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Activation of the Proapoptotic Death Receptor DR5 by Oligomeric Peptide and Antibody Agonists

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The cell-extrinsic apoptotic pathway triggers programmed cell death in response to certain ligands that bind to cell-surface death receptors. Apoptosis is essential for normal development and homeostasis in metazoans, and furthermore, selective activation of the cell-extrinsic pathway in tumor cells holds considerable promise for cancer therapy. We used phage display to identify peptides and synthetic antibodies that specifically bind to the human proapoptotic death receptor DR5. Despite great differences in overall size and structure, the DR5-binding peptides and antibodies shared a tripeptide motif, which was conserved within a disulfide-constrained loop of the peptides and the third complementarity determining region of the antibody heavy chains. The X-ray crystal structure of an antibody in complex with DR5 revealed that the tripeptide motif is buried at the core of the interface, confirming its central role in antigen recognition. We found that certain peptides and antibodies exhibited potent proapoptotic activity against DR5-expressing SK-MES-1 lung carcinoma cells. These phage-derived ligands may be useful for elucidating DR5 activation at the molecular level and for creating synthetic agonists of proapoptotic death receptors.

Keywords: phage display; apoptosis; antibody library; peptide library; protein engineering

Introduction

Apoptosis, or programmed cell death, is essential for normal development and homeostasis in metazoans[1]. In mammalian cells, apoptosis is initiated by two main signaling pathways.[2,3] The cell-intrinsic pathway is typically triggered in response to DNA damage or other forms of cell distress, and involves activation of pro-apoptotic members of the *BCL2* gene superfamily, which in turn cause the mitochondria to release apoptogenic factors into the cytosol.[4–8] This results in the formation of an "apoptosome" complex that recruits and activates the apoptosis-initiating protease caspase-9, which in turn activates "executioner" proteases caspase-3, -6 and -7.[9,10] Cellular damage induced by chemotherapy and irradiation triggers apoptosis in tumor cells through the cell-intrinsic pathway. However, this pathway is usually dependent on p53 function, and many human cancers evade apoptosis through mutations that cause inactivation of p53.[3,11] In contrast, the cell-extrinsic apoptotic pathway is typically initiated by ligand-induced oligomerization of cell-surface death receptors through a process that is independent of p53.[2] Oligomerization of death receptors can lead to the assembly of a death-inducing signaling complex (DISC) and activation of caspase-8 and caspase-10, which initiate apoptosis by activating the same set of executioner

Abbreviations used: Apo2L, apoptosis-inducing ligand 2; CDR, complementarity determining region; CRD, cysteine-rich domain; DcR, decoy receptor; DISC, death-inducing signaling complex; DR, death receptor; ECD, extracellular domain; ELISA, enzyme-linked immunosorbent assay; Fab, antigen-binding fragment; hDR, human death receptor; mAb, monoclonal antibody; OPG, osteoprotegerin; PLAD, preligand assembly domain; TNF, tumor necrosis factor; TNFR, TNF receptor; TRAIL, TNF-related apoptosis-inducing ligand.

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caspases that are activated by caspase-9 in the cell-intrinsic pathway.

Selective activation of apoptosis is currently being investigated in clinical trials for cancer therapy.[2,3,11–13] The tumor necrosis factor (TNF) gene superfamily member Apo2L/TRAIL† is a promising candidate for cancer therapy, because it initiates apoptosis through the cell-extrinsic pathway independently of p53 and is highly selective for tumor cells over normal cells.[2,14,15] Apo2L/TRAIL is a type II transmembrane protein,[16,17] but it also exists in a soluble form produced by proteolytic cleavage at the cell surface[18] or as a recombinant protein.[14,16] Apo2L/TRAIL binds at least five natural receptors,[12] including two apoptosis-signaling death receptors (DR4 and DR5)[19–22] and three non-signaling decoy receptors (DcR1, DcR2 and OPG).[19,21,23–26]

