple). A, control cells. B, cells incubated with 20 nmol/L anti-CD19:K for 30 minutes. C, cells incubated as in B then washed and cultured in medium alone for an additional 6 hours. Bar, 5 μm.
specific for the p85 fragment of poly(ADP-ribose) polymerase, which is generated by the action of the apoptotic protease caspase 3, detected an 85-kDa band in lysates from cells incubated with the conjugate for 3 hours (Fig. 5D).

**Actions of Anti-CD33:K on Myeloid Leukemia Cell Lines.** Anti-CD33:K induced killing of the THP1 myeloid leukemia cell line, with an IC_{50} of 6.5 nmol/L (Fig. 6A). In contrast, up to 70 nmol/L of unconjugated anti-CD33 was ineffective (data not shown) and micromolar levels of D-(KLAKLAK)₂ were required to induce cell death (IC_{50} 14 μmol/L; Fig. 2B).

In contrast to THP1, the HL60, U937, and K562 myeloid cell lines were unaffected by up to 70 nmol/L anti-CD33:K (Fig. 6A). In the case of K562 cells, the resistance is accounted for by the absence of cell surface expression of CD33. However, U937 and HL60 cells expressed CD33 at levels comparable to THP1 (data not shown). Furthermore, THP1 cells were readily killed by anti-CD33 conjugated to either D-(KLAKLAK)₂ or to calicheamycin (Fig. 6B). In contrast, HL60 cells were killed by anti-CD33 calicheamycin but was entirely resistant to anti-CD33:K (Fig. 6C). As expected, CD33-negative Jurkat and Raji cells were entirely resistant to anti-CD33:K (data not shown).

**Actions of Conjugates on Fresh Chronic Lymphocytic Leukemia and Acute Myeloid Leukemia Cells In vitro.** We finally carried out a limited number of experiments using freshly isolated CLL and AML cells. Each of 12 CLL isolates studied were efficiently killed following 48 hours of incubation with anti-CD19:K, with IC_{50} values below 5 nmol/L. Three representative examples are shown in Supplementary Fig. 2A. In contrast, killing of AML cells isolated from five patients by anti-CD33:K was less efficient, between 100% and 40% of the cells remaining resistant to up to 50 nmol/L conjugate following a 48-hour incubation (Supplementary Fig. 2B).

**Discussion**

Conjugation of the amphipathic peptide D-(KLAKLAK)₂ to an anti-CD19 antibody generated a reagent which efficiently killed three of three malignant B lymphoid cell lines as quantified by MTT assay. The killing of these lines was additionally confirmed by clonogenic assay and by manual counting of viable cells. A combination of immunofluorescence, flow cytometric, and Western blot experiments support a mechanism whereby the anti-CD19:K reagent was rapidly internalized and the peptide moiety released from the targeting antibody. However, technical limitations prevented the direct demonstration of free peptide in targeted cells. Therefore, the precise mechanism of cell killing remains unclear at present, although clear evidence of apoptosis induction...
was obtained by demonstrating the caspase-mediated cleavage of poly(ADP-ribose) polymerase.

In contrast, a peptide/anti-CD33 conjugate killed only one of four myeloid cell lines tested here. The resistance of one of these lines (K562) could be accounted for by the lack of expression of cell surface CD33. However, the remaining two resistant lines (HL60 and U937) expressed abundant CD33, suggesting that resistance to killing resulted from a cell type–dependent inability of internalized peptide to access and/or activate the cell death machinery. This interpretation was corroborated by our demonstration that the anti-CD33 K-resistant HL60 cell line was nevertheless susceptible to anti-CD33 conjugated to a different toxin, calicheamicin.

Limited studies using malignant cells freshly isolated from CLL or AML patients additionally confirmed the conclusion that targeting of amphipathic peptides to B lymphoid tumors was more successful than targeting to malignant myeloid cells. Whereas targeting of toxic moieties via linkage to anti-CD19 would affect normal B lymphoid cells and malignant populations in vivo, these reagents would be expected to spare progenitor populations that would allow repopulation of the B-lymphocyte compartment.

Whereas micromolar levels of the peptide itself was uniformly toxic to all of the cell lines tested here, linkage to antibodies resulted in at least a 100-fold increase in efficiency of killing of the targeted cells. The reagents generated by our protocol showed IC50 values in the low nanomolar range. The increased efficiency was accompanied by high selectivity, since neither conjugate tested here killed cells that did not express the appropriate cell surface target molecule.

Numerous toxins, including ricin and Pseudomonas exotoxin, have been used in targeting strategies. However, amphipathic peptides may offer some potential advantages. First, construction using D-amino acids would render these species resistant to proteolytic degradation, both in the plasma and within cellular compartments (14). Second, they can be synthesized economically by purely chemical procedures. Third, induction of vascular leak syndrome is often the dose-limiting factor in the therapeutic use of many natural toxins. A conserved three amino acid sequence has been identified as a cause of this syndrome (22). The absence of this motif from the amphipathic peptide suggests that its use in targeted strategies may avoid limitations resulting from vascular leakage.

Finally, amphipathic peptides offer the prospect of constructing completely synthetic targeted reagents, thus avoiding the use of potentially hazardous biomolecules. Peptides that bind selectively to specific immunoglobulins (23) or other unidentified cell surface structures (24) of malignant B lymphoid cells have been identified by phage display technology. Design of peptides containing the amphipathic peptide linked to such a targeting sequence offers the prospect of synthesizing novel clone-specific targeted reagents.

In summary, we have shown that internalization of the amphipathic peptide D-(KLAKLAK)2 into malignant B lymphoid cells efficiently induces cell killing. However, this strategy was less successful against myeloid leukemia cells. We therefore suggest that this peptide sequence may be of value in the synthesis of novel targeted reagents for the treatment of a subset of hematopoietic malignancies.

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