Structural analyses of Apo2L/TRAIL have revealed a typical TNF-like fold and trimeric quaternary structure.[27,28] Furthermore, the structure in complex with DR5 reveals that, like other members of the TNF superfamily, the Apo2L/TRAIL trimer binds to three receptors,[29–31] suggesting that a trimeric receptor complex is the functional unit for signaling.[12] In addition to providing insights into biological function, structure analyses have guided the *in vitro* evolution of receptor-selective mutants of Apo2L/TRAIL, which were used to show that DR5 may contribute more than DR4 to apoptosis signaling.[32] These results also confirmed findings with agonist monoclonal antibodies (mAbs), which showed that apoptosis can be initiated by engagement of DR5 alone, albeit less potently, even in cancer cells that express both DR5 and DR4.[33–35]

Using phage-displayed libraries of synthetic antibodies built on a human framework,[36,37] we were able to obtain the first structure of an antigen-binding fragment (Fab) in complex with a TNF receptor (TNFR) family member.[38] Herein, we have extended these studies and obtained anti-hDR5 antibodies and peptides that act as potent agonists of cell death when presented in oligomeric form. The crystal structure of one such Fab in complex with hDR5 reveals a binding epitope that overlaps with, but differs significantly from, the epitope for binding to Apo2L/TRAIL.

Our results demonstrate that the biological activity of Apo2L/TRAIL can be mimicked by synthetic peptides and antibodies that recognize hDR5 by very different structural mechanisms. These ligands will be useful tools for helping to elucidate the molecular details of the cell-extrinisic apoptotic pathway, and furthermore, they may prove useful as therapeutic agents.

† The polypeptide chain is identified by a letter in lower case italics preceding each residue type and number, as follows: *a*, Apo2L/TRIAL; *d*, hDR5; *h*, antibody heavy chain; *l*, antibody light chain.

Results

Anti-hDR5 peptides

We used phage display to identify peptidic ligands for hDR5. We first used a panel of highly diverse naïve peptide libraries to select parental binding clones. Subsequently, we used biased libraries to increase the affinity and reduce the size of the parental clones. The selection process was monitored by DNA sequencing to ascertain the sequences of the selected peptides, and by the use of competitive phage enzyme-linked immunosorbent assays (ELISAs) to estimate affinities for the displayed peptides.

Selection of parental anti-hDR5 peptides

We pooled phage from a panel of 18 polyvalent peptide-phage libraries (Table 1) to obtain a library with a combined diversity of 3.8×10^{11} independent sequences. The pool contained mainly peptides with pairs of cysteine residues capable of forming intramolecular disulfides to facilitate the presentation of structurally constrained loops of variable length. Following four rounds of selection for binding to immobilized hDR5 extracellular domain (hDR5-ECD), we obtained two unique peptide-phage clones that exhibited binding to the hDR5-ECD in phage ELISAs, and the binding was competitive with the binding of Apo2L/TRAIL (data not shown). Alignment of the two sequences revealed significant homology (Figure 1(a)). Most notably, both peptides contained three cysteine residues with identical spacings; two of the cysteine residues were fixed in the naïve library, but the

Table 1. Naïve peptide-phage libraries

Library[a]	Design[b]	Diversity[c]
1	X_8	2.6×10^{10}
2	X_{20}	1.2×10^{10}
3	$X_4CX_2GPX_4CX_4$	2.0×10^{10}
4	CX_2GPX_4C	2.5×10^{10}
5	$X_7CX_4CX_7$	2.5×10^{10}
6	$X_7CX_5CX_6$	1.4×10^{10}
7	$X_6CX_6CX_6$	2.5×10^{10}
8	$X_6CX_7CX_5$	2.1×10^{10}
9	$X_5CX_8CX_5$	1.9×10^{10}
10	$X_5CX_9CX_4$	2.0×10^{10}
11	$X_4CX_{10}CX_4$	2.5×10^{10}
12	$X_2CX_4CX_2$	2.1×10^{10}
13	$X_2CX_5CX_2$	2.2×10^{10}
14	$X_2CX_6CX_2$	1.5×10^{10}
15	$X_2CX_7CX_2$	2.1×10^{10}
16	$X_2CX_8CX_2$	2.1×10^{10}
17	$X_2CX_9CX_2$	2.2×10^{10}
18	$X_2CX_{10}CX_2$	2.4×10^{10}

[a] Libraries were displayed on gene-8, under the control of the P_{tac} promoter.
[b] Fixed residues are denoted by the single-letter amino acid code, while X denotes a randomized position where an NNK degenerate codon encodes all 20 natural amino acids.
[c] The libraries were pooled to achieve a total diversity of 3.8×10^{11}